The Fission Yeast Ubiquitin-Conjugating Enzymes UbcP3, Ubc15, and Rhp6 Affect Transcriptional Silencing of the Mating-Type Region

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Genes transcribed by RNA polymerase II are silenced when introduced near the mat2 or mat3 mating-type loci of the fission yeast Schizosaccharomyces pombe. Silencing is mediated by a number of gene products and cis-acting elements. We report here the finding of novel trans-acting factors identified in a screen for high-copy-number disruptors of silencing. Expression of cDNAs encoding the putative E2 ubiquitin-conjugating enzymes UbcP3, Ubc15 (ubiquitin-conjugating enzyme), or Rhp6 (Rad homolog pombe) from the strong nmt1 promoter derepressed the silent mating-type loci mat2 and mat3 and reporter genes inserted nearby. Deletion of rhp6 slightly derepressed an ade6 reporter gene placed in the mating-type region, whereas disruption of ubcP3 or ubc15 had no obvious effect on silencing. Rhp18 is the S. pombe homolog of Saccharomyces cerevisiae Rad18p, a DNA-binding protein that physically interacts with Rad6p. Rhp18 was not required for the derepression observed when UbcP3, Ubc15, or Rhp6 was overproduced. Overexpressing Rhp6 active-site mutants showed that the ubiquitin-conjugating activity of Rhp6 is essential for disruption of silencing. However, high dosage of UbcP3, Ubc15, or Rhp6 was not suppressed by a mutation in the 26S proteasome, suggesting that loss of silencing is not due to an increased degradation of silencing factors but rather to the posttranslational modification of proteins by ubiquitination. We discuss the implications of these results for the possible modes of action of UbcP3, Ubc15, and Rhp6.

Transcriptional silencing is observed in the mating-type region of Schizosaccharomyces pombe. The mating-type region occupies approximately 30 kb in the right arm of chromosome II and contains three cassettes, mat1, mat2-P, and mat3-M (Fig. 1A) (reviewed in references 2 and 25). The mating-type genes located at the mat1 locus are transcribed and determine the mating type of the cells. The mating type can be switched by duplicative transposition of the genes and promoters contained within the silent locus mat2-P or mat3-M (39). Silencing affects a region of approximately 16 kb that contains mat2 and mat3 and whose limits are controlled by boundary elements called IR-L and IR-R (inverted repeat left and right [59, 84]). This domain is a “cold spot” for recombination as well as for transcription and adopts a particular chromatin structure with features similar to those of heterochromatin (59).

Many parallels can be drawn between the position effects exerted on the donor loci of the fission yeast mating-type region and the heterochromatic inactivation of gene expression in higher eukaryotes. Position effects are exerted on the mating-type region by a number of trans-acting factors that are homologous to heterochromatin-associated proteins in higher eukaryotes (16, 36). Several chromodomain proteins affect silencing: the two HP1 homologs Swi6 (switching deficient [44]) and Chp2 (chromodomain protein [83]) and the SUV39H1 homolog Clr4 (cryptic locus regulator [20, 33, 80]). Like SUV39H1, Clr4 contains a SET domain in addition to its chromodomain and displays histone H3 methyltransferase activity in vitro and in vivo (55, 62). Methylation of histone H3 lysine 9 by Clr4 creates a binding site for Swi6 (5, 55). Likewise, methylation of histone H3 lysine 9 by SUV39H1 creates a binding site for mammalian HP1 proteins (43). Clr3 and Clr6, two histone deacetylase homologs, are also required for silencing the mating-type region (20, 26, 80). Histones are underacetylated in the mating-type region and at centromeres (19, 60). Hence, hypoacetylation is another shared feature between S. pombe heterochromatin and heterochromatin in higher eukaryotes. These conserved histone acetylation and methylation patterns stress the fundamental role of histone modifications in the establishment of chromatin structure and transcriptional states (72).

DNA polymerase α was recently shown to play a role in transcriptional silencing in fission yeast (56). Other factors that affect silencing in the mating-type region are Clr1, a zinc-finger protein (G. Thon and A. Klar, unpublished observations); Clr2, which has no described homologs in other systems (7); Rik1 (recombination in K [81]), a protein with homology to UV-damage DNA-binding proteins; and the products of the esp1-3 genes (enhancer of Swi6 phenotype [81]). It is likely that mutations in additional trans-acting silencers will be identified, like esp2 (centromere suppressor of position effect), which causes partial derepression of transcription in the mating-type region (17). One striking feature of the trans-acting factors characterized so far is that single mutations or deletions do not abolish silencing but merely reduce it, suggesting that these factors act in overlapping pathways.
Chromosomal deletion analyses have identified a number of cis-acting elements that are required for transcriptional silencing at the donor loci and have confirmed that redundant mechanisms contribute to silencing. Deletion of a 1.5-kb DNA fragment on the centromere proximal side of mat2 (80) or of a 0.2-kb subelement, called REII (3), causes a small silencing defect. The presence of a mat2 silencing element was previously indicated by deletion analysis of a mat2-containing plasmid (18). When a chromosomal deletion of that silencing element is combined with a mutation in rik1, chap2, swi6, clr1, clr2, clr3, or clr4, full derepression of mat2 and its flanking regions occurs (80, 83) (Thon, unpublished). Deletions introduced near the mat3 cassette behave in a manner similar to that of deletion of the mat2 silencer (79). These synergistic effects indicate the existence of two epistasis groups of silencing factors (80). Together with further genetic analyses, they suggest that factors encoded by the esp genes act via the mat2- and mat3-enhancers, whereas rik1, chap2, swi6, clr1, clr2, clr3, and clr4 act via an element located in the K region between mat2 and mat3. Some cross talk is likely to occur between the two pathways, although the details of the interactions are not known (27, 54, 81).

