Single-Cell Observations Reveal Intermediate Transcriptional Silencing States

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Summary

Analysis of transcriptional silencing in Saccharomyces has provided valuable insights into heterochromatin formation and function. However, most of these studies revealed only the average behaviors of populations of cells. Here, we examined transcriptional silencing by monitoring individual yeast cells carrying distinguishable fluorescent reporter genes inserted at two different silent loci. These studies showed that two silent loci in a single cell behave independently, demonstrating that heterochromatin formation is locus autonomous. Furthermore, some silencing mutants with an intermediate phenotype, such as sir1, consist of two distinct populations, one repressed and one derepressed, while other mutants, such as those inactivating the SAS-I histone H4 K16 acetylase, consist of cells all with an intermediate level of expression. Finally, both establishment and decay of silencing can be influenced by specific gene activators, with establishment occurring stochastically over several generations. Thus, quantifying silencing in individual cells reveals aspects of silencing not evident from population-wide measurements.

Introduction

The heritable features of an organism derive not only from the sequence of their chromosomal DNA but also from the way in which that DNA is packaged by chromatin. Certain regions of genomes are packaged in chromatin that both prevents expression of the underlying DNA and templates its own persistence over multiple cell divisions (Grewal and Moazed, 2003). Such chromatin-based epigenetic repression accounts for such diverse phenomena as X chromosome inactivation and chromosomal imprinting in mammals, position effect variegation in Drosophila, and transcriptional silencing in fungi.

The budding yeast Saccharomyces cerevisiae displays three types of chromatin-based epigenetic repression: mating type silencing, telomere position effect, and rDNA silencing (Rusche et al., 2003). Mating type genes, which at the active MAT locus determine the mating type of the cell, are permanently repressed when resident at either of two cryptic loci, HML or HMR, as a result of packaging in a repressive chromatin. Repression requires small cis-acting sites, or silencers, flanking the two loci and genes, SIR1 through SIR4, whose products participate in and modify the chromatin covering HML and HMR. Repression also involves extensive modification of the histones across the loci, especially deacetylation of histone tails and elimination of methylation of certain core residues (Braunstein et al., 1993; Ng et al., 2003; Suka et al., 2001; van Leeuwen et al., 2002; Zhang et al., 2003). Similarly, genes residing near telomeres, or exogenous genes inserted near telomeres, are on average more repressed than the same genes inserted at nontelomeric sites on the chromosome (Gottschling et al., 1990). Repression requires telomeric sequences and the corresponding telomeric binding proteins, Ku and Rap1, as well as all the genes, except SIR1, required for mating type silencing (Luo et al., 2002; Mishra and Shore, 1999). rDNA silencing is mechanistically related to these two phenomena in that the histone deacetylase Sir2 and a nucleolar protein Net1 suppress expression of RNA polymerase II-dependent genes inserted within the rDNA repeat (Smith and Boeke, 1997; Straight et al., 1999). A simple model for mating type silencing and telomere position effect is that protein complexes bound to silencers and to telomeres recruit the Sir2/3/4 complex, which both modifies and polymerizes along the chromatin to establish a repressive state (Liou et al., 2005).

While deletion of SIR2, SIR3, or SIR4 completely eliminates silencing at telomeres and at the mating type loci, mutation of other genes—SIR1, SAS or any of several genes involved in chromatin deposition—can yield partial expression of one or both silent mating type loci (Enomoto and Berman, 1998; Pillus and Rine, 1989; Xu et al., 1999). Although an intermediate phenotype could reflect partial expression in every cell in the population, studies of HML expression in a sir1 strain revealed that intermediate expression resulted from the presence in the culture of two distinct populations, one phenotypically repressed or fully active. Even in wild-type cells, telomeres impose a similar epigenetic phenotype, which is most clearly demonstrated by the red-white sectoring pattern of colonies carrying ADE2 insertion in a telomeric region (Gottschling et al., 1990). In this case, red/white sectoring signifies the persistence of the Ade phenotype in a cell’s progeny—Ade2+ cells are red and Ade2- cells are white—sufficient to form a discrete sector of the colony of all the same phenotype. Surprisingly, sir1 cells with ADE2 inserted into HMR did not exhibit a similar sectoring phenotype (Sussel et al., 1993), suggesting that the sir1-induced intermediate silencing phenotype at HMR may not be epigenetic. Also, whether the partial silencing phenotype arising from mutations in other genes accrues from an epigenetic process has not been resolved.

In order to address the nature of partial silencing phenotypes, we developed a method for observing and quantifying the silencing state of multiple loci in individual cells in a population. The current tools of molecular biology—such as DNA microarray analysis or chromatin...
immunoprecipitation—provide a measure only of the average behavior of cells within the population examined. While this often reflects the actual process ongoing in a cell, in many cases measurement of the average behavior blurs or distorts what actually occurs in individual cells. In this report, we describe the application of microscopy methods in conjunction with fluorescent reporter probes to examine transcriptional silencing at the HML and HMR on an individual cell basis. Studies using this assay have allowed us to address directly the nature of intermediate silencing states in a variety of mutants and to uncover aspects of heterochromatin formation and decay not previously accessible by population-based analyses.

