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Chromatin assembly factor Asf1p-dependent occupancy of the SAS histone acetyltransferase complex at the silent mating-type locus *HMLα*

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ABSTRACT

Transcriptional repression of the silent mating-type loci *HMLα* and *HMR*a in *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, *sas* mutant phenotypes include loss of *SIR1*-dependent silencing of *HMLα*. To gain insight into the mechanism of the SAS complex mediated silencing at *HMLα*, we investigated the expression and chromatin structure of the α2 gene in the *HMLα* locus. We found that deletion of *SAS2* in combination with a null allele of *SIR1* changed the chromatin structure of the precisely positioned nucleosome, which includes the mRNA start site of the α2 gene and derepressed α2 transcription. The Sas2p HAT domain was required for this silencing. Furthermore, chromatin immunoprecipitation analysis revealed that the SAS complex was associated with the *HMLα* locus, and *ASF1* (which encodes chromatin assembly factor Asf1p), but not *SIR1* and *SIR2*, was necessary for this localization. These data suggest that the HAT activity and *ASF1*-dependent localization of the SAS complex are required for *SIR1*-dependent *HMLα* 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 *Saccharomyces cerevisiae*, silenced loci include the *HMLα* and *HMR***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 *SIR2*, *SIR3* or *SIR4* gene results in loss of silencing at *HM* loci, and a *SIR1*-disrupted strain is composed of mixed populations of silenced and unsilenced cells (9).

Something About Silencing (*SAS*) *2* was identified as an enhancer of *sir1* epigenetic *HMLα* 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 *HMLα*, deletion of *SAS2* has very little effect on silencing. However, deletion of *SAS2* combined with deletion of *SIR1* causes a severe silencing defect (10,17). Normal *HMR***a** silencing is unaffected by *SAS2* deletion, but *sas2* mutation suppresses the silencing defect caused by mutations in silencer elements of *HMR***a** (10,17). To determine the role of *SAS2* in rDNA silencing, a strain in which the *URA3* gene is integrated at the rDNA locus was used. The *sas2* deletion strain showed more effective *URA3* repression, indicating that the deletion of *SAS2* increased rDNA repression (12). In the case of telomeres, loss of *SAS2* causes hypoacetylation in adjacent sub-telomeric regions, leading to the recruitment of

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Sir3p to these regions and inactivation of gene expression (15,16). Therefore, *sas2* mutations reduce silencing of *HML*α (when combined with deletion of *SIR1*) but improve silencing at *HMR***a** 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* genetically function in the same pathway to repress the *HML*α locus (11). In the present study, we show that *SAS2* is essential for the organization of the chromatin structure at *HML*α in a *sir1* mutant and that *ASF1* is required for the recruitment of the SAS complex to the *HML*α locus.

MATERIALS AND METHODS

Yeast strains, plasmids and manipulations

The strains used in this study are listed in Table 1 and were either published previously 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).

Table 1. Yeast strains used in this study

Strain[a]	Genotype	Source
W303-1a	MATa *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 *sir4Δ::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 sas5Δ::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 *sir3Δ::HIS3MX6*	
YSM64	a *sir2Δ::HIS3MX6*	
YSM85	a *SAS2-13Myc:kanMX6 sir1Δ::HIS3MX6*	
YSM87	a *SAS2-13Myc:kanMX6 asf1Δ::HIS3MX6*	
YSM90	a *SAS2-13Myc:kanMX6 sir2Δ::HIS3MX6*	
YSM104	a *SAS2-13Myc:kanMX6 asf1Δ::HIS3MX6 sir1Δ::TRP*	
YSM112	W303-1a [pRS416/CEN/URA3 (pS14)]	
YSM113	YJW265 [pRS416/CEN/URA3 (pS14)]	
YSM114	YSM87 [pRS416/CEN/URA 3(pS14)]	
YSM115	YSM87 [Ycp50/CEN/URA3/ASF1 (pLS27)]	
YSM116	YSM104 [pRS416/CEN/URA3 (pS14)]	
YSM117	YSM104 [Ycp50/CEN/URA3/ASF1 (pLS27)]	
YSM118	W303-1a [pRS416/CEN/URA3 (pS15)]	
YSM119	W303-1b [pRS416/CEN/URA3 (pS15)]	
YSM120	YJW252 [pRS416/CEN/URA3 (pS15)]	
YSM121	YJW258 [pRS416/CEN/URA3 (pS15)]	
YSM122	YJW258 [pRS416/CEN/URA3/ PSAS2-SAS2 (pS126)]	
YSM123	YJW258 [pRS416/CEN/URA3/ PSAS2-SAS2-M1 (pS136)]	
YJW251[b]	α *his4*	D. Rivier