![Figure 1](https://example.com/fig1.png)

**FIG. 1.** Screen for high-copy-number disruptors of silencing. (A) Mating-type region of the tester strain PG1608. (B) Haploid meiosis phenotypes of PG1608 overexpressing ubcP3, rhp6, ubc15, or no cDNA (−). Colonies grown on MSA plus uracil plus adenine were stained with iodine. (C) Silencing of ura4 in transformed PG1608 cells was estimated by plating 10-fold serial dilutions of cell suspensions on medium lacking uracil (AA-thiamine-leucine-uracil [+ uracil]) or containing 5-FOA (FOA-thiamine-leucine [+ FOA]) and on complete medium (AA-thiamine-leucine). (D) Multiple-sequence alignment showing the homology between UbcP3, Ubc15, and Rhp6. Black shading indicates identical residues, while gray shading indicates conserved residues.
grounds give rise to a strong derepression of transcription in the mating-type region and at centromeric locations (26). However, no cumulative effect is observed when a rhp6 mutant allele is combined with a clr2, clr4, or swi6 mutant allele. Taken together, these results indicate that Clr6 functions in a pathway different from that ofClr1 and Clr3 and suggest that Clr6 may act via the silencing elements centromere proximal to mat2 and mat3 (26).

Here we report the results of experiments designed to identify novel factors affecting position effects in *trans*. A screen for genes that cause disruption of silencing when overexpressed was performed. The screen was expected to reveal genes whose products or enzymatic activities are required for silencing in precise dosage. Such screens were pioneered in *Saccharomyces cerevisiae* (48), and they were subsequently conducted in *S. pombe* to identify factors involved in centromere function (29, 34), cytoskeletal dynamics (14), or cell shape (10). The approach seemed well suited to the study of position-effect variegation, a phenomenon particularly sensitive to gene dosage. Our screen led to the identification of UbcP3, Ubc15, and Rhp6, three members of a large family of proteins in *S. pombe* that cause disruption of silencing when overexpressed. We propose that *ubcP3*, *ubc15*, and *rhp6* are involved in centromere function (29, 34), cytoskeletal dynamics, and particularly sensitive to gene dosage. Our screen led to the identification of factors involved in centromere function (29, 34), cytoskeletal dynamics, and particularly sensitive to gene dosage.

### MATERIALS AND METHODS

**DNA sequences of *ubcP3*, *ubc15*, and *rhp6*.** The sequences described in this study are accessible via cosmid numbers at the Sanger Centre fission yeast genomic database or via accession numbers at SWISS-PROT: SPBP16F5 and Q9HDP3 (*ubcP3*), SPBC1105, Q9YS8 (1) (ubc15), and SPAC15B11 and P23566 (rhp6).

**Strains and media.** The *S. pombe* strains used in this study are listed in Table 1 with their genotypes and origins. *S. pombe* cells were propagated and tested using the following media: yeast extract supplemented (YES) (81); minimum salts-arginine (MSA) (15) supplemented with 100 μg of adenine, 100 μg of uracil, and 200 μg of t-leucine per liter as indicated; medium containing amino acids and supplements (AA) (66); substituting yeast nitrogen base lacking thiamine for yeast nitrogen base when indicated); fluoroorotic acid (FOA)-thiamine-leucine (81; substituting yeast nitrogen base lacking thiamine for yeast nitrogen base); and FOA (1 g of 5-FOA and 50 μg of uracil per liter). The *Escherichia coli* strain DH5 (30) was used for cloning plasmids. It was propagated in *S. pombe* by the lithium acetate method (51). Transformants were grown to saturation at 33°C. Series of 10-fold dilutions were spotted onto MSA supplemented with adenine and uracil and grown at 33°C. Spor colonies were identified by iodine staining and streaked on YES plates. Subsequently, the YES plates were replica plated onto MSA supplemented with adenine and uracil and onto MSA supplemented with adenine and uracil and onto MSA supplemented with adenine.

**Silencing assay.** Spot tests were performed to evaluate the expression levels of reporter genes placed at silenced loci in the mating-type region. *S. pombe* cultures were grown to saturation at 33°C. Series of 10-fold dilutions were spotted onto plates of YES, AA lacking appropriate supplements, supplemented MSA, or 5-FOA as indicated. Plates were incubated at 33°C for 4 to 6 days until full growth was achieved except for *ubc22* strains, which were incubated at 28°C.

**Plasmid recovery and identification.** Plasmids were recovered from *S. pombe* in *E. coli* DH5 using the miniprep DNA extraction procedure previously described by Moreno et al. (51). Sequence data were obtained by ABI prism big dye terminator cycle sequencing (PE Applied Biosystems, Perkin-Elmer) using primers specific for the nmt1 promoter in the *S. pombe* expression vector PREPSX (25, 46, 47). The library was transformed into *S. pombe* by the lithium acetate method (51). Transformants were plated onto MSA supplemented with adenine and uracil and grown at 33°C. Spor colonies were identified by iodine staining and streaked on YES plates. Subsequently, the YES plates were replica plated onto MSA supplemented with adenine and uracil and onto MSA supplemented with adenine and uracil and onto MSA supplemented with adenine.

