Chromatin assembly factor Asf1p-dependent occupancy of the SAS histone acetyltransferase complex at the silent mating-type locus \textit{HML}\textsubscript{a}

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ABSTRACT

Transcriptional repression of the silent mating-type loci \textit{HML}\textsubscript{a} and \textit{HMR}\textsubscript{a} in \textit{Saccharomyces cerevisiae} is regulated by chromatin structure. Sas2p is a catalytic subunit of the SAS histone acetyltransferase (HAT) complex. Although many HATs seem to relieve chromosomal repression to facilitate transcriptional activation, \textit{sas} mutant phenotypes include loss of \textit{SIR1}-dependent silencing of \textit{HML}\textsubscript{a}. To gain insight into the mechanism of the SAS complex mediated silencing at \textit{HML}\textsubscript{a}, we investigated the expression and chromatin structure of the \textit{a2} gene in the \textit{HML}\textsubscript{a} locus. We found that deletion of \textit{SAS2} in combination with a null allele of \textit{SIR1} changed the chromatin structure of the precisely positioned nucleosome, which includes the mRNA start site of the \textit{a2} gene and derepressed \textit{a2} transcription. The Sas2p HAT domain was required for this silencing. Furthermore, chromatin immunoprecipitation analysis revealed that the SAS complex was associated with the \textit{HML}\textsubscript{a} locus, and \textit{ASF1} (which encodes chromatin assembly factor Asf1p), but not \textit{SIR1} and \textit{SIR2}, was necessary for this localization. These data suggest that the HAT activity and \textit{ASF1}-dependent localization of the SAS complex are required for \textit{SIR1}-dependent \textit{HML}\textsubscript{a} silencing.

INTRODUCTION

Silencing affects gene repression in a regional rather than promoter- or sequence-specific manner (1). The structure of the chromatin itself can affect gene expression, and changes in chromatin structure can result from the modification of histone tails as well as from the action of chromatin-remodeling complexes (2). A number of factors have been identified that contribute to transcriptional regulation by covalent modification of histones. In many cases, a relationship between histone acetylation and gene activation has been revealed by the identification of transcriptional co-activators, such as dedicated histone acetyltransferases (HATs) (3,4). Histone acetylation is reversed by histone deacetylases (HDACs), and many repression phenomena are regulated by HDACs (5). Moreover, HATs have been shown to contribute to repression and activation (4).

In \textit{Saccharomyces cerevisiae}, silenced loci include the \textit{HML}\textsubscript{a} and \textit{HMR}\textsubscript{a} mating-type loci, the telomere regions and the ribosomal DNA repeats (2). A variety of proteins, including the silent information regulator (Sir) proteins, are required to silence the mating information genes at silent loci (6). One of the Sir proteins, Sir2p, possesses HDAC activity important for silencing (7,8). Disruption of the \textit{SIR2}, \textit{SIR3} or \textit{SIR4} gene results in loss of silencing at \textit{HM} loci, and a \textit{SIR1}-disrupted strain is composed of mixed populations of silenced and unsilenced cells (9).

Something About Silencing (\textit{SAS}) 2 was identified as an enhancer of \textit{sir1} epigenetic \textit{HML}\textsubscript{a} silencing defects (10). Sas2p is a member of the MYST (MOZ, Ybf2/Sas3, Sas2 and TIP60) family of HATs and forms a complex, termed the SAS complex, with Sas4p and Sas5p (11–14). The SAS complex mainly acetylates histone H4 at lysine 16 (12–16). The role of SAS2 in silencing is different for each silenced locus. At \textit{HML}\textsubscript{a}, deletion of \textit{SAS2} has very little effect on silencing. However, deletion of \textit{SAS2} combined with deletion of \textit{SIR1} causes a severe silencing defect (10,17). Normal \textit{HMR}\textsubscript{a} silencing is unaffected by \textit{SAS2} deletion, but \textit{sas2} mutation suppresses the silencing defect caused by mutations in silencer elements of \textit{HMR}\textsubscript{a} (10,17). To determine the role of \textit{SAS2} in rDNA silencing, a strain in which the \textit{URA3} gene is integrated at the rDNA locus was used. The \textit{sas2} deletion strain showed more effective \textit{URA3} repression, indicating that the deletion of \textit{SAS2} increased rDNA repression (12). In the case of telomeres, loss of \textit{SAS2} causes hypoacetylation in adjacent sub-telomeric regions, leading to the recruitment of...
Sir3p to these regions and inactivation of gene expression (15,16). Therefore, sas2 mutations reduce silencing of HMLα (when combined with deletion of Sir1) but improve silencing at HMRα when accompanied by a weakened silencer element, hypoacetylated sub-telomeric regions, or the rDNA locus. To understand the differing roles of Sas2p, it is important to investigate the biochemical mechanisms by which these factors regulate gene silencing in each silent locus.

