

# The yeast SAS (something about silencing) protein complex contains a MYST-type putative acetyltransferase and functions with chromatin assembly factor ASF1

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It is well established that acetylation of histone and nonhistone proteins is intimately linked to transcriptional activation. However, loss of acetyltransferase activity has also been shown to cause silencing defects, implicating acetylation in gene silencing. The something about silencing (Sas) 2 protein of *Saccharomyces cerevisiae*, a member of the MYST (MOZ, Ybf2/Sas3, Sas2, and TIP60) acetyltransferase family, promotes silencing at *HML* and telomeres. Here we identify a ~450-kD SAS complex containing Sas2p, Sas4p, and the tf2f-related Sas5 protein. Mutations in the conserved acetyl-CoA binding motif of Sas2p are shown to disrupt the ability of Sas2p to mediate the silencing at *HML* and telomeres, providing evidence for an important role for the acetyltransferase activity of the SAS complex in silencing. Furthermore, the SAS complex is found to interact with chromatin assembly factor Asf1p, and *asf1* mutants show silencing defects similar to mutants in the SAS complex. Thus, ASF1-dependent chromatin assembly may mediate the role of the SAS complex in silencing.

[Key Words: SAS; silencing; MYST; acetyl transferase; ASF1; chromatin assembly factor]

Received April 27, 2001; revised version accepted July 24, 2001.

Transcriptional silencing is a form of position-dependent and gene-independent transcriptional repression in which chromatin structure is altered over large regions of the genome. In *Saccharomyces cerevisiae*, silenced loci include the *HML* and *HMR* mating-type loci, the telomeres, and genes within the ribosomal DNA. Many gene products have been identified that contribute to silencing (Sherman and Pillus 1997; Lustig 1998). Silent information regulator (Sir) proteins 1–4 are well-studied factors that play important functions in transcriptional silencing (Cockell et al. 1998). Sir2p, Sir3p, and Sir4p are components of a multiprotein complex, and mutations in any of these genes result in the complete derepression of the silent mating and telomere loci. In contrast to mutations in *SIR2*, *SIR3*, or *SIR4*, mutations in *SIR1* only lead to a partial derepression of the silent-mating

loci. This is because in *sir1* mutants, there is an epigenetic phenomenon in which some cells have fully silenced *HM* loci, whereas others have fully expressed loci (Pillus and Rine 1989). Telomeric silencing, however, appears to be independent of Sir1p (Aparicio et al. 1991).

In a yeast genetic screen conducted to identify enhancers of *sir1* epigenetic silencing defects, *something about silencing* (SAS) 2 was identified (Reifsnyder et al. 1996). The genetic experiments showed that *SAS2* has opposite regulatory effects, depending on the silenced locus. Sas2p promotes silencing at *HML* and telomeres but weakens it at an *HMR* locus with mutations in *HMR-E* silencer elements (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997). Recently, the *SAS4* and *SAS5* genes were also isolated as negative regulators of silencing at *HMR*-containing defective *HMR-E* silencer elements (Xu et al. 1999a,b). *SAS4* and *SAS5* were also found to be positive regulators of silencing at *HML* and telomeres, and similar to *SAS2*, each gene interacts genetically with *SIR1* in silencing at *HML*. Strains containing null alleles of all combinations of *sas2*, *sas4*, and *sas5* were no more

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.907201>.

defective for *HML* silencing than any of the single deletion strains (Xu et al. 1999b). These genetic experiments suggest that *SAS2*, *SAS4*, and *SAS5* function in the same genetic pathway.

Sas2p is a member of the MYST (Moz, Ybf2/Sas3, Sas2, and TIP60) family of acetyltransferases. MYST-related proteins have been identified from yeast to humans and include the following: human MOZ (Borrow et al. 1996), MORF (Champagne et al. 1999), TIP60 (Yamamoto and Horikoshi 1997), and HBO1 (Iizuka and Stillman 1999); *Drosophila* MOF (Smith et al. 2000); and yeast Sas2p (Reifsnnyder et al. 1996), Sas3p (Takechi and Nakayama 1999; John et al. 2000), and Esa1p (Smith et al. 1998). These MYST-related proteins show a high degree of sequence conservation in the acetyl-coenzyme A (acetyl-CoA) binding and zinc finger regions. Most MYST proteins have been shown to possess histone acetyltransferase (HAT) activity (Sternier and Berger 2000). Substrate specificity of the MYST acetyltransferases has also been investigated. For instance, in addition to HAT activity, TIP60 possesses autoacetylation activity (Creaven et al. 1999). Though Sas2p and MOZ contain the highly conserved acetyl-CoA binding motif, the histone acetylation activity and substrate specificity of these proteins have not been identified.

Many acetyltransferases are components of large multiprotein complexes in which associated subunits are often required for specific HAT activity and function in transcription (Howe et al. 1999; Brown et al. 2000). The complexes containing Sas3p, Esa1p, dMOF, and hTIP60 have been purified and characterized (Allard et al. 1999; Ikura et al. 2000; John et al. 2000; Smith et al. 2000). The human TIP60 complex contains additional subunits possessing ATPase, DNA helicase, and DNA-binding activities (Ikura et al. 2000). Sas3p is the catalytic subunit of the HAT complex NuA3, which also contains the TBP-associated factor TAF<sub>II</sub>30. Sas3p mediates the interaction between NuA3 and Spt16p, a component of the yeast CP complex (Cdc68/Pob3) that functions in transcription elongation and DNA replication (John et al. 2000). NuA4 is ~1.3 MD in size, and five Esa1p-interacting subunits have been identified (Allard et al. 1999; Eisen et al. 2000; Galarneau et al. 2000). Characterization of these subunits provides us with important informa-

tion about the role of HAT complexes in gene expression.

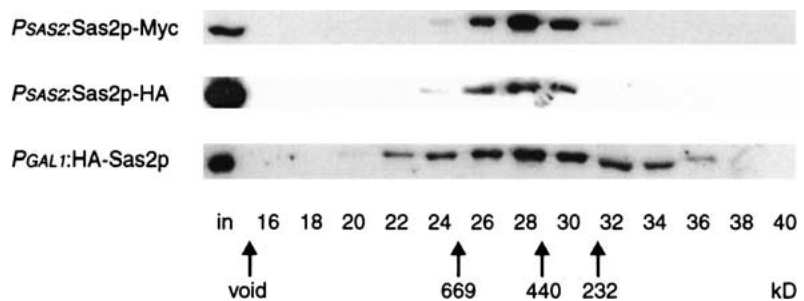
Histone acetylation is important for the regulation of gene silencing. Deletion of *GCN5* enhances telomere silencing (Sun and Hampsey 1999). In contrast, deletion of *SAS2* leads to derepression of *HML* and a telomere proximal reporter gene (Reifsnnyder et al. 1996). Mutations in *HAT1*, which encodes a subunit of a type B HAT, weaken telomeric silencing in the presence of certain histone tail mutations (Kelly et al. 2000). Furthermore, loss of the Nat1p-Ard1p cytoplasmic N-terminal acetyltransferase leads to silencing defects (Mullen et al. 1989). The significance of acetylation of histones and other proteins relevant to silencing remains unclear. Although *SAS2* has been genetically associated with silencing, it is not known how the specific aspects of *SAS2*-mediated silencing are conferred. Here we identify and characterize the native Sas2p complex and investigate the effect of mutations in the Sas2p acetyl-CoA binding site on silencing in vivo. We also show that the Sas2 complex interacts with chromatin assembly factor Asf1p, and deletion of both *ASF1* and *SIR1* causes derepression of *HML*. Further, we show that mutations in *ASF1*, like those in *SAS* genes, improve silencing at mutated *HMR* loci.

## Results

### *The native Sas2 complex is ~450 kD in size*

To gain further insight into the function of Sas2p, we sought to investigate whether it was a component of a multiprotein complex. To determine the molecular size of the native Sas2p-containing complex, Sas2p was tagged at its C terminus with the 13-Myc epitope by homologous recombination at the *SAS2* chromosomal locus. Whole cell extracts were prepared from a strain expressing Sas2p-Myc (YJW265) and separated by Superose 6 size exclusion chromatography. Fractions were monitored for the presence of Sas2p-Myc by Western blot analysis. Only one protein peak was detected, which eluted in fractions 26 to 30 and corresponded to a molecular mass of ~450 kD (Fig. 1, upper panel). To confirm these results, two other strains that expressed either a C-terminal 3-HA epitope tagged Sas2p (YJW214) or an

**Figure 1.** Detection of a 450-kD Sas2p-containing complex. Yeast extracts were separated by Superose 6 size exclusion chromatography, and fractions were analyzed for Sas2p by Western blot analysis (anti-Myc or anti-HA monoclonal antibodies). (Top panel) Superose 6-separated whole cell extracts prepared from strain YJW265 expressing Sas2p-Myc. (Middle panel) Whole cell extracts prepared from strain YJW214, expressing Sas2p-HA. The Sas2p-HA complexes were enriched by Mono Q anion-exchange chromatography before separation by Superose 6. (Bottom panel) Superose 6-separated whole cell extracts prepared from strain YJW213 expressing the *GAL1*-regulated HA-Sas2p. Arrows mark peak fractions for molecular weight standard proteins.