### TABLE 1. *S. pombe* strains used in this study

| Strain | Mating-type region | Auxotrophic markers | Others |
|--------|--------------------|---------------------|--------|
| FY340  | *h*<sup>+/N</sup> | leu1-32  | TM1(H fragilis) ∶ *ura4* R.I. |
| HU52   | *h*<sup>0</sup> mat3-M(ECORV)∶*ade6* | leu1-32  | leu1-32 ura4-D3/E ade6-M210 |
| PB141  | *h*<sup>0</sup> mat3-M(ECORV)∶*ade6* | leu1-32  | leu1-32 ura4-D18 ade6-DN/N |
| PG9    | *h*<sup>0</sup> mat3-M(ECORV)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG506  | mat1-Mstk10∶mat3-M(ECORV)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG505  | *h*<sup>0</sup> mat3-M(ECORV)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG1141 | *h*<sup>0</sup> mat3-M(ECORV)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG1247 | *h*<sup>0</sup> mat3-M(ECORV)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG1598 | mat1-Mstk1∶mat2-P(Xbal)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG1608 | mat1-Mstk1∶Δ(Bgl II-Bss III)∶mat2-P(Xbal)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG1786 | mat1-Mstk1∶Δ(Bgl II-Bss III)∶mat2-P(Xbal)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG1789 | mat1-Mstk1∶mat2-P(Xbal)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG1899 | *h*<sup>0</sup> mat3-M(ECORV)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG2875 | *h*<sup>0</sup> | leu1-32 ura4-DS/E ade6-M210 | ubc22∶kan<sup>R</sup> TM1(H fragilis) ∶ *ura4* R.I. |
| PG2876 | *h*<sup>0</sup> | leu1-32 ura4-DS/E ade6-M210 | ubc22∶kan<sup>R</sup> TM1(H fragilis) ∶ *ura4* R.I. |
| PG2883 | mat1-Mstk10∶mat2-P(Xbal)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG2887, PG2888 | *h*<sup>0</sup> mat3-M(ECORV)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG2889, PG2890 | *h*<sup>0</sup> mat2-P(Xbal)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG2891, PG2892 | *h*<sup>0</sup> Δ(Bgl II-Bss III)∶mat2-P(Xbal)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PG2895 | *h*<sup>0</sup> mat3-M(ECORV)∶*ade6* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| PI131  | *h*<sup>0</sup> mat3-M(ECORV)∶*ura4* | leu1-32  | leu1-32 ura4-D18 ade6-M210 |
| SP837  | *h*<sup>0</sup> | leu1-32 ura4-DS/E ade6-M210 | leu1-32 ura4-DS/E ade6-M210 |

**Genomic DNA library and screen.** An *S. pombe* cDNA library was provided by C. J. Norbury (ICRF, Cell Cycle Group, Oxford, United Kingdom) and B. Edgar. In this library, *S. pombe* cDNAs are placed under the control of the nmt1 promoter in the *S. pombe* expression vector PREPSX (25, 46, 47). The library was transformed into *S. pombe* by the lithium acetate method (51). Transformants were plated onto MSA supplemented with adenine and uracil and grown at 33°C. Spor colonies were identified by iodine staining and streaked on YES plates. Subsequently, the YES plates were replica plated onto MSA supplemented with adenine and uracil and onto MSA supplemented with adenine and uracil and onto MSA supplemented with adenine.

**Mating-type region and at centromeric locations (26).**
CAAGGTTCAGTCG, and native Pfu polymerase (Strategene). rps4-1 was amplified by PCR using the forward primer 5′-CCGGATCCTGCGACATGTCGAGTTCTAAAAAGC, the reverse primer 5′-CCGGATCCTGCGACATGTCGAGTTCTAAAAAGC, and native Pfu polymerase (Strategene). Both PCR products were digested with SalI and BamHI and cloned into pREP3X digested with SalI and BamHI using T4 DNA Ligase (Roche). The resulting clones were checked by sequencing, and silencing phenotypes were assayed in S. pombe.

Construction of ubcP3 and ubc15 disruption strains. Disruptions were carried out in an SP837/PG1141 diploid strain. The disruption constructs were prepared in pBluescript SK (Strategene). ubcP3 upstream and downstream flanking sequences, of 800 and 1,000 bp, respectively, were amplified from genomic DNA by PCR using the upstream forward primer 5′-CCGGATCCTGCGACATGTCGAGTTCTAAAAAGC, and native Pfu polymerase (Strategene). Both PCR products were digested with SalI and BamHI and cloned into pREP3X digested with SalI and BamHI using T4 DNA Ligase (Roche). The resulting clones were checked by sequencing, and silencing phenotypes were assayed in S. pombe.

RESULTS

A screen for S. pombe cDNAs that in elevated dosage disrupt transcriptional silencing in the mating-type region. The preponderance of evidence indicates that transcriptional silencing is mediated by a large multicompartment structure. Overexpression of one component may perturb the stoichiometric relationship between subunits and cause defects in silencing because of improper assembly or maintenance of the repressive state. To identify such gene products with potential roles in the formation of repressive chromatin, we screened an S. pombe cDNA library (B. Edgar and C. Norbury) for cDNAs that in elevated dosage cause derepression of genes residing in the silent part of the mating-type region. The cDNAs were expressed from the strong, inducible nmt1 promoter (no message on thiamine [46]). Since silencing defects can be masked by a redundancy of the silencing mechanisms, the cDNA library was introduced into a partially derepressed strain, PG1608. In PG1608 cells, silencing is reduced by the deletion of a cis-acting element adjacent to mat2 (Fig. 1A). Further derepression of the region such as that caused by mutations in trans-acting factors of the S. pombe mat158 strain group gives rise to easily observable phenotypes. Because they contain the unswitchable mat1-Msmt-0 allele, PG1608 cells express the M mating type stably. Expression of the P mating-type genes residing in the normally silent mat2 cassette in conjunction with the M mating-type genes from mat1-M causes cells to undergo haploid meiosis. Haploid meiosis can be detected by exposure to iodine vapors, upon which spore-containing colonies stain darkly, whereas colonies containing no spores are not stained. Stable mat1-M cells in which silencing is not compromised appear totally Spoj in this assay. PG1608 cells are stained very lightly.