Results

sir1 Cells Exhibit Bistable Expression of the HML and HMR Mating Type Loci

To address the nature and dynamics of transcriptional silencing, we have used fluorescent reporters to quantify simultaneously in individual living cells the expression state of two loci subject to transcriptional silencing. We constructed strains in which a gene encoding a fluorescent protein of one color, under the control of the yeast URA3 promoter, is inserted at one silent mating type locus while a gene encoding a different fluorescent protein, also under control of the URA3 promoter, is inserted at another silent mating type locus. For ease of observation, both reporter proteins are tagged with a nuclear localization signal so that proteins expressed from these reporter genes concentrate in the nucleus.

As shown in Figure 1, our reporter constructs respond to transcriptional silencing in a manner similar to that of the genes normally resident at the silent mating type loci. In examining individual cells by deconvolution microscopy, we have never observed a SIR+ cell carrying these constructs to exhibit expression above background (<1/10^5 cells). This is consistent with FACS analysis, which yields a pattern of fluorescence intensity distribution of SIR+ hml::P_{URA3}-YFP or hmr::P_{URA3}-CFP indistinguishable from cells not carrying any fluorescent reporter (data not shown). Similarly, deletion of sir3, which completely eliminates silencing of HML and HMR, yields expression of the fluorescent reporter protein in all cells and at a level identical to that obtained from cells carrying the same reporter inserted at the active MAT locus (data not shown). Thus, these reporter
constructs accurately reveal the silencing state of the loci in which they are inserted. Moreover, directly comparing expression of two \textit{HML} reporter constructs in \textit{sir3} diploid cells revealed a high level of concordance between the outputs of the two reporters (Figure 2C). Thus, while the constructs when fully expressed exhibit extrinsic noise from cells size variation and cell-wide fluctuations in biosynthetic components (Raser and O’Shea, 2004), they show no stochastic variation linked to the constructs themselves.

In \textit{sir1} strains, expression of mating type loci as revealed by the fluorescent reporter genes exhibits a bimodal pattern. As evident from visual inspection of the micrographs, some cells express \textit{HML} or \textit{HMR} at high levels while others express the loci at low levels (Figure 1). This pattern is independent of the stability of the fluorescent reporter protein (see Figure S1 in the Supplemental Data available with this article online). We confirmed this qualitative impression by quantifying the fluorescence intensity in individual cells in populations of \textit{SIR}+, \textit{sir3}, and \textit{sir1} strains, as described in Experimental Procedures. As shown in Figure 2A, \textit{SIR}+ and \textit{sir3} \textit{hml::P}_{\text{URA3}}-\text{YFP} cells exhibit essentially Gaussian distributions of fluorescence intensity centered around 175 and 2200 units, respectively. On the other hand, \textit{sir1 hml::P}_{\text{URA3}}-\text{YFP} cells exhibit two peaks of intensity centered around 300 and 1800 units, a pattern that statistically fits a bimodal distribution better than a unimodal distribution ($p < 10^{-25}$) but does not fit a trimodal distribution better than bimodal ($p = 0.81$). This pattern was confirmed by FACS analysis, showing clearly the bimodal distribution of \textit{HML} expression for \textit{sir1} cells (Figure 2B). Because the proportion of cells in the active state for \textit{HMR} ($\approx 90\%$) is substantially higher than that for \textit{HML} ($\approx 40\%$), determining the distribution pattern of expression of \textit{HMR} in this strain cannot be done as rigorously as that for \textit{HML}, even though the pattern better fits a bimodal than a unimodal distribution ($p = 0.09$) (Figure 2A). However, in a \textit{sir1 ppr1} strain in which repression of \textit{HMR} is greater than in the \textit{sir1 PPR1} strain (see below and Figure 3), the expression pattern fits a bimodal distribution significantly better than a unimodal

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**Figure 2. Intermediate Silencing Phenotypes of \textit{sir1} and \textit{sas2} Cells Are Different**

(A) Distribution of fluorescence intensity per cell as determined from microscopic analysis. The total fluorescence intensity over individual cells was determined both at 470 nm and 535 nm for more than 100 cells for each strain, all of which carry \textit{hml::P}_{\text{URA3}}-\text{YFP} \textit{hmr::P}_{\text{URA3}}-\text{CFP}. Strains are the same as those in Figure 1. Average fluorescence intensity over each cell was calculated as described in the Supplemental Data. Plotted are the fractions of cells within a specified average intensity range.

(B) Distribution of fluorescence intensity per cell as determined by FACS analysis. Cells from strains carrying \textit{hml::P}_{\text{URA3}}-\text{YFP} (Y3401-Y3407) or \textit{hmr::P}_{\text{URA3}}-\text{YFP} (Y3408-Y3414) and the indicated silencing mutation were analyzed by FACS. Pattern for \textit{SIR}+ is shown in black and for \textit{sir3} in red. Approximately 10^6 cells were analyzed for each strain.