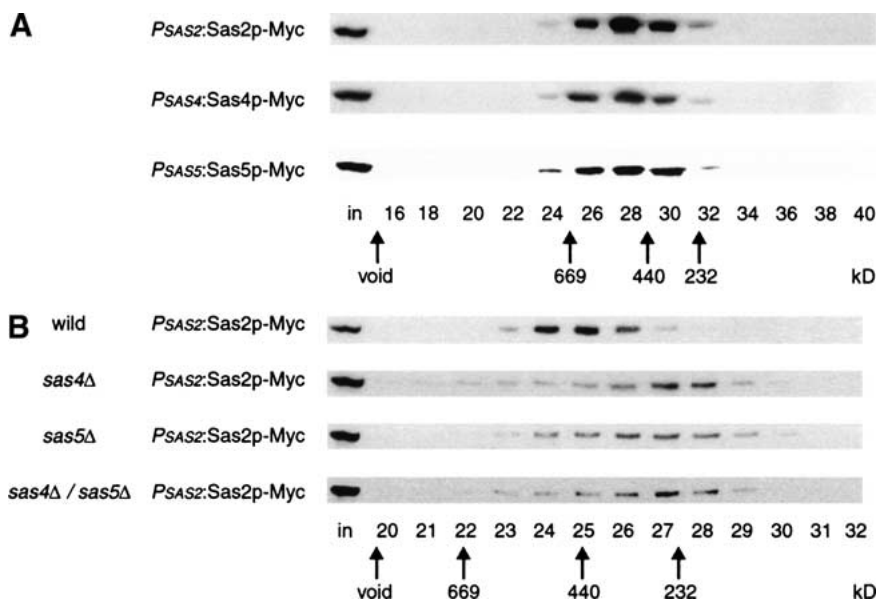
N-terminal 3-HA tagged Sas2p (YJW213) regulated by the *GAL1* promoter were constructed. The Sas2p-HA complexes were enriched by Mono Q anion-exchange before separation by Superose 6 to improve detection of the native complex with the HA-antibody. Again, the Sas2p-HA signal eluted in fractions 26 to 30 as observed for Sas2p-Myc (Fig. 1, middle panel). Similarly, when extracts prepared from the *GAL*-regulated HA-Sas2 strain were separated by Superose 6, the HA-Sas2p also peaked in fractions 26 to 30 (Fig. 1, lower panel). These results indicate that the type and the position of the epitope tags do not affect the size of the Sas2 complex. Although the HA-Sas2p signal was spread out, this was likely caused by the overproduction of Sas2p by the strong *GAL1* promoter. The results from the three chromosomally integrated Sas2p-tagged strains indicate that the size of the native Sas2 complex is ~450 kD.

#### *Sas4p and Sas5p are components of the Sas2 complex*

Genetic experiments have shown that *SAS2*, *SAS4*, and *SAS5* are positive regulators of silencing at *HML* and telomeres and that the role of these *SAS* genes in silencing of *HML* is not redundant (Xu et al. 1999b). These results suggest that Sas4p and Sas5p function in the same pathway as Sas2p. To investigate the size of the Sas4 and Sas5 complexes, C-terminal 13-Myc-tagged strains were generated (YJW228 and YJW229). Separation of whole cell extracts from the Sas4-Myc and Sas5-Myc strains by gel filtration showed that the Sas4p- and Sas5p-containing complexes are similar in size to the Sas2 complex (Fig. 2A). This raises the possibility that Sas2p, Sas4p, and Sas5p may in fact be components of the same complex. Accordingly, deletion of either *SAS4* or *SAS5* might alter the size of the Sas2 complex. To examine this, whole cell extracts from wild type and *sas4Δ*, *sas5Δ*, and *sas4Δ sas5Δ* strains expressing Sas2p-

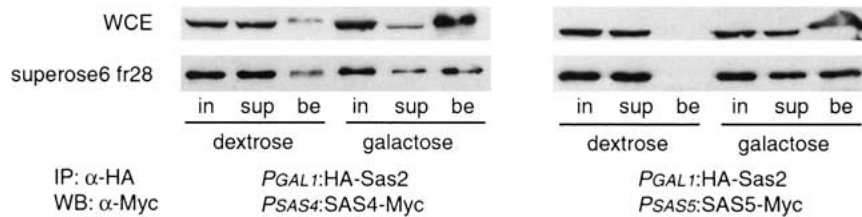
Myc were fractionated by Superdex 200 size exclusion chromatography (Fig. 2B). The Sas2p-Myc signal from the three deletion strains was more spread out and ran at significantly lower apparent molecular weights, although the Sas2 complex was present in all of the strains. The peak fractions of the Sas2p-Myc were shifted from fraction 25 to fractions 27, 26, and 27 by the deletion of the *SAS4*, *SAS5*, and *SAS4/SAS5* genes, respectively. These results show that mutations in *SAS4* and *SAS5* alter the size or conformation of the Sas2 complex and suggest that Sas4p and Sas5p are components of the Sas2 complex.

To confirm the presence of Sas4p and Sas5p in the Sas2 complex, double-tagged yeast strains containing *GAL*-regulated HA-Sas2p and Sas4p-Myc or Sas5p-Myc were generated (YJW230 and YJW231). Coimmunoprecipitation experiments were performed with whole cell extracts and peak Superose 6 fractions from the double-tagged strains grown in dextrose or galactose medium. Sas2p was immunoprecipitated with HA-antibody, and coprecipitation of Sas4p and Sas5p was monitored by anti-Myc Western analysis. As shown in Figure 3, Sas4p and Sas5p coprecipitated with Sas2p from both the whole cell extracts and the peak fractions of Superose 6 only when HA-Sas2 expression was induced with galactose. Control experiments performed with extracts and Superose 6 fractions prepared from strains grown in dextrose medium show that efficient coprecipitation of Sas4p and Sas5p was dependent on their interaction with Sas2p. The low level of Sas4p-Myc detected in the bead fraction when the strain was grown on dextrose is likely caused by leakiness of the *GAL1* promoter. The cofractionation of Sas2p with Sas4p and Sas5p and the immunoprecipitation experiments indicate that Sas4p and Sas5p are indeed components of the Sas2 complex. Thus, we named the Sas2 complex, which also contains Sas4p and Sas5p, simply the SAS complex.



**Figure 2.** Sas4p and Sas5p are components of the Sas2 complex. (A) Extracts were prepared from strains YJW265, YJW228, and YJW229 expressing the C-terminal Myc-tagged Sas2, Sas4, and Sas5 proteins, respectively. The sizes of the Sas2, Sas4, and Sas5 complexes were determined by analysis of elution profiles from Superose 6. (B) Comparison of the Sas2p-Myc elution profiles from Superdex 200 size exclusion chromatography following fractionation of whole cell extracts prepared from wild type (YJW265) and *sas4Δ* (YJW269), *sas5Δ* (YJW270), and *sas4Δ sas5Δ* (YJW271) mutant strains. Shown are Western blots of column fractions probed with Myc-antibodies.

**Figure 3.** Sas4p and Sas5p coimmunoprecipitate with Sas2p. Coimmunoprecipitation experiments were performed with both whole cell extracts (WCE) and Superose 6 fraction 28 prepared from double-tagged strains HA-Sas2p, Sas4p-Myc (YJW230) and HA-Sas2p, Sas5-Myc (YJW231; indicated at the bottom of the panel). Protein fractions were incubated with HA-antibodies, and 10% of input (in), 10% of supernatant (sup), and bead (be) fractions from the immunoprecipitates were probed for Myc-tagged proteins.



#### Purification and characterization of the SAS complex

To further characterize the SAS complex, it was purified to homogeneity. For this purpose, a double-tagged yeast strain harboring chromosomally integrated Sas4p-Myc and a plasmid expressing Sas2p tagged with an N-terminal 6xHis and a C-terminal Flag and regulated by the *GAL10* promoter was used (YJW276). This *SAS2* expression plasmid was also used for the mating assays and could fully complement the *sas2* deletion (see Fig. 5B). The SAS complex was purified on the basis of the Sas4p-Myc signal, using the anti-Myc Western blot analysis as outlined in Figure 4A. The Flag affinity-purified SAS complex was fractionated by Superdex 200, and each fraction was analyzed by silver stain and Western blot analysis (Fig. 4B). The major Sas2p peak (fraction 23, 24) corresponds to a molecular mass of ~450 kD, similar to the complex characterized in Figures 1 and 2. Western blot analysis revealed that Sas4p-Myc (p100) was only found in the 450-kD Sas2 complex (Fig. 4B, middle panel). A significant portion of Sas2p was also detected at higher molecular weights (e.g., fraction 20). Whether this is a unique complex containing Sas2p or an artifact of overexpression of Sas2p is under investigation. As a control, we performed mock purification from whole cell extracts prepared from the wild-type parental strain containing an empty expression vector (YJW243). No protein bands were detected in the Flag eluate from the control strain by silver staining (data not shown).