[a]Strains, except YJW251, are isogenic with W303-1a or W303-1b.
[b]YJW251 is a lawn strain for mating assays.

RNA blots

A 40 μg aliquot of total RNA prepared from logarithmically growing cells was separated on 1.0% 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 (OD_{600} = 1). The nuclear pellet from a 1 liter culture was resuspended in 2.4 ml digestion buffer (10 mM HEPES, pH 7.5, 0.5 mM $MgCl_2$ and 0.05 mM $CaCl_2$). 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, proteinase K digestion and phenol–chloroform extraction. The purified DNA was resuspended in 0.1× 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′-TATGTCTAGTATG-CTGGATTTAAACTCAT-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 (OD_{600} = 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

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′-ATGCTCAGCTAGACGTTTTTCTTTC-3′ and 5′-TATGTCTAGTATGCTGGATTTAAACTCAT-3′; for the *ACT1* promoter region, 5′-CTTTTTCTTCCACGTCCTCTT-GC-3′ and 5′-TGGGATGGTGCAAGCGC-3′; and for the subtelomeric chromatin at 7.5 kb from the end of chromosome VI, 5′-TCATGGTCTTGACAACTTTATGCG-3′ and 5′-TATC-TGACGTGAAAGTTCAGCGC-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 [^{32}P]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.