Approximately 105 transformants expressing cDNAs from the aforementioned library were screened for an increase in the intensity of iodine staining. Thirty-seven transformants with increased sporulation were isolated and subjected to a second screen. In PG1608 cells, the S. pombe ura4 gene is inserted at an XbaI site approximately 400 bp centromere distal to mat2 (Fig. 1A). The ura4 gene placed at that location is stringently repressed in a wild-type background and partially derepressed in PG1608 (80) (Fig. 1C). The 37 transformants displaying increased haploid meiosis were examined for their ability to grow in the absence of uracil. Twelve isolates showed a plasmid-dependent sporulation phenotype and enhanced ability to grow in the absence of uracil. Plasmids were recovered from these isolates and sequenced. They could be divided into three classes: class 1 with eight clones containing rhp6 (Rad homolog pombe 6 [63]), which encodes the S. pombe Rad6 homolog; class 2 with three clones containing ubc15, which encodes a hitherto-uncharacterized putative E2 ubiquitin-conjugating enzyme; and class 3, consisting of one clone containing ubcP3, which encodes a protein previously identified on the basis of its ability to bind ubiquitin (61) (Fig. 1D). In addition to ubcP3, the last clone also contained a cDNA (rps4-1) encoding the ribosomal protein S4. The two cDNAs were cloned individually in pREP3X and overexpressed separately in PG1608. All effects originally observed using the hybrid clone were found to be due to overexpression of ubcP3. Figure 1B shows the sporulation phenotypes of cells overexpressing ubcP3, ubc15, or rhp6, and Fig. 1C shows their ability to grow in the absence of uracil and in the presence of 5-FOA, a toxicogenic substrate of the Ura4 enzyme.

Effect of high UbcP3, Ubc15, or Rhp6 dosage on the expression of the S. pombe ade6 and ura4 genes placed near mat2 or mat3. To determine whether the silencing defects caused by overexpressing ubcP3, ubc15, or rhp6 were specific to genes residing at or near the mat2 locus or to cells with the 1.5-kb deletion flanking mat2-P, these cDNAs were overexpressed in a number of different backgrounds. The S. pombe ura4 gene is repressed in a wild-type background when placed at the mat2 centromere-distal XbaI site (PG1789 [80]) or when placed at an EcoRV site located approximately 150 bp away from mat3-M (PG1899) (Fig. 2A and B [82]). We transformed PG1879 and PG1899 with the pREP3X plasmid lacking an insert or containing ubcP3, ubc15, or rhp6. Expression of the cDNAs was induced by depletion of thiamine, and the silencing phenotypes were assayed by spot tests and Northern blot analysis. In the Northern blot analysis, the amount of ura4 transcript originating from the mating-type region was compared with the amount of transcript originating from a truncated ura4 allele, ura4-DS/E (1). As shown in Fig. 2D and 3 (lanes labeled mat2-P and Δ1.5 kb mat2-P), expression of
pressed (Fig. 2E and F). In summary, derepression of silencing by high dosage of UbcP3, Ubc15, or Rhp6 affects the genes contained within mat2-P, ura4 inserted near mat2-P, and ura4 and ade6 inserted near mat3-M. Derepression can occur when all cis-acting elements are present, albeit to a lesser degree than when one of the cis-acting elements is deleted (Fig. 3, compare mat2-P and ∆1.5 kb mat2-P lanes; see also below). Finally, derepression is observed in both switchable (PG1899 and PB141) and nonswitchable (PG1608 and PG1789) backgrounds.

**Combination of cis- and trans-acting mutations: epistasis analysis.** As previously mentioned, silencing factors that repress transcription in the mating-type region can be assigned to one of two epistasis groups. swi6, clr1, clr2, clr3, and clr4 belong to one group that acts in part via the K region; clr6, esp1, esp2, and esp3 belong to a second group that possibly exerts its effect via the mat2/mat3 elements (26, 81). To determine whether ubcP3, ubc15, or Rhp6 could be assigned to one of the two pathways, an epistasis analysis was performed. The fact that the cDNA clones were obtained in a partially derepressed strain caused by deletion of the element in front of mat2 suggested that the silencing defects caused by overexpressing ubcP3, ubc15, or Rhp6 might be exerted via the swi6, clr1, clr2, clr3, and clr4 pathway. If this was true, combining overexpression of ubcP3, ubc15, or Rhp6 with a mutation in swi6, clr1, clr2, clr3, or clr4 should not give rise to cumulative derepression. Indeed, overexpression of ubcP3, ubc15, or Rhp6 in swi6-115 (Fig. 4A and B), clr1-5 (Fig. 4C and D), clr4-681, clr3-735, or clr2-760 (data not shown) cells does not change their level of haploid meiosis. Furthermore, in a manner similar to the derepression caused by mutations in the Swi6 epistasis group (22, 27, 81), high dosage of ubcP3, ubc15, or Rhp6 induced high levels of haploid meiosis in nonswitchable esp3-1 cells (Fig. 4C and D) and derepressed transcription in a ∆K1 strain (Fig. 4E and F). These phenotypes are consistent with ubcP3, ubc15, and Rhp6 affecting silencing via the swi6, clr1, clr2, clr3, and clr4 pathway.