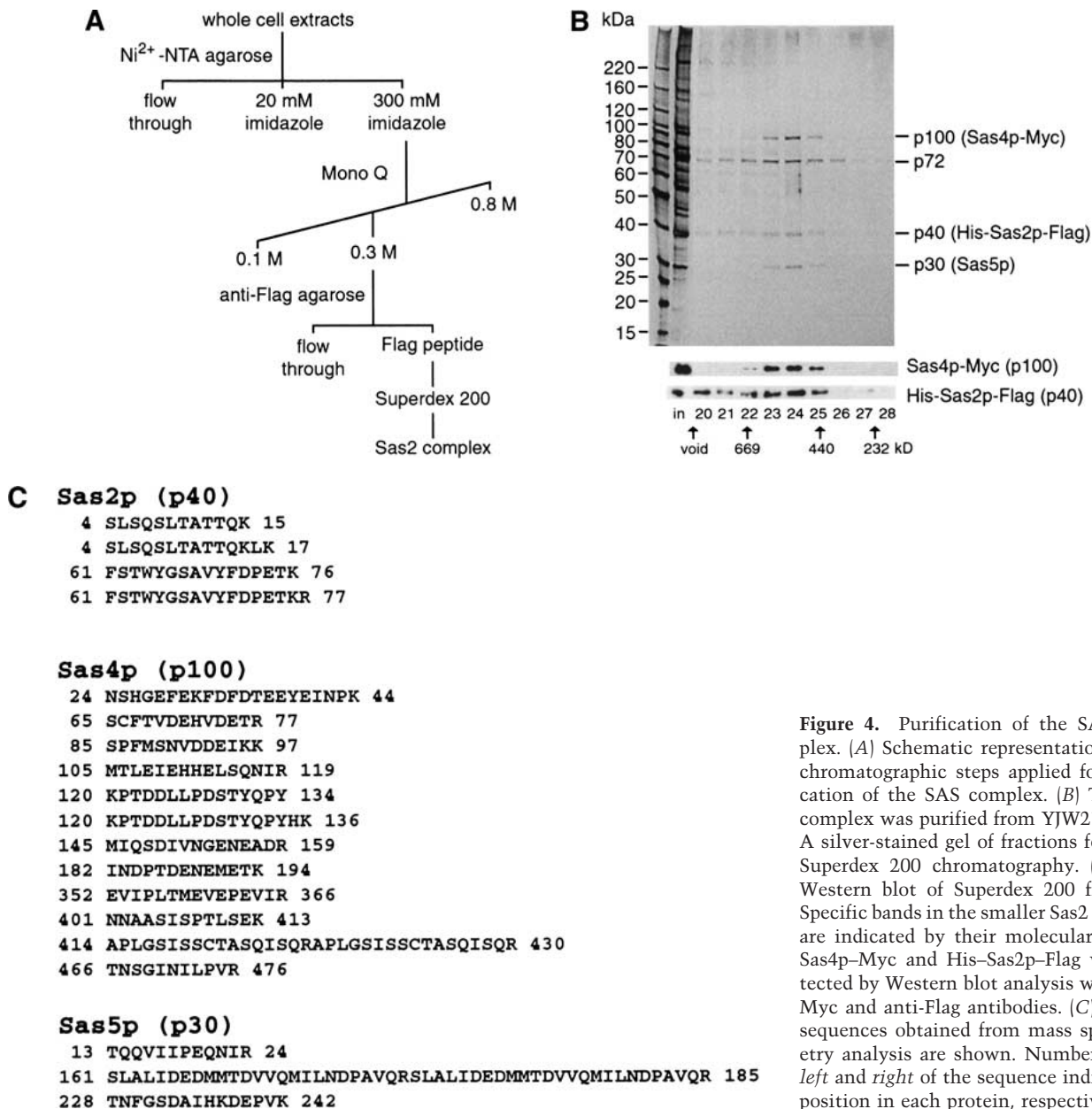
Three major bands (p100, p72, and p30) cofractionated with the Sas2p (p40) peak fraction. To identify the co-eluting proteins, the p100, p72, p40, and p30 bands were excised and analyzed by mass spectrometry. Twelve, four, and three peptides obtained from p100, p40, and p30 corresponded to the Sas4p, Sas2p, and Sas5p, respectively (Fig. 4C). This finding confirmed that Sas2p, Sas4p, and Sas5p co-associate in a unique and distinct complex. Thirteen peptide sequences from p72 correspond to six different yeast proteins. Experiments are in progress to confirm which, if any, of these proteins are bona fide subunits of the SAS complex.

#### The conserved acetyl-CoA binding motif of Sas2p is required for HML and telomere silencing

The yeast Sas2, Sas3, and Esa1 proteins all show a significant degree of conservation in the MYST-related ace-

tyl-CoA binding site (Fig. 5A). However, for some MYST proteins (including Sas2p), HAT activity has not been described yet. To test for Sas2p HAT activity, we performed HAT assays with the purified SAS complex. Partially and highly purified fractions of the SAS complex failed to acetylate free or nucleosomal histones in vitro (data not shown). Recombinant Sas2p could not be assayed because it was insoluble. It is possible that Sas2p might require other cofactors to acetylate histones or that it acetylates nonhistone proteins. Either possibility would suggest that the highly conserved acetyl-CoA binding motif in Sas2p should be essential for its function. Mutations within the predicted acetyl-CoA binding pocket in Sas3p (M1 429GYG to AAA; M2 426QR to AA) have been shown to greatly reduce HAT activity of the NuA3 complex (John et al. 2000). By comparison, a Sas3p (M3 434LM to AA) mutation in the hydrophobic pocket somewhat distal to the acetyl-CoA binding site did not affect NuA3 HAT activity (John et al. 2000). To investigate the role of the Sas2p putative acetyl-CoA binding site in vivo, we mutated conserved amino acids that are predicted to be important for acetyl-CoA binding and examined their effect on silencing at the silent mating-type loci and at a telomere. For this purpose, we constructed point mutants in *SAS2*, which corresponded to *SAS3* mutants M1, M2, and M3 (Fig. 5A).

The *SAS2* gene was originally isolated as an enhancer of the epigenetic silencing defects of yeast *sir1* mutants. *SAS2* also contributes to silencing at the telomeres (Reifsnnyder et al. 1996). The combined deletion of *sir1* and *sas2* genes causes a much more severe silencing defect at *HML* than does the deletion of *sir1* alone. To investigate the relevance of the Sas2p putative acetyl-CoA binding site on *HML* silencing, wild-type and mutated *SAS2* alleles were transformed into the *sir1 sas2* double-mutant strain (Fig. 5B). We constructed three types of expression vectors. Two of them contain the native *SAS2* promoter on either CEN or 2 $\mu$  plasmids, and the other is regulated by the *GAL10* promoter. As judged by mating assays, the *sas2-M3* mutant restored *HML* silencing to wild-type levels when overexpressed and to intermediate levels when expressed from a CEN plasmid (Fig. 5B). However, mutant strains carrying the *sas2-M1* mutation could no longer restore the mating-type silencing (Fig. 5B). Thus, residues within the acetyl-CoA binding domain (M1), not distal (M3), are absolutely required for the *SAS2* silencing function. This result indicates that the con-



**Figure 4.** Purification of the SAS complex. (A) Schematic representation of the chromatographic steps applied for purification of the SAS complex. (B) The SAS complex was purified from YJW276. (Top) A silver-stained gel of fractions following Superdex 200 chromatography. (Bottom) Western blot of Superdex 200 fractions. Specific bands in the smaller Sas2 complex are indicated by their molecular weight. Sas4p-Myc and His-Sas2p-Flag were detected by Western blot analysis with anti-Myc and anti-Flag antibodies. (C) Peptide sequences obtained from mass spectrometry analysis are shown. Numbers at the left and right of the sequence indicate the position in each protein, respectively.

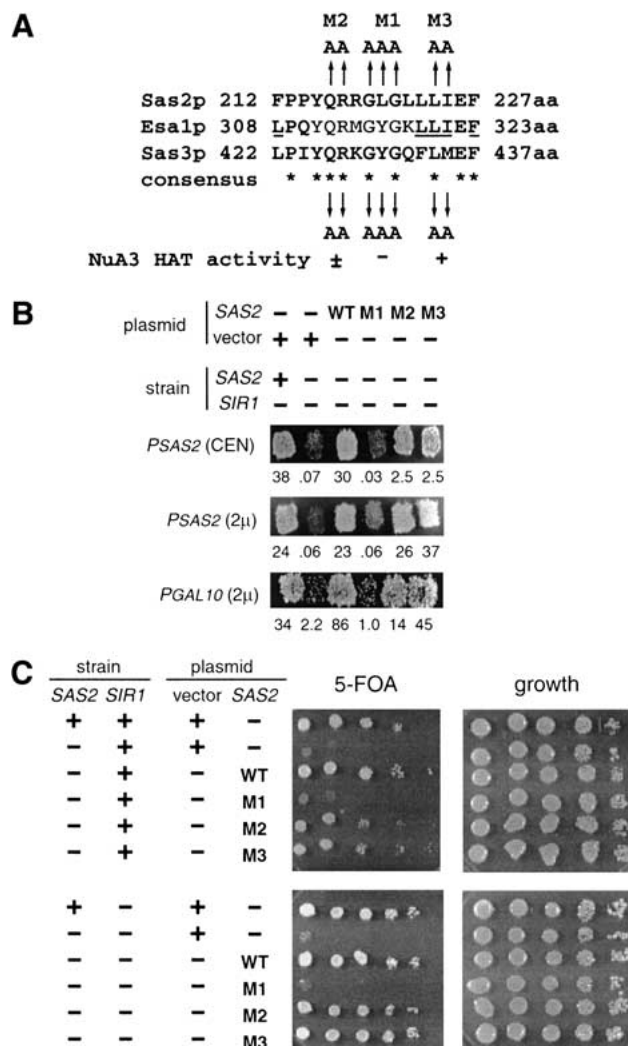
served acetyl-CoA binding motif in Sas2p is necessary for the *HML* silencing in vivo and strongly suggests that Sas2p possesses acetyltransferase activity in vivo.