(C) Average fluorescence intensities from more than 100 cells from each indicated strain were determined at 470 nm and 535 nm, and the value obtained at 470 nm for each cell is plotted versus the value obtained at 535 nm for that same cell. (Left panels) \textit{hml::P}_{\text{URA3}}-\text{YFP}/\textit{hml::P}_{\text{URA3}}-\text{CFP} cells, (right panels) \textit{hmr::P}_{\text{URA3}}-\text{YFP}/\textit{hmr::P}_{\text{URA3}}-\text{CFP} cells. Strains were the following: \textit{SIR}+/\textit{SIR}+ (Y3415 and Y3419), green diamonds; \textit{sir3}/\textit{sir3} (Y3416 and Y3420), blue squares; \textit{sir1}/\textit{sir1} (Y3417 and Y3421), red triangles; and \textit{sas2}/\textit{sas2} (Y3422 and Y3426), orange triangles.
distribution \(p < 10^{-5}\). These results reveal that two different silent loci in a sir1 strain exhibit different proportions of activation versus repression. Moreover, the results confirm the previous interpretation that sir1 cells consist of a mixture of two discrete subpopulations of cells, one with active and the other with repressed silent mating type loci.

**Different Loci Are Silenced Independently in the Same sir1 Cell**

The study by Pillus and Rine (1989) demonstrating the epigenetic nature of sir1 cells concluded that silencing is a property of the cell rather than a property of the locus. That is, they found that 20% of cells with one copy of HML were phenotypically repressed for the locus, i.e., sensitive to growth inhibition by \(x\) factor. Since derepression of either of the two copies of HML in a cell is sufficient to render a cell resistant to \(x\) factor, then, if silencing were a property of the locus, only 4% of cells carrying two copies of HML should be sensitive to growth inhibition by \(x\) factor. Since Pillus and Rine observed that 20% of such cells were sensitive to \(x\) factor, they concluded that silencing was a property of the cell, not the locus. Sussel et al. (1993) reported similar results examining HMR using an ADE2 reporter system. Thus, both these studies conclude that silencing states of two HML loci or two HMR loci in sir1 cells are correlated.

We used our fluorescence reporter assay to determine whether two different loci in individual sir1 cells are silenced independently or in concert. We counted the number of sir1 hml::P_{URA3}-YFP hmr::P_{URA3}-CFP cells that exhibited each of the four classes of repression or expression at HML and HMR (Table 1). Based on the proportion of cells derepressed for HML (39%) and the proportion derepressed for HMR (86%) observed in this experiment, we could predict the proportion of cells expected to be derepressed for both or repressed for both, assuming that the two loci behaved

<table>
<thead>
<tr>
<th>Straina</th>
<th>Expression Stateb</th>
<th>YFP</th>
<th>CFP</th>
<th>Number Observed</th>
<th>Number Predictedc</th>
</tr>
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<tbody>
<tr>
<td>hml::P_{URA3}-YFP</td>
<td>+</td>
<td>-</td>
<td>629</td>
<td>618</td>
<td></td>
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<tr>
<td>hmr::P_{URA3}-CFP</td>
<td>-</td>
<td>+</td>
<td>77</td>
<td>66</td>
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<tr>
<td>hml::P_{URA3}-YFP</td>
<td>+</td>
<td>-</td>
<td>387</td>
<td>398</td>
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<tr>
<td>hmr::P_{URA3}-CFP</td>
<td>-</td>
<td>+</td>
<td>92</td>
<td>103</td>
<td></td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>29</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>hmr::P_{URA3}-CFP</td>
<td>-</td>
<td>+</td>
<td>16</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>12</td>
<td>10</td>
<td></td>
<td></td>
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<tr>
<td>-</td>
<td>-</td>
<td>57</td>
<td>55</td>
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Table 1. Locus Independence of Silencing in sir1 Strains

\(^a\)Data from haploid strain Y3403 (sir1 hml::P_{URA3}-YFP hmr::P_{URA3}-CFP) and diploid strain Y3417 (sir1/sir1 hml::P_{URA3}-YFP hmr::P_{URA3}-CFP).

\(^b\)Cells with fluorescence intensity >3σ above background were scored positive for expression of the indicated fluorescent protein.

\(^c\)Calculated by multiplying the total number of observed cells by the fraction of YFP-expressing (or nonexpressing) cells and by the fraction of CFP-expressing (or nonexpressing) cells. Chi-squared calculation for the haploid strain returns \(p = 0.06\), thus failing to reject the null hypothesis that the observed values are different than the predicted random association. Data from experiments with five separate haploid cultures yielded \(p\) values of 0.84, 0.25, 0.21, 0.10, 0.06, and 0.04. Chi-squared test for the diploid strain returns \(p = 0.38\).
completely independently (Table 1, column 4). Qualitatively, the presence of substantial numbers of HML-derepressed HMR-repressed and HML-repressed HMR-derepressed cells demonstrated that the two loci are not regulated in unison. Furthermore, applying a chi-square test to these data indicates that one cannot exclude the null hypothesis that the two loci are regulated independently. Thus, we conclude that two silent loci in the same cell behave independently in a sir1 background.