We previously showed that the chromatin assembly factor Asf1p interacts with the SAS complex, and ASF1 and SAS2 functionally in the same pathway to repress the hMLα locus (11). In the present study, we show that SAS2 is essential for the organization of the chromatin structure at HMLα in a sir1 mutant and that ASF1 is required for the recruitment of the SAS complex to the HMLα locus.

**MATERIALS AND METHODS**

**Yeast strains, plasmids and manipulations**

The strains used in this study are listed in Table 1 and were either previously published or were created for this study by using standard yeast manipulations (18,19). Expression plasmids of wild-type and mutant alleles of SAS2 and ASF1 were described previously (11). Mating assays were performed as described previously (11,20).

**RNA blots**

A 40 μg aliquot of total RNA prepared from logarithmically growing cells was separated on 1% agarose–formaldehyde gels and transferred to Hybond-N+ membranes (Amersham Biosciences, Piscataway, NJ). Specific messages were detected using randomly labeled α2 and SCR1 probes.

**High-resolution micrococcal nuclease mapping**

Preparation of nuclei was carried out as described previously (21,22). Briefly, nuclei were isolated from yeast cells, which were grown to mid-log phase (OD600 = 1). The nuclear pellet from 1 liter culture was resuspended in 2.4 ml digestion buffer (10 mM HEPES, pH 7.5, 0.5 mM MgCl2 and 0.05 mM CaCl2). The suspension was divided into 400 μl portions, each of which was digested at 37°C for 10 min by using increasing concentrations (0–16 U/ml) of micrococcal nuclease (MNase; Amersham Biosciences). The reaction was terminated by adding EDTA, and the DNA was purified after treatment with RNase, protease K digestion and phenol–chloroform extraction. The purified DNA was resuspended in 0.1x TE (1 mM Tris–HCl, pH 8.0, 0.1 mM EDTA). MNase cleavage sites were detected by multiple rounds of Taq DNA polymerase-based primer extension. The primer (5’-TATGCTGTTAGT-GCTGATTAAAAACTCAT-3’) was end-labeled by T4 polynucleotide kinase. The cycling program was 94°C for 1 min, 53°C for 2 min and 72°C for 2 min for 35 cycles, and was followed by a 10 min chase at 72°C. The products were electrophoresed on a 6% polyacrylamide–8 M urea gel. The gel was dried and used to expose X-ray film. Relative MNase sensitivity was expressed graphically after scanning the autoradiogram and analyzing the scan by the NIH Image program (version 1.62).