Unexpectedly, the *sas2-M2* mutant was also able to restore *HML* silencing (Fig. 5B). By analogy to the Esa1p crystal structure, residues corresponding to M1 and M2 interact with acetyl-CoA. The crystal structure of Esa1p/CoA indicates that the M1 mutation, which includes two Gly to Ala mutations, would likely disrupt the conformation of the  $\alpha 3$  helix and loop observed to interact with acetyl-CoA (Yan et al. 2000). In contrast, the M2 mutation residues are predicted to make modest interactions with acetyl-CoA. Removing side-chains of Esa1p Gln312 and Arg313 would be expected to weaken the Esa1p/CoA interactions only slightly. We therefore pre-

dict that the mutant Sas2-M2 might retain weak acetylation activity that is sufficient for restoring silencing. This evidence is also supported by telomere silencing experiments (described below).

It was formally possible that the *sas2-M1* mutant did not rescue silencing because of a lack of protein expression or a failure to become incorporated into the SAS complex. To rule this out, the SAS complex was partially purified from strains (YJW275, 278–280) carrying wild-type and mutant His-Sas2p-Flag expression constructs. Anti-Flag Western blot of fractions from anion exchange and size exclusion columns indicated that the abundance and the size of the mutant SAS complexes were unchanged (data not shown).

To investigate the effect of the mutations in the con-



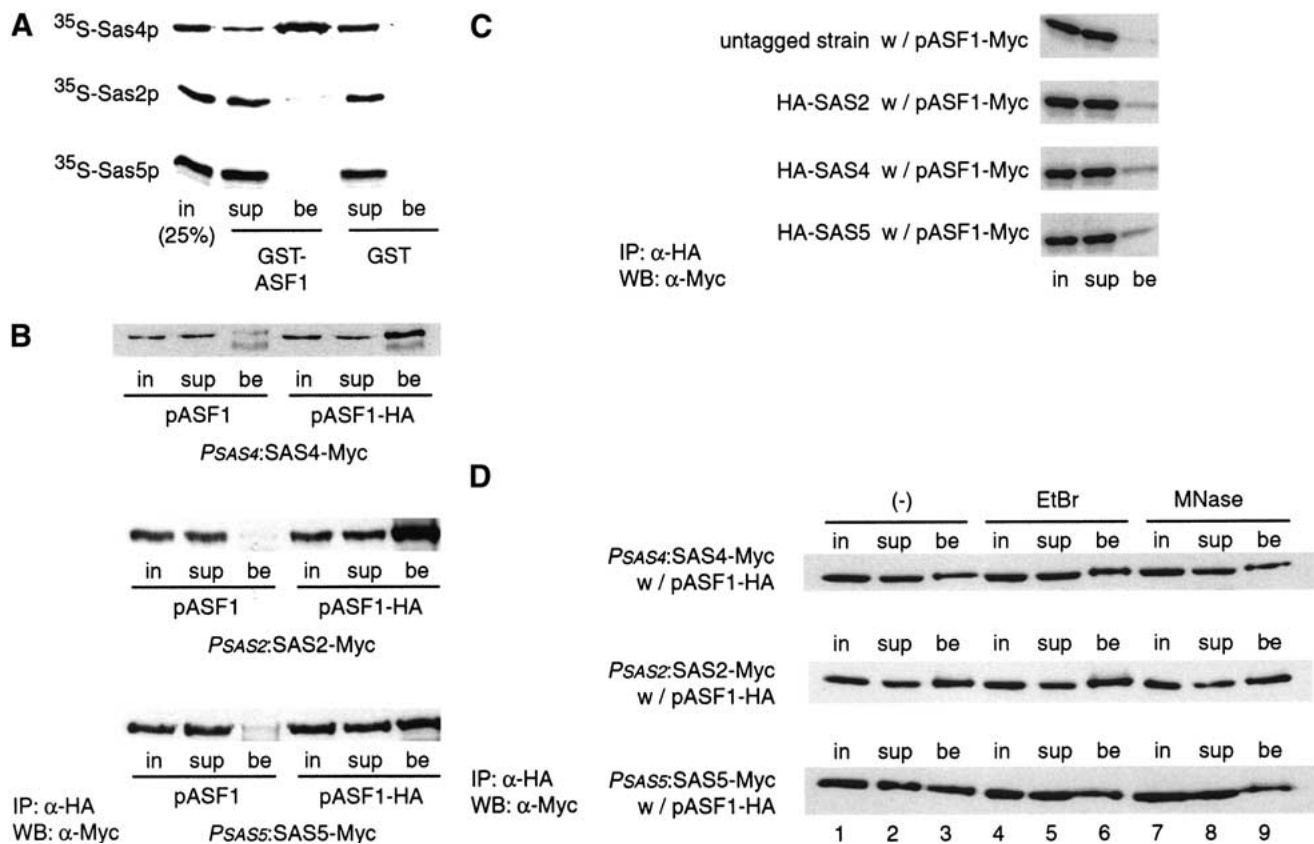
**Figure 5.** The conserved acetyl-CoA binding motif of Sas2p is required for *HML* and telomeric silencing. (A) Sequence comparison of acetyl-CoA binding motifs of Sas2p, Sas3p, and Esa1p. The positions of the mutated amino acid residues are shown. The residues of Esa1p involved in acetyl-CoA binding are indicated in gray, and underlined amino acids represent buried residues (Yan et al. 2000). (B) The effect of the SAS2 acetyl-CoA binding site mutations on silencing at *HML* was assayed by measuring mating efficiencies. Strains expressing wild-type and mutant alleles of SAS2 from CEN-based plasmids containing the SAS2 promoter (YJW348, YJW350, YJW352, YJW353, YJW354, and YJW355; ordered from left to right), 2 $\mu$ -based plasmids also containing the SAS2 promoter (YJW349, YJW351, YJW356, YJW357, YJW358, and YJW359), and 2 $\mu$ -based plasmids regulated by the *GAL10* promoter (YJW281, YJW282, YJW283, YJW284, YJW285, and YJW286) were patched onto plates lacking uracil and replica plated onto minimal medium spread with a lawn of *MAT $\alpha$*  cells. Quantitative mating analyses of the same strains are expressed as a percentage of diploids formed per viable cells. (C) To evaluate the expression of a telomere-proximal *URA3* gene, serial dilutions of saturated cultures were inoculated onto plates lacking leucine in the presence and absence of 5-FOA. Silencing was compared for the following strains (top to bottom): YJW324, YJW328, YJW330, YJW369, YJW370, YJW371, YJW326, YJW332, YJW334, YJW375, YJW376, and YJW377.

served acetyl-CoA binding site on telomeric silencing, plasmids containing wild-type and mutated SAS2 alleles were transformed into a *sas2* deletion strain containing the *URA3* gene inserted into the *ADH4* locus adjacent to an artificial telomere. The silencing efficiency was measured by 5-fluoroorotic acid (5-FOA) sensitivity. 5-FOA is toxic to *Ura*<sup>+</sup> cells. Lack of telomeric silencing, and thus expression of the *URA3* gene, results in sensitivity to 5-FOA, whereas strains that repress *URA3* transcription are resistant to 5-FOA. The wild-type strain containing the empty expression vector grew on 5-FOA plates because the *URA3* gene was repressed. In contrast, the *sas2* deletion strain was sensitive to 5-FOA, in agreement with a previous report by Reifsnyder et al. (1996). Although the plasmid containing the wild type and the innocuous mutant *sas2-M3* gene fully complemented the deletion of SAS2, the *sas2-M1* plasmid showed no detectable complementation activity and behaved like the empty vector. These results were obtained from both *SIR1* wild-type and *sir1* deletion strains. Interestingly, the mutant *sas2-M2* showed only partial restoration of silencing in *SIR1* cells but restored wild-type levels of silencing in *sir1* deletion strains. This is surprising because Sir1p does not play a major role in the silencing of telomeric reporter genes (Aparicio et al. 1991). Perhaps deletion of *SIR1* releases Sir2p, Sir3p, and Sir4p from the *HM* loci and thus improves telomeric silencing.

Taken together, the mating-type loci and telomere silencing experiments indicate that the conserved acetyl-CoA binding site in Sas2p is required for *HML* and telomeric silencing. The severity of the effect of different mutations correlates with their predicted effect on acetyl-CoA binding. These results suggest that the acetyltransferase activity of Sas2p is necessary for its function in the silencing in vivo.

#### *Asf1p* interacts with the SAS complex

Sas4p (amino acids 339–481) was found in a two-hybrid screen with chromatin assembly factor Asf1 (anti-silencing function 1; Sutton et al. 2001). *ASF1* was originally identified as a gene that derepresses transcriptional silencing when overexpressed (Le et al. 1997; Singer et al. 1998). *Drosophila* *ASF1* has been shown to be a component of the replication-dependent chromatin assembly factor (RCAF) complex (Tyler et al. 1999). Human Asf1p associates with acetylated histone H3 and H4, functioning as a histone chaperone for chromatin assembly in vitro (Munakata et al. 2000). To determine whether Asf1p interacts with Sas4p in vitro, GST pull-down assays were performed (Fig. 6A). <sup>35</sup>S-labeled Sas4p interacted with GST-Asf1p but not with GST alone. By comparison, <sup>35</sup>S-labeled Sas2p and Sas5p did not bind to GST-Asf1p. These results suggest that only Sas4p interacts with Asf1p in the absence of the other two subunits. To provide evidence that Asf1p interacts with Sas4p in vivo, immunoprecipitation experiments were performed (Fig. 6B). Whole cell extracts from strains expressing HA-tagged or nontagged Asf1p with Sas4-Myc protein were incubated with HA-antibody and precipitated. Immuno-