Since the study from Pillus and Rine (1989) was based on strains carrying two copies of HML, we also tested concordance of silencing in sir1/sir1 hmr::P<sup>URA3</sup>YFP/hmr::P<sup>URA3</sup>CFP diploid cells. The data from one such experiment is presented in Figure 2C. As with HML versus HMR, the presence of cells repressed for one of the loci while derepressed for the other demonstrates that the two HML loci are not obligatorily coregulated. Furthermore and consistent with the previous experiment, we found that the two loci exhibited statistically random expression patterns (Table 1). Similar results were obtained with sir1/sir1 hmr::P<sup>URA3</sup>YFP/hmr::P<sup>URA3</sup>CFP diploid cells (Figure 2C), although, since the proportion of active HMR loci in a sir1 background is higher than that of HML, a greater proportion of cells have both loci active. Nonetheless, the expression states of the two HMR loci in the sir1/sir1 strain are statistically uncorrelated. Thus, in contrast to the results reported by Pillus and Rine, we observe that two HML loci or two HMR loci in the same sir1 cell are silenced independently.

**Sas2 Cells Exhibit Intermediate Expression of Silent Loci**

The products of SAS2, SAS4, and SAS5 comprise a MYST family histone acetyl transferase complex, termed SAS-I, that acetylates lysine 16 of histone H4 (Osada et al., 2001; Suka et al., 2002; Sutton et al., 2003). Loss of Sas2, Sas4, or Sas5 activity results in hypoacetylation of this residue in vivo, especially within subtelomeric regions, with a concomitant increase in the spread of Sir3 and Sir-dependent silencing inward from the telomeres. This observation is consistent with the fact that deacetylation of histone H4 lysine 16 is correlated with and required for transcriptional silencing (Braunstein et al., 1993; Johnson et al., 1990; Park and Szostak, 1990). Similarly, deletion of any of these genes enhances silencing at the DNA locus and at mutant HMR loci with partially defective silencer elements (Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996). In contrast to these observations, deletion of SAS2, SAS4, or SAS5 causes a reduction in the efficiency of silencing at HML such that some of the cells behave as though the locus were phenotypically repressed and some as though it were phenotypically active (Xu et al., 1999). Moreover, in conjunction with sir1, deletion of SAS2 causes a complete loss of silencing at HML but suppresses the sir1 defect at HMR (Xu et al., 1999). Thus, the SAS complex paradoxically has both pro-silencing and antasilencing activity.

Using our dual reporter strain, we examined the role of the SAS-I complex on silencing HML and HMR. As evident from Figure 2, deletion of sas2 diminished silencing at HML and, to a significantly lesser extent, at HMR. However, in contrast to the situation with sir1 mutants, expression pattern of the silent loci in sas2 cells was not bimodal but rather unimodal (p < 10<sup>-5</sup>) with a peak at an intermediate level between fully repressed and fully active. The same results were obtained with sas4 and sas5 mutants (Figure 2B). These results were confirmed by analysis of diploid cells with the two homologous silent mating type loci marked with different reporters (Figure 2C). Thus, the intermediate phenotype of the population of sas2 cells is a consequence of intermediate expression of each cell in the population. Also in contrast with the results with sir1 mutant, HML is more affected than HMR. Finally, for both HML and HMR, sas2 sir1 mutants do not silence as well as does either mutant alone (Figure 2B and data not shown). Thus, as measured by this reporter system, sas2 and sir1 exhibit a synthetic defect in silencing the mating type loci.

**Transcription ActivatorsCompete with the Silencing Apparatus for Expression of a Target Promoter**

Aparicio and Gottschling (1994) reported that the extent of silencing of a URA3 gene inserted into telomeric sites could be diminished by increasing levels of Ppr1, a transcriptional activator for URA3. Moreover, they showed that, at least for cells arrested at M phase, activation of PPR1 could induce the transition of a telomere positioned URA3 gene from a silenced to an active state. This would suggest that Ppr1 can antagonize the silencing apparatus, but whether by blocking establishment during S phase or by promoting decay of the silenced state could not be resolved. Since the URA3 promoter controls transcription of the reporter constructs used in our studies, we could investigate this question using our strains. As evident from Figure 3 (compare with Figure 2A), the level of expression of both YFP and CFP in the sir3 ppr1 strain is reduced to 40%-50% of that in a sir3 PPR1 strain, consistent with the role of Ppr1 in stimulating transcription from the URA3 promoter. Moreover, the proportion of silenced sir1 cells, both at HML and HMR, is increased in ppr1 versus PPR1 strains. That is, 86% of ppr1 sir1 cells exhibit silencing of the HML locus and 26% exhibit silencing of the HMR locus, whereas only 40% and 7% of sir1 PPR1 cells are silenced for HML and HMR, respectively. Finally, the mean expression of both HML and HMR in a ppr1 sas2 strain is significantly lower than that in a PPR1 sas2 strain such that, for instance, HMR locus appears fully silenced in this background. Accordingly, Ppr1 enhances both the basal level of activity of the URA3 promoter and the resistance of the promoter to silencing.