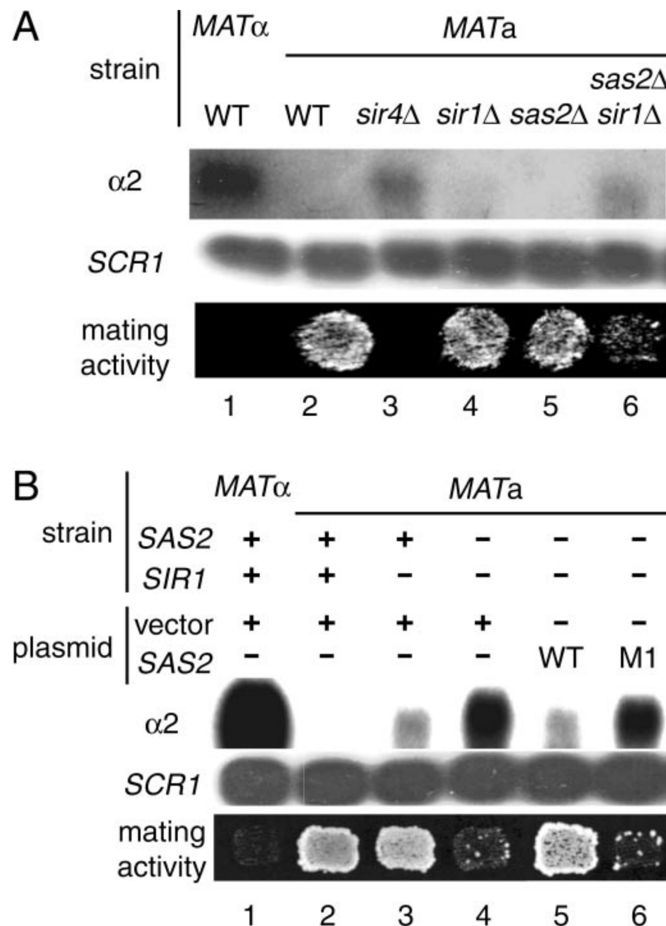

Figure 1. Deletion of *SAS2* and *SIR1* in a *MAT***a** strain leads to derepression of *HMLα*2 expression and a non-mating. (**A**) RNA from either wild-type (WT, W303-1b and W303-1a), *sir4Δ* (YJW257), *sir1Δ* (YJW252), *sas2Δ* (YJW253) or *sas2Δsir1Δ* (YJW258) strains was hybridized by northern blotting to a probe specific for either the α2 or *SCR1* gene. RNA loading was standardized to *SCR1*. A qualitative mating assay was performed by patches, which were replica-plated to a lawn of *MATα* cells. WT and mutant strains are presented at the top of the panel. (**B**) A *sas2sir1* double-deletion strain was transformed with a plasmid carrying the WT or a mutant form of the *SAS2* gene (M1) under the control of its own promoter. Strains analyzed (ordered from left to right) were YSM119, YSM118, YSM120, YSM121, YSM122 and YSM123.

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 **a** 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 *MAT***a** 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 MAT***a** 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 *sas2* 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 1A). These results suggest that *SAS2* is essential for the organization of the nucleosome precisely positioned over the transcriptional initiation site of the α2 in a *sir1*-mutant strain.

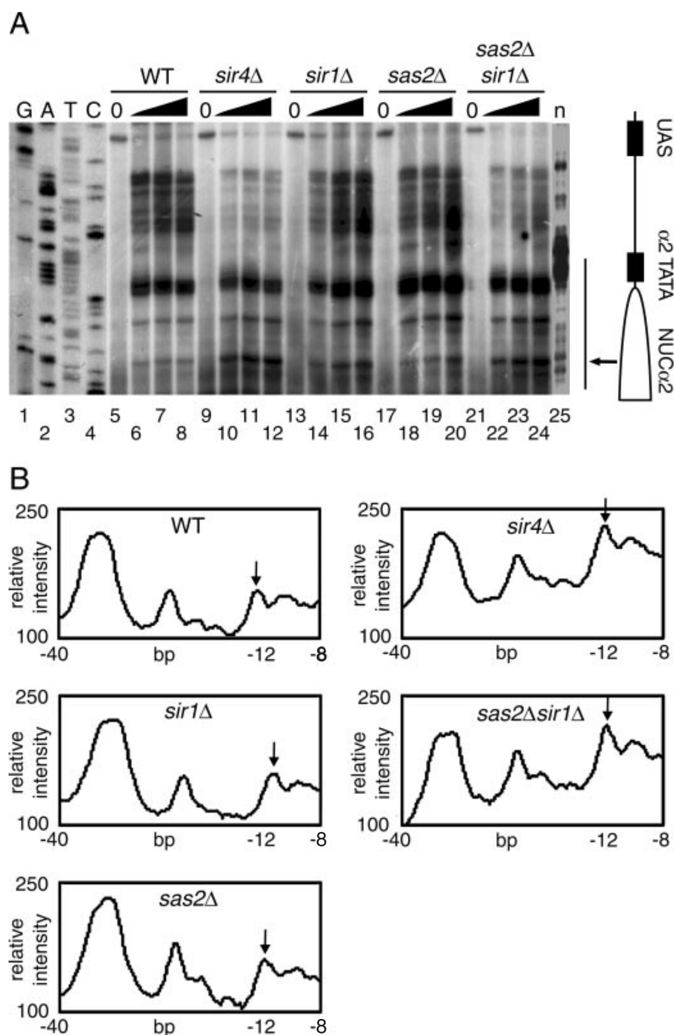

Figure 2. High-resolution MNase mapping of the promoter region of the α2 gene at the *HMLα* locus. (**A**) The chromatin structure of the Crick strand was mapped by primer-extension analysis of MNase-digested sites with the primer located at the coding region of the α2 gene. Strains used were as in Figure 1A. Extensions of undigested (0) and dose-dependent-digested chromatin are indicated. The 'n' lane shows the naked DNA digested with MNase (lane 25). The G, A, T and C columns indicate dideoxynucleotide-terminated sequencing reactions (lanes 1–4). The ellipse corresponds to the inferred position of the nucleosome, and boxes show the positions of the TATA-box and UAS regions. Arrow indicates sites in the α2 gene whose nuclease-sensitivity was increased in mutants relative to WT. The vertical bar indicates the region used for scanning to express the relative intensity in Figure 2B. (**B**) The relative MNase sensitivity expressed graphically after scanning and analyzing the autoradiogram in (**A**) by using NIH Image 1.62 software. Increased levels of nuclease-sensitive sites of the α2 gene in mutants (*sir4Δ* and *sas2Δsir1Δ*) relative to WT are indicated by arrows. The position from the transcription start site is shown at the bottom of each panel.