**Chromatin immunoprecipitation assay**

The chromatin immunoprecipitation (ChIP) assay was performed essentially as described previously (23,24). A 50 ml culture of yeast (OD600 = 1) was treated with formaldehyde (final concentration of 1%) for 30 min at 20°C, and 2.5 ml of 2 M glycine was added to stop the cross-linking reaction. Cells were harvested and disrupted by vortexing in the presence of glass beads, and the lysate was sonicated to generate DNA fragments that ranged in size from 200 to 800 bp. To immunoprecipitate Myc-tagged proteins and Sir2p, we incubated anti-Myc antibody (9E10, Roche, Indianapolis, IN) and anti-Sir2p antibody (Santa Cruz Biotech., Santa Cruz, CA), respectively, with the extract overnight at 4°C, and the extract–antibody mixture then was incubated for an additional 3–4 h with protein G Sepharose beads (Amersham Biosciences). In some experiments, Myc-blocking peptide (Roche, final concentration 313 μg/ml) was added. Immunoprecipitates were washed with 1 ml each of lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml pepstatin A), lysis buffer supplemented with 250 mM NaCl (for Myc-tagged Sas proteins) or 500 mM

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**Table 1. Yeast strains used in this study**

| Strain | Genotype | Source |
|--------|----------|--------|
| W303-1a | MATα ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 | J. Workman |
| W303-1b | MATα ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 | J. Workman |
| YJW228 | a SAS4-13Myc:kanMX6 | J. Workman |
| YJW229 | a SAS5-13Myc:kanMX6 | J. Workman |
| YJW252 | a sir1Δ:LEU2 | D. Rivier |
| YJW253 | a sas2Δ:TRP1 | D. Rivier |
| YJW257 | a sir1Δ:URA3 lys2Δ:hisG | D. Rivier |
| YJW258 | a sas2Δ:TRP1 sir1Δ:LEU2 | D. Rivier |
| YJW265 | a SAS2-13Myc:His3MX6 | J. Workman |
| YJW269 | a SAS2-13Myc:His3MX6 sas4Δ:kan | J. Workman |
| YJW270 | a SAS2-13Myc:kanMX6 sas4Δ:His3 | J. Workman |
| YJW271 | a SAS2-13Myc:TRP sas4Δ:kan sas5Δ:His3 | J. Workman |
| YJW433 | a asf1Δ:His3MX6 | J. Workman |
| YJW435 | a asf1Δ:His3 sir1Δ:LEU2 | J. Workman |
| YJW436 | a asf1Δ:His3 sas2Δ:TRP1 | J. Workman |
| YS480 | a sir1Δ:His3MX6 | J. Workman |
| YSM64 | a sir2Δ:His3MX6 | J. Workman |
| YSM65 | a SAS2-13Myc:kanMX6 sir1Δ:His3MX6 | J. Workman |
| YSM87 | a SAS2-13Myc:kanMX6 sas4Δ:His3 | J. Workman |
| YSM90 | a SAS2-13Myc:kanMX6 sas2Δ:His3MX6 | J. Workman |
| YSM104 | a SAS2-13Myc:kanMX6 asf1Δ:His3MX6 sas2Δ:TRP1 | J. Workman |

*Strains, except YJW251, are isogenic with W303-1a or W303-1b. YJW251 is a lawn strain for mating assays.*
NaCl (for Sir2p), LiCl–detergent wash buffer (250 mM LiCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycholate) and TE. DNA was eluted with elution buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA and 1% SDS). After reversal of the formaldehyde-induced cross-links, 1/5000 of input DNA and 1/45 of each immunoprecipitated DNA were used as templates for amplification by PCR. The sequences of primers for PCR were as follows: for the HMLα region, 5'-ATGCTCAAGCTAGAGTCTTTCTTTC-3' and 5'-TATGCTCAGTAGCTGGATTTAAACTCAT-3'; for the ACT1 promoter region, 5'-CTTTTTCTCAGTCCTCTTCGC-3' and 5'-TGGAATGGTGCAAGCGC-3'; and for the sub-telomeric chromatin at 7.5 kb from the end of chromosome VI, 5'-CTACGTTCCTTGACCAACTTTATTGCG-3' and 5'-TATCCTGACGTGAAGTTCAGGC-3'. Amplification was performed in a 20 μl reaction volume. The number of PCR cycles yielding product within the linear range was determined by analysis of 2-fold serial dilutions of the starting materials, and PCR products were separated on a 6% polyacrylamide gel and were detected by autoradiography. For quantitative analysis, 0.025 μl of [32P]dCTP (110 TBq/mmol; Amersham Biosciences) was added to the PCR. After electrophoresis, the gel was dried, and the radioactivity corresponding to a specific band was measured by a bioimage analyzer (model BAS 1800II, Fuji Film, Tokyo, Japan).