These results confirm that a transcriptional activator and the silencing apparatus compete for expression of a target locus. To determine whether the presence of the activator blocks establishment of silencing or accelerates decay of silencing, we examined how the kinetics of loss or acquisition of silencing were affected by Ppr1. We determined the rates of conversion between silenced and active HM loci in a sir1 strain by repeatedly interrogating fields of cells immobilized on agar under a coverslip (Figure 4A). The initial percent of repressed and derepressed cells at the two loci and the rates of conversion between the two states are presented in Table 2. Considering the level of repression at HML or
HMR as a dynamic equilibrium achieved by a balanced transition between repressed (R) and derepressed (DR) states, we can consider an equilibrium constant as the ratio of DR to R cells, which should be equal to the rate of conversion from DR to R cells divided by the rate of conversion from R to DR cells. For both HMR and HML, these two determinations yield remarkably consistent values. Thus, the proportion of repressed and derepressed in the population arises as an equilibrium distribution dictated by the rates of interconversion between the two states.

We also determined the rates of interconversion between the expression states at HML and HMR in a ppr1 background. As shown in Table 2, these values also yield relatively consistent equilibrium values. Moreover, the data indicate both that the rate of repressed to derepressed state at both loci is lower in ppr1 versus PPR1 cells and that the rate of derepressed to repressed is higher in ppr1 versus PPR1 cells. Thus, the presence of Ppr1 affects interconversion in both directions between the two expression states at both loci.

By following the expression pattern over time of populations of cells obtained by sorting sir1 cells into two populations based on expression state of the HML locus, we confirmed the equilibrium process suggested by single-cell analysis described above. In this case,

Table 2. Rates of Interconversion between Active and Repressed Mating Type Loci in sir1 Cells

<table>
<thead>
<tr>
<th>Locus</th>
<th>PPR1</th>
<th>Repressed (R)</th>
<th>Derepressed (DR)</th>
<th>R → DR (k₁, gen⁻¹)</th>
<th>DR → R (k₋₁, gen⁻¹)</th>
<th>K₀D (DR/R)</th>
<th>K₀R (k₁/k₋₁)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HML</td>
<td>+</td>
<td>40</td>
<td>60</td>
<td>0.13</td>
<td>0.084</td>
<td>1.5</td>
<td>1.57</td>
</tr>
<tr>
<td>HML</td>
<td>-</td>
<td>86</td>
<td>14</td>
<td>0.04</td>
<td>0.12</td>
<td>0.17</td>
<td>0.35</td>
</tr>
<tr>
<td>HMR</td>
<td>+</td>
<td>7</td>
<td>93</td>
<td>0.19</td>
<td>0.013</td>
<td>14.0</td>
<td>14.2</td>
</tr>
<tr>
<td>HMR</td>
<td>-</td>
<td>26</td>
<td>74</td>
<td>0.054</td>
<td>0.027</td>
<td>2.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Data obtained from strains Y3403 (sir1Δ hml::P_UTIL3-YFP hmr::P_UTIL3-CFP) and Y3425 (sir1Δ hml::P_UTIL3-YFP hmr::P_UTIL3-CFP ppr1Δ), as described in the legend to Table 1 and Experimental Procedures. Gen⁻¹ = per generation.
we used a strain expressing GFP at the HML locus, which has higher fluorescence intensity than YFP or CFP. We sorted live sir1 HML::P<sub>URA3</sub>-GFP cells, collecting the brightest and dimmest 10% of the population. We then grew each of these subpopulations, sampling approximately every generation and measuring by FACS analysis the expression of HML in the population of cells. The bimodal nature of the expression pattern of HML in the initial sir1 culture is evident from these FACS data, presented in Figure 4B. Moreover, the subset of cells initially all repressed for HML gradually gave rise to a mixed population of repressed and derepressed cells, as eventually did the population of cells initially fully derepressed for the locus (Figure 4B). By determining the proportion of repressed and derepressed cells as a function of generations of outgrowth of the fully repressed subset of cells, we were able to estimate the rate of conversion from repressed to derepressed at 0.35 per generation and the rate from derepressed to repressed at 0.15 per generation (Figure 4C). While these values are two to three times higher than those determined by microscopic examination, due perhaps to the different growth conditions, they accurately predict the proportion of repression to derepression cells in this particular sir1 culture.

Establishment of Silencing Is Stochastic

Miller and Nasmyth (1984) showed many years ago that establishment of silencing, following transfer from nonpermissive to permissive temperature of a strain carrying the temperature sensitive sir3-8 allele, required progression through the cell cycle. Subsequent work has yet to identify the particular cell cycle step through which the cell must pass in order to establish silencing (Lau et al., 2002; Martins-Taylor et al., 2004). Moreover, recent work has suggested that complete silencing requires progression through multiple cell cycles (Katan-Khayovich and Struhl, 2005), although this study did not distinguish whether repression was stochastic with each cell cycle or whether all cells progressed through intermediate stages of repression over several generations before attaining full repression.