**Figure 6.** The SAS complex interacts with Asf1p. (A) Individually expressed  $^{35}\text{S}$ -labeled Sas proteins were incubated with GST or GST-Asf1 fusion proteins coupled to glutathione Sepharose beads. Proteins that remained bound to the beads fraction (be) were subjected to SDS-PAGE. The amount of input (in) and supernatant (sup) are equivalent to 25% of the reaction in the assay. (B) Coimmunoprecipitation experiments were performed with whole cell extracts prepared from the double-tagged strains expressing the Sas4p-Myc (*top*, YJW416 and YJW417), Sas2p-Myc (*middle*, YJW414 and YJW415), Sas5p-Myc (*bottom*, YJW418 and YJW419), and Asf1p or Asf1p-HA (indicated at the *bottom* of the panel). Whole cell extracts were incubated with anti-HA antibodies, and 0.5% of input (in), 0.5% of supernatant (sup), and bead (be) fractions from the immunoprecipitates were probed for the Myc-tagged proteins. (C) The HA-tagged Sas proteins coimmunoprecipitate Asf1p. The Myc-tagged ASF1p expression plasmid was transformed into the untagged strain and the chromosomally integrated HA-tagged SAS2, SAS4, and SAS5 strains (YJW457, YJW458, YJW459, and YJW460). Results are as described in B. (D) The interaction between the SAS complex and ASF1p is not mediated via DNA/chromatin in the extracts. Coimmunoprecipitation experiments were performed in untreated WCE (lanes 1–3), presence of ethidium bromide (EtBr) (lanes 4–6), or presence of MNase (lanes 7–9). Whole cell extracts were prepared from strains expressing the Sas4p-Myc (*top*, YJW417), Sas2p-Myc (*middle*, YJW415), Sas5p-Myc (*bottom*, YJW419). Results are as described in Figure 6B except that 1% of input and supernatant was loaded.

precipitated Sas4-Myc protein was only detected on the beads from the strain expressing HA-tagged Asf1p, indicating that Asf1p interacts with Sas4p at physiological levels. We have shown that Sas4p is a component of the SAS complex (Figs. 2–4). If Asf1p interacts with the SAS complex, Sas2p and Sas5p should also coprecipitate with Asf1p-HA. Immunoprecipitation experiments using Asf1p-HA and nontagged Asf1p showed that Asf1p-HA interacts with both the Sas2p-Myc and Sas5p-Myc (Fig. 6B). To provide further support for the Asf1-SAS complex interaction, reciprocal immunoprecipitation experiments were performed (Fig. 6C). For these experiments, a plasmid expressing Asf1p, containing a C-terminal Myc tag regulated by the native promoter, was transformed into strains harboring chromosomally integrated HA-Sas2p, HA-Sas4p, and HA-Sas5p (YJW458, YJW459, and

YJW460). As a control, Asf1p-Myc expression plasmid was transformed into the untagged strain (YJW457). Extracts from strains expressing untagged Sas proteins or HA-tagged Sas proteins were precipitated with HA-antibody, and coprecipitation of Asf1p was detected by anti-Myc Western blot analysis. Consistent with our previous findings, all of HA-tagged Sas proteins coimmunoprecipitated Asf1p-Myc. These results indicate that Asf1p interacts with the native SAS complex in vivo. The fraction of Myc-tagged Asf1p that coprecipitated with HA-Sas proteins is less than the fraction of Myc-tagged Sas proteins that coprecipitated with Asf1-HA (Fig. 6B,C). This indicates that only a fraction of Asf1p is associated with the SAS complex and is consistent with the fact that Asf1p is also found in other complexes (e.g., with Rad53) that function in processes independent of silenc-

ing (e.g., DNA repair; Le et al. 1997; Tyler et al. 1999; Emili et al. 2001; see below).

To rule out the possibility that the interaction between the SAS complex and ASF1 is mediated via DNA/chromatin, extracts were treated before immunoprecipitation with either ethidium bromide (EtBr) or micrococcal nuclease (MNase). These reagents disrupt DNA-protein interaction by either distorting DNA structure by intercalation (EtBr) or by degrading DNA (MNase). The efficiency of interaction between Asf1 and Sas proteins was not influenced by the addition of either EtBr or MNase (Fig. 6D), indicating that the interaction between the native SAS complex and Asf1p is not mediated via DNA/chromatin.

#### The effect of ASF1, SIR1, and SAS2 disruption on sensitivity to mutagens

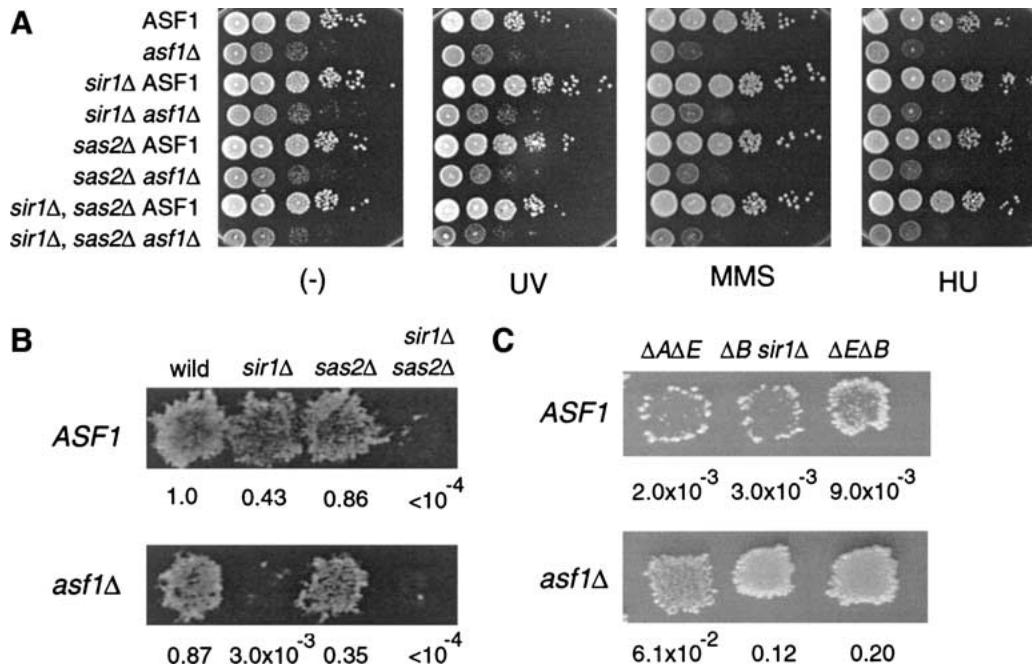
*asf1* mutants show multiple phenotypes, including slow growth, a weak sensitivity to ultraviolet light, a sensitivity to the DNA alkylating reagent methyl methane sulfonate (MMS), and a sensitivity to hydroxyurea (HU), a chemical inhibitor of ribonucleotide reductase that blocks DNA replication by impairing dNTP synthesis (Le et al. 1997; Tyler et al. 1999; Emili et al. 2001). The functional interaction between *ASF1* and *RAD53* (DNA damage checkpoint protein gene) may play a role in the

control of DNA repair (Emili et al. 2001; Hu et al. 2001). *ASF1* also contributes to gene silencing. Overexpression of *ASF1* reduces silencing at mating-type loci, telomeres, and ribosomal DNA (Le et al. 1997; Singer et al. 1998). Though the *asf1* mutant showed no significant defect in the silencing at mating-type loci and telomeres, deletion of *ASF1* in combination with a null allele of *CAC1* or *CAC2*, which encode subunits of chromatin assembly factor 1 (CAF-1), weakens gene silencing (Tyler et al. 1999; Sutton et al. 2001).

To test for a role of the SAS complex and Sir1p in DNA repair, we examined the effects of disruption of *ASF1* in combination with a null allele of *SIR1* and/or *SAS2* on sensitivity to mutagens (Fig. 7A). Although *asf1* mutants grow slowly, no additional effects on growth by disruption of *ASF1* in *sas2* and/or *sir1* deletion strains were observed. Mutation of *SIR1* and/or *SAS2* in both *ASF1* and *asf1* strains did not enhance the sensitivity to DNA damaging agents or the DNA replication inhibitor. These results indicate that *SIR1* and *SAS2* are not involved in *ASF1*-mediated DNA repair and DNA replication and suggest that interaction between the SAS complex and Asf1p is important for different *ASF1* functions.