**The antisilencing activity of Rhp6 requires an intact active site.** The silencing defects caused by high dosage of UbcP3, Ubc15, or Rhp6 could be a result of these ubiquitin-conjugating enzymes titrating away silencing complexes from their cognate site of action by direct association. If this was the case, a catalytically inactive E2 ubiquitin-conjugating enzyme would most likely confer an effect on silencing similar to that of a catalytically active enzyme. To test this possibility, we created active-site mutants of Rhp6. In the process of covalently attaching ubiquitin to lysines in target proteins, ubiquitin-conjugating enzymes form a transient thioester bond with ubiquitin at an active-site cysteine (22). We mutated the predicted active-site cysteine of Rhp6, cysteine 88, by site-directed mutagenesis. Cysteine 88 is the only cysteine present in Rhp6. By analogy with *S. cerevisiae* Rad6p active-site mutants (75, 76), Rhp6(C88A) with its active-site cysteine changed to alanine should be unable to form a thioester conjugate with ubiquitin, whereas Rhp6(C88S), with a change of cysteine to serine, should be able to bind ubiquitin but should be unable to transfer it to target proteins. Overexpressed Rhp6 carrying either of the two point mutations, Rhp6(C88S) or Rhp6(C88A), caused cell elongation (not shown) and slow growth (Fig. 5B, complete) but failed to derepress transcription (Fig. 5B, uracil

**FIG. 2.** High dosage of UbcP3, Rhp6, or Ubc15 generally affects silencing in the mating-type region. (A) Mating-type region of the switchable (h*) strain PG1899. (B) Silencing in PG1899 was assayed by plating serial dilutions of cells transformed with plasmids containing no cDNA (−), ubcP3, ubc15, or Rhp6 on the same media as in Fig. 1C. (C) Mating-type region of the nonswitchable strain PG1789. (D) Silencing was assayed in PG1789 by spotting serial dilutions of cells on the same media as in Fig. 1C. (E) Mating-type region of PB141. (F) Silencing of ade6 placed near mat3-M was assayed by plating dilution series of transformed PB141 cells on medium lacking adenine (PM plus uracil) (− adenine) and complete medium (PM plus uracil plus adenine).

**ubcP3, ubc15, or Rhp6 increased expression of ura4 placed at the XbaI site near mat2-P.** Similarly, expression of ura4 placed at the EcoRV site near mat3 increased upon induction of ubcP3, ubc15, or Rhp6 expression (Fig. 2B and Fig. 3, lanes labeled mat3-M). Expression of the full-length ura4 gene randomly integrated in the genome (R.I. [1]) was not similarly affected by overexpression of the ubiquitin-conjugating enzymes (Fig. 3, lanes labeled R.I.), indicating that the effect observed near mat2 or mat3 is on silencing per se. Furthermore, the *S. pombe* ade6 gene placed at the EcoRV site was also derepressed when ubcP3, ubc15, or Rhp6 was overexpressed (Fig. 2E and F). In summary, derepression of silencing by high dosage of UbcP3, Ubc15, or Rhp6 affects the genes contained within mat2-P, ura4 inserted near mat2-P, and ura4 and ade6 inserted near mat3-M. Derepression can occur when all cis-acting elements are present, albeit to a lesser degree than when one of the cis-acting elements is deleted (Fig. 3, compare mat2-P and ∆1.5 kb mat2-P lanes; see also below). Finally, derepression is observed in both switchable (PG1899 and PB141) and nonswitchable (PG1608 and PG1789) backgrounds.

**Combination of cis- and trans-acting mutations: epistasis analysis.** As previously mentioned, silencing factors that repress transcription in the mating-type region can be assigned to one of two epistasis groups. swi6, clr1, clr2, clr3, and clr4 belong to one group that acts in part via the K region; clr6, esp1, esp2, and esp3 belong to a second group that possibly exerts its effect via the mat2/mat3 elements (26, 81). To determine whether ubcP3, ubc15, or Rhp6 could be assigned to one of the two pathways, an epistasis analysis was performed. The fact that the cDNA clones were obtained in a partially derepressed strain caused by deletion of the element in front of mat2 suggested that the silencing defects caused by overexpressing ubcP3, ubc15, or Rhp6 might be exerted via the swi6, clr1, clr2, clr3, and clr4 pathway. If this was true, combining overexpression of ubcP3, ubc15, or Rhp6 with a mutation in swi6, clr1, clr2, clr3, orclr4 should not give rise to cumulative derepression. Indeed, overexpression of ubcP3, ubc15, or Rhp6 in swi6-115 (Fig. 4A and B), clr1-5 (Fig. 4C and D), clr4-681, clr3-735, or clr2-760 (data not shown) cells does not change their level of haploid meiosis. Furthermore, in a manner similar to the derepression caused by mutations in the Swi6 epistasis group (22, 27, 81), high dosage of ubcP3, ubc15, or Rhp6 induced high levels of haploid meiosis in nonswitchable esp3-1 cells (Fig. 4C and D) and derepressed transcription in a ∆K1 strain (Fig. 4E and F). These phenotypes are consistent with ubcP3, ubc15, and Rhp6 affecting silencing via the swi6, clr1, clr2, clr3, and clr4 pathway.

