Cell, Vol. 86, 667–677, August 23, 1996, Copyright ©1996 by Cell Press

A Deubiquitinating Enzyme Interacts with SIR4 and Regulates Silencing in S. cerevisiae

Danesh Moazed and Alexander D. Johnson
Department of Microbiology
University of California
San Francisco, California 94143

Summary

The SIR2, SIR3, and SIR4 proteins are required for silencing of transcription at the silent mating type loci and at telomeres in yeast. Using protein affinity chromatography, we show that SIR2, SIR3, and two proteins of 69 and 110 kDa tightly associate with SIR4. Surprisingly, the 110 kDa SIR4-binding protein is identical to UBP3, one of several previously described yeast enzymes that deubiquitinate target proteins. Deletion of the *UBP3* gene results in markedly improved silencing of genes inserted either near a telomere or at one of the silent mating type loci, indicating that UBP3 is an inhibitor of silencing. We discuss possible roles for UBP3 in controlling the activity or assembly of the SIR protein complex.

Introduction

Eukaryotic chromosomes typically contain large transcriptionally inactive domains of chromatin. These domains, which are often located adjacent to centromeres and telomeres, are called heterochromatin. Accumulating evidence from a wide variety of organisms ranging from human to yeast suggests that these specialized chromatin structures play a role both in chromosome stability and in the regulation of gene expression (Laurenson and Rine, 1992; Karpen, 1994). For example, during development of somatic cells of female mammals, one of the two X chromosomes is condensed into heterochromatin and transcriptionally inactivated (Grant and Chapman, 1988). In Drosophila melanogaster and Schizosaccharomyces pombe, large centromeric regions are heterochromatic, and in both organisms, mutations that disrupt heterochromatin result in decreased chromosome stability (Allshire et al., 1995; Kellum and Alberts, 1995). Moreover, position effects on gene expression are often observed when DNA rearrangements bring genes close to heterochromatin (Spofford, 1976). These rearrangements often result in transcriptional inactivation of genes juxtaposed to heterochromatin. The structural constituents of heterochromatin and the mechanisms that regulate its formation and propagation are poorly understood.

Studies of cell-type control in the budding yeast, Saccharomyces cerevisiae, have provided a link between regulation of cell type and heterochromatin in an organism that is amenable to both genetic and biochemical approaches. In S. cerevisiae, mating type genes located at *MAT* are transcribed and determine the mating type of the cells (for reviews, see Herskowitz et al., 1992; Johnson, 1995). Identical copies of the mating type genes (including their promoters) are located at two other loci, *HML* and *HMR*, also known as the silent loci; however, these copies are not transcribed. This position effect on gene expression, which in yeast is referred to as silencing, shares many properties with heterochromatic inactivation of gene expression in higher eukaryotes. Genetic studies have identified a number of *trans*-acting factors that are necessary for silencing including the products of the *SIR1*, *SIR2*, *SIR3*, and *SIR4* genes (Rine and Herskowitz, 1987). *SIR1* is only required for the establishment of silencing at the *HML* and *HMR* loci, whereas *SIR2*, *SIR3*, and *SIR4* are required for both the establishment and maintenance of silencing (Pillus and Rine, 1989). NAT1 and ARD1, subunits of a heterodimeric N-terminal acetyl transferase, are also required for silencing, but play a more important role at *HML*, since little or no derepression of *HMR* is observed in *nat1ard1* double mutants (Mullen et al., 1989; Whiteway et al., 1987). In addition, the highly conserved N-termini of histones H3 and H4 are essential for silencing and have been shown to interact with the SIR3 and SIR4 proteins (Kayne et al., 1988; Johnson et al., 1990; Thompson et al., 1994; Hecht et al., 1995), providing convincing evidence for a direct role of chromatin structure in silencing.

In yeast as in other eukaryotes, transcription of genes inserted near the telomeres is repressed (Gottschling et al., 1990). With the exception of *SIR1*, telomeric silencing requires all of the gene products that are necessary for silencing *HML* and *HMR*, suggesting that similar mechanisms are responsible for repression at all these locations (Aparicio et al., 1991).

Silencing is directed by specific *cis*-acting sequence elements; at the *HM* loci these sequences are termed silencers (Brand et al., 1985, 1987). Analysis of these elements has identified additional proteins that are involved in silencing. Each silencer is composed of three distinct elements, two of which are bound by the abundant nuclear proteins RAP1 and ABF1 (Shore and Nasmyth, 1987; Halfter et al., 1989; Rhode et al., 1989). The third element which is a consensus autonomously replicating sequence (ARS) binds to a multisubunit complex called the origin recognition complex (ORC; Bell and Stillman, 1992). Genetic evidence for the involvement of these DNA-binding proteins in silencing has been obtained by the isolation of mutations in *RAP1*, *ABF1*, and ORC subunits that disrupt silencing (Kurtz and Shore, 1991; Foss et al., 1993; Loo et al., 1995). In addition, the RAP1 protein has been shown to interact with SIR3 (Moretti et al., 1994), and in a similar fashion, the other silencer binding proteins are thought to participate in recruiting the SIR proteins to the silencer (Laurenson and Rine, 1992). RAP1 is also thought to recruit the SIR proteins to telomeres since telomeric repeat sequences in S. cerevisiae are composed of RAP1 binding sites (Buchman et al., 1988; Longtine et al., 1989). How the SIR proteins repress transcription once recruited to the silencer and what determines the size of the repressed domain are unknown.

In contrast to some of the other proteins that function in silencing, the SIR proteins are specific to silencing

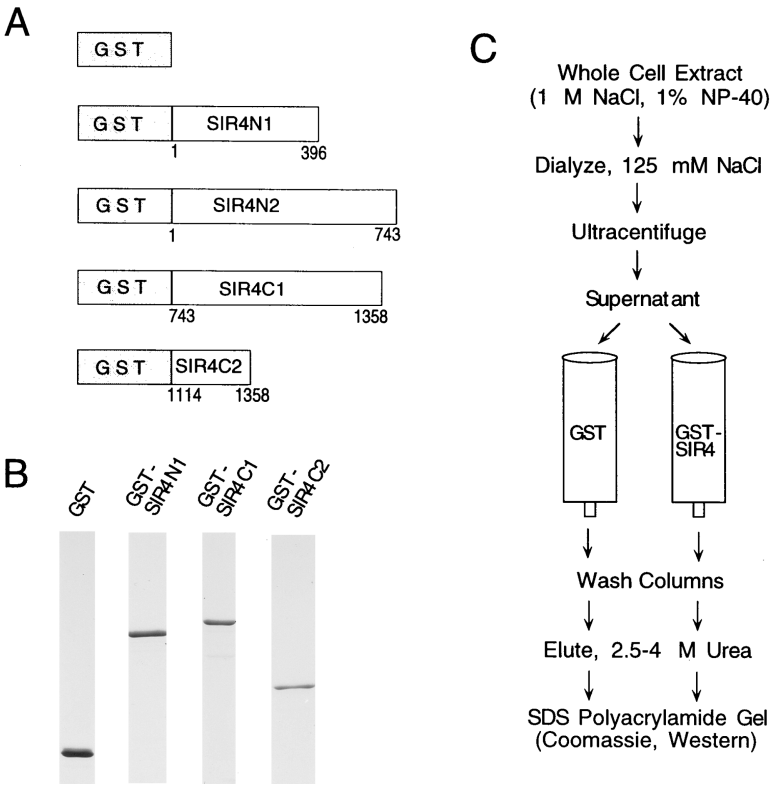

Figure 1. Strategy for Affinity Purification of SIR4-Interacting Proteins

(A) The GST-SIR4 fusion proteins used in the studies described here.

(B) Coomassie-stained SDS polyacrylamide gel of ~1 μg of purified GST fusions.

(C) Scheme for the affinity purification experiments.

and are absolutely required for this process, yet little is known about their biochemical properties and their interactions with other proteins. In order to identify structural or regulatory components that act together with the SIR proteins, we have undertaken a biochemical approach to the study of silencing and used protein affinity chromatography to identify proteins that interact with the SIR products. Here we report the results of experiments that utilize a SIR4 affinity column to detect interactions between the SIR4 protein and other proteins in yeast. We found that silencing proteins SIR2 and SIR3, in addition to two other proteins of 69 and 110 kDa, tightly bound to an affinity column containing the C-terminal half of SIR4. We have identified the 110 kDa protein as UBP3 (Baker et al., 1992), a member of a large family of yeast enzymes involved in removing ubiquitin from proteins. Deletion of the *UBP3* gene resulted in a marked increase in silencing at the telomeres and the *HML* locus, suggesting that UBP3 is an inhibitor of silencing. We propose that UBP3 regulates silencing by controlling either the activity or the assembly of the SIR protein complex.