To address this latter point, we examined establishment of repression in a sir3-8 hml::P<sub>URA3</sub>-YFP strain following transition of cells from nonpermissive to permissive temperature. To avoid interference by perdurance of the reporter protein after establishment of repression, we examined whether cells had an active or repressed HML locus at various times after the temperature shift by photobleaching a field of cells and then interrogating them 2 hr later to determine whether they had resynthesized YFP. Cells that were repressed at the time of photobleaching would remain dim while those that had an active HML locus would recover fluorescence. As an internal control, we mixed the test cells with a population of sir3Δ hml::P<sub>URA3</sub>-YFP cells. These control cells are distinguishable from sir3-8 cells, since these sir3-8 cells express a protein localized to the spindle pole body and fluorescently tagged with RFP.

To examine establishment of silencing, we grew sir3-8 hml::P<sub>URA3</sub>-YFP cells at 30°C to derepress the silent loci and then shifted cells to 23°C, mixed them with sir3Δ hml::P<sub>URA3</sub>-YFP cells, and mounted them on an agar slab for microscopy. At various times following the temperature shift, cells were exposed to 500 nm light to photobleach the resident YFP. As evident from the results from this experiment, an example of which is shown in Figure 5, YFP repopulated almost all the sir3Δ cells. In contrast, not all sir3-8 cells regained YFP fluorescence, and the proportion of cells in which YFP was restored decreased with increasing time (Figure 5B), consistent with cells establishing silencing at HML over time. However, different cells acquired silencing at different times. For instance, for three independent cells shown in Figure 5A (upper series), all initially in G1, two became silent within 3 hr while the other retained an active HML locus. Even more dramatically, Figure 5A (lower series) shows a single cell that at 5 hr gave rise to a mother cell that had budded and a daughter cell that had budded. As evident in the figure, the mother cell lineage became repressed while the daughter cell lineage retained an active HML locus. These results clearly demonstrate that onset of silencing is a stochastic process that occurs over more than one generation.

By way of comparison, we also determined the kinetics of the onset of silencing by determining when cells acquired sensitivity to α factor inhibition. MATA HMLα sir3-8 cells are resistant to α factor inhibition when grown at 30°C as a result of the coexpression of MATA and HMLα. At 23°C, such cells are sensitive, since HMLα is silenced. Accordingly, we grew the strain at 30°C, shifted to 23°C, and assessed when cells became sensitive to α factor arrest. Consistent with the results above, different cells silenced HML at different generations following the temperature shift. Moreover, as shown in Figure 5B, the onset of silencing as measured in this assay, while slightly delayed perhaps due to the fact that cells are sensitive to α factor arrest only during the G1 phase of the cell cycle, closely followed that as measured by loss of YFP synthesis.

Discussion

By measuring the dynamics of silencing in individual cells, we have been able to draw several conclusions regarding the mechanism of silencing that have not been accessible from studies of populations of cells.

Silencing Is Locus Specific

We find that the expression state of one locus in a cell is completely independent of the silencing state of another locus. This is true whether the two loci reside at distinct sites in the genome—as for HML and HMR—or at the same site on homologous chromosomes. This result extends observations by Renauld et al. (1993), who noted that two telomeric reporters could assume distinct silencing states in a cell, although they did not address whether silencing of the two sites was independent. Our results stand in contrast to conclusions by Pillus and Rine (1989) on concordance of silencing at two HML loci and by Sussel et al. (1993) on the concordance of silencing at two HMR loci. However, in both of these previous studies, the two loci were marked with the same reporter so that the silencing state of each locus could not be independently determined. Accordingly, the conclusions of concordant silencing in these two studies were based on the untested assumption of the additive nature of the phenotypes of partially silenced...
alleles, a critique not applicable to our studies since we can observe the expression states of the two loci independently. Finally, the fact that the silencing states of two loci in a cell are completely uncorrelated suggest that locus-specific stochastic events leading to formation or decay of silencing far outweigh any cell-wide fluctuations in silencing components that might have been expected to influence the extent of silencing in the cell (Hecht et al., 1996; Raser and O’Shea, 2004; Renaud et al., 1993).

We also observed that HML and HMR exhibit differential sensitivities to mutants affecting silencing. This difference is not the consequence of one locus being more readily silenced than the other, since HMR was more derepressed in sir1 mutants while HML was more derepressed in sae2 mutants. Thus, silencing is not a monolithic process but rather can apparently proceed through mechanistically distinct pathways that are differentially sensitive to different genetic perturbations.

Transcriptional Activators Compete with the Silencing Apparatus Both in Establishment and Maintenance of the Silent Loci
We determined the rates of initiation and decay of silencing in sir1 mutants. These values are significantly faster than those calculated for Pillus and Rine for silencing α1 and α2 at HML. Furthermore, elimination of Ppr1, the transcriptional activator for URA3, decreased the transition rate both from repressed to derepression and from derepressed to repressed of the silenced URA3 promoter in our constructs. Both these results suggest that different promoters have different sensitivities to silencing. Previous work has demonstrated that some promoters contain regions that function as
barriers to silencing, rendering them completely resistant to silencing (Bi and Broach, 1999). However, the promoters in our current studies are not resistant to silencing but rather exhibit different proportions of silent and active loci, suggesting that the silencing machinery functions in competition with the particular transcriptional activation machinery resident at the specific promoter.