**The antisilencing activity of Rhp6 requires an intact active site.** The silencing defects caused by high dosage of UbcP3, Ubc15, or Rhp6 could be a result of these ubiquitin-conjugating enzymes titrating away silencing complexes from their cognate site of action by direct association. If this was the case, a catalytically inactive E2 ubiquitin-conjugating enzyme would most likely confer an effect on silencing similar to that of a catalytically active enzyme. To test this possibility, we created active-site mutants of Rhp6. In the process of covalently attaching ubiquitin to lysines in target proteins, ubiquitin-conjugating enzymes form a transient thioester bond with ubiquitin at an active-site cysteine (22). We mutated the predicted active-site cysteine of Rhp6, cysteine 88, by site-directed mutagenesis. Cysteine 88 is the only cysteine present in Rhp6. By analogy with *S. cerevisiae* Rad6p active-site mutants (75, 76), Rhp6(C88A) with its active-site cysteine changed to alanine should be unable to form a thioester conjugate with ubiquitin, whereas Rhp6(C88S), with a change of cysteine to serine, should be able to bind ubiquitin but should be unable to transfer it to target proteins. Overexpressed Rhp6 carrying either of the two point mutations, Rhp6(C88S) or Rhp6(C88A), caused cell elongation (not shown) and slow growth (Fig. 5B, complete) but failed to derepress transcription (Fig. 5B, uracil...
and + 5-FOA). Hence, the ubiquitin-conjugating activity of Rhp6 is required for its effects on silencing when overproduced. These effects are therefore not caused by a titration of silencing complexes via direct binding to Rhp6.

**Effect of disrupting ubcP3, ubc15, or rhp6 on silencing in the mating-type region.** We constructed two diploid strains, one containing a ubcP3 (ubc15) allele disrupted with ura4 and one containing a ubc15 allele entirely replaced with ura4. Examining the progeny of these diploids showed that neither ubcP3 nor ubc15 is essential for cell viability. To assay for silencing phenotypes, ubcP3::ura4 or ubc15::ura4 was crossed into strains with ade6 integrated near mat3-M with or without deletion of the mat3-M silencer. Disruption of ubcP3 or ubc15 did not cause obvious defects in silencing of ade6 at mat3-M (data not shown). In addition, the ubcP3::ura4 ubc15::ura4 double mutant failed to reveal a silencing defect (data not shown). Furthermore, h90 ubcP3::ura4 and h90 ubc15::ura4 cells had mating and sporulation phenotypes indistinguishable from those of wild-type h90 cells (data not shown).

We used the ubc2::kan1 allele described in reference 40 to test whether Rhp6 depletion had an effect on silencing. Northern blot analyses revealed a small increase in the amount of ura4 transcript originating from sites within the mating-type region in ubc2::kan1 strains (Fig. 6), indicating that Rhp6 participates in the repression of marker genes placed near the silent cassettes in wild-type strains.

**The derepression caused by high dosage of UbcP3, Ubc15, or Rhp6 does not require rhp18.** The S. cerevisiae RAD18 gene encodes a protein with single-stranded-DNA-binding activity that interacts with the ubiquitin-conjugating enzyme Rad6p and functions in postreplication repair (4). Similarly, stable hRad18p-HHR6A and hRad18p-HHR6B protein complexes are formed when the human Rad6p homologs HHR6A and HHR6B are coexpressed with human hRAD18 in yeast cells (88). Rhp18, the S. pombe Rad18 homolog, could possibly be involved in the silencing defects observed in our study by providing an interface between ubiquitin-conjugating enzymes and DNA. In order to test this possibility, we overexpressed ubcP3, ubc15, or rhp6 in rhp18::ura4 cells containing ade6 near the mat3-M cassette (86) (Fig. 7A). Expression of ade6 was first assayed by plating cells on medium lacking adenine and on medium containing a low concentration of adenine on which cells lacking Ade6 accumulate a red pigment (Fig. 7B). Expression of ade6 was further analyzed by Northern blotting, using a truncated ade6 transcript originating from the ade6- DN/N allele (19) as internal control (Fig. 7C). Derepression of ade6 occurred in rhp18::ura4 cells, indicating that the silencing defects caused by overproduction of UbcP3, Ubc15, or Rhp6 do not depend on Rhp18.

**The derepression caused by high dosage of Ubc15 or Rhp6 can occur in a 26S proteasome mutant.** Polyubiquitination can target proteins for degradation via the 26S proteasome (22). Degradation recycles misfolded proteins and participates in cell cycle control (22, 41, 53, 85). Elevated doses of UbcP3, Ubc15, or Rhp6 might therefore cause the observed silencing defects by increasing turnover of silencing factors via the 26S proteasome. If this was the case, defects in the 26S proteasome might prevent derepression from occurring or at least attenuate it. In order to test this hypothesis, we assayed whether mts3-1, a thermosensitive mutation in the 19S regulatory cap of the 26S proteasome (68), suppressed the silencing defects caused by high dosage of UbcP3, Ubc15, or Rhp6. mts3-1 cells displayed a partial growth defect at the semipermissive temperature of 30°C, and they grew very poorly on FOA-containing plates, preventing use of this medium to assess expression of ura4. Overexpression of UbcP3 also proved toxic to mts3-1 cells, precluding any test of silencing (Fig. 8). Overexpression of Ubc15 or Rhp6 could be assayed, and Fig. 8 shows that it
caused derepression of transcription even when the 26S proteasome was compromised, leading to increased growth in the absence of uracil. This indicates that disruption of silencing by Ubc15 or Rhp6 overproduction is not due to the degradation of silencing factors.

**DISCUSSION**

In this study, we present the identification of a group of proteins that influence transcriptional silencing in fission yeast and an initial characterization of their mode of action. These proteins belong to the family of ubiquitin-conjugating enzymes. One of them, Rhp6, was previously implicated in transcriptional repression (70), as was its *S. cerevisiae* homolog Rad6p (11, 32). Both Rhp6 and Rad6p have been extensively characterized because of their role in DNA damage repair.

Two other ubiquitin-conjugating enzymes identified by our screen, UbcP3 and Ubc15, were not obtained in previous mutant searches. Their effects on silencing are similar to those of Rhp6, which raises the possibility that the three ubiquitin-conjugating enzymes act on related substrates.