Results

We used protein affinity chromatography to identify proteins in yeast extracts that specifically bind to SIR4. N- and C-terminal domains of the SIR4 protein were fused to Glutathione S-Transferase (GST-SIR4N1, GST-SIR4N2, GST-SIR4C1, and GST-SIR4C2, Figures 1A and 1B), expressed in E. coli, and purified using glutathione-agarose. Truncations of the SIR4 protein were used because full-length SIR4 was unstable in E. coli and could not be purified. Each purified protein was cross-linked to an activated resin and used to select specific proteins from a yeast extract by affinity chromatography as illustrated in Figure 1C. The extract used in these studies was prepared by lysis of yeast cells in a buffer containing 1 M NaCl and 1% Triton-X 100 or NP-40, conditions that are required for optimal solubilization of the SIR proteins (D. M., unpublished data). This extraction buffer is also likely to solubilize any SIR-interacting protein(s). In order to promote protein–protein interactions during chromatography, the salt concentration in the extract was reduced to 125 mM by dialysis prior to loading the extract on columns. The extract was loaded on a GST-SIR4 affinity column and a GST control column run in parallel. Each column was then washed extensively (40 column volumes), and the bound protein was eluted using 2.5–4 M urea. Preliminary experiments showed that a specific set of proteins eluted from the GST-SIR4C1 (C-terminal 615 amino acids of SIR4 fused to GST) affinity column when compared to columns containing either GST alone, GST-SIR2, GST-SIR4N1, or GST-SIR4N2 (N-terminal 396 or 743 amino acids of SIR4 fused to GST, respectively), and the results from the GST-SIR4C1 column were pursued.

SIR2, SIR3, and Two Proteins of 69 and 110 kDa Bind to an Affinity Column Containing the C-terminal Half of SIR4

Figure 2A shows a Coomassie-stained polyacrylamide gel of proteins that eluted at 2.5 M urea from the GST-SIR4C1 column compared with those that eluted under the same conditions from a control GST-SIR2 column. Two successive peak elution fractions for each column

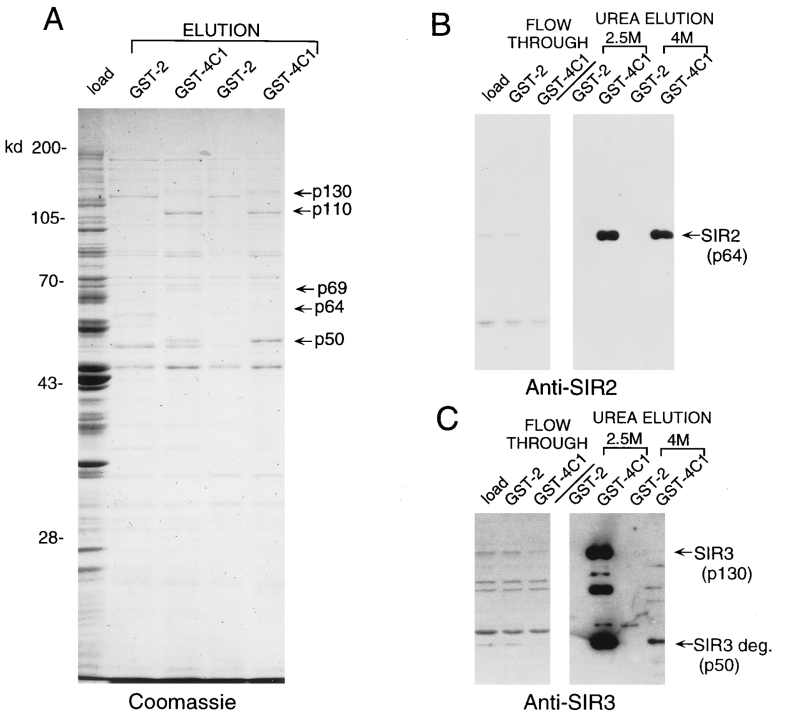

Figure 2. Affinity Purification of Yeast Proteins on a GST-SIR4 Column Containing the C-terminal 615 Amino Acids of SIR4

(A) Coomassie-stained gel of the load and two successive 2.5 M urea elution fractions from each GST-SIR2 (GST-2) and GST-SIR4C1 (GST-4C1) columns; elution fractions 3 and 4 (out of a total of 10) for each column are shown. Arrows point to proteins that specifically elute from the GST-SIR4C1 column. Position of molecular weight markers is indicated on the far left.

(B and C) Western blots of load, flowthrough, and elution fractions, probed with anti-SIR2 (B) and anti-SIR3 (C) antibodies, showing the specific binding and elution of the respective proteins from the GST-SIR4C1 column (lanes labeled GST-4C1).

are shown. Five proteins of 130, 110, 69, 64, and 50 kDa specifically eluted from the GST-SIR4C1 column (designated p130, p110, p69, p64, and p50, respectively, Figure 2A).

The relative migration of p130 and p64 in SDS polyacrylamide gels appeared very close to that expected for SIR3 and SIR2, suggesting that p130 and p64 might correspond to SIR3 and SIR2. We therefore probed Western blots of samples from the load, flowthrough, and elution fractions of this experiment with anti-SIR2 and anti-SIR3 antibodies. The Western results showed that both SIR2 and SIR3 proteins specifically eluted from the GST-SIR4C1 column at 2.5 M urea (Figures 2B and 2C). In addition, both proteins were nearly completely depleted from the flowthrough of the GST-SIR4C1 column (Figures 2B and 2C). To confirm the identification of SIR2 and SIR3, a blot was successively probed with anti-SIR2 and anti-SIR3 antibodies to visualize SIR2 and SIR3, respectively, and then stained with Coomassie to visualize total protein. This experiment showed that p130 and p64 precisely overlapped the SIR3 and SIR2 signals, respectively (data not shown). Consistent with these results, p64 and SIR2 displayed very similar elution profiles from the GST-SIR4C1 column. Both proteins elute inefficiently at 2.5 M urea as broad peaks and continue to elute from the column at 4 M urea (Figure 2B). In addition to the 130 kDa band, the SIR3 antibody recognized a 50 kDa band in both the crude extract and the elution fraction of the GST-SIR4C1 column. This 50 kDa band appears to be a degradation product of SIR3 because it was absent in whole cell extracts that are prepared by rapid lysis of yeast cells in SDS sample buffer (data not shown). The 50 kDa band precisely overlapped the p50 protein, suggesting that p50 was a degradation product of SIR3. This conclusion is consistent with the variability in the ratio of p130 and p50 in different experiments and with the parallel behavior of the SIR3 protein on Western blots in these experiments.

Moretti et al. (1994) had previously demonstrated a SIR3-SIR4 interaction using the two-hybrid method. The elution of SIR2 and SIR3 from the GST-SIR4 affinity column observed here provides evidence that both SIR2 and SIR3 interact with SIR4, either directly or through only a small number of additional proteins.

SIR3, p110, and p69 also bound to an affinity column composed of the C-terminal 244 amino acids of SIR4 fused to GST (GST-SIR4C2, Figure 3A). No detectable SIR2 bound to this smaller GST-SIR4 column, but nearly all of the SIR3 protein in the load was depleted from the flowthrough of this column (data not shown). However, SIR3 eluted very inefficiently from the GST-SIR4C2 column using 2.5 M urea, and was barely detectable in the 2.5 M urea elution fraction by staining with Coomassie (Figure 3A).