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 it 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). Meijsing 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* region relative to *ACT1* (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

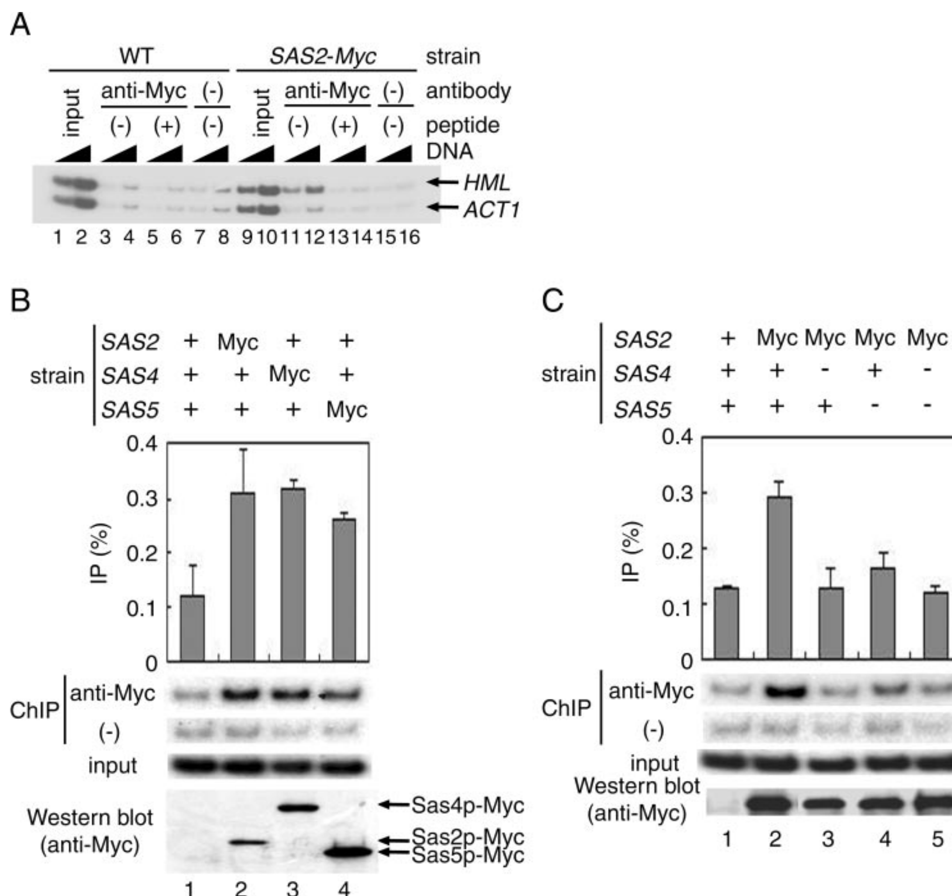

Figure 3. The SAS complex occupancy at the promoter in the *HMLα* locus. (**A**) Association of Sas2p with the *HMLα* region is detected by the ChIP assay. Sonicated chromatin was prepared from wild-type (WT) and Sas2p–Myc-expressing (YJW265) strains. Immunoprecipitation was carried out using monoclonal 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 [^{32}P]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.

complex, termed the SAS complex (11,13,14). We next asked whether Sas4p and Sas5p associate with the *HMLα* 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 in the *HMLα* 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 *HMLα* 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 *HMR***a** 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 *SIR1* or *SIR2* deletion strains. Western blot analysis revealed that Sas2p–Myc was expressed efficiently in both *SIR1* and *SIR2*

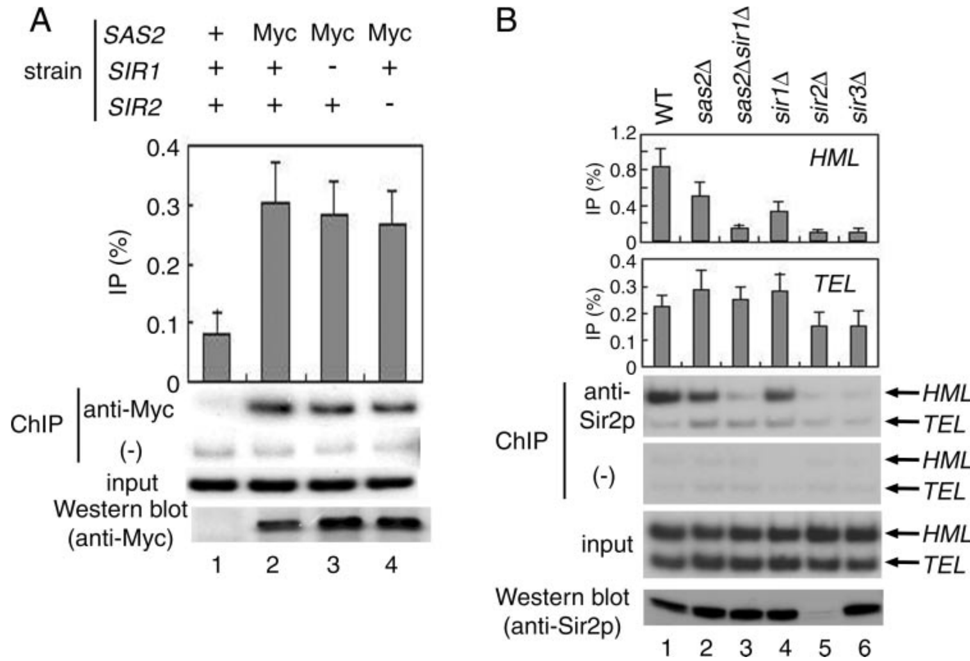

Figure 4. (**A**) *SIR1* and *SIR2* are not required for the recruitment of Sas2p to the promoter in the *HMLα* locus. ChIP assay was performed with chromatin prepared from *sir1Δ* (YSM85) and *sir2Δ* (YSM90) strains that expressed the C-terminal Myc epitope-tagged Sas2p. (**B**) Effect of deletion of *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.

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*.

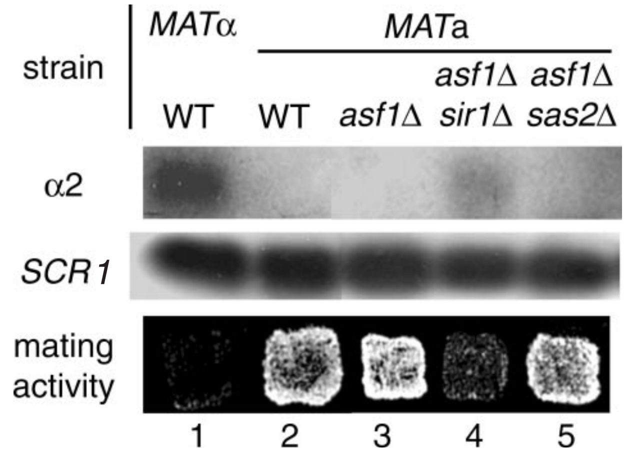

Figure 5. *ASF1* and *SAS2* function in the same pathway in *HMLα* silencing. Disruption of *ASF1* in combination with a null allele of *SIR1*, but not of *SAS2*, derepressed α2 expression. RNA from either wild-type (WT: W303-1b and W303-1a), *asf1Δ* (YJW433), *asf1Δsir1Δ* (YJW435) or *asf1Δsas2Δ* (YJW436) 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 replica-plated to a lawn of α 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 repression occurred in the *asf1 sir1* double-deletion strain but not in the *asf1*Δ*sas2*Δ 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, *asf1*Δ, and *asf1*Δ*sir1*Δ 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 the association of Sas2p with the *HMLα* locus in the *asf1* mutants is not due to a decrease in Sas2p expression or disruption of the SAS complex.

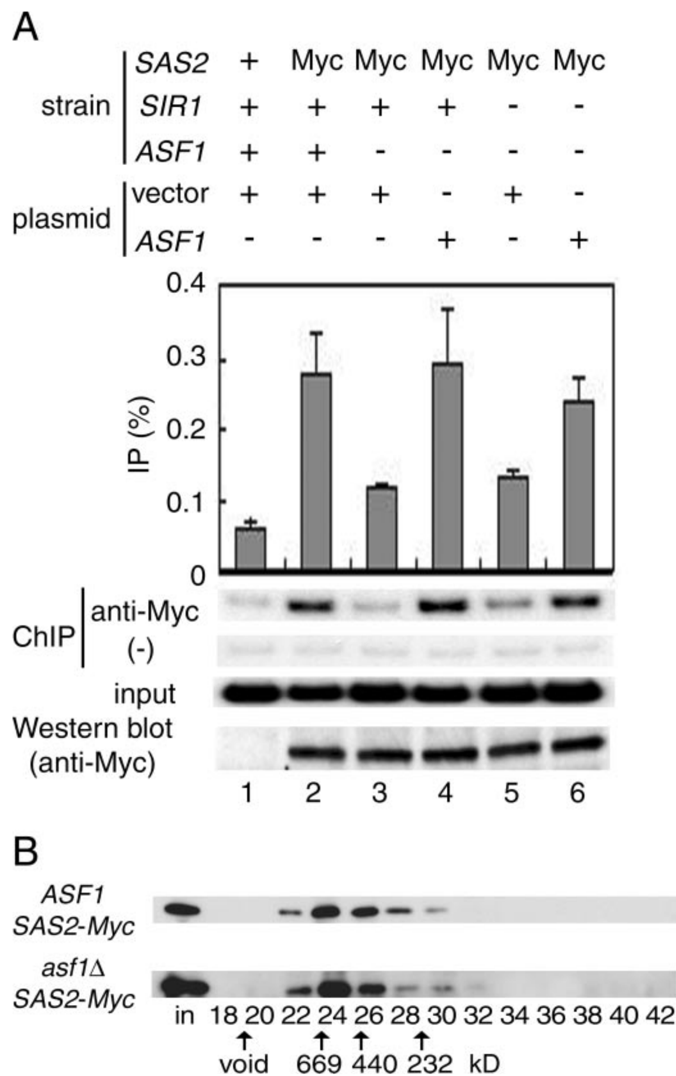

Figure 6. Sas2 occupancy at the *HMLα* region is dependent on *ASF1*. (**A**) Chromatin was prepared from the Sas2p–Myc-expressing strains containing either an empty vector or a CEN-based *ASF1* plasmid expressed from its own promoter. Strains for the ChIP assay (ordered from left to right) were YSM112, YSM113, YSM114, YSM115, YSM116 and YSM117. Results are shown as in Figure 3B. (**B**) Comparison of the Sas2p–Myc elution profiles from Superdex 200 size exclusion chromatography after fractionation of whole-cell extracts prepared from wild-type (YJW265) and *asf1Δ* (YSM87) mutant strains. Shown are western blots of column fractions probed with the anti-Myc antibody.

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 *HMR***a** silencing is unaffected by *SAS2* deletion, but *sas2* mutations suppress the silencing defect caused by mutation in the silencer elements of *HMR***a** (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 residue in the silenced domain and is held in this region. Sas2p might contribute to regulating the histone H4 lysine 16 acetylation state at the chromosome level as well as at the locus level. Deletion of *ASF1* likely would bring about the same phenomenon, because we found that Sas2p occupancy at the *HMLα* locus was dependent on *ASF1*. Other investigators have shown that the association of Sir proteins at the *HMLα* silencer is somewhat reduced in *sir1*-mutant cells (29). The deletion of

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 *HML*α 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 *HML*α region might require physical interaction with Asf1p. In the present study, we found that the SAS complex is associated with the *HML*α region, but not the *ACT1* promoter. However, Moshkin *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 TFIID 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 Asf1 proteins. The mechanism of the recruitment specificity of the SAS complex is still unknown. *HML*α binding factors other than Sir proteins may enhance the SAS complex association with the *HML*α 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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