**Ubiquitin-conjugating enzymes and silencing in S. pombe.**

Overexpression of UbcP3, Ubc15, or Rhp6 impaired silencing of genes residing within or near the *mat2* or *mat3* mating-type cassettes, whereas disruption of *ubcP3* or *ubc15* did not significantly affect silencing. Deletion of *rhp6* had pleiotropic phenotypes, including a partial loss of silencing of marker genes introduced in the mating-type region. The lack of phenotype of the *ubcP3* or *ubc15* disruptions and the relatively small effect of the *rhp6* deletion suggest that ubiquitin-conjugating enzymes might affect silencing redundantly. Fourteen putative E2 ubiquitin-conjugating enzymes have been identified in fission yeast (Sanger Centre fission yeast genomic database). Our data do not rule out the possibility that ubiquitin-conjugating enzymes other than UbcP3, Ubc15, and Rhp6 affect transcriptional silencing. Ubc4, for example, a ubiquitin-conjugating enzyme that causes chromosome loss when overexpressed (34), could influence position effects, a possibility remaining to be investigated. Remarkably, UbcP3, Ubc15, Rhp6 and Ubc4 are more closely related to each other than to any other putative ubiquitin-conjugating enzyme in *S. pombe*. UbcP3 and Ubc15 being most similar with 50% identical amino acids (Fig. 1D).

A point mutation in the 5′ splice junction at the second intron of *rhp6/sng1* was previously reported to cause a 10-fold reduction in mature *rhp6* mRNA level (70). That mutation derepresses the *mat2*-P and *mat3*-M genes but not heterologous reporter genes inserted in the silent regions flanking the donor cassettes (70). In addition, Singh and coworkers (70) observed derepression only in cells that were able to switch their mating type, which suggests a role for *rhp6* in reassembly of chromatin after the DNA replication that accompanies mating-type switching. We found that *rhp6* overexpression caused derepression not only of the mating-type genes contained within the silent cassettes but also of heterologous genes introduced near the cassettes and that the derepression occurred both in switchable and nonswitchable cells. These results are not incompatible with those of Singh et al. (70). Ubiquitination of a substrate by Rhp6 might be required for the spreading of a silencing factor from regions that flank the cassettes into the...
freshly used donor cassettes. Reduced concentrations of Rhp6, such as those caused by the sng1 mutation, would not allow spreading of the silencing factor, whereas overproduction of Rhp6 might cause ubiquitination of an excess of substrate and targeting of the silencing factor to novel sites. This would lead to depletion of the factor not only within the donor cassettes but more generally in the entire region that is normally silenced. Our observation that deletion of rhp6 reduces silencing of a ura4 marker placed near mat3-M more difficult to reconcile with the conclusions of Singh et al. (70). That derepression might not have occurred or might have escaped detection in cells containing a residual amount of Rhp6.

Ubiquitin-processing enzymes and silencing. S. cerevisiae RAD6/UBC2 is required for silencing at telomeres and the HM loci (32). RAD6 is also required for transcriptional silencing of Ty1 elements in the RDN1 locus (11). These effects of Rad6p can be explained in terms of alterations of chromatin structure. Gross structural alterations of chromatin are also consistent with the role of RAD6 and rhp6 in sporulation (24, 50, 63) and with the role of the mouse Rad6p homolog HHR6B in the histone-to-protamine transition during spermatogenesis (65, 42). Overexpression of rhp6 mutated at cysteine 88 did not disrupt silencing (this study), and active-site mutants of RAD6 (32) are silencing deficient, suggesting that in both cases the ubiquitination event itself is necessary for silencing. Other ubiquitin-processing enzymes play a role in transcriptional silencing.

E(var)3-64E, a putative ubiquitin-specific protease, affects position-effect variegation in Drosophila melanogaster (31). In S. cerevisiae, the ubiquitin hydrolases Ubp3p and Dot4p, respectively, counteract (49) and enhance (37, 69) silencing.
lencing. Both Ubp3p and Dot4p interact with the silencing factor Sir4p, and these deubiquitinating enzymes may regulate silencing by controlling either the activity or the assembly of SIR protein complexes, possibly helping restrict transcriptional repression to normally silenced loci (37, 49). In the present study we show that, in addition to Rhp6, two hitherto-uncharacterized putative E2 ubiquitin-conjugating enzymes, UbcP3 and Ubc15, influence silencing in the mating-type region of S. pombe. Collec-