#### *asf1* mutants affect HML and HMR silencing

To investigate whether Asf1p and the SAS complex function together, we tested the mating efficiencies of strains



**Figure 7.** DNA damage sensitivity and mating efficiencies of *asf1*, *sir1*, and *sas2* mutants. (A) Effects of disruption of *ASF1* and *SIR1* and/or *SAS2* on sensitivity to mutagens. Serial dilutions of wild type and mutant strains were spotted and grown on complete media in the absence (-), or presence of ultraviolet radiation (100 J/m<sup>2</sup>), MMS (0.006%), or HU (25 mM). Strains analyzed were W303-1a (wild type), YJW433 (*asf1*Δ), YJW252 (*sir1*Δ), YJW435 (*asf1*Δ *sir1*Δ), YJW253 (*sas2*Δ), YJW436 (*asf1*Δ *sas2*Δ), YJW258 (*sas2*Δ *sir1*Δ), and YJW437 (*asf1*Δ *sas2*Δ *sir1*Δ). (B) *ASF1* contributes to silencing at *HML*. Mating assays are shown with quantitative mating assay results given below. Strains shown are W303-1a (wild type), YJW252 (*sir1*Δ), YJW253 (*sas2*Δ), YJW258 (*sas2*Δ *sir1*Δ), YJW433 (*asf1*Δ), YJW435 (*asf1*Δ *sir1*Δ), YJW436 (*asf1*Δ *sas2*Δ), and YJW437 (*asf1*Δ *sas2*Δ *sir1*Δ). (C) Deletion of *ASF1* restores silencing to strains with mutations in *HMR-E*. Quantitative mating assay results are expressed relative to a value of 1.0 for wild-type W303-1b. Strains shown are YAB53 (ΔAΔE), YAB197 (ΔB*sir1*Δ), YGM75 (ΔEΔB), SY557 (*asf1*ΔΔAΔE), SY558 (*asf1*ΔΔB *sir1*Δ), and SY560 (*asf1*ΔΔEΔB).



harboring a null allele of *ASF1* in combination with null alleles of *SIR1* and/or *SAS2* (Fig. 7B). Similar to deletion of either *SIR1* or *SAS2*, deletion of *ASF1* results in a very slight reduction in silencing at *HML*, as indicated by quantitative mating analysis. On the other hand, the mating ability of a *asf1 sir1* double mutant was three orders of magnitude less than that of wild type or single deleted strains and was almost the same as that of double *sas2 sir1* or triple *asf1 sas2 sir1* deletion strains. A strain containing null alleles of both *ASF1* and *SAS2* was only slightly more defective than strains individually deleted for these genes. These results suggest that both Asf1p and the SAS complex function in a pathway that enhances the epigenetic silencing defects of *sir1* mutants.

In contrast to their effect on silencing at *HML*, deletions of *SAS2*, *SAS4*, and *SAS5* improve silencing at an *HMR* locus that has mutations in E silencer elements (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997; Xu et al. 1999a). We tested the effect of deletion of *ASF1* on *HMR* silencing by measuring the mating ability of three *MAT $\alpha$*  strains with mutated *HMR*-E elements (Fig. 7C). In all three cases, loss of *ASF1* resulted in a substantial improvement of mating, as has been previously reported for the *SAS* genes.

## Discussion

We identified and characterized a SAS complex that is ~450 kD in size and contains Sas2p, Sas4p, and Sas5p. There are several lines of evidence that lead us to this conclusion. First, size exclusion chromatography showed that complexes containing Sas4p and Sas5p are similar in size to the protein complex containing Sas2p (Fig. 2A). Second, deletion of *SAS4* and/or *SAS5* alters the size of the Sas2 complex (Fig. 2B). Third, Sas4p and Sas5p coimmunoprecipitated with Sas2p (Fig. 3). Fourth, a highly purified Sas2 complex contains Sas4p and Sas5p (Fig. 4B). Finally, the mass spectrometry data confirmed that Sas4p and Sas5p are components of the Sas2 complex (Fig. 4C). The existence of Sas2p, Sas4p, and Sas5p in the same complex explains recent studies, which have showed that the *SAS2*, *SAS4*, and *SAS5* have similar functions in silencing of *HML* (Xu et al. 1999b).

In addition to the characterization of the subunits of the SAS complex, we have shown that the conserved acetyl-CoA binding site in Sas2p is required for *HML* and telomeric silencing (Fig. 5). This strongly suggests that acetyltransferase activity of the SAS complex is critical for silencing in vivo. However, the substrate of the SAS complex is currently unknown. We could not detect any HAT activity in partially or highly purified preparations of the SAS complex using free or nucleosomal histones (data not shown). Although these are negative results, they do suggest that the substrate of Sas2 might be proteins other than histones. We have provided strong evidence that the acetyl-CoA binding domain is essential for the silencing function of Sas2p in vivo. The identification of the substrate of the SAS complex will enable us to address the silencing mechanism mediated by the Sas2-dependent acetylation.

## *SAS complex links DNA replication and silencing to chromatin modification*

It has been shown that the restoration of silencing at the *HMR* locus with a mutated silencer by a *sas2* mutant required Orc2p and Orc5p, which are subunits of the origin recognition complex (ORC; Ehrenhofer-Murray et al. 1997). ORC is an initiator complex for DNA replication but also affects transcriptional silencing (Dutta and Bell 1997). HBO1 (histone acetyltransferase binding to ORC) is a member of the MYST acetyltransferases family and has been identified as an interaction partner of the human ORC1 protein (Iizuka and Stillman 1999). HBO1 also interacted with the androgen receptor and a transcriptional repression domain of HBO1 was identified (Sharma et al. 2000). Genetic interactions between *SAS2* and ORC-encoding genes suggest that HBO1 might be functionally related to the Sas2 protein. Thus, the Sas2 complex might play a key role in linking DNA replication and gene silencing to chromatin modification.

## *Sas5p is a tf2f domain-containing protein*

It remains unclear how *SAS4* and *SAS5* mediate *SAS2*-dependent silencing. Sas4p and Sas5p might be essential for the structural integrity of the SAS complex, because deletion of the *SAS4* and/or *SAS5* genes altered the size of the SAS complex. Sas4p has no obvious homology with other known proteins. A BLAST search (Altschul et al. 1997) reveals that an N-terminal stretch of ~100 amino acids in Sas5p has homology with proteins that have been identified in *S. cerevisiae*, *S. pombe*, *Caenorhabditis elegans*, *Drosophila*, *Arabidopsis*, and humans. This domain is ~60% homologous in all these proteins and is registered as the tf2f domain in the NCBI Conserved Domain Database. These tf2f domain-containing proteins include yeast TAF<sub>II</sub>30p. The prototype of this family, TAF<sub>II</sub>30, is a component of at least five yeast transcription-related complexes, NuA3, TFIID, TFIIF, SRB-mediator complex, and SWI/SNF (Henry et al. 1994; Cairns et al. 1996; Moqtaderi et al. 1996; Gustafsson et al. 1998; John et al. 2000). It is likely that all members of the tf2f family are involved in chromatin modification/remodeling processes. However, the exact role of these proteins in chromatin modification/remodeling is unclear. Deletion of *TAF<sub>II</sub>30* does not alter the structural integrity or the catalytic activity of the SWI/SNF complex (Cairns et al. 1996). Similarly, the deletion of *TAF<sub>II</sub>30* results in modest decreases in NuA3 HAT activity and does not significantly alter the size of NuA3 (John et al. 2000). The Sas5p tf2f domain is similar to that in the human GAS41, ENL, and AF-9. GAS41 has been found to be amplified in low-grade gliomas (Fischer et al. 1997). Recently, GAS41 was also identified as a binding partner of NuMA, a component of the nuclear matrix in interphase cells (Harborth et al. 2000). The interaction between NuMA and GAS41 may provide a link between nuclear architecture and gene expression. Human ENL and AF-9 proteins are implicated in human acute leukemia, because *ENL* and *AF-9* were found fused