Identification of the 110 kDa SIR4-Binding Protein as UBP3

The GST-SIR4C2 column provided a higher yield of the p110 and p69 proteins than did the GST-SIR4C1 column (Figures 2A and 3A). The eluate from the GST-SIR4C2 column was therefore used to further characterize the p110 protein. Following electrophoresis and transfer to blotting membrane, the p110 band was excised and used to obtain peptide sequence data (courtesy of C. Turk, S. Zhou, and R. Tjian). Two peptide sequences were obtained for the p110 protein purified on the GST-SIR4C2 column; a third and shorter peptide sequence was obtained using the p110 band purified on the larger GST-SIR4 column (GST-SIR4C1). A search of the protein data bases revealed perfect matches between all three

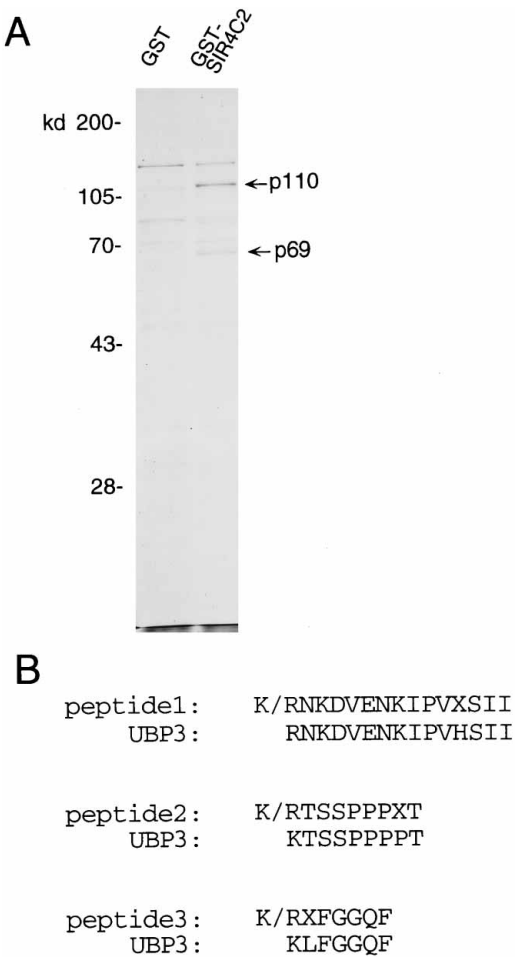

Figure 3. Binding of p110 and p69 to a GST-SIR4 Column Containing the Extreme C-terminus of SIR4 and the Identification of p110 as UBP3

(A) Coomassie-stained SDS polyacrylamide gel of 2.5 M urea elution fraction from affinity columns bearing either GST or the C-terminal 244 amino acids of SIR4 fused to GST.

(B) Comparison of protein microsequence data from the p110 protein band with the UBP3 amino acid sequence (Baker et al., 1992). Amino acid sequence is indicated using the single letter code. K/R, inferred from the known position of cleavage by trypsin; X denotes ambiguous positions in peptide sequences.

peptides and a single protein, UBP3 (Figure 3B), indicating that p110 is UBP3. The calculated molecular weight of UBP3 is 101,916 daltons, consistent with its migration as a 110 kDa protein in SDS polyacrylamide gels. We confirmed the interaction of UBP3 with SIR4 observed in the affinity column experiments (Figures 2 and 3) by showing that an HA-tagged UBP3 isolated from yeast is associated with GST-SIR4C2 but not with GST (data not shown). UBP3 is a member of a large family of yeast enzymes that are involved in removing ubiquitin from other proteins (Baker et al., 1992). Ubiquitin usually marks proteins for destruction by the proteasome but is also believed to play regulatory roles distinct from protein degradation (Varshavsky et al., 1987; Finley et al., 1989). The identification of UBP3 as a SIR4-binding protein implicates ubiquitin in silencing.

Silencing in *ubp3* Mutant Strains

We next carried out experiments to test the possible role of *UBP3* in silencing. UBP3 is one of three ubiquitin processing enzymes that were originally isolated based on their ability to cleave a ubiquitin-β-galactosidase fusion protein produced in E. coli (Baker et al., 1992). Other experiments and information obtained from sequence comparisons with the yeast genome indicate the existence of at least seventeen UBPs in yeast (Papa and Hochstrasser, 1993). *ubp3* mutant strains (obtained by replacement of the *UBP3* coding region with *HIS3*) have a slight growth defect, but no detectable defect in their ability to deubiquitinate a test substrate composed of ubiquitin-β-galactosidase (Baker et al., 1992). The lack of a significant defect in the deubiquitinating activity in *ubp3* cells may be due to redundancy in the functions of deubiquitinating enzymes, but is also consistent with a specific role for UBP3 that does not involve general protein degradation.

As discussed in the introduction, the SIR proteins are required for silencing at both telomeres and the *HM* loci (Aparicio et al., 1991). We tested the effect of a *UBP3* deletion on silencing of a *URA3* gene inserted near a telomere (Gottschling et al., 1990; Aparicio et al., 1991). The degree of repression can be measured by assessing growth on media lacking uracil (−URA) or containing 5-fluoro-orotic acid (5-FOA). The product of the *URA3* gene converts 5-FOA into a lethal substance. Repression of *URA3* results in better growth on 5-FOA plates and poor growth on −URA plates, while expression of *URA3* produces the opposite result. We deleted most of the *UBP3* coding sequence in strains which contain the *URA3* gene either near a telomere on the left arm of chromosome VII, UCC1001, or at an internal position within the *ADH4* locus, UCC1003 (Aparicio et al., 1991), to generate strains DM237 (*URA3-TEL*, *ubp3Δ::HIS3*) and DM239 (*URA3*-Internal, *ubp3Δ::HIS3*), respectively, and measured the ability of these strains to grow on complete, 5-FOA, and −URA media. Deletion of *UBP3* resulted in about 100 times less growth on −URA plates and slightly better growth on 5-FOA plates when the *URA3* gene was located near the telomere (Figure 4A, left panels), indicating that silencing is more effective in the *ubp3Δ::HIS3* strain. As a control, no growth defect was observed on −URA plates when *URA3* was located at an internal chromosomal positon (Figure 4A, right panels), indicating that this effect depends on the position of the *URA3* gene at the telomere and is not caused by a general defect in the ability of the *ubp3Δ::HIS3* strain to grow on −URA medium. These results indicate that telomeric silencing is markedly increased in the *ubp3* deletion strain and support a role for UBP3 in the regulation of silencing as first suggested by its interaction with SIR4.

To demonstrate that the increased telomeric repression observed in the *ubp3Δ::HIS3* strain is not specific to *URA3*, we showed that deletion of *UBP3* also decreased the expression of the *TRP1* gene when inserted near telomere VIIL (Figure 4B) (Gottschling et al., 1990). As a control we also tested the effect of deleting the *SIR2* gene on telomeric repression of *TRP1*. Telomeric repression of *TRP1* results in poor growth of cells on −TRP media. As previously reported (Aparicio et al.,

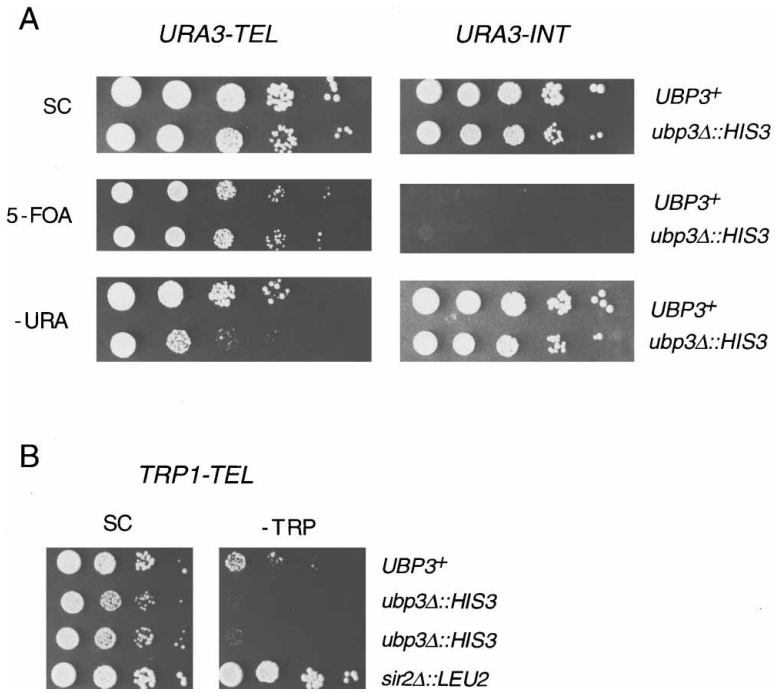

Figure 4. Increased Telomeric Silencing in *ubp3* Mutant Cells

(A) Silencing of a telomere proximal *URA3* gene (*URA3-TEL*) in *UBP3*$^+$ (UCC1001) and a *ubp3Δ::HIS3* strain (DM237); panels on the right (*URA3-INT*) show that deletion of *UBP3* has no effect on the expression of *URA3* at an internal chromosomal position. Ten-fold serial dilutions of cultures from each strain were plated on synthetic complete (SC), 5-FOA, and −URA plates.