Previous work by Aparicio and Gottschling (1994) showed that induction of Ppr1 could activate a previously silenced URA3 locus. This indicates that a transcriptional activator can gain access to a silenced promoter and disrupt the repressive chromatin structure across the locus, at least during certain phases of the cell cycle. Similarly, Sekinger and Gross (2001) demonstrated that heat shock factor as well as TBP and RNA polymerase II are bound at a heat shock promoter even when it is packaged in silent chromatin. In a more recent study, Chen and Widom (2005) conclude that transcriptional activators can gain access to silenced chromatin but that TBP and RNA polymerase are excluded. Our results are consistent with the conclusion that transcriptional activators have access to promoters in silent chromatin. We found that the rate of derepression of our silenced loci in a sir1 strain are 3- to 4-fold higher in a PPR1 than in a ppr1 background, indicating that the Ppr1 protein can gain access to, and disrupt, a preexistent heterochromatic structure. Our results further indicate that the presence of PPR1 also reduces the rate at which the URA3 promoter becomes silenced. This would suggest that the presence of an active transcriptional complex at the promoter, perhaps through recruitment of chromatin-modifying activities promoting active chromatin, tends to preclude establishment of silent chromatin. Thus, silencing apparatus and the transcriptional activation machinery compete both in formation and in reactivation of silent chromatin.

Previous data on silencing in sir1 mutants has been interpreted to indicate that Sir1 predominantly plays a role in establishment of silencing (Rusche et al., 2003). Our measurements returned relatively robust rates of decay of the silent state in sir1 cells. We also showed that establishment of silencing is stochastic, often requiring several generations before converting from an active state to a repressed state. These two observations would indicate that, were the rates of decay of Sir1 cells the same as that in sir1 cells, then we would have observed a significant number of derepressed cells in our wild-type strains. Since we do not, we conclude that Sir1 must play a role in maintenance, likely by promoting persistence of the repressed chromatin over the silent loci.

Loss of SAS-1 Acetyl Transferase Activity Causes Uniform Intermediate Expression of Silent Loci
Sas2, along with Sas4 and Sas5, comprises a histone acetylase complex with specificity for histone H4 K16 both in vitro and in vivo (Kimura et al., 2002; Suka et al., 2002; Sutton et al., 2003). Since acetylation of this residue precludes silencing, one might have predicted that mutational inactivation of this complex would enhance silencing. Previous work has shown that this is not uniformly the case. Although deletion of SAS2 enhances silencing in subtelomeric regions and suppresses silencer defects at HMR, silencing at telomeres and at HML is diminished in sas2 mutants (Ehrenhofer-Murray et al., 1997; Reifsnnyder et al., 1996; Xu et al., 1999). These opposite effects on silencing have been explained by suggesting that the loss of H4 K16 acetylation promotes genome-wide spreading of silencer proteins, thereby diluting the effective concentration of silencer proteins at normally silenced loci. This might be expected to cause loss of silencing at some of the more sensitive loci, as has been suggested for the differential effects on silencing of eliminating Dot1 histone methyl transferase (van Leeuwen et al., 2002).

Our results suggest that, while this mechanism may be operative, elimination of SAS-1 acetyl transferase activity has an even more unanticipated effect. In particular, in contrast with the pattern of silencing seen in sir1 strains, HML expression in sas2 cells is not biphasic but rather exhibits a uniform but intermediate expression in all cells. That is, all cells exhibit the same level of expression of the reporter at HML, which is greater than that in fully repressed cells but significantly less than that in fully active cells. Thus, the loss of SAS-1 histone acetyl transferase activity does not affect the persistence of the silent state but rather the effectiveness of the silent state. The reduced effectiveness of silent chromatin in sas2 mutants might result from altered histone modification affecting access or activity of the transcriptional machinery. We note, though, that histone H4 K16 is generally unacetylated in silent chromatin so that loss of SAS acetylase activity should have little effect on acetylation patterns in silent chromatin. Moreover, expression of our reporter constructs in sir3 sas2 cells is identical to that in sir3 cells, indicating that loss of SAS-1 acetylase activity does not generally affect transcriptional activity of our reporters. An alternative explanation is that the diminished local concentration of silencer proteins due to spreading of silencing to other regions would yield fully persistent but porous silent chromatin, i.e., heterochromatin with holes in it. In this latter context, we note that several histone deposition mutants—cac1 and asf1—also exhibit intermediate silencing phenotypes, which might be explained in the same manner and might account for the unusual phenotype response in these mutants (Enomoto and Berman, 1998; Meijsing and Ehrenhofer-Murray, 2001). In either event, our observations clearly demonstrate that intermediate silencing phenotypes are not always the result of distinct epigenetic states in the population but that partially active heterochromatin states can exist.

Onset of Silencing Proceeds in a Stochastic Manner
Miller and Nasmyth (1984) demonstrated that establishment of silencing requires progression through the cell cycle. However, Katan-Khaykovich and Struhl (2005) have provided evidence that establishment of full repression requires several generations of growth, although without resolving whether this reflects a stochastic process in an all-or-none formation of silent chromatin at each generation or the need for a succession of intermediate steps accomplished over several generations. The fact noted above that intermediate stages of silencing can exist lends weight to the latter hypothesis. Our results demonstrate that establishment of silencing is clearly stochastic, such that cells at the
same stage of growth undergo a different number of cell divisions before acquiring a repressed state. Even different progeny from a single cell acquire the repressed state after different numbers of cell divisions. Thus, once the cell has become permissive for silencing, it has some likelihood, but not an absolute certainty, of silencing the target locus in the next generation. These results suggest caution in interpreting prior and future experiments examining chromatin changes as a function of cell cycle leading to establishment of silencing, since all the cells do not necessarily act in concert.

