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Regulation of Antisense Transcription by NuA4 Histone Acetyltransferase and Other Chromatin Regulatory Factors

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NuA4 histone lysine (K) acetyltransferase (KAT) promotes transcriptional initiation of TATA-binding protein (TBP)-associated factor (TAF)-dependent ribosomal protein genes. TAFs have also been recently found to enhance antisense transcription from the 3′ end of the *GAL10* coding sequence. However, it remains unknown whether, like sense transcription of the ribosomal protein genes, TAF-dependent antisense transcription of *GAL10* also requires NuA4 KAT. Here, we show that NuA4 KAT associates with the *GAL10* antisense transcription initiation site at the 3′ end of the coding sequence. Such association of NuA4 KAT depends on the Reb1p-binding site that recruits Reb1p activator to the *GAL10* antisense transcription initiation site. Targeted recruitment of NuA4 KAT to the *GAL10* antisense transcription initiation site promotes *GAL10* antisense transcription. Like NuA4 KAT, histone H3 K4/36 methyltransferases and histone H2B ubiquitin conjugase facilitate *GAL10* antisense transcription, while the Swi/Snf and SAGA chromatin remodeling/modification factors are dispensable for antisense, but not sense, transcription of *GAL10*. Taken together, our results demonstrate for the first time the roles of NuA4 KAT and other chromatin regulatory factors in controlling antisense transcription, thus illuminating chromatin regulation of antisense transcription.

Noncoding RNAs have been implicated in various cellular processes such as X-chromosome inactivation, genomic imprinting, dosage compensation, heterochromatin formation, metabolism, development, and differentiation (1–5). There are several classes of noncoding RNAs, which include microRNAs, small nuclear RNAs, small interfering RNAs, Piwi-interacting RNAs, and natural antisense transcripts (6). About 72% of genes in human and mouse are associated with antisense transcription (7, 8). Antisense transcripts arise from the strand opposite to the sense strand and play regulatory functions in interfering with the stability of sense transcripts, and hence gene expression. Therefore, a number of studies have been focused on the use of antisense oligonucleotides in regulation of gene expression and treatment of diseases without permanently altering the genes. In fact, antisense oligonucleotides are in various clinical trials for treatment of diseases such as cancers, hypertension, respiratory illness, and HIV infection (9–13).

Despite great potentials of antisense transcripts/transcription in disease pathogenesis and treatment, it is not clearly understood how antisense transcription is initiated. Recently, we have demonstrated that, like in sense transcription, RNA polymerase II is targeted to the 3′ end of the *GAL10* coding sequence by an activator Reb1p or Reb1p-binding site and general transcription factors (GTFs) such as transcription factor IID (TFIID) (which is composed of TATA-binding protein [TBP] and a set of TBP-associated factors [TAFs]), TFIIB, and Mediator to initiate antisense transcription (14). Further, we have shown that the Gal4p activator and proteasome that facilitate *GAL10* sense transcription are dispensable for *GAL10* antisense transcription (14), supporting that *GAL10* sense and antisense transcriptions are initiated independently and differently. These recent results shed much light on the initiation of antisense transcription (14). However, the involvement of the chromatin structure/dynamics and the associated factors in regulation of antisense transcription remains poorly understood.

Here, we have carried out experiments to analyze chromatin regulation of antisense transcription. We have taken the advantage of the *GAL* gene cluster, which consists of three genes, namely, *GAL1*, *GAL7*, and *GAL10* (Fig. 1A). These genes are induced for sense transcription in galactose-containing growth medium (14). However, long noncoding antisense transcripts are generated from the 3′ end of the *GAL10* coding sequence in dextrose-containing growth medium (Fig. 1A), which is repressive to *GAL* sense transcription (14–16). Therefore, using this *GAL* gene cluster, the chromatin regulation of *GAL10* antisense transcription in dextrose-containing growth medium can be clearly analyzed without any interference from the sense transcription, hence contributing to our understanding of the regulation of antisense transcription by epigenetic factors. We initially focused our studies on NuA4 histone lysine (K) acetyltransferase (KAT), as NuA4 KAT is known to facilitate TAF-dependent sense transcription of the ribosomal protein genes (17–19), while its role in regulation of TAF-dependent *GAL10* antisense transcription remains unknown. Here, we find that NuA4 KAT is associated with the 3′ end of the *GAL10* coding sequence (i.e., *GAL10* antisense transcription initiation site) (Fig. 1A) for histone H4 acetylation and antisense transcription. However, NuA4 KAT-regulated *GAL10* antisense transcription is not controlled by TOR (target of rapamycin), while TOR regulates sense transcription of NuA4 KAT-dependent ribosomal protein genes. Like NuA4 KAT, histone H3 K4 methyltransferase (Set1p) and histone H3 K36 methyltransferase (Set2p), which are essential for H3 K4 and K36 methylation, re-

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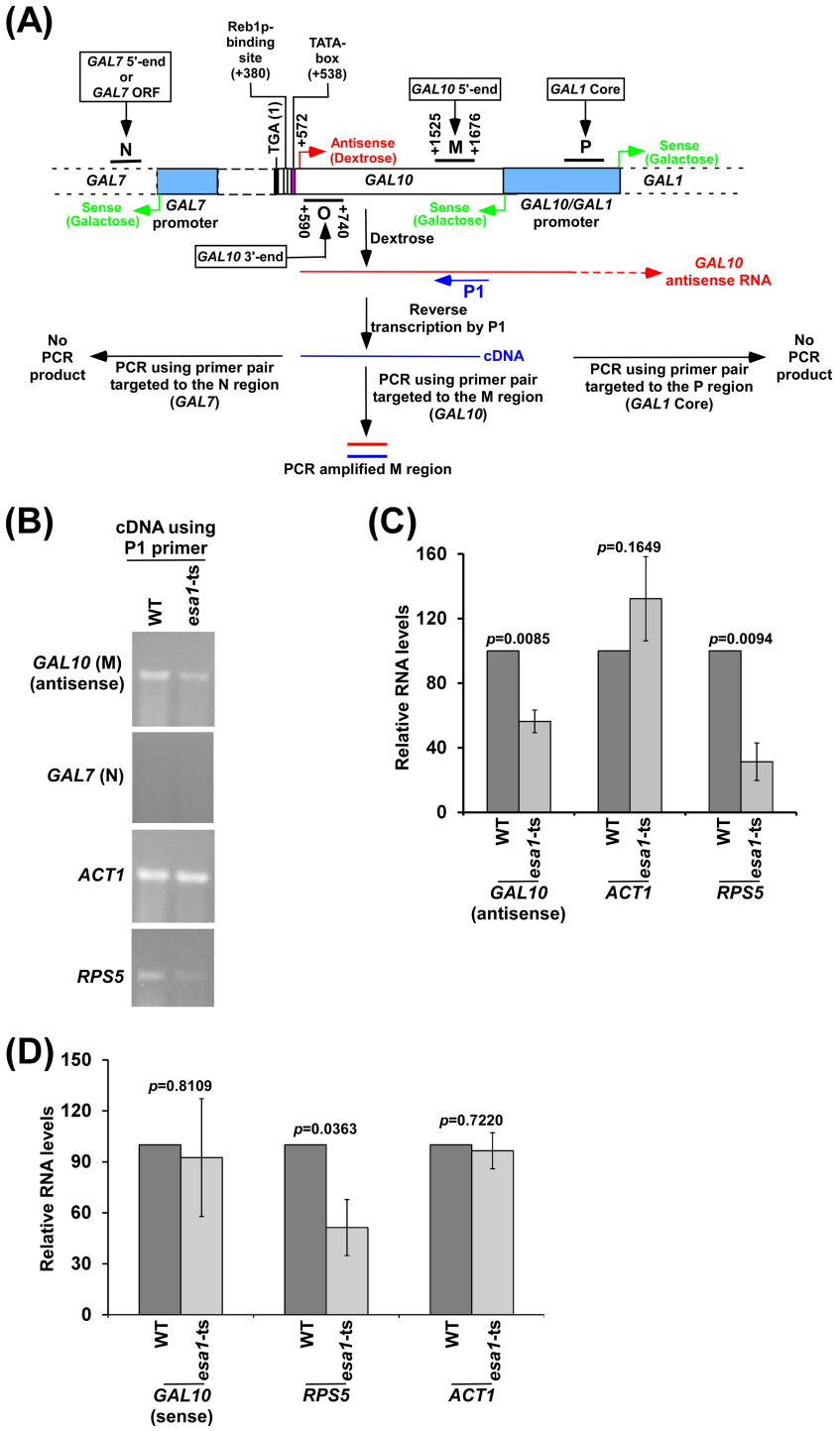


FIG 1 NuA4 KAT promotes *GAL10* antisense transcription. (A) Schematic diagram showing the experimental strategy for analysis of the *GAL10* antisense transcript. The P1 primer targeted toward the 5′ end of the *GAL10* antisense transcript was extended by AMV reverse transcriptase-based reverse transcription at 42°C, and subsequently the extended primer was amplified by primer pairs targeted to the coding regions, M and N, of *GAL10* and *GAL7*, respectively. The numbers are presented with respect to the position of the translational stop codon (TGA) of *GAL10*. Regions P, M, O, and N represent *GAL1* core, *GAL10* 5′ end, *GAL10* 3′ end, and *GAL7* 5′ end (or *GAL7* open reading frame [ORF]), respectively, in the ChIP assay. (B and C) Analysis of the *GAL10* antisense transcript in the *esa1*-ts mutant and wild-type (WT) strains in dextrose-containing growth medium. The RNA level in the wild-type strain was set to 100, and the relative RNA level in the mutant strain was plotted in panel C. (D) Sense transcription analysis of *GAL10*, *RPS5*, and *ACT1* in the *esa1*-ts and wild-type strains in galactose-containing growth medium by RT-PCR.

spectively, facilitate *GAL10* antisense transcription. Further, we find that histone H2B ubiquitin conjugase Rad6p (which is essential for histone H2B ubiquitylation) promotes *GAL10* antisense transcription. However, the Swi/Snf (*swi*tch/sucrose *non*fermentable) chromatin remodeling factor and SAGA (Spt-Ada-Gcn5-acetyltransferase) chromatin modification factor are dispensable for *GAL10* antisense transcription, while these factors have stimulatory functions in *GAL10* sense transcription. Collectively, our results demonstrate the roles of different chromatin modification/regulatory factors in controlling *GAL10* antisense transcription, thus significantly advancing our knowledge of the chromatin regulation of antisense transcription, as presented below.

MATERIALS AND METHODS

Plasmids. The plasmid pFA6a-13Myc-KanMX6 (20) was used for genomic tagging of the Esa1p and Eaf5p components of NuA4 KAT, Set1p, and Rad6p. The plasmids PRS406 (21), PRS403 (21), and pFA6a-13Myc-HIS3MX6 (20) were used for PCR-based disruption of *SET1*, *SET2*, *EAF1*, and the RING (*r*eally *i*nteresting *n*ew *g*ene) domain of *BRE1*.

Strains. A yeast (*Saccharomyces cerevisiae*) strain harboring a temperature-sensitive (ts) mutation in Esa1p (LPY3291) and its isogenic wild-type equivalent (LPY3498) were obtained from the Pillus laboratory (Lorraine Pillus, University of California, San Diego, CA) (22). A yeast strain harboring a null mutation in *SWI2* (MSY143) and its wild-type equivalent (FY406) were obtained from the Struhl laboratory (Kevin Struhl, Harvard Medical School, Boston, MA) (23). A yeast strain harboring a null mutation in *RAD6* (STY2; Δ*rad6* in FM392) and its wild-type equivalent (STY1; FM392) were obtained from the Shilatifard laboratory (Ali Shilatifard, Stowers Institute for Medical Research; purchased from Research Genetics). The *PAF1* deletion mutant strain (DY7014) in the W303a background was obtained from the Stillman laboratory (David Stillman, University of Utah Health Sciences Center) (24). A yeast strain harboring a null mutation in *SPT20* (FY1097) and its isogenic wild-type equivalent (FY67) were obtained from the Winston laboratory (Fred Winston, Harvard Medical School, Boston, MA) (25). A yeast strain carrying mutations in the Reb1p-binding site at the 3′ end of the *GAL10* coding sequence and its isogenic wild-type equivalent were obtained from the Tollervey laboratory (David Tollervey, University of Edinburg, United Kingdom) (15). A yeast strain (YKH045) expressing Flag-tagged histone H2B and hemagglutinin (HA)-tagged ubiquitin was obtained from the Osley laboratory (Mary Ann Osley, University of New Mexico School of Medicine) (26). The Δ*set3*, Δ*rpd3*, and wild-type (BY4741) strains were from the Davie laboratory (Judith K. Davie, Southern Illinois University School of Medicine; purchased from Open Biosystems). *SET1* was deleted in the wild-type strain (W303a) by the PCR-based gene disruption method to generate ASY16. *SET2* was deleted in the wild-type strain (ZDY2; derived from W303a) (27) by the PCR-based gene disruption method to generate SLY7 (28). The RING domain of Bre1p was deleted in YKH045 to generate BUY57 as done previously (29). Multiple Myc epitope tags were added at the original chromosomal loci of *SET1* and *RAD6* in the wild-type strain (W303a) to generate the PSY4 and PSY2 strains, respectively. Multiple Myc epitope tags were added at the original chromosomal locus of *EAF5* in the wild-type strain (W303a) to generate RSY70. Likewise, Eaf5p was tagged with multiple Myc epitopes at its C terminus in the BUY24 strain (which was derived from W303a by deleting *EAF1*) to generate RSY69. Multiple Myc epitope tags were added at the original chromosomal locus of *ESA1* in the wild-type strain (Sc599) (30) to generate BUY12 (17). Multiple Myc epitope tags were added at the original chromosomal locus of *ESA1* in the yeast strain carrying mutations in the Reb1p-binding site at the 3′ end of the *GAL10* coding sequence and its isogenic wild-type equivalent to generate BUY46 and BUY45, respectively. All these strains with their genotypes are listed in Table S1 in the supplemental material.

Growth media. Yeast cells were grown in yeast extract-peptone plus 2% dextrose (YPD) up to an optical density at 600 nm (OD_{600}) of 1.0 at 30°C prior to harvesting for analysis of *GAL10* antisense transcripts or formaldehyde-based *in vivo* cross-linking for chromatin immunoprecipitation (ChIP) experiments. For experiments in the wild-type and ts mutant strains, yeast cells were grown in YPD medium at 23°C up to an OD_{600} of 0.85 and then transferred to 37°C for 1 h before cross-linking or harvesting for RNA analysis. For analysis of rapamycin response, yeast cells were grown in YPD medium up to an OD_{600} of 1.0 and then treated with 100 nM rapamycin (Sigma) for 30 min prior to harvesting.

Antibodies. Various antibodies were used in the ChIP and Western blot analyses. These are anti-Rpb1 (8WG16; Covance), anti-Myc (9E10; Santa Cruz Biotechnology, Inc.), anti-HA (F-7; Santa Cruz Biotechnology, Inc.), anti-histone H3 (Ab-1791; Abcam), anti-H3 K4 trimethylation (Ab-8580; Abcam), anti-H3-K36 trimethylation (Ab-9050; Abcam), anti-Flag (F3165; Sigma), and anti-acetylated histone H4 (06866; Millipore) antibodies.

ChIP assay. The ChIP assay was performed as described previously (17, 28). Briefly, yeast cells were treated with 1% formaldehyde, collected, and resuspended in lysis buffer. Following sonication, cell lysate (400 μl of lysate from 50 ml of yeast culture) was precleared by centrifugation, and then 100 μl of lysate was used for each immunoprecipitation. Immunoprecipitated protein-DNA complexes were treated with proteinase K, the cross-links were reversed, and DNA was purified. Immunoprecipitated DNA was dissolved in 20 μl of TE 8.0 (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA), and 1 μl of immunoprecipitated DNA was analyzed by PCR (a total of 23 cycles). The PCR mixture contained [α-^{32}P]dATP (2.5 μCi for 25 μl of reaction mixture), and the PCR products were detected by autoradiography after separation on a 6% polyacrylamide gel. As a control, "input" DNA was isolated from 5 μl of lysate without going through the immunoprecipitation step and was dissolved in 100 μl of TE 8.0. To compare the PCR signal arising from the immunoprecipitated DNA with that from the input DNA, 1 μl of input DNA was used in the PCR analysis. For ChIP analysis of histone H3, histone H2B, and Myc-tagged Esa1p, Eaf5p, Set1p, Rad6p, and Rpb1p, the protocol described above was modified as described previously (17, 28). Serial dilutions of input and immunoprecipitated DNA samples were used to assess the linear range of PCR amplification as described previously (18). The data presented here are within the linear range of PCR analysis. The primer pairs used for PCR analysis were as follows: *GAL10* 3′ end, 5′-CTATGTTCAGTTAGTTTGGCTAGC-3′ and 5′-TTGATGCTCTGCATAATAATGCCC-3′; *GAL10* 5′ end, 5′-CTACGAGATTCCCAAATATGATTCC-3′ and 5′-TAACGCAAGATAGCAAACTTCCAAC-3′; *GAL7* 5′ end, 5′-AAAGTGCAATCTGTGAGAGGCAATT-3′ and 5′-TTTTCTCTTGCTTCTCTGGAGAGAT-3′; *GAL1* core, 5′-ATAGGATGATAATGCGATTAGTTTTTTAGCCTT-3′ and 5′-GAAAATGTTGAAAGTATTAGTTAAAGTGGTTATGCA-3′; *RPS5* UAS, 5′-AGAAACAATGAACAGCCTTGAGTTCTC-3′ and 5′-GCAGGGCCATTCTCATCTGA-3′; Chr.-V, 5′-GGCTGTCAGAATATGGGGCCGTAGTA-3′ and 5′-CACCCCGAAGCTGCTTTCACAATAC-3′; *ADH1* 5′ end, 5′-CTGGTTACACCCACGACGGTTCTT-3′ and 5′-CAGACTTCAAAGCCTTGTAGACG-3′; and 18S ribosomal DNA (rDNA), 5′-GAGTCCTTGTGGCTCTTGGC-3′ and 5′-AATACTGATGCCCCCGACC-3′.

Autoradiograms were scanned and quantitated by the National Institutes of Health ImageJ (version 1.62) program. Immunoprecipitated DNAs were quantitated as the ratio of immunoprecipitate to input in the autoradiogram. The average ChIP signal from the biologically independent experiments is reported with standard deviation (SD) (Microsoft Office Excel 2003). The Student *t* test in Microsoft Excel 2003 (with tail = 2 and types = 3) was used to determine the *P* values for statistical significance of the changes in the ChIP signals. The changes were considered to be statistically significant at a *P* value of <0.05. ChIP signals were determined for the upstream activating sequence (UAS), the core promoter (core), toward the 5′ end of the coding sequence (5′ end), toward the 3′ end of the coding sequence (3′ end), and an inactive region within chromosome V (Chr.-V).

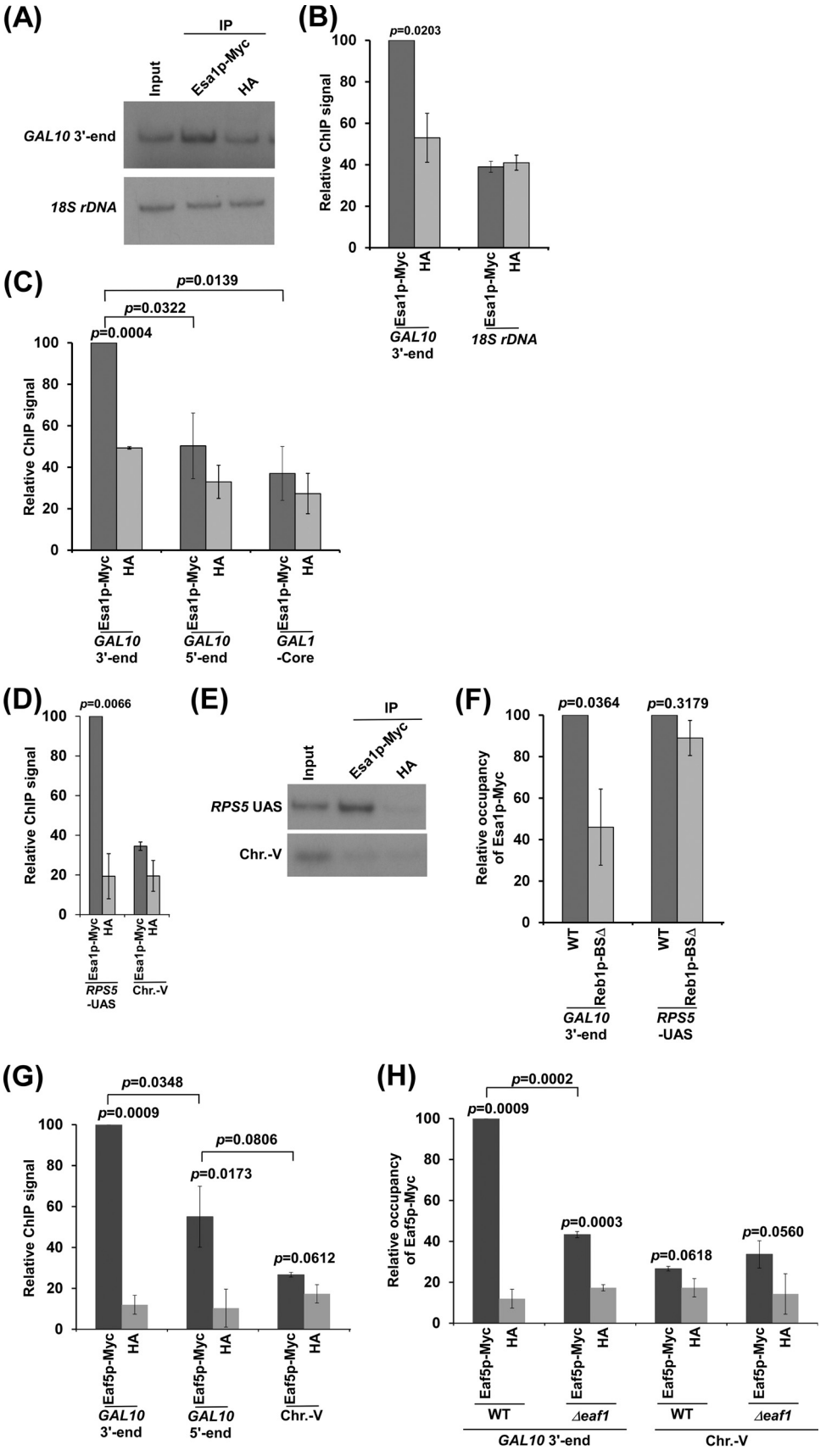
ChDIP assay. To determine histone H2B ubiquitylation at *GAL10*, we performed a chromatin double-immunoprecipitation (ChDIP) assay as described previously (31). Briefly, 400 μl lysate from 50 ml yeast culture was first immunoprecipitated using anti-Flag antibody and protein A/G Plus-agarose beads. Following elution of anti-Flag immunoprecipitate by Flag peptide (Sigma), the eluate was immunoprecipitated using an anti-HA antibody, and immunoprecipitated DNA sample was dissolved in 10 μl TE 8.0, of which 1 μl was used for PCR analysis. The "input" DNA was isolated from 5 μl lysate and suspended in 100 μl TE 8.0, of which 1 μl was used for PCR analysis.

RNA preparation. Total RNA was prepared from yeast cell culture as described by Peterson et al. (32). Briefly, 10 ml of yeast culture was harvested and then suspended in 100 μl of RNA preparation buffer (500 mM NaCl, 200 mM Tris-HCl, 100 mM Na_2-EDTA, and 1% SDS) along with 100 μl of phenol-chloroform-isoamyl alcohol and a 100-μl volume-equivalent of glass beads (acid washed) (Sigma). Subsequently, the yeast cell suspension was vortexed at maximum speed (10 in a VWR Mini-vortex mixer) (catalog number 58816-121; VWR) five times (30 s each). The cell suspension was put on ice for 30 s between pulses. After vortexing, 150 μl of RNA preparation buffer and 150 μl of phenol-chloroform-isoamyl alcohol were added to the yeast cell suspension, followed by vortexing for 15 s at maximum speed on a VWR mini-vortex mixer. The aqueous phase was collected following a 5-min centrifugation at maximum speed in a microcentrifuge machine. Total RNA was isolated from the aqueous phase by ethanol precipitation.

Analysis of *GAL10* antisense transcript. Reverse transcription-PCR (RT-PCR) analysis of the *GAL10* antisense transcript was performed according to standard protocols (14). Briefly, total RNA was prepared from yeast culture as described above. Equal amounts (15 to 30 μg) of total RNA were used in the reverse transcription assay for both the wild-type and mutant strains. RNA was treated with RNase-free DNase (M610A; Promega), and then reverse transcribed into cDNA using primer P1 (Fig. 1A) targeted to the *GAL10* antisense transcript (P1, 5′-CTACGAGATTC CCAAATATGATTCC-3′) as described in the protocol supplied by Promega (A3800; Promega). Reverse transcription was carried out at 42°C using avian myeloblastosis virus (AMV) reverse transcriptase. PCR was performed within the linear range using synthesized first-strand cDNA (or the extended P1 primer shown in Fig. 1A) as the template and primer pairs targeted to the *GAL10* coding sequence (represented as region M in Fig. 1A), *GAL1* core promoter (marked as region P in Fig. 1A), and *GAL7* coding sequence (marked as region N in Fig. 1A). Primer pairs targeted to the *ACT1*, *RPL2B*, *RPS5*, *PYK1*, and *ADH1* coding sequences were also used to amplify the above cDNAs. RT-PCR products were separated by 2.2% agarose gel electrophoresis and visualized by ethidium bromide staining. RT-PCR experiments were carried out three times. These experiments are biologically independent. The average signal from these biologically independent experiments is reported with SD (Microsoft Excel 2003). Student's *t* test (with tail = 2 and types = 3) was used to determine *P* values for statistical significances of the changes in the RT-PCR signals. The changes were considered to be statistically significant at a *P* value of <0.05. The primer pairs used in the PCR analysis of cDNAs were as follows: *GAL7* (N), 5′-AAAGTGCAATCTGTGAGAGGCAATT-3′ and 5′-TTTTCTCTTGCTTCTCTGGAGAGAT-3′; *GAL10* (M), 5′-CTACGA GATTCCCAAATATGATTCC-3′ and 5′-TAACGCAAGATAGCAAACT TCCAAC-3′; *ACT1*, 5′-TCCACCACTGCTGAAAGAGAAATTG-3′ and 5′-AATAGTGATGACTTGACCATCTGGA-3′; *RPL2B*, 5′-GTGCTTTC CACAAGTACAGATTGAA-3′ and 5′-TTTGACCAGAAACGGCACCTC TAGA-3′; *RPS5*, 5′-AGGCTCAATGTCCAATCATTGAAAG-3′ and 5′-CAACAACTTGGATTGGGTTTTGGTC-3′; *ADH1*, 5′-CGGTAACAGA GCTGACACCAGAGA-3′ and 5′-ACGTATCTACCAACGATTTGACC C-3′; *PYK1*, 5′-AAGTTTCCGATGTCGGTAACGCTAT-3′ and 5′-TTG GCAAGTAAGCGATAGCTTGTTC-3′; *GAL1* (P), 5′-ATAGGATGATA ATGCGATTAGTTTTTTAGCCTT-3′ and 5′-GAAAATGTTGAAAGTA TTAGTTAAAGTGGTTATGCA-3′; and 18S rDNA, 5′-GAGTCCTTGT GGCTCTTGGC-3′ and 5′-AATACTGATGCCCCCGACC-3′.

RESULTS

NuA4 KAT facilitates antisense transcription from the 3′ end of the *GAL10* coding sequence. NuA4 KAT has been previously shown to be required for recruitment of TAFs (or TFIID) to the promoters of the ribosomal protein genes for transcriptional initiation (17). However, the role of NuA4 KAT in antisense transcriptional initiation remains unknown. We have recently demonstrated the roles of TAFs in antisense transcriptional initiation from the 3′ end of the *GAL10* coding sequence (14). Like sense transcription of the ribosomal protein genes, the TAF-dependent antisense transcription from *GAL10* may also require NuA4 KAT. To test this, we analyzed the role of NuA4 KAT in antisense transcription from the 3′ end of the *GAL10* coding sequence. In this direction, the *ESA1* (which is an integral component of NuA4 with KAT activity) wild-type and ts strains were grown in dextrose-containing medium (which induces *GAL10* antisense transcription and represses *GAL10* sense transcription [14]) at 23°C up to an OD_{600} of 0.85 and then switched to 37°C for 1 h for ts inactivation of Esa1p prior to harvesting for *GAL10* antisense transcript analysis, as done previously (17). Total RNAs from the wild-type and ts mutant strains were isolated and analyzed as described in our recent publication (14) and schematically shown in Fig. 1A. Briefly, a primer (P1) targeted toward the 5′ end of the *GAL10* antisense transcript was used for synthesis of cDNA, and subsequently, cDNA was amplified using a primer pair encompassing region M in the *GAL10* coding sequence. Such analysis would generate the *GAL10* antisense transcript but not sense transcript in dextrose-containing growth medium. However, the use of the P1 primer in the cDNA synthesis can also detect sense transcripts of other genes (e.g., *ACT1*, *ADH1*, *RPS5*, and *RPL2B*) that are expressed in dextrose-containing growth medium, since the P1 primer can randomly hybridize to RNAs (including mRNAs and rRNAs) at 42°C during cDNA synthesis via matched (A·T and G·C) as well as mismatched (G·T, G·A, A·C, T·C, G·G, A·A, T·T, and C·C) base pairs (33–35), as discussed previously (14). We found that *GAL10* antisense transcription was significantly decreased in the *esa1*-ts mutant strain in comparison to the wild-type equivalent (Fig. 1B and C; see Fig. S1A and B in the supplemental material). Likewise, Esa1p promoted sense transcription of the NuA4-dependent *RPS5* gene (Fig. 1B and C). However, sense transcription of the NuA4-independent *ACT1* gene (19) was not changed in the *esa1*-ts mutant strain (Fig. 1B and C). Further, as a control, we used a primer pair targeted to the *GAL7* coding sequence (region N) in the above PCR analysis. We did not observe a PCR signal (Fig. 1B), as the *GAL7* primer pair (which was successfully used in our previous studies and generated a PCR signal when genomic DNA was used as a template [see Fig. S1C in the supplemental material]) (14) was located upstream of the *GAL10* antisense initiation site (Fig. 1A) (14–16) and *GAL7* sense transcription does not occur in dextrose-containing growth medium (14). Likewise, using a primer pair (at region P) upstream of the P1 primer (Fig. 1A) in the above RT-PCR analysis, we did not observe a PCR signal (see Fig. S1D in the supplemental material), as the primer pair (which amplified the P region when genomic DNA was used as a template [see Fig. S1C in the supplemental material]) at region P is located outside the P1-generated cDNA of the *GAL10* antisense transcript. The absence of the PCR signals at the N region of *GAL7* or the P region (Fig. 1A and B; see Fig. S1D in the supplemental material) supports that there was no residual


(A)
IP
Input
Esa1p-Myc
HA
GAL10 3'-end
18S rDNA
(B)
p=0.0203
Relative ChIP signal
100
80
60
40
20
0
Esa1p-Myc
HA
GAL10 3'-end
18S rDNA
(C)
p=0.0139
p=0.0322
p=0.0004
Relative ChIP signal
GAL10 3'-end
GAL10 5'-end
GAL1 -Core
(D)
p=0.0066
Relative ChIP signal
RPS5 -UAS
Chr.-V
(E)
IP
Input
Esa1p-Myc
HA
RPS5 UAS
Chr.-V
(F)
p=0.0364
p=0.3179
Relative occupancy of Esa1p-Myc
WT
Reb1p-BSΔ
GAL10 3'-end
RPS5 -UAS
(G)
p=0.0348
p=0.0009
p=0.0806
p=0.0173
p=0.0612
Relative ChIP signal
Eaf5p-Myc
HA
GAL10 3'-end
GAL10 5'-end
Chr.-V
(H)
p=0.0002
p=0.0009
p=0.0003
p=0.0618
p=0.0560
Relative occupancy of Eaf5p-Myc
WT
Δeaf1
GAL10 3'-end
Chr.-V

DNA contamination in the above RT-PCR analysis. Collectively, our results support that NuA4 KAT facilitates *GAL10* antisense transcription. However, *GAL1/10* sense transcription is not regulated by NuA4 KAT (19) (Fig. 1D), while *RPS5* and other ribosomal protein genes are dependent on NuA4 KAT for sense transcription (17–19) (Fig. 1D). Consistent with these results, NuA4 KAT was not found to be associated with the *GAL1/10* promoter (19) but was associated with *RPS5* and other ribosomal protein genes (17–19).

NuA4 KAT associates with the 3′ end of the *GAL10* coding sequence. To determine whether NuA4 KAT plays a direct role in facilitating antisense transcription from the 3′ end of the *GAL10* coding sequence, we analyzed the association of Myc epitope-tagged Esa1p with the *GAL10* 3′ end in dextrose-containing growth medium. We found significant association of Myc-tagged Esa1p with the 3′ end of the *GAL10* coding sequence (region O in Fig. 1A) in comparison to the nonspecific anti-HA antibody (or background signal) (Fig. 2A and B). As a negative control, we also analyzed the association of Myc-tagged Esa1p with 18S rDNA (or the gene transcribed by RNA polymerase I) and found that the Myc-tagged Esa1p ChIP signal was same as that of anti-HA (or background) (Fig. 2A and B). Further, the PCR analysis for 18S rDNA was carried out within the linear range, as the ratio of the Myc-tagged Esa1p and HA signals was not significantly altered at various dilutions (see Fig. S2 in the supplemental material). Thus, our results support that Esa1p is associated with the *GAL10* 3′ end where antisense transcription is initiated. However, similar recruitment of Esa1p was not observed at the 5′ end of the *GAL10* coding sequence (region M in Fig. 1A) or *GAL1* core promoter (region P in Fig. 1A) in dextrose-containing growth medium (Fig. 2C). NuA4 KAT associates with the UASs of the NuA4-dependent ribosomal protein genes for TAF-dependent sense transcription (17–19). Thus, as a positive control, we showed that Esa1p is associated with the UAS of a ribosomal protein gene, *RPS5*, but not with an inactive region within chromosome V (Chr.-V) (Fig. 2D and E), consistent with previous studies (17, 18). Collectively, our results demonstrate that Esa1p associates with the *GAL10* antisense transcription initiation site. Such association of NuA4 KAT with *GAL10* is dependent on the Reb1p-binding site (Fig. 2F) that recruits Reb1p to the *GAL10* antisense transcription initiation site to trigger transcription (15). As a control, we showed that the targeting of NuA4 KAT to the *RPS5* UAS is not altered in the absence of the Reb1p-binding site (Fig. 2F), since the Reb1p-binding site is absent at the *RPS5* promoter/UAS. At the *RPS5* promoter/UAS, recruitment of NuA4 KAT depends on the RPG box that targets the activator Rap1p (17–19). Thus, our results support that NuA4 KAT is targeted by Reb1p at the *GAL10* antisense transcription initiation site. Consistently, the Eaf5p component of NuA4 KAT is predominantly recruited to the 3′ end of the *GAL10* coding sequence (Fig. 2G). Further, NuA4 KAT's overall structural integrity is maintained by its Eaf1p component (18, 36, 37). In agreement with this fact, we find that the recruitment of the Eaf5p component of NuA4 KAT to the 3′ end of the *GAL10* coding sequence is impaired in the Δ*eaf1* strain (Fig. 2H). Similarly, NuA4 KAT components are not recruited to the promoters of the ribosomal protein genes in the absence of Eaf1p (18).

Since NuA4 KAT is associated with the *GAL10* antisense transcription initiation site, it is likely to regulate targeted histone H4 acetylation. To test this, we analyzed the level of histone H4 acetylation at the 3′ end of the *GAL10* coding sequence in the wild-type and *esa1*-ts mutant strains at the nonpermissive temperature. We found that histone H4 acetylation at the 3′ end of the *GAL10* coding sequence is impaired in the *esa1*-ts mutant strain in comparison to the wild-type equivalent (Fig. 3A to C). Such a decrease of histone H4 acetylation is not due to the eviction of histone H3/H4 tetramer, since the level of histone H3 (a representative component of histone H3/H4 tetramer) at the *GAL10* antisense transcription initiation site is not reduced in the *esa1*-ts mutant strain (Fig. 3B and C). Further, we found that the 5′ end of the *GAL10* coding sequence is also acetylated at histone H4 in an Esa1p-dependent manner (Fig. 3C). Similarly, Esa1p-dependent histone H4 acetylation is also observed at the *GAL7* coding sequence (Fig. 3C) and inactive region within Chr.-V (18) in dextrose-containing growth medium. The presence of such histone H4 acetylation at the 5′ ends of the *GAL10* and *GAL7* coding sequences or Chr.-V is likely due to nontargeted global histone H4 acetylation by piccolo-NuA4 (picNuA4) (which is composed of Esa1p, Yng2p, and Epl1p [38, 39]), as previous studies (38, 39) demonstrated the role of picNuA4 in global histone H4 acetylation in a nontargeted fashion. Consistently, we also observed nontargeted histone H4 acetylation at the promoters of the NuA4-dependent ribosomal protein genes in the absence of NuA4 or Eaf1p (18), and such acetylation is impaired in the *esa1*-ts mutant (18), hence supporting the role of picNuA4 in nontargeted histone H4 acetylation in the absence of NuA4. Here, we observed a high level of histone H4 acetylation predominantly at the 3′ end of the *GAL10* coding sequence (Fig. 3D), and such acetylation was impaired in the absence