(B) Silencing of a telomere proximal *TRP1* gene (*TRP1-TEL*) in *UBP3*$^+$ (JRY 4469), *ubp3Δ::HIS3* (DM313), and *sir2Δ::LEU2* (JRY 4470) strains. Ten-fold serial dilutions of cultures were plated on SC and −TRP plates.

1991), deletion of *SIR2* resulted in improved growth on −TRP plates due to loss of silencing. On the other hand, deletion of *UBP3* resulted in a near complete loss of growth on −TRP plates (Figure 4B). We also tested the effect of deleting *UBP3* on silencing of a telomeric *ADE2* gene in a colony color assay and found that deletion of *UBP3* increased the repression of a telomere proximal *ADE2* gene (data not shown). Therefore, the effect of the *UBP3* deletion on telomeric repression is not gene specific.

Since the *SIR* genes are also required for silencing at *HML* and *HMR*, it would be expected that a deletion of *UBP3* would also affect silencing at these loci. *Decreased* silencing at *HML* and *HMR* results in a mating defect that is caused by the expression of both **a** and α mating type gene products and is simple to detect by a mating assay (Sprague, 1991). However, in wild-type strains the *HM* loci are very efficiently repressed, and an increase in repression would not be predicted to have an effect on mating. In fact, we found that deletion of *UBP3* had no significant effect on mating efficiency (D. M., unpublished data). To test whether mutations in *UBP3* also increase silencing at the *HM* loci, we measured the effect of deleting *UBP3* in a strain where the *URA3* gene has been inserted at *HML* and is under the control of the *HML* silencers (UCC3515; Singer and Gottschling, 1994). Deletion of *UBP3* resulted in 100- to 1000-fold reduced growth on −URA plates when the *URA3* gene was located at *HML* (Figure 5). Therefore, UBP3 is a negative regulator of silencing at both the telomeres and the *HML* locus.

Levels of the SIR2, SIR3, and SIR4 Proteins in *ubp3* Mutant Strains

Silencing is sensitive to the dosages of the SIR3 and SIR4 proteins. Overexpression of SIR3 results in more efficient telomeric silencing (Renauld et al., 1993), whereas overexpression of SIR4 or its C-terminal domain disrupts silencing (Ivy et al., 1986; Marshall et al., 1987). It is possible that the improvement in silencing in *ubp3* strains could be due to changes in the levels of the SIR proteins. In order to test this idea, we analyzed the levels of the SIR2, SIR3, and SIR4 proteins in extracts prepared from wild-type (JRY2334), *sir2Δ::HIS3* (JRY 3433), *sir3Δ::TRP1* (JRY3289), *SIR4Δ::HIS3* (JRY3411), and *ubp3Δ::HIS3* (DMY228, *UBP3* disrupted in JRY2334) strains by Western analysis. No significant change was observed in the levels of SIR3 and SIR4 in the *ubp3Δ::HIS3* strain compared to its isogenic parent strain; but the levels of the SIR2 protein were reduced in the mutant strain (data not shown). We note that the reduced levels of SIR2 do not provide a straightforward explanation

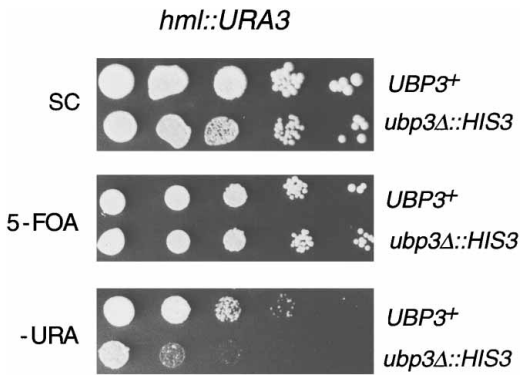

Figure 5. Deletion of the *UBP3* Gene Results in Increased Silencing of *URA3* Inserted at *HML*

Silencing of *URA3* inserted at the *HML* locus in a *UBP3*$^+$ (UCC3515) and a *ubp3Δ::HIS3* (DM298) strain. Ten-fold serial dilutions of cultures from each strain were plated on synthetic complete (SC), 5-FOA, and −URA plates.

for the increased silencing observed in *ubp3* deletion strains. It is unlikely that deletion of *UBP3* influences silencing by modulating the steady-state levels of the SIR2, SIR3, or SIR4 proteins.

Discussion

We have used protein affinity chromatography to identify proteins that physically interact with the yeast silencing protein SIR4. One of the SIR4 binding proteins was identified as UBP3, a yeast protein previously shown to be a deubiquitining enzyme (Baker et al., 1992). In cells with a deletion of *UBP3*, the efficiency of silencing at both the telomeres and the *HML* locus is greatly increased, suggesting that *UBP3* opposes (negatively regulates) silencing. This finding suggests that ubiquitin itself plays a positive role in silencing. Below we discuss possible mechanisms for the regulation of silencing by ubiquitin and UBP3.

Our results also provide evidence for the physical association of the SIR proteins. Based on genetic interactions between certain alleles of the *SIR2*, *SIR3*, and *SIR4* genes, Rine and Herskowitz (1987) proposed that the SIR proteins are components of a multiprotein complex. Two-hybrid studies have demonstrated that the SIR3 and SIR4 proteins interact, but provided no evidence for the interaction of SIR2 with either SIR3 or SIR4 (Moretti et al., 1994). Because of the unique role of SIR2 in reducing recombination levels at ribosomal DNA repeats and because of its role in promoting general histone deacetylation in yeast (Gottlieb and Esposito, 1989; Braunstein et al., 1993), there was some question as to whether SIR2 is part of the same complex as SIR3 and SIR4. We have identified SIR2 and SIR3 as two of the four major proteins that elute from the GST-SIR4C1 column, providing evidence that SIR2, as well as SIR3, is associated with SIR4.

The extreme C-terminus of SIR4, used as an affinity probe in the experiments described here, is a functionally important domain of the protein. Overexpression of this domain disrupts silencing, and this phenotype is suppressed by overexpression of SIR3 (Ivy et al., 1986; Marshall et al., 1987). Deletion of the C-terminal 167 amino acids of SIR4 results in a loss of silencing at the *HM* loci and telomeres and promotes an increase in life span (Kennedy et al., 1995). In addition, this domain has the potential to form an extended coiled-coil and bears weak similarity to the mammalian nuclear lamins (Diffley and Stillman, 1989). We have shown that this same region of SIR4 (the C-terminal 244 amino acids) is sufficient for its interaction with SIR3, as well as two other proteins, UBP3 and an unknown 69 kDa protein. The interactions we observed are summarized in Figure 6.

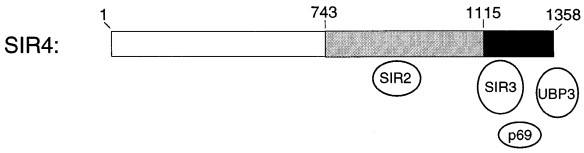

Figure 6. Summary of Protein Affinity Column Results

SIR3, UBP3, and p69 bind to the extreme C-terminus of SIR4 (shaded black, amino acids 1115–1358). Overexpression of this domain of SIR4 disrupts silencing (Ivy et al., 1986; Marshall et al., 1987), and its deletion results in longer life span (Kennedy et al., 1995). Binding of SIR2 to SIR4 requires the larger C-terminal half of SIR4 (shaded gray and black, amino acids 743–1358).

Negative Regulation of Silencing by *UBP3*

Gene silencing is a stochastic phenomenon that gives rise to clonal populations of cells where the expression of silencer proximal genes is either on or off. In yeast, this epigenetic aspect of silencing was first discovered in *sir1* mutant cells in which defects in the establishment of silencing at the *HM* loci give rise to mixed but stable populations of cells (Pillus and Rine, 1989). Similar to the situation in *sir1* mutant cells, telomeric silencing produces a mixed population of cells where the expression of telomere proximal genes is either on or off (Sandell and Zakian, 1992). The mechanisms that control the frequency or the extent of inactivation are unknown, although overexpression of the SIR3 protein has been shown to increase the efficiency and extent of spreading of silencing from a yeast telomere (Renauld et al., 1993). On the other hand, silencing can be relieved at particular times during the yeast life cycle by an unknown mechanism(s). For example, cell age may contribute to a breakdown of silencing at the *HM* loci (Smeal et al., 1996), and repression from yeast silencers at novel locations is sensitive to environmental conditions such as the carbon source in the growth media (Shei and Broach, 1995). In the latter case, derepression occurs when cells are switched from media containing a fermentable carbon source to media containing a nonfermentable carbon source (Shei and Broach, 1995). Negative regulation of silencing by UBP3 provides a possible mechanism for the reversal of silencing in these instances.