**Table 1.** Yeast strains

Strain	Genotype	Source
W303-1a <sup>a</sup>	<i>MATaade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100</i>	A. Sachs
YJW213	<i>aHis3MX6:PGAL1-3HA-SAS2</i>	
YJW214	<i>aSAS2-3HA:His3MX6</i>	
YJW219	<i>akanMX6:PGAL1-3HA-SAS4</i>	
YJW220	<i>akanMX6:PGAL1-3HA-SAS5</i>	
YJW228	<i>aSAS4-13Myc:kanMX6</i>	
YJW229	<i>aSAS5-13Myc:kanMX6</i>	
YJW230	<i>aHis3MX6:PGAL1-3HA-SAS4-SAS4-13Myc:kanMX6</i>	
YJW231	<i>aHis3MX6:PGAL1-3HA-SAS2 SAS5-13Myc:kanMX6</i>	
YJW243	W303-1a [pESC/URA (pS99)]	
YJW252	JRY4622 ( <i>a sir1Δ::LEU2</i> )	D. Rivier
YJW253	DRY1655 ( <i>a sas2Δ::TRP1</i> )	D. Rivier
YJW254	DRY1656 ( <i>a sas4Δ::kan</i> )	D. Rivier
YJW255	DRY1657 ( <i>a sas5Δ::HIS3</i> )	D. Rivier
YJW258	DRY1658 ( <i>a sir1Δ::LEU2 sas2-Δ1::TRP1</i> )	D. Rivier
YJW263	DRY1663 ( <i>a sas4Δ::kanMX4 sas5Δ::HIS3</i> )	D. Rivier
YJW265	<i>a SAS2-13Myc:His3MX6</i>	
YJW269	<i>a SAS2-13Myc:His3MX6 sas4Δ::kan</i>	
YJW270	<i>a SAS2-13Myc:kanMX6 sas5Δ::HIS3</i>	
YJW271	<i>a SAS2-13Myc:TRP1 sas4Δ::kanMX4 sas5Δ::HIS3</i>	
LPY1382	<i>a sas2Δ::TRP1</i>	L. Pillus
YJW275	LPY1382 [pESC/URA/PGAL10-6xHIS-SAS2-FLAG (pS116)]	
YJW276	YJW228 [pESC/URA/PGAL10-6xHIS-SAS2-FLAG (pS116)]	
YJW278	LPY1382 [pESC/URA/PGAL10-6xHIS-SAS2-M1-FLAG (pS117)]	
YJW279	LPY1382 [pESC/URA/PGAL10-6xHIS-SAS2-M2-FLAG (pS118)]	
YJW280	LPY1382 [pESC/URA/PGAL10-6xHIS-SAS2-M3-FLAG (pS119)]	
YJW281	YJW252 [pESC/URA (pS99)]	
YJW282	YJW258 [pESC/URA (pS99)]	
YJW283	YJW258 [pESC/URA/PGAL10-6xHIS-SAS2-FLAG (pS116)]	
YJW284	YJW258 [pESC/URA/PGAL10-6xHIS-SAS2-M1-FLAG (pS117)]	
YJW285	YJW258 [pESC/URA/PGAL10-6xHIS-SAS2-M2-FLAG (pS118)]	
YJW286	YJW258 [pESC/URA/PGAL10-6xHIS-SAS2-M3-FLAG (pS119)]	
YJW348	YJW252 [pRS416/CEN/URA (pS15)]	
YJW349	YJW252 [pRS426/2μ/URA (pS23)]	
YJW350	YJW258 [pRS416/CEN/URA (pS15)]	
YJW351	YJW258 [pRS426/2μ/URA (pS23)]	
YJW352	YJW258 [pRS416/CEN/URA/PSAS2-SAS2 (pS126)]	
YJW353	YJW258 [pRS416/CEN/URA/PSAS2-SAS2-M1 (pS136)]	
YJW354	YJW258 [pRS416/CEN/URA/PSAS2-SAS2-M2 (pS137)]	
YJW355	YJW258 [pRS416/CEN/URA/PSAS2-SAS2-M3 (pS138)]	
YJW356	YJW258 [pRS426/2μ/URA/PSAS2-SAS2 (pS127)]	
YJW357	YJW258 [pRS426/2μ/URA/PSAS2-SAS2-M1 (pS142)]	
YJW358	YJW258 [pRS426/2μ/URA/PSAS2-SAS2-M2 (pS143)]	
YJW359	YJW258 [pRS426/2μ/URA/PSAS2-SAS2-M3 (pS144)]	
YJW414	YJW265 [pRS426/2μ/URA3/ASF1 (pASB89)]	
YJW415	YJW265 [pRS426/2μ/URA3/ASF1-3HA (pASB90)]	
YJW416	YJW228 [pRS426/2μ/URA3/ASF1 (pASB89)]	
YJW417	YJW228 [pRS426/2μ/URA3/ASF1-3HA (pASB90)]	
YJW418	YJW229 [pRS426/2μ/URA3/ASF1 (pASB89)]	
YJW419	YJW229 [pRS426/2μ/URA3/ASF1-3HA (pASB90)]	
YJW433	<i>aasf1Δ::HIS3</i>	
YJW435	<i>aasf1Δ::HIS3 sir1Δ::LEU2</i>	
YJW436	<i>aasf1Δ::HIS3 sas2Δ::TRP1</i>	
YJW437	<i>aasf1Δ::HIS3 sas2Δ::TRP1 sir1Δ::LEU2</i>	
YJW457	W303-1a [pRS424/2μ/TRP1/PASF1-ASF1-9Myc (pASB92)]	
YJW458	YJW213 [pRS424/2μ/TRP1/PASF1-ASF1-9Myc (pASB92)]	
YJW459	YJW219 [pRS424/2μ/TRP1/PASF1-ASF1-9Myc (pASB92)]	
YJW460	YJW220 [pRS424/2μ/TRP1/PASF1-ASF1-9Myc (pASB92)]	
W303-1b <sup>b</sup>	<i>MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100</i>	R. Rothstein
YGM75	<i>hmrΔEΔB</i>	G. Micklem
SY560	<i>hmrΔEΔB asf1::His5+</i>	

**Table 1.** (Continued)

Strain	Genotype	Source
YAB53	<i>hmrΔAΔE</i>	A. Brand
SY557	<i>hmrΔAΔE asf1::His5<sup>+</sup></i>	
YAB197	<i>hmrΔB sir1::LEU2</i>	A. Brandt
SY558	<i>hmrΔB sir1::LEU2 asf1::His5<sup>+</sup></i>	
YJW251 <sup>c</sup>	JRY2728 <i>MATα his4</i>	D. Rivier
AY1281 <sup>c</sup>	<i>MATα his1</i>	K. Arndt
AY1283 <sup>c</sup>	<i>MATα his1</i>	K. Arndt
YJW287 <sup>d</sup>	LPY917/UCC1001 ( <b>a</b> <i>TEL(VIIL) adh4::URA3 ade2-101<sup>oc</sup> his3-Δ200 leu2-Δ1 lys2-801<sup>am</sup> trp1-Δ1 ura3-52</i> )	L. Pillus
YJW288	LPY1285 <b>a</b> <i>sir1Δ::HIS3</i>	L. Pillus
YJW289	LPY2058 <b>a</b> <i>sas2Δ::TRP1</i>	L. Pillus
YJW290	LPY2062 <b>a</b> <i>sas2Δ::TRP1 sir1Δ::HIS3</i>	L. Pillus
YJW324	YJW287 [pRS425/2μ/LEU (pS20)]	
YJW326	YJW288 [pRS425/2μ/LEU (pS20)]	
YJW328	YJW289 [pRS425/2μ/LEU (pS20)]	
YJW330	YJW289 [pRS425/2μ/LEU/PSAS2-SAS2 (pS123)]	
YJW332	YJW290 [pRS425/2μ/LEU (pS20)]	
YJW334	YJW290 [pRS425/2μ/LEU/PSAS2-SAS2 (pS123)]	
YJW369	YJW289 [pRS425/2μ/LEU/PSAS2-SAS2-M1 (pS139)]	
YJW370	YJW289 [pRS425/2μ/LEU/PSAS2-SAS2-M2 (pS140)]	
YJW371	YJW289 [pRS425/2μ/LEU/PSAS2-SAS2-M3 (pS141)]	
YJW375	YJW290 [pRS425/2μ/LEU/PSAS2-SAS2-M1 (pS139)]	
YJW376	YJW290 [pRS425/2μ/LEU/PSAS2-SAS2-M2 (pS140)]	
YJW377	YJW290 [pRS425/2μ/LEU/PSAS2-SAS2-Me (pS141)]	

<sup>a</sup>Strains below are isogenic with W303-1a.

<sup>b</sup>Strains below are isogenic with W303-1b.

<sup>c</sup>YJW251, AY1281, and AY1283 are lawn tester strains for mating assays.

<sup>d</sup>Strains below are isogenic with YJW287.

to the *ALL-1* gene in leukemias with translocations (Tkachuk et al. 1992; Negrini et al. 1993). Further analysis of the function of Sas5p in Sas2p-mediated silencing might help us understand how human *tf2f* proteins such as ENL, AF-9, and GAS41 contribute to tumorigenesis in humans.

#### *ASF1 and SAS2 enhance the silencing defect at HML in a sir1 mutant*

We showed that chromatin assembly factor Asf1p interacts with components of the SAS complex in vivo. Individually, *asf1* mutants do not show significant derepression of silencing at mating-type loci (Le et al. 1997; Singer et al. 1998; Tyler et al. 1999), but the double *asf1 sir1* deletion strain displayed a much more severe silencing defect at *HML* than did deletion of either gene alone. This phenotype of *asf1* is similar to that which is observed for *sas* genes on silencing at *HML* (Xu et al. 1999b). Furthermore, *asf1* mutations, like *sas* mutations, restored silencing at mutated *HMR* silencers. These genetic results suggest that *ASF1* and *SAS2/4/5* function in the same pathway to regulate silencing at *HML* and *HMR*. Although *asf1*, *sas2*, *sas4*, and *sas5* mutants have very similar silencing phenotypes at *HML* and *HMR*, they have drastically different properties with respect to telomeric silencing. Although *asf1* mutants show little or no telomeric silencing defect (Singer et al. 1998), *sas2*, *sas4*, and *sas5* mutants are totally defective in telomeric silencing (Reifsnnyder et al. 1996).

Nucleosome assembly may be important to generate the silent state, because deletion of CAF-1 subunits leads to a significant decrease in silencing at telomeres and a subtle weakening of silencing at *HML* and *HMR* (Enomoto and Berman 1998). *CAC1* encodes the largest subunit of CAF-1 that assembles newly synthesized histones onto replicated DNA. A *cac1* mutation enhanced the mating defect of a *sir1* deletion strain. CAF-1 contributes to the maintenance, but not the re-establishment, of silencing at the mating loci (Enomoto and Berman 1998). Mutations in the genes for either chromatin assembly factor, CAF-1 or Asf1p, enhance the silencing defect of *sir1* mutants. *ASF1* function also might be involved in the maintenance of silent mating type genes. Because *ASF1* and *SAS2/4/5* genetically function in the same pathway to repress the *HML* locus, the interaction between the SAS complex and Asf1p might have a role for maintenance.

#### Materials and methods

##### *Yeast strains and manipulations*

Strains used in this study are described in Table 1. All of the chromosomal integrated tagged-strains were generated by the one-step PCR-mediated technique as described elsewhere (Longtine et al. 1998). The template plasmids were kindly provided by Dr. Peter Philippsen (Institut für Angewandte Microbiologie, Biozentrum, Universität Basel, Switzerland). A DNA fragment used to replace the *ASF1* open reading frame (ORF) with the *S. pombe his5<sup>+</sup>* gene was synthesized by PCR using

synthetic oligonucleotides and the plasmid pME3 as described (Wach et al. 1997). Standard yeast manipulations were performed as described (Guthrie and Fink 1991).