Experimental Procedures

Strains
Strains and strain construction are described in Supplemental Data. Standard molecular biology techniques, yeast methods, media, and plasmid transformation were used.

Single-Cell Imaging and Analysis
Cells were grown overnight in synthetic complete (SC) medium to early logarithmic phase at room temperature. One to two milliliters of culture was spun down, resuspended in small volume of fresh medium. About 15 µl of cells were spotted onto a 24 × 60 mm cover slip (Corning), and a piece of agar from SC plates was placed on top. The agar was sealed with silicon gel to reduce evaporation. Cells were imaged on a DeltaVision restoration microscope workstation (Applied Precision) based on an inverted microscope (Eclipse TE600; Nikon), using an oil-immersed objective at 100× magnification and a CCD camera (CoolSNAP HQ; Roper Scientific). To detect YFP, CFP, GFP, or mCherry fluorescence, filter sets from Chroma Technologies Corp. were used. The images were acquired from six (haploid cells) or eight (diploid cells) 1 µm z stacks, deconvolved, and compressed into a single projection using the image software SoftWoRx (Applied Precision). Before each experiment, the microscope camera was calibrated using the calibration slide provided with the DeltaVision workstation. The optimum parameters for measuring fluorescent intensities were determined and normalized with sir3Δ cells included in all experiments and then applied to all other images.

The fluorescent intensity of the individual cells was obtained (raw cell intensity data) from the output of SoftWoRx. Linear regression (ANOVA) models were used to adjust the raw cell intensity data to remove background variations across images and strains (test strain, positive control [sir3Δ], and negative control [wt]). Normal mixture models were used in the analysis of the adjusted cell intensity data to detect bimodality. See Supplemental Data for more details.

Time-lapse imaging was performed on a DeltaVision epifluorescence microscope (Applied Precision, Issaquah, WA) with a 100× oil immersion lens, taking z stack sections every hour. The resulting images were deconvolved and projected to 2D. To calculate switching rates, the total numbers of phenotypic switches of each class (from HML repressed to HML derepressed, e.g.) were determined over the course of an experiment, which provided the numerators for rate calculation. Simultaneous switches of closely related cells were scored as a single switch that exhibited phenotypic lag. Since the expression states of the population are in equilibrium in these cultures, we could determine the number of switching opportunities—the denominator in the rate calculation—as the total number of cells of a particular class (HML repressed, e.g.) present at the end of the experiment minus the number of such cells at the beginning of the experiment.

For photobleaching experiments, 2 stack sections were taken as indicated, and cells were then exposed for 100 s to 500 nm light, which was sufficient to reduce YFP fluorescence to background levels (data not shown).

FACS Analysis and Cell Sorting
Cells were grown overnight in SC medium to early logarithmic phase at room temperature. About 3 × 10⁶ cells were harvested by centrifugation, washed in 10 mM Tris buffer twice, and resuspended in 1 ml 10 mM Tris buffer on ice for further FACS analysis or cell sorting. FACS analysis for YFP or GFP fluorescence was performed in a four-color, single laser FACScan machine (BD Biosciences). Data acquisition and analysis were performed using the CellQuest software (BD Biosciences).

The cells from strain Y3430 were sorted based on the GFP fluorescent intensity in a FACS Vantage SE machine (BD Biosciences). Viable cells were gated by their forward and side scatter characteristics, and gates were set to sort top 10% GFP-positive and bottom 10% GFP-negative cell populations. Around 10⁵ cells were collected for each population of cells. Sorted cells were concentrated to 10⁶ cells/ml in fresh SC medium at time point T0. About 15,000 cells were analyzed by FACS at each time point, and the culture density at each time point was determined by counting the number of cells in a hemacytometer. Data were extracted for analysis using FlowJo software (TreeStar, San Carlos, California). In the time-course analysis of repressed cells becoming derepressed, the percentage of repressed cells in each sample was estimated by fitting the FACS data to a bimodal distribution of two normal populations using maximum likelihood estimates as described in Supplemental Data.

Supplemental Data
Supplemental Data include supplemental text, one figure, one table, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/23/2/219/DC1/.

Acknowledgments
We would like to thank Trisha Davis, Sean Clark, Patricia Melloy, David Rivier, Judith Berman, and Roger Tsien for strains and plasmids. We also thank Dr. Minge Xie for invaluable assistance in the statistical analysis of our data. Dr. Peter Houston for instruction and assistance with microscopy, and Christina DeCoste for extensive support in FACS sorting and analysis. This work was supported by grant GM 48540 from the National Institutes of Health.

Received: November 15, 2005
Revised: March 23, 2006
Accepted: May 16, 2006
Published: July 20, 2006

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