Furthermore, silenced chromatin domains may need to be disassembled during the S phase of the cell cycle in order for replication and repair enzymes to gain access to DNA. For example, most Drosophila somatic tissues are composed of cells with polytene nuclei that form as a result of altered cell cycles in which many rounds of DNA replication occur without cytokinesis (Spradling and Orr-Weaver, 1987). Heterochromatic DNA in the polytene nuclei is as much as 1000-fold underreplicated (Gall et al., 1971), suggesting that heterochromatin limits access to the replication machinery. A mechanism may therefore exist to disassemble heterochromatin to allow DNA replication. In yeast, UBP3 may be involved in such a disassembly process.

Possible Mechanisms of UBP3 Action

The observation that silencing is improved in cells lacking UBP3 suggests that ubiquitin itself, the substrate for UBP3 and other ubiquitin hydrolases, is a positive regulator of silencing. Two general models for the involvement of ubiquitin and UBP3 in the regulation of silencing can be formulated (Figure 7). Ubiquitin is a highly conserved 76 amino acid polypeptide whose best known function is in marking proteins for destruction by the proteasome (Hershko, 1988). Ubiquitination could in principle be involved in regulating silencing by promoting the degradation of an inhibitor of silencing. According to this model (Figure 7A), UBP3 would negatively regulate silencing by removing the ubiquitin moiety from

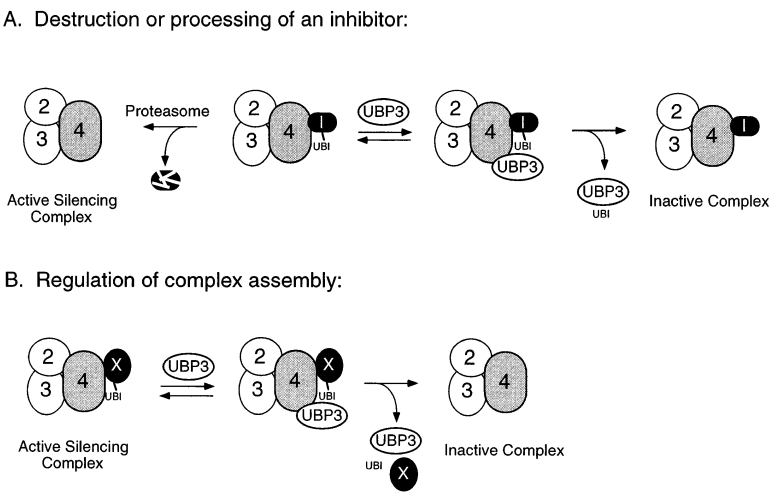

Figure 7. Two Possible Mechanisms That Can Account for the Increased Levels of Silencing Observed in *ubp3* Mutant Strains

(A) UBP3 stabilizes an inactive SIR protein complex by binding to SIR4 and deubiquitinating an inhibitor (I, black oval) of the complex. Activation of the complex requires the destruction or processing of I by the proteasome pathway.

(B) Alternatively, ubiquitination of a component of the SIR protein complex could be essential for the activity of this complex (X, black oval). Deubiquitination of X results in its inactivation or dissociation from the complex. UBI, ubiquitin.

this inhibitor and thereby stabilizing the inhibitor. Examples of this kind of regulation by the ubiquitin-proteasome pathway occur in two other systems: p65, an inhibitor of the NF-κB transcriptional regulator, and p27, an inhibitor of cell cycle progression, are both ubiquitinated and destroyed by the proteasome (Palombella et al., 1994; Pagano et al., 1995). A variant of the model in Figure 7A is that an inhibitory domain in one of the SIR proteins or another silencing protein has to be removed to activate the protein, as is also the case in the regulation of NF-κB activity. NF-κB is synthesized as an inactive 105 kDa precursor protein; following phosphorylation and ubiquitination, the precursor protein is processed by the proteasome to generate an active 50 kDa protein that is competent for entry into the nucleus and DNA binding (Fan and Maniatis, 1991; Palombella et al., 1994). The activity of NF-κB is therefore regulated both by the destruction of an inhibitory subunit (p65, mentioned above) and by the destruction of an inhibitory domain in a precursor protein by the ubiquitin-proteasome pathway.

In contrast to its role in targeting proteins for degradation or processing, ubiquitin also appears to promote macromolecular assembly and function, and a second model for the role of ubiquitin in silencing is based on this idea (Figure 7B). UBP3 might inhibit silencing by removing a ubiquitin moiety that is involved in the assembly or function of a SIR protein complex (Figure 7B). While UBP3 physically interacts with SIR4, it could act (remove ubiquitin) on SIR4 itself or any other protein that interacts with SIR4. These include SIR2, SIR3, and the p69 protein identified here, and any of the four core histones, since SIR4 interacts with the N-termini of both histones H3 and H4. With regard to histones, we note that histones H2A and H2B are two of the major ubiquitinated proteins in mammalian cells, but attempts to detect ubiquitinated H2A and H2B in S. cerevisiae have been unsuccessful (Swerdlow et al., 1990). An example of ubiquitin functioning to regulate complex assembly occurs in ribosome biogenesis. Several yeast ribosomal proteins are synthesized as C-terminal extensions of ubiquitin (Finley et al., 1989). The N-terminal ubiquitin moiety is important for the proper assembly of 40S ribosomal subunits, as the synthesis of these proteins without the N-terminal ubiquitin extension results in the formation of defective ribosomal subunits. In other examples, the N-termini of the yeast DNA repair protein, RAD23 and its human homolog, xeroderma pigmentosum group C protein, are very similar to ubiquitin (Watkins et al., 1993; Masutani et al., 1994), and one of the subunits of the human transcription elongation factor SIII is a ubiquitin-like protein (Garrett et al., 1995); in neither case do these ubiquitin-like sequences appear to be involved in protein degradation. More recently, regulatory roles for ubiquitin in the endocytosis of the yeast pheromone receptor and in the activation of an IκBα kinase complex have been demonstrated (Chen et al., 1996; Hicke and Riezman, 1996).

Ubiquitin Processing Enzymes as a New Class of Biological Regulators

A large number of ubiquitin processing enzymes (UBPs) have been identified in S. cerevisiae. The current estimate for the total number of these enzymes based on sequence homologies in the publicly accessible sequenced portion of the yeast genome stands at 20 (~80% of the genome, Saccharomyces Genome Database). Therefore, members of this group of enzymes have the potential for regulating different cellular processes by acting on a number of different substrates. Recently, the Drosophila *faf* gene, which is required for determination of photoreceptor cell fate during eye development, was identified as a ubiquitin processing enzyme (Huang et al., 1995). In addition, Spierer and colleagues have isolated mutations in a ubiquitin processing enzyme that enhance position effect variegation in Drosophila (P. Spierer, personal communication). As silencing in both yeast and Drosophila is similarly enhanced by mutations in particular ubiquitin processing enzymes, the regulation of silencing by these enzymes appears to be an evolutionarily conserved process.

Experimental Procedures

Strains, Plasmids, and Antibodies

Protease deficient strains BJ2168, *MAT***a** *leu2 pep4–3 prb1–112 prc1–407 trp1 ura3–52 gal1* and BJ5459, *MAT***a** *ura3–52 trp1 lys2–801 leu2-Δ1 his3-Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL* (Jones, 1984) were used for preparing extracts for affinity chromatography experiments. A set of isogenic strains provided by J. Rine was used

to test the specificity of affinity purified SIR2, SIR3, and SIR4 antisera: JRY2334 (W3031a), *MAT***a** *ade2–1 can1–100 his3–11 leu2–3.112 trp1 ura3–1 GAL*; JRY3433, JRY2334 with *sir2*Δ*::HIS3*; JRY3289, JRY2334 with *sir3*Δ*::TRP1*, and JRY3411, JRY2334 with *sir4*Δ*::HIS3*. A *UBP3* deletion strain (YRB175) and its isogenic parent strain (YRB116) were provided by R. Baker (Baker et al., 1992). A *ubp3* deletion strain (DM228) was also constructed by replacing most of the *UBP3* coding region with *HIS3* in strain JRY2334 using pRB206 digested with SacI/SphI (Baker et al., 1992). The following strains from the Gottschling laboratory were used to measure telomeric or *HML* silencing (Aparicio and Gottschling, 1994; Singer and Gottschling, 1994). UCC1001, *MAT***a** *ura3–52 lys2–801amber ade2–101ochre trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 TELadh4::URA3*; UCC1003, *MAT***a** *ura3–52 lys2–801amber ade2–101ochre trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 adh4::URA3*, and UCC3515, *MAT***a** *lys2–801 trp1-*Δ*63 hml::URA3 ade2–101 his3-*Δ*200 leu2-*Δ*1 ura3–52*. The *UBP3* coding sequence was replaced by *HIS3* in strains UCC1001, UCC1003, and UCC3515 (Aparicio and Gottschling, 1994; Singer and Gottschling, 1994) to generate strains DM237, DM239, and DM298. Strains JRY4469 (*MAT***a** *ade2–1 his3–11 leu2–3,112 trp1–1 ura3*Δ*::LEU2 TEL-VII::URA3::TRP1*), JRY4470 (*MAT***a** *ade2–1 his3–11 leu2–3,112 trp1–1 ura3*Δ*::LEU2 sir2*Δ*::LEU2 TEL-VII::URA3::TRP1*) were a gift from Andrew Dillin and Jasper Rine. Strain DM313 was generated by replacement of *UBP3* with *HIS3* in strain JRY4469.