#### Plasmid construction

The C-terminal Flag-tagged Sas2p, Sas4p, and Sas5p expression vectors, pS100, pS120, and pS104, were constructed by fusing the *SAS2*, *SAS4*, and *SAS5* ORFs, which were amplified by PCR, and the products were subcloned into the *SpeI* site of the Flag-tagged protein expression vector pESC/URA (pS99; Stratagene). For the N-terminal 6xHis-tagged and the C-terminal Flag-tagged Sas2p, Sas4p, and Sas5p expression vectors pS116, pS128, and pS129, the double-stranded oligonucleotide containing 6xHis-tagged sequence was ligated into the *NotI* site of pS100, pS120, and pS104. Plasmids expressing Sas2p regulated by the endogenous promoter were generated by PCR amplification of the *SAS2*-coding sequences along with 1 kb upstream region sequence from yeast genomic DNA (pS123, pS126, pS127). The amplified product was cloned into pRS416 (pS15), pRS425 (pS20), and pRS426 (pS23). All fragments generated by PCR were verified by sequencing. Mutations within the putative acetyl-CoA binding site of *SAS2* (pS117–119, pS136–pS144) were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) following manufacturer's protocols, and mutations were confirmed by sequencing. ASB90 (*ASF1*-[HA]<sub>3</sub> in pRS426) was created as follows. First, PCR mutagenesis was used to replace the stop codon at the 3' end of the *ASF1* ORF in pLS67 (*ASF1* in pUC18) with a *NotI* site to create pASB31. A fragment encoding a triple repeat of the HA epitope flanked by *NotI* restriction sites was inserted into the *NotI* site of pASB31 to create pASB35. An *XbaI*-*EcoRI* fragment encoding the C terminus of Asf1p in plasmid pLS27 (full length *ASF1* in YCp50) was replaced with an *XbaI*-*EcoRI* fragment from pASB35 to create pASB41 (*ASF1*-[HA]<sub>3</sub> in YCp50). A *SalI*-*EcoRI* fragment from pASB41 containing *ASF1*-[HA]<sub>3</sub> was cloned into pRS426 to create ASB90. ASB92 (*ASF1*-[Myc]<sub>9</sub>) was created as follows. A *NotI* fragment containing 13 tandem copies of the Myc epitope was inserted into the *NotI* site of ASB31 so that the Myc tag was in-frame with the C terminus of Asf1p. A *NdeI* fragment that encodes the Myc-tagged C terminus of Asf1p was then used to replace the sequences for the untagged C terminus of *ASF1* in pRS424. ASB89 (*ASF1* in pRS426) was created by cloning a *Bam*HI-*Hind*III fragment containing *ASF1* from pLS27 into pRS426. ASB92 *ASF1*[Myc]<sub>9</sub> in pRS424. To create the GST-Asf1p fusion protein expression vector (pS173), the ORF of *ASF1* gene was subcloned into the *Bam*HI and *EcoRI* sites of the glutathione S-transferase (GST) expression vector pGEX-2T (Amersham-Pharmacia).

#### Purification of the SAS complex

Whole cell extracts were prepared essentially as a previously published procedure (Eberharter et al. 1998). Approximately 4 mg and 0.4 mg of whole cell extracts were loaded onto a 24-mL Superose 6 HR 10/30 and a 2.4-mL Superdex 200 PC 3.2/30 column (Amersham-Pharmacia), respectively, equilibrated in buffer C (40 mM HEPES at pH 7.8, 500 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL leupeptin, and 0.5 μg/mL pepstatin A). For Figure 1, whole cell extracts from two liters of SAS2-HA strain (YJW214) were mixed with 1.6 mL of Ni<sup>2+</sup>-NTA agarose (QIAGEN) overnight. The unbound fraction was loaded onto a 20-mL MonoQ HR 16/10 column (Amersham-Pharmacia). The bound fraction was eluted in a 25-column-volume gradient from 0.1 M to 0.5 M NaCl in buffer B (50 mM Tris at pH 8.0, 10% glycerol, 0.1%

Tween 20, and protease inhibitors described above). Fractions containing Sas2-HA were concentrated over a 1-mL MonoQ HR 5/5 column (Amersham-Pharmacia) using the same gradient as described above. The Sas2-HA fractions were subjected to gel filtration by a Superose 6. The fractions were precipitated with TCA before gel electrophoresis. All gel filtration columns were calibrated using a high-molecular-weight filtration calibration kit (Amersham-Pharmacia).

For mass spectrometry analyses, whole cell extracts were prepared from 12 L of yeast strain YJW276. The extracts were bound to 10 mL of Ni<sup>2+</sup>-NTA agarose resin overnight. Then resin was washed with the extraction buffer (40 mM HEPES at pH 7.5, 350 mM NaCl, 10% glycerol, 0.1% Tween-20, and protease inhibitors) and 20 mM imidazole solution containing 100 mM NaCl, 10% glycerol, 0.1% Tween-20, and protease inhibitors described above. Bound proteins were eluted with 30 mL of 300 mM imidazole solution. The imidazole eluate was loaded onto a 1-mL MonoQ column. Retained proteins were eluted in a 10-column-volume gradient from 0.1 M to 0.8 M NaCl in buffer B. Dithiothreitol (DTT) and anti-Flag M2 affinity resin was added to the fraction containing Sas4p-Myc. Beads were washed extensively with buffer B containing 0.5 M NaCl and 1 mM DTT, and bound proteins were eluted with buffer B containing 0.5 M NaCl, 1 mM DTT and 0.7 mg/mL Flag peptide (Sigma). The eluate was subjected to a 2.4-mL Superdex 200 PC 3.2/30 gel filtration column equilibrated in the buffer C.

#### Western blotting and antibodies

Ten microliters of each fraction were electrophoresed on a SDS-PAGE gel, transferred to nitrocellulose, and detected by ECL Western blotting analysis detection system (Amersham-Pharmacia). Anti-Myc (clone 9E10, Boehringer), horseradish peroxidase-conjugated anti-HA (clone 3F10, Boehringer), and anti-Flag M2 (Sigma) antibodies were used.

#### Immunoprecipitations and GST pull-down assays

One microliter of the anti-HA antibody (clone 16B12, Covance) was mixed with 400 μg of whole cell extracts or 72 μL of Superose 6 fractions that were diluted to appropriate salt concentration of 150 mM NaCl with buffer and incubated on ice for 8 h. Twenty microliters of protein G-Sepharose beads were added to the sample and incubated at 4°C for 8 h. In some experiments, ethidium bromide (EtBr) was added (50 μg/mL), and the extracts were incubated on ice for 30 min. Precipitates were removed by centrifugation at 4°C for 10 min, and the supernatant was used for immunoprecipitation experiments. For micrococcal nuclease (MNase) treatment, extracts were treated with 0.4 units of MNase, 0.5 mM CaCl<sub>2</sub>, and 0.5 mM MgCl<sub>2</sub> at 27°C for 10 min. Beads were washed with extraction buffer or Buffer C containing 150 mM NaCl before gel electrophoresis.

GST fusion proteins were expressed in *Escherichia coli* as described by the manufacturer (Amersham-Pharmacia) and cross-linked to glutathione sepharose 4B with dimethylpiperimidate (Harlow and Lane 1988). The N-terminal 6xHis-tagged and the C-terminal Flag-tagged Sas2, Sas4, and Sas5 proteins were individually expressed from pS116, pS128, and pS129 with T3 polymerase using TNT-coupled reticulocyte lysate system (Promega). Five microliters of the reticulocyte lysate reaction containing [<sup>35</sup>S]methionine-labeled Sas proteins were incubated in buffer B containing 350 mM NaCl at 4°C overnight with GST-fusion proteins cross-linked to glutathione Sepharose 4B. Beads were washed with buffer B containing 350 mM NaCl before gel electrophoresis.

### Mass spectrometry

The Sas2 complex fraction was electrophoresed through a 4% to 15% SDS–polyacrylamide gradient gel. Silver-stained protein bands were excised and digested in gel trypsin. Proteins were identified by microcolumn high-performance liquid chromatography electrospray ionization tandem mass spectrometry and database searching (Grant et al. 1998).

### Mating and telomeric silencing assays

Mating assays were performed as described previously (Sprague 1991). The patches from the strains containing pRS416- and pRS426-based plasmids were replica plated to a lawn of  $\alpha$ -cells (AY1283) grown on dextrose plates. Galactose plates were used for mating assays with the strains carrying the pESC plasmids. Quantitative mating assay was performed as described previously (Ehrenhofer-Murray et al. 1997). Serial dilutions of test strains were mixed with  $1.0 \times 10^7$  cells of a MAT $\alpha$  lawn (AY1283 or YJW251) or a MATa lawn (AY1281) and plated onto selective medium and rich medium to determine the number of viable cells. For the experiments in Figure 5, mating efficiency was expressed as a percentage of diploid cells formed per viable cells. For the experiments in Figure 7, mating efficiency was expressed as the number of diploids formed per viable cells and was normalized to the efficiency of an isogenic wild-type strain. Mating efficiencies are the average of three independent experiments. For the telomeric silencing assay, three microliters of 10-fold serial dilutions of the saturated cultures were spotted on plates lacking leucine (for plasmid selection) and 5-FOA containing plates.

### Acknowledgments

We thank members of the Workman, Simpson, and Reese Laboratories at Penn State for valuable discussions. We are also grateful to Peter Philippsen for plasmids, Lorraine Pillus for providing the LPY strains, and David H. Rivier for a generous gift of DRY strains. We thank Song Tan for structure analysis of the HAT domain mutants, Jean Bucaria for excellent technical assistance, and Thomas Kusch for useful advice and critical reading of the manuscript. We also thank Tsutomu Nishihara for encouragement of this work. Support for this work was provided by grants from NIGMS to J.L.W. and to R.S., and NCCR Yeast Center Grant RR11823 to J.Y.R. C.E.B. is a Leukemia and Lymphoma Society Postdoctoral Fellow. J.L.W. is an Associate Investigator of the HHMI.

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