FIG. 7. Disruption of silencing caused by overexpression of ubcP3, ubc15, or rhp6 does not require rhp18. (A) Mating-type region of Hu52 and PG2895. (B) Silencing of ade6 near mat3-M was tested by plating dilution series of rhp18Δ (Hu52) or rhp18Δ::ura4 (PG2895) cells transformed with plasmids containing no cDNA (−), ubcP3, ubc15, or rhp6 on medium lacking adenine (AA-thiamine-leucine-adenine) (− adenine - T), on medium poor in adenine (AA-thiamine-leucine + 12 mg of adenine per liter) (Low adenine - T), and on complete medium (AA-acid-thiamine-leucine). (C) A Northern blot prepared with RNA from the strains displayed in panel B was hybridized to a probe for the ade6 open reading frame. “ade6” indicates the full-length ade6 transcript originating from the mating-type region, while “ade6-DN/N” indicates the truncated ade6 transcript originating from the ade6-DN/N allele.
Histones as potential substrates. In higher eukaryotes, histone H2A, histone H2B, and histone H3 can be ubiquitinated and a number of studies suggest that histone ubiquitination contributes to the organization of chromatin (reviewed in references 9, 12, 71, and 87). Ubiquitination of histone H2A and histone H2B has been proposed to regulate chromosome condensation, nucleosomes being deubiquitinated at metaphase and rapidly reubiquitinated at anaphase (45, 52). Ubiquitination of histone H2B, and to a lesser extent of histone H2A, is in some cases associated with transcriptional activity (13, 57). Histone H3 ubiquitination occurs during the maturation of rat spermatids concomitantly with extensive chromatin remodeling (12). The purified S. cerevisiae Rad6p protein can ubiquitinate histone H2A, H2B, and H3 in vitro (28, 74). Rad6p ubiquitinates histone H2B in vivo in S. cerevisiae, and mutation of the conserved ubiquitination site in H2B confers mitotic and meiotic defects (64). However, a silencing-proficient mutant (rad6-149) is unable to ubiquitinate histone H2B to detectable levels, indicating that histone H2B ubiquitination does not directly mediate silencing in S. cerevisiae (64 and references therein). Comparison of S. cerevisiae histone H2A and histone H2A from higher eukaryotes, which can be ubiquitinated at lysine 119 (58, 77), suggested which lysine might be ubiquitinated in yeast. Mutating this lysine, however, produced cells that were indistinguishable from the wild type under a variety of conditions, indicating that ubiquitination of histone H2A does not play a major role in S. cerevisiae cell growth, sporulation, or resistance to heat, stress, or UV radiation (77). This conclusion was confirmed by the examination of a quadruple mutant of histone H2A, in which lysine 119 as well as three neighboring lysines were changed to arginine (64). However, neither study tested whether mutating lysines of histone H2A or histone H2B influenced transcriptional silencing. Future studies will reveal whether and which nucleosomal proteins are ubiquitinated in S. pombe and whether ubiquitination of histones influences silencing.

Repair. Transcriptional silencing, DNA repair, and recombination are related by their dependence on chromatin structure. Deletions of chromatin assembly factors (CAC1, CAC2, or CAC3) cause sensitivity to UV and reduce telomeric silencing in S. cerevisiae (21, 38, 73). The S. cerevisiae Rad6p interacts with Rad18p, which is a key factor in the RAD6 DNA repair pathway. Rad18p may target Rad6p to damaged DNA by its ability to bind to single-stranded DNA (4). RAD18 is not required for silencing of telomeric markers in S. cerevisiae (32), but its deletion further reduces telomeric silencing in cells lacking CAC1 (24). Here we show that disruption of silencing by overexpressing ubcP3, ubc15, or rhp6 does not require rhp18. Further experiments are required to determine whether Rhp18p is required for UbcP3, Ubc15, or Rhp6 effects on silencing. In a wild-type background, a requirement that would be overruled when the UbcP3, Ubc15, or Rhp6 dosage is increased. High dosage of UbcP3, Ubc15, or Rhp6 or disruption of ubcP3 or ubc15 had no effect of UV sensitivity (data not shown), suggesting that the effects exerted on silencing are not directly related to the UV DNA damage response.

Protein degradation. A large number of proteins are modified covalently by the enzymatic attachment of ubiquitin to side chains of lysines, a modification that often leads to proteolytic degradation (53, 85). Covalently bound polyubiquitin chains are recognized by one or more subunits of the 26S proteasome as the first step in the process that leads to the degradation of the ubiquitinated protein and recycling of the 76-amino-acid ubiquitin molecules (22, 85). Mutations in mts2 and pad1 encoding 19S regulatory cap subunits of the 26S proteasome were reported to cause increased repression of centromeric ura4 (35), indicating that silencing factors might normally be degraded by the proteasome. We found that derepression caused by high dosage of Ubc15 or Rhp6 can occur in a strain carrying a mutation in the 19S regulatory cap of the 26S proteasome (mts3-I [68]). This suggests that disruption of silencing by Ubc15 or Rhp6 is not caused by increased degradation of silencing factors via the 26S proteasome. However, we cannot rule out that some silencing factors were degraded at the semipermissive temperature because of residual activity of the proteasome.

Models for the role of ubiquitin-conjugating enzymes in silencing. Figure 9 presents four mechanisms by which a high dosage of ubiquitin-conjugating enzymes could reduce transcriptional silencing. The silenced chromatin state is repre-
sent in Fig. 9A. In the first model (Fig. 9B), the turnover of histones or chromatin-associated silencing factors is increased when E2-ubiquitinating enzymes are in abundance. Our finding that derepression of silencing occurs in a proteasome mutant argues against this model. In the second model (Fig. 9C), the derepression results from the ubiquitin-conjugating enzymes directly titrating silencing complexes away from their site of action by protein-protein associations, as is, for example, the case in the titration of Sir4p by overexpressed Dot4p in S. cerevisiae (37). This model predicts that catalytically inactive ubiquitin-conjugating enzymes would be able to derepress silencing when overexpressed, arguing against a direct titration model in the case of Rhp6. Indirect titration might be another mechanism by which disruption of silencing occurs (Fig. 9D). In this model, ubiquitin modification of chromatin components or histones normally targets silencing factors to their site of action and directs the assembly of higher-order chromatin structures. Overexpression of ubiquitin-conjugating enzymes leads to excessive ubiquitination and to the titration of silencing factors. Finally (Fig. 9E), increased ubiquitination might directly prevent the formation of heterochromatin. Our results globally support the last two models, the small reduction of silencing observed in the absence of Rhp6 arguing in favor of the model in Fig. 9D in the case of Rhp6. Additional experiments are needed to identify the substrates and elucidate the mechanisms of action of UbcP3, Ubc15, and Rhp6.

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