Antibodies that recognize the SIR2, SIR3, and SIR4 proteins were generous gifts from Amy Axelrod and Jasper Rine. A trpE-SIR2 fusion protein was used to produce both mouse monoclonal and rabbit polyclonal antibodies against the SIR2 protein. Rabbit antibodies that recognize the SIR3 protein were produced against a β-galactosidase-SIR3 fusion protein; anti-SIR4 antibodies were produced against a β-galactosidase-SIR4 fusion protein containing the C-terminal 615 amino acids of SIR4. The SIR2, SIR3, and SIR4 antisera were affinity purified on columns prepared by cross-linking GST-SIR2, GST-SIR3, and GST-SIR4 to AffiGel 10, respectively (D. M., A. D. J., A. Axelrod, and J. Rine, unpublished data). The specificity of each affinity purified antibody was confirmed by Western analysis of whole cell extracts from wild-type (JRY2334), *sir2*Δ*::HIS3* (JRY3433), *sir3*Δ*::TRP1* (JRY3289), and *sir4*Δ*::HIS3* (JRY3411) strains.

Expression and Purification of GST-SIR4 Proteins

GST-SIR4 fusion proteins were generated by in-frame ligation of *SIR4* coding sequences with GST in the pGEX series of expression plasmids (Smith and Johnson, 1988, Pharmacia). An EcoRI site was inserted after the ATG start codon of SIR4 using oligonucleotide-directed mutagenesis. An oligonucleotide was synthesized to insert an EcoRI site after the first ATG codon of SIR4. Using this and a second oligonucleotide complementary to an internal HindIII site in SIR4, a 534 bp fragment of SIR4 containing amino acids 2 to 177 was PCR (polymerase chain reaction) amplified and subcloned into the EcoRI–HindIII site of pRS316 (Sikorski and Hieter, 1989) to generate pDM116. This fragment was sequenced to show that it was free of PCR errors. A SIR4 HindIII fragment containing the remainder of the SIR4 coding sequence including the stop codon and 170 bp of the 3′ untranslated region was then subcloned into the HindIII site of pDM116 to produce pDM118, a plasmid containing the entire SIR4 coding sequence with an EcoRI site preceding the second codon. An EcoRI–XhoI fragment from pDM118 was inserted into the EcoRI–XhoI site of pGEX-2T-1 to generate pDM150 (GST-SIR4), and an EcoRI–PvuII fragment (SIR4 amino acids 2–396) from pDM118 was subcloned into pGEX-4T-1 digested with EcoRI and SmaI to produce pDM137 (GST-SIR4N1). pDM145 (GST-SIR4N2), SIR4 amino acids 2 to 743 fused to GST, was produced by ligation of a 2.2 kb SmaI fragment from pDM118 into the SmaI site of pGEX-3X. A SmaI–ClaI fragment of the SIR4 gene, encoding amino acids 743 to 1358 of SIR4 was blunt-ended and ligated into the SmaI site of pGEX-2T to yield pDM119 (GST-SIR4C1). pDM149 (GST-SIR4C2, SIR4 amino acids 1114 to 1358 fused to GST) was produced by ligation of a 1.6 kb PvuII fragment of SIR4 into the SmaI site of pGEX-4T-2.

DH5α cells containing the above expression plasmids were grown in 1.5× LB media to an A600 of 0.6–0.8 and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 1–4 hr at 30°C. The cells were harvested, washed with ice-cold phosphate-buffered saline, and stored at −20°C. All the following steps were performed at 4°C. Cell pellets (~10 g) were resuspended in 80 ml of lysis buffer (20 mM Tris–HCl [pH 8.0], 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine) containing 200 μg/ml of lysozyme. Following resuspension on ice, dithiothreitol (DTT) was added to 10 mM and Triton X-100 or NP-40 to 1%, and the NaCl concentration was raised to 350 mM (some of the GST-SIR4 fusion proteins precipitated at lower salt concentrations). The lysate was sonicated to reduce viscosity and centrifuged in a Ti60 rotor at 35,000 rpm for 1 hr. The supernatant was loaded onto a 5 ml glutathione agarose (Sigma) column or incubated with the resin in batch on a mixer for 1 hr. The column was washed with each of the following: 10 ml of lysis buffer; 100 ml 20 mM Tris–HCl (pH 8.0), 1 M NaCl, 1 mM DTT, 0.1% Tween-20; and then with 20 ml 20 mM HEPES–KOH (pH 7.6), 350 mM NaCl, 1 mM DTT. Protein was eluted using 10 mM glutathione in 50 mM HEPES–KOH (pH 7.6), 350 mM NaCl, 1 mM DTT. The peak fractions were pooled and dialyzed against 50 mM HEPES–KOH (pH 7.6), 350 mM NaCl, 20% glycerol, quick frozen in aliquots, and stored at −70°C. This protocol yielded about 50 mg of GST and 10–20 mg of each GST-SIR4 fusion protein.

Protein Affinity Chromatography

Purified GST or GST-SIR4 fusion proteins were crosslinked to AffiGel 10 (BioRad) in 50 mM HEPES (pH 7.6), 350 mM NaCl, 20% glycerol (buffer C). Two to four mg of each protein was added to 1 ml of AffiGel 10 in a volume of 2 ml and mixed using an end-over-end mixer at 4°C for 1–2 hr. This usually resulted in 70%–90% coupling. The beads were then washed with buffer C and resuspended in 0.1 M Tris–HCl (pH 8.0), 350 mM NaCl and incubated on ice for 1 hr. The affinity resin was then transferred to a small column and washed successively with 20 column volumes of each of the following: buffer C containing 1 mM DTT; buffer C containing 1 mM DTT, 2.5 M urea; buffer C containing 1 mM DTT, 0.75 M urea; and buffer C containing 1 mM DTT. BSA-AffiGel 10 columns were constructed in a similar manner by cross-linking 4 mg of BSA (bovine serum albumin) per ml of AffiGel 10. These columns were used to clear the extract prior to its application to the GST or GST-SIR4 affinity columns and to reduce the background of nonspecific proteins (Kellogg and Alberts, 1992). Usually a 10 ml precolumn was used for each 1 ml affinity column. Prior to loading of the extract, each column was washed with 20 column volumes of buffer D (50 mM HEPES [pH 7.6], 125 mM NaCl, 1% NP-40, 5 mM $Mg(OAc)_2$, 1 mM DTT, 5 mM benzamidine–HCl, 1 mM PMSF, 1 μg/ml each leupeptin, bestatin, and pepstatin).