Determination of the molecular size of the SAS-containing complex

Whole-cell extracts were prepared as described previously (11,25). Approximately 0.4 mg of each whole-cell extract was loaded onto a 2.4 ml Superdex 200 PC 3.2/30 column (Amersham Biosciences) that had been equilibrated in buffer containing 50 mM Tris–HCl, pH 8.0, 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. A 10 μl aliquot of each fraction was electrophoresed in an SDS–polyacrylamide gel, transferred to nitrocellulose membrane and detected with the ECL western blotting analysis detection system (Amersham Biosciences). Anti-Myc antibody (9E10, Roche) was used.

RESULTS

HMLα2 was derepressed in a sas2 sir1-deleted strain

The α2 protein, which is a repressor of transcription of a-specific genes, is encoded by MATα and an essential factor for the regulation of mating-type-specific genes in α cells (26). The silent α information is also stored at the HMLα locus in both α and α cells. Previous work showed that the deletion of either SIR1 or SAS2 results in a very slight reduction of mating activity in a MATα strain, as indicated by quantitative mating analysis. The combined deletion of SIR1 and SAS2 strain causes a much more severe mating defect than that of the wild-type strain or the single-deletion strains (10,11). To show that the double deletion of SAS2 and SIR1 directly affects silencing at HMLα, we performed northern blotting analysis to detect the level of α2 mRNA in wild-type and deletion strains. α2 was transcribed not only in MATα cells but also in sir4Δ and sas2Δsir1Δ MATα cells (Figure 1A).

We previously showed that wild-type SAS2, but not SAS2 with a mutation in the conserved HAT domain, could restore the mating activity of the sas2Δsir1Δ strain. Furthermore, this mutation of amino acids 219–221 (GLG) to alanine residues (termed SAS2-M1) abolished the HAT activity of Sas2p but did not affect the formation of the SAS complex (11,13). To determine whether Sas2p HAT activity is required for the repression of α2 expression, wild-type and mutant SAS2 alleles were transformed into the sas2Δsir1Δ strain (Figure 1B). The mating activity and RNA level of α2 in the sir1 mutant were the same as those of the sas2 sir1 double-deletion strain carrying the wild-type SAS2 expression plasmid (Figure 1B, compare lanes 3 and 5). SAS2-M1 failed to restore mating activity and α2 repression to the sas2 sir1 double-mutant strain (Figure 1B, compare lanes 4 and 6). These results suggest that the Sas2p HAT activity is required for the repression of α2 expression from HMLα in a sir1 mutant.
A strain with deletions of SIR1 and SAS2 shows loss of nucleosome organization at HMLα

The unique and highly organized chromatin structure of HMLα has been determined by high-resolution chromatin mapping analysis using MNase, which preferentially cuts the linker DNA between nucleosomes (27). Although a precisely positioned nucleosome (NUCα2) covers the transcription start site of the α2 gene at the HMLα locus, the promoter region of the α2 gene is nucleosome-free [(27); see also Figure 2A, lanes 6–8 and Figure 2B]. We used MNase mapping to examine the effect of α2 mutations on the chromatin structure of HMLα. Deletion of SIR4, which is essential for HMLα silencing, resulted in increased nuclease sensitivity of one site, indicated by the arrow, compared with that of the wild type (Figure 2A, compare lanes 6–8 with lanes 10–12). Although we used downstream and reverse-strand primers in the attempt to visualize the positioning of NUCα2 clearly, we failed to obtain sufficient quantities of primer-extension products. For easy comparison of the MNase sensitivity, the lane treated with the highest concentration of MNase was selected for scanning (Figure 2A, lanes 8, 12, 16, 20 and 24). Relative MNase sensitivity is shown in Figure 2B after scanning and analyzing with the NIH Image software. The intensity of the induced sensitivity of the site highlighted in Figure 2A is the same in sas2 sir1 double-deleted cells as in SIR4-deleted cells (compare lanes 10–12 with lanes 22–24) and stronger than that of wild-type or singly deleted strains (compare lanes 6–8, 14–16 and 18–20 with lanes 22–24). The MNase sensitivity of this site in different mutants correlates inversely with their mating activity and directly with the level of α2 mRNA (Figure 2A). These results suggest that SAS2 is essential for the organization of the nucleosome precisely positioned over the transcriptional initiation site of the α2 gene in a sir1-mutant strain.

Interestingly, the nuclease sensitivity of the region between the UAS and TATA-box regions was decreased in sas2Δsir1Δ and sir4Δ strains. This region is generally less nuclease sensitive at MATα than at HMLα (27), and is thought that in α2-positive cells, the protection of this region in chromatin from the nuclease might result from an association of transcription factors, including Rap1p, which bind to UAS (27). Overall, chromatin in this region is less accessible to nuclease in sas2Δsir1Δ strain than in wild-type strains.

The SAS complex associates with the HMLα locus

Genetic experiments revealed that combining the null allele of SIR1 with that of SAS2, SAS4, SAS5 or ASF1 results in the reduction of silencing at HMLα (11,17). Meijising and Ehrenhofer-Murray (12) reported that Sas2p is physically present at the rDNA locus, but not ACT1. To determine whether Sas2p or the SAS complex is located at the HMLα locus, we performed ChIP analysis using strains expressing Sas2p–Myc. Immunoprecipitated DNA was amplified by PCR with a primer pair spanning the α1 and α2 promoter regions in the HMLα locus (Figure 3A). We used ACT1, a gene whose transcription is not regulated by SAS2. As a negative control, because the ACT1 mRNA level in the sas2 mutant was same as that in the wild-type strain (15). Two-fold serial dilutions of the input and the immunoprecipitated DNA were performed to verify that the amount of PCR product was dependent on the starting material. Input DNA also was used as a template to confirm that these regions were amplified equally by PCR. In a SAS2–Myc strain, Sas2p–Myc associated with the promoter regions of the α1 and α2 in the HMLα locus relative to the ACT1 locus (Figure 3A). This association was completely competed by adding a Myc-blocking peptide (compare lanes 11 and 12 with lanes 13 and 14).

We previously purified a complex containing Sas2p and showed that Sas4p and Sas5p were components of this
complex, termed the SAS complex (11,13,14). We next asked whether Sas4p and Sas5p associate with the HMLa locus. ChIP analyses using SAS4–Myc and SAS5–Myc revealed that Sas4p–Myc and Sas5p–Myc were recruited together with Sas2p–Myc to the promoter regions of the α1 and α2 genes in the HMLa region (Figure 3B). This finding supports the possibility that these Sas proteins associate with chromatin as a complex. Accordingly, deletion of either SAS4 or SAS5 might disrupt the association of Sas2p with the HMLa locus.

To evaluate whether this disruption occurs, we performed ChIP using cells expressing Sas2p–Myc and deleted for SAS4, SAS5 or both genes (Figure 3C). The amount of amplified PCR products from the three deletion strains was markedly lower than from the wild-type strain, although Sas2p–Myc was expressed efficiently in all of the SAS2–Myc strains. These results show that mutations in SAS5 and especially SAS4 inhibit the association of Sas2p–Myc with the promoter regions of the α1 and α2 in the HMLα region and that Sas2p–Myc was recruited to this region as a component of the SAS complex.

**SIR1 and SIR2 are not required for the recruitment of Sas2p to the promoters in the HMLα locus**

Four Sir proteins localize to HMLα and HMRα and are important for silencing. Sir1p binds to Orc1p, one of the silencer binding proteins, and helps to recruit Sir4p. Sir2p, Sir3p and Sir4p form a complex and spread in both directions from the silencers (1,28). A physical interaction between Sas and Sir proteins has not been reported, although all of them localize to the HMLα locus. To understand the role of the Sir proteins in the association of Sas2p with the HMLα locus, we first asked whether loss of Sir1p or Sir2p results in disruption of this association. To do this, Sas2p was tagged with Myc in Sir1Δ (YJW269) or Sir2Δ (YJW270) strains. Western blot analysis revealed that Sas2p–Myc was expressed efficiently in both Sir1Δ and Sir2Δ deletion strains.

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**Figure 3.** The SAS complex occupancy at the promoter in the HMLα locus. (A) Association of Sas2p with the HMLα region is detected by the ChIP assay. Sonicated chromatin was prepared from wild-type (WT) and Sas2p–Myc-expressing (YJW265) strains. Immunoprecipitation was carried out using monoclonal antibodies to the Myc tag (lanes 3–6 and 11–14), and normal IgG was used as a negative control (lanes 7, 8, 15 and 16). Myc-blocking peptide was added in some immunoprecipitation experiments (lanes 5, 6, 13 and 14). Input and immunoprecipitated DNA were amplified by PCR using primer pairs spanning the promoter region of α1 and α2 genes or ACT1. PCR products were resolved on a 6% polyacrylamide gel and visualized by autoradiography. (B) Localization of subunits of the SAS complex to the promoter of the α1 and α2 genes. Soluble chromatin was prepared from the strains that expressed the C-terminal Myc epitope-tagged Sas2p (YJW265), Sas4p (YJW228) or Sas5p (YJW229), and immunoprecipitated with or without anti-Myc antibody. Final DNA extractions were amplified with [32P]dCTP. The PCR product was separated on the polyacrylamide gel and quantitated with a bioimage analyzer after drying the gel. ChIP efficiency is reported as a percentage of immunoprecipitated material (top panel). Data are presented as the mean ± SD from three independent experiments. Input DNA (input) was equally amplified by PCR in WT and Sas2p–Myc-expressing strains. Myc-tagged proteins were detected by western blotting (bottom panel). (C) SAS4 or SAS5 or both are required for the recruitment Sas2p to the HMLα locus. ChIP assay was performed with chromatin prepared from sas4Δ (YJW269), sas5Δ (YJW270) and sas4Δsas5Δ (YJW271) strains that expressed the C-terminal Myc epitope-tagged Sas2p. Results are shown as in Figure 3B.
deletion strains (Figure 4A). We prepared chromatin fractions from these strains and subjected them to ChIP analysis for the Myc epitope. As shown previously, Sas2p–Myc associated with the HMLα region in wild-type cells. Loss of SIR1 or SIR2 did not affect this association (Figure 4A). This finding indicates that SIR1 and SIR2 are not required for the recruitment of Sas2p to the promoters in the HMLα locus.

Disruption of SAS2 in a wild-type strain increased the spreading of Sir proteins to the sub-telomeric region (15,16). Deletion of SAS2 in a sir1 mutant also may lead to spreading of the finite number of Sir2p molecules into sub-telomeric regions, resulting in a decrease in Sir2p occupancy at the HMLα locus. To test this hypothesis, we asked whether loss of SAS2 results in increased or decreased Sir2p association. We observed localization of Sir2p in the regions of the HMLα locus and sub-telomeric chromatin 7.5 kb from the end of chromosome VI in the wild-type and deletion strains (Figure 4B). Consistent with previous observations, the Sir2p association detected within the HMLα locus and sub-telomeric regions in the wild-type strain was greater than that in a sir2 deletion strain (Figure 4B, compare lanes 1 and 5) and was slightly increased in the sub-telomeric region by SAS2 deletion (16,29). Interestingly, although Sir2p was expressed efficiently in all of the strains except a sir2 deletion strain, the disruption of SAS2 or SIR1 decreased the amount of Sir2p localization at the HMLα locus (Figure 4B). In the combination of SAS2 deletion with the null allele of SIR1, the Sir2p association at the HMLα locus was completely lost, similar to that in the SIR2 and SIR3 deletion strains. This indicates that although Sas2p association at the HMLα locus does not require SIR1 or SIR2, Sir2p localization is partially dependent on SAS2.

**Figure 4.** (A) SIR1 and SIR2 are not required for the recruitment of Sas2p to the promoter in the HMLα locus. ChIP assay was performed with chromatin prepared from sir1Δ (YSM85) and sir2Δ (YSM90) strains that expressed the C-terminal Myc epitope-tagged Sas2p. (B) Effect of deletion of SAS2 and SIR genes on the Sir2p occupancy. Soluble chromatin was prepared from wild-type and deletion strains, and immunoprecipitated with or without anti-Sir2p antibody. Strains analyzed (ordered from left to right) were W303-1a, sas2Δ (YJW253), sas2Δsir1Δ (YJW258), sir1Δ (YJW252), sir2Δ (YSM64), and sir3Δ (YS480). Results are shown as in Figure 3B.

**Figure 5.** ASF1 and SAS2 function in the same pathway in HMLα silencing. Disruption of ASF1 in combination with a null allele of SIR1, but not of SAS2, derepressed α2 expression. RNA from either wild-type (WT: W303-1b and W303-1a), asf1Δ (YJW433), asf1Δsir1Δ (YJW435), or asf1Δsas2Δ (YJW436) was hybridized by northern blotting to a probe specific for either the α2 or SCR1 gene. A qualitative mating assay was performed by patches, which were replicated to a lawn of cells.

**α2 expression in the asf1 sir1 deletion strain**

We and others (11,12) previously showed that the SAS complex physically interacts with Asf1p and these factors function in a pathway that enhances the epigenetic silencing defects of sir1 mutants. To learn more about the function of ASF1 in the HMLα silencing, we measured the expression of α2 in an ASF1 deletion strain (Figure 5). Deletion of ASF1 results in a very slight reduction in silencing at HMLα as indicated by
quantitative mating analysis (11), and the α2 mRNA level in the asf1 mutant was indistinguishable from that of the wild-type strain. We previously showed that the combined deletion of ASF1 and SIR1 caused much more severe silencing defects at HMLα than does the deletion of ASF1 alone (11). Derepression of α2 expression occurred in the asf1 sir1 double-deletion strain but not in the asf1Δsas2Δ strain. Loss of mating activity correlated with the increased α2 expression in the asf1 sir1 deletion strain (Figure 5).

**Loss of ASF1 disrupts the recruitment of the SAS complex to the HMLα locus**

We showed that SIR1 and SIR2 are not required for the recruitment of Sas2p to the promoters in the HMLα locus (Figure 4). Next, we investigated whether ASF1 is required for Sas2p–Myc recruitment. Loss of ASF1 markedly decreased Sas2p association with the HMLα locus (Figure 6A). This decrease was restored by a plasmid carrying ASF1 (compare lanes 3 and 4). In a sir1 mutant, the effect of ASF1 on the Sas2p recruitment was the same as for the SIR1 wild-type strain. These results indicate that ASF1, but not SIR1, is required for the recruitment of Sas2p to the HMLα locus. Sas2p expression levels in whole-cell extracts from wild-type, asf1Δ, and asf1Δsir1Δ strains were indistinguishable, and disruption of ASF1 did not affect the size of the SAS complex (Figure 6A and B). These data indicate that loss of association of Sas2p with the HMLα locus in the asf1 mutant is not due to a decrease in Sas2p expression or disruption of the SAS complex.

**DISCUSSION**

Deletion of SIR1 in combination with a null allele of either SAS2 or ASF1 causes a much more severe silencing defect at HMLα than does deletion of either gene alone (11), but the role of these factors in silencing was unclear. We showed that the combination of mutation of SAS2 with SIR1 induced derepression of α2 expression and changed the precisely positioned nucleosome that includes the transcriptional initiation site of the α2, and that the HAT activity of Sas2p is critical for this effect. Furthermore, ChIP assays revealed specific association of the SAS complex with the HMLα locus, and the SAS complex recruitment required ASF1 but not SIR1 and SIR2.

The effect of the disruption of SAS2 on silencing is different among loci. For example, normal HMRα silencing is unaffected by SAS2 deletion, but sas2 mutations suppress the silencing defect caused by mutation in the silencer elements of HMRα (10,17). Deletion of SAS2 leads to loss of hyperacetylation of histone H4 at lysine 16 in regions adjacent to telomeres. This results in the spreading of Sir3p away from the telomeres into these sub-telomeric regions, leading to repression of gene expression in the sub-telomeric region (15,16). However, deletion of SAS2 causes the loss of silencing at the telomeres themselves, presumably because of titration of Sir proteins away from this locus.

In present study, Sas2p was found to be associated with the HMLα locus, and Sir2p was not required for this Sas2p association. Disruption of SAS2 increased the spreading of Sir proteins to the sub-telomeric region (15,16). Deletion of SAS2 in sir1 mutants also led to the spreading of the finite number of Sir2p molecules into sub-telomeric regions and resulted in a decrease in Sir2p occupancy at the HMLα locus. We also showed that the Sas2p HAT activity is essential for α2 repression. Acetylation of lysine 16 of histone H4 might be a landmark for Sir2p assembly; once lysine 16 of histone H4 is acetylated by Sas2p, Sir2p recognizes and deacetylates that mark for Sir2p assembly: once lysine 16 of histone H4 is acetylated by Sas2p, Sir2p recognizes and deacetylates that mark for Sir2p assembly. The effect of the disruption of SAS2 on silencing is different among loci. For example, normal HMRα silencing is unaffected by SAS2 deletion, but sas2 mutations suppress the silencing defect caused by mutation in the silencer elements of HMRα (10,17). Deletion of SAS2 leads to loss of hyperacetylation of histone H4 at lysine 16 in regions adjacent to telomeres. This results in the spreading of Sir3p away from the telomeres into these sub-telomeric regions, leading to repression of gene expression in the sub-telomeric region (15,16). However, deletion of SAS2 causes the loss of silencing at the telomeres themselves, presumably because of titration of Sir proteins away from this locus.
SIR1 in combination with null alleles of either SAS2 or ASF1 may decrease the association of Sir proteins to a much greater extent than that seen after deletion of SIR1 only, thereby causing a much more severe silencing defect at HMLα than that seen after deletion of either gene alone.

We previously showed that Sas4p, one of the subunits of the SAS complex, directly interacts with Asf1p (11). Therefore, recruitment of the SAS complex to the HMLα region might require physical interaction with Asf1p. In the present study, we found that the SAS complex is associated with the HMLα region, but not the ACT1 promoter. However, Mosskin et al. (30) showed that Drosophila Asf1 associated with multiple sites, including heterochromatic and transcriptionally active regions. Furthermore, asf1 mutants are defective in the repression of histone gene transcription during the cell cycle and in cells arrested in the early S phase (31). Finally, Asf1 interacts with bromodomain-containing subunits of TFIIID and the Brahma complex, a member of the SWI/SNF ATP-utilizing chromatin-remodeling factors (30,32). These results indicate that ASF1 affects transcriptional control through a variety of mechanisms. The estimated numbers of Asf1p and Sas4p molecules per yeast cell are 6230 and 768, respectively (33). This distribution suggests that the SAS complex interacts with a subset of Asf1p proteins. The mechanism of the recruitment specificity of the SAS complex is still unknown. HMLα binding factors other than Sir proteins may enhance the SAS complex association with the HMLα locus. Alternatively, Asf1p-associated factors that selectively bind to Asf1p within transcriptionally active regions may inhibit the interaction between the SAS complex and Asf1p. To address this possibility, we are purifying the factors that interact with the SAS complex and Asf1p.

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