Protease deficient yeast strains BJ2168 or BJ5459 were grown to A660 of 1–1.5, harvested by centrifugation, washed with cold distilled water, frozen in liquid nitrogen, and stored at −70°C. The frozen cell pellet (15–30 g) was resuspended in one volume of 2× buffer L (250 mM Tris–HCL [pH 7.5], 20% glycerol, 2 mM EDTA, 20 mM $Mg(OAc)_2$, 2 M NaCl), and after addition of BSA to 0.5 mg/ml, PMSF to 1 mM, benzamidine to 5 mM, DTT to 2 mM, NP-40 to 1%, and leupeptin, bestatin and pepstatin to 1 μg/ml each, the cells were disrupted by agitation with glass beads in a Biospec bead beater equipped with an ice/salt cooling jacket, using 10–12 ten-second pulses (88 ml chamber filled with 35 ml 0.5 mm glass beads). The glass beads were washed with 5–10 ml 1× buffer L containing protease inhibitors, and the wash was added to the cell lysate. The lysate was centrifuged for 30 min at 15,000 rpm in an SS34 rotor. The supernatant (~100 ml) was dialyzed against buffer D (20 mM HEPES [pH 7.6], 55 mM NaCl, 5 mM $Mg(OAc)_2$, 10% glycerol, 1 mM DTT, 1 mM EDTA, 0.5 mM PMSF, 5 mM benzamidine) until its conductivity was equal to that of the buffer D containing 125 mM NaCl. After addition of leupeptin, pepstatin, and bestatin to 1 μg/ml each and ultracentifugation in a type 35 rotor at 33,000 rpm for 1 hr, the supernatant was divided in half and loaded onto a 1 ml control GST column and a 1 ml GST-SIR4 affinity column at a flow rate of 4–7 ml per hour; each 1 ml affinity column was attached to a 10 ml BSA column and the extract passed through the BSA column before the GST affinity column. The affinity columns were then detached from the precolumns and washed with 40 column volumes (40 ml) of buffer D containing 0.5% NP-40, 1 μg/ml each leupeptin,

bestatin, and pepstatin, and then with five column volumes of the same buffer without NP-40 or protease inhibitors. The bound protein was then eluted using 10 ml buffer D with 2.5 M urea (without NP-40 or protease inhibitors) and 10 one-milliliter fractions were collected. The column was then eluted using 5 ml of the same buffer but with 4 M urea, and 5 one-milliliter fractions were collected. These concentrations of urea were more efficient than 1 M NaCl for elution of proteins from GST-SIR4 affinity columns. Each fraction was diluted using one volume of cold water containing 50 mg of deoxycholate and precipitated with 10% trichloroacetic acid (TCA). Each TCA pellet was washed with cold acetone, resuspended in 10–50 μl 1.5× SDS sample buffer, and incubated at 65°C for 10 min. Protein was then separated on 8.5% SDS polyacrylamide gels and visualized using Coomassie or silver.

Western Transfer and Protein Sequencing

Peak fractions from one or two affinity columns were pooled, and protein was separated on an 8.5% SDS polyacrylamide gel (Tall Mighty, Hoefer) and transferred to PVDF membrane (ImmobilonP, Millipore) using a BioRad Transblotter in 1× SDS gel running buffer containing 0.01% SDS and 10% methanol at 250 mA for 50 min at 4°C. The membrane was stained with 0.2% Ponceau S (Sigma) in 1% acetic acid to visualize protein bands. The desired protein bands were excised, washed with water, and stored in water at −20°C. Protein sequencing was performed by Chris Turk (UC San Francisco) and Sharleen Zhou (laboratory of Robert Tjian, UC Berkeley) by Edman degradation of tryptic peptides. The peptide sequences were used to search the nonredundant protein and DNA databases at the National Center for Biotechnology Information (NCBI) using the BLAST programs.

Measurements of Silencing at Telomeres and *HM* Loci

Telomeric silencing was measured as previously described (Gottschling et al., 1990; Aparicio et al., 1991). Strains containing the *URA3* gene near a telomere (UCC1001 and DM237) or at an internal position (UCC1003 and DM239) were grown on yeast extract–peptone–dextrose (YEPD) plates for 3 days. Single colonies were inoculated into fresh liquid YEPD media and grown overnight; fresh cultures were inoculated from the overnights and grown to an A660 of 0.6–1.0. Ten-fold serial dilutions of these cultures were plated on complete synthetic media (SC), 5-FOA, and −URA plates, and the plates were incubated at 30°C for 2–3 days. Similar results were obtained when dilutions of the overnight cultures were plated directly. 5-FOA resistance of strains with a telomeric *URA3* varies from 0.1% to 90% (Gottschling et al., 1990; Wiley and Zakian, 1995); in our experiments, 60%–90% of UCC1001 (*URA3-TEL*, VIIL) cells gave rise to 5-FOA-resistant colonies. Decreased silencing results in a loss of growth on 5-FOA plates, and measurements of 5-FOA resistance provide a large range for measuring defects in silencing. Loss of growth on −URA, on the other hand, provides a larger range for measuring increased levels of silencing than 5-FOA resistance and was primarily used in our experiments to judge increased levels of silencing. To measure silencing of a telomeric *TRP1* gene, strain JRY4469 (*TEL-VII::URA3::TRP1*), JRY4470 (*sir2Δ::LEU2 TEL-VII::URA3::TRP1*) and DM313 (*ubp3Δ::HIS3 TEL-VII::URA3::TRP1*) were grown in YEPD media as described above and were plated on complete synthetic (SC) and −TRP media.

Acknowledgments

Correspondence should be addressed to D. M. We are indebted to Jasper Rine and members of his laboratory for support, encouragement, and advice throughout the course of this project, and to Sharleen Zhou, Robert Tjian, and Chris Turk for protein microsequencing. We thank Pierre Spierer for communicating results prior to publication, Amy Axelrod and Jasper Rine for their generous gift of anti-SIR antibodies, and Jasper Rine, Rohan Baker, Andrew Dillin, Catherine Fox, and Dan Gottschling for gifts of yeast strains and plasmids. We thank Martha Arnaud, Burk Braun, Kelly Komachi, José Pérez-Martin, Michael Redd, and Madhu Wahi for valuable comments on the manuscript, and Madhu Wahi and Dominik Escher for helpful discussions. This work was supported by grants from the National Institutes of Health.

Received May 6, 1996; revised July 3, 1996.

References

Allshire, R.C., Nimmo, E.R., Ekwall, K., Javerzat, J.P., and Cranston, G. (1995). Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. Genes Dev. *9*, 218–233.

Aparicio, O.M., and Gottschling, D.E. (1994). Overcoming telomeric silencing: a trans-activator competes to establish gene expression in a cell cycle-dependent way. Genes Dev. *8*, 1133–1146.

Aparicio, O.M., Billington, B.L., and Gottschling, D.E. (1991). Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell *66*, 1279–1287.

Baker, R.T., Tobias, J.W., and Varshavsky, A. (1992). Ubiquitin-specific proteases of Saccharomyces cerevisiae. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. J. Biol. Chem. *267*, 23364–23375.

Bell, S.P., and Stillman, B. (1992). ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature *357*, 128–134.

Brand, A.H., Breeden, L., Abraham, J., Sternglanz, R., and Nasmyth, K. (1985). Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell *41*, 41–48.

Brand, A.H., Micklem, G., and Nasmyth, K. (1987). A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell *51*, 709–719.

Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D., and Broach, J.R. (1993). Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev. *7*, 592–604.

Buchman, A.R., Kimmerly, W.J., Rine, J., and Kornberg, R.D. (1988). Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in Saccharomyces cerevisiae. Mol. Cell. Biol. *8*, 210–225.

Chen, Z.J., Parent, L., and Maniatis, T. (1996). Site-specific phosphorylation of IkBa by a novel ubiquitination-dependent protein kinase activity. Cell *84*, 853–862.

Diffley, J.F., and Stillman, B. (1989). Transcriptional silencing and lamins [letter]. Nature *342*, 24.

Fan, C.M., and Maniatis, T. (1991). Generation of p50 subunit of NF-kappa B by processing of p105 through an ATP-dependent pathway. Nature *354*, 395–398.

Finley, D., Bartel, B., and Varshavsky, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature *338*, 394–401.

Foss, M., McNally, F.J., Laurenson, P., and Rine, J. (1993). Origin recognition complex (ORC) in transcriptional silencing and DNA replication in S. cerevisiae. Science *262*, 1838–1844.

Gall, J.G., Cohen, E.H., and Polan, M.L. (1971). Repetitive DNA sequences in Drosophila. Chromosoma *33*, 319–344.

Garrett, K.P., Aso, T., Bradsher, J.N., Foundling, S.I., Lane, W.S., Conaway, R.C., and Conaway, J.W. (1995). Positive regulation of general transcription factor SIII by a tailed ubiquitin homolog. Proc. Natl. Acad. Sci. USA *92*, 7172–7176.

Gottlieb, S., and Esposito, R.E. (1989). A new role for a yeast transcriptional silencer gene, *SIR2*, in regulation of recombination in ribosomal DNA. Cell *56*, 771–776.

Gottschling, D.E., Aparicio, O.M., Billington, B.L., and Zakian, V.A. (1990). Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. Cell *63*, 751–762.

Grant, S.G., and Chapman, V.M. (1988). Mechanisms of X-chromosome regulation. Annu. Rev. Genet. *22*, 199–233.

Halfter, H., Kavety, B., Vandekerckhove, J., Kiefer, F., and Gallwitz, D. (1989). Sequence, expression and mutational analysis of BAF1,

a transcriptional activator and ARS1-binding protein of the yeast Saccharomyces cerevisiae. EMBO J. *8*, 4265–4272.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S.M., and Grunstein, M. (1995). Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell *80*, 583–592.

Hershko, A. (1988). Ubiquitin-mediated protein degradation. J. Biol. Chem. *263*, 15237–15240.

Herskowitz, I., Rine, J., and Strathern, J.N. (1992). Mating-type determination and mating-type interconversion in *Saccharomyces cerevisiae*. In The Molecular and Cellular Biology of the Yeast *Saccharomyces*, E.W. Jones, J.R. Pringle, and J. R. Broach, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 583–656.

Hicke, L., and Riezman, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell *84*, 277–287.

Huang, Y., Baker, R.T., and Fischer-Vize, J.A. (1995). Control of cell fate by a deubiquitinating enzyme encoded by the fat facets gene. Science *270*, 1828–1831.

Ivy, J.M., Klar, A.J., and Hicks, J.B. (1986). Cloning and characterization of four SIR genes of Saccharomyces cerevisiae. Mol. Cell. Biol. *6*, 688–702.

Johnson, A.D. (1995). Molecular mechanisms of cell-type determination in budding yeast. Curr. Biol. *5*, 552–558.

Johnson, L.M., Kayne, P.S., Kahn, E.S., and Grunstein, M. (1990). Genetic evidence for an interaction between SIR3 and histone H4 in the repression of the silent mating loci in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA *87*, 6286–6290.

Jones, E.W. (1984). The synthesis and function of proteases in Saccharomyces: genetic approaches. Annu. Rev. Genet. *18*, 233–270.

Karpen, G.H. (1994). Position-effect variegation and the new biology of heterochromatin. Curr. Opin. Genet. Dev. *4*, 281–291.

Kayne, P.S., Kim, U.J., Han, M., Mullen, J.R., Yoshizaki, F., and Grunstein, M. (1988). Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell *55*, 27–39.

Kellogg, D.R., and Alberts, B.M. (1992). Purification of a multiprotein complex containing centrosomal proteins from the Drosophila embryo by chromatography with low-affinity polyclonal antibodies. Mol. Biol. Cell *3*, 1–11.

Kellum, R., and Alberts, B.M. (1995). Heterochromatin protein 1 is required for correct chromosome segregation in Drosophila embryos. J. Cell Sci. *108*, 1419–1431.

Kennedy, B.K., Austriaco, N.R., Jr., Zhang, J., and Guarente, L. (1995). Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae. Cell *80*, 485–496.

Kurtz, S., and Shore, D. (1991). RAP1 protein activates and silences transcription of mating-type genes in yeast. Genes Dev. *5*, 616–628.

Laurenson, P., and Rine, J. (1992). Silencers, silencing, and heritable transcriptional states. Microbiol. Rev. *56*, 543–560.

Longtine, M.S., Wilson, N.M., Petracek, M.E., and Berman, J. (1989). A yeast telomere binding activity binds to two related telomere sequence motifs and is indistinguishable from RAP1. Curr. Genet. *16*, 225–239.

Loo, S., Laurenson, P., Foss, M., Dillin, A., and Rine, J. (1995). Roles of ABF1, NPL3, and YCL54 in silencing in Saccharomyces cerevisiae. Genetics *141*, 889–902.

Marshall, M., Mahoney, D., Rose, A., Hicks, J.B., and Broach, J.R. (1987). Functional domains of SIR4, a gene required for position effect regulation in Saccharomyces cerevisiae. Mol. Cell. Biol. *7*, 4441–4452.

Masutani, C., Sugasawa, K., Yanagisawa, J., Sonoyama, T., Ui, M., Enomoto, T., Takio, K., Tanaka, K., van der Spek, P.J., Bootsma, D., et al. (1994). Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast RAD23. EMBO J. *13*, 1831–1843.

Moretti, P., Freeman, K., Coodly, L., and Shore, D. (1994). Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. Genes Dev. *8*, 2257–2269.

Mullen, J.R., Kayne, P.S., Moerschell, R.P., Tsunasawa, S., Gribskov, M., Colavito-Shepanski, M., Grunstein, M., Sherman, F., and Sternglanz, R. (1989). Identification and characterization of genes and mutants for an N-terminal acetyltransferase from yeast. EMBO J. *8*, 2067–2075.

Pagano, M., Tam, S.W., Theodoras, A.M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P.R., Draetta, G.F., and Rolfe, M. (1995). Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science *269*, 682–685.

Palombella, V.J., Rando, O.J., Goldberg, A.L., and Maniatis, T. (1994). The ubiquitin–proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell *78*, 773–785.

Papa, F.R., and Hochstrasser, M. (1993). The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. Nature *366*, 313–319.

Pillus, L., and Rine, J. (1989). Epigenetic inheritance of transcriptional states in S. cerevisiae. Cell *59*, 637–647.

Renauld, H., Aparicio, O.M., Zierath, P.D., Billington, B.L., Chhablani, S.K., and Gottschling, D.E. (1993). Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. Genes Dev. *7*, 1133–1145.

Rhode, P.R., Sweder, K.S., Oegema, K.F., and Campbell, J.L. (1989). The gene encoding ARS-binding factor I is essential for the viability of yeast. Genes Dev. *3*, 1926–1939.

Rine, J., and Herskowitz, I. (1987). Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae. Genetics *116*, 9–22.

Sandell, L.L., and Zakian, V.A. (1992). Telomeric position effects in yeast. Trends Cell Biol. *2*, 10–14.

Shei, G.J., and Broach, J.R. (1995). Yeast silencers can act as orientation-dependent gene inactivation centers that respond to environmental signals. Mol. Cell. Biol. *15*, 3496–3506.

Shore, D., and Nasmyth, K. (1987). Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell *51*, 721–732.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19–27.

Singer, M.S., and Gottschling, D.E. (1994). TLC1: template RNA component of Saccharomyces cerevisiae telomerase. Science *266*, 404–409.

Smeal, T., Claus, J., Kennedy, B., Cole, F., and Guarente, L. (1996). Loss of transcriptional silencing causes sterility in old mother cells in S. cerevisiae. Cell *84*, 633–642.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene *67*, 31–40.

Spofford, J.B. (1976). Position effect variegation in Drosophila. In The Genetics and Biology of Drosophila, M. Ashburner and E. Novitski, eds. (New York: Academic Press), pp. 955–1018.

Spradling, A., and Orr-Weaver, T. (1987). Regulation of DNA replication during Drosophila development. Annu. Rev. Genet. *21*, 373–403.

Sprague, G.F. (1991). Assay of yeast mating reaction. In Guide to Yeast Genetics and Molecular Biology, C. Guthrie and G.R. Fink, eds. (San Diego: Accademic Press, Inc.), pp. 77–93.

Swerdlow, P.S., Schuster, T., and Finley, D. (1990). A conserved sequence in histone H2A which is a ubiquitination site in higher eucaryotes is not required for growth in Saccharomyces cerevisiae. Mol. Cell. Biol. *10*, 4905–4911.

Thompson, J.S., Ling, X., and Grunstein, M. (1994). Histone H3 amino terminus is required for telomeric and silent mating locus repression in yeast. Nature *369*, 245–247.

Varshavsky, A., Bachmair, A., and Finley, D. (1987). The N-end rule

of selective protein turnover: mechanistic aspects and functional implications. Biochem. Soc. Trans. *15*, 815–816.

Watkins, J.F., Sung, P., Prakash, L., and Prakash, S. (1993). The Saccharomyces cerevisiae DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. Mol. Cell. Biol. *13*, 7757–7765.

Whiteway, M., Freedman, R., Van Arsdell, S., Szostak, J.W., and Thorner, J. (1987). The yeast ARD1 gene product is required for repression of cryptic mating-type information at the HML locus. Mol. Cell. Biol. *7*, 3713–3722.

Wiley, E.A., and Zakian, V.A. (1995). Extra telomeres, but not internal tracts of telomeric DNA, reduce transcriptional repression at Saccharomyces telomeres. Genetics *139*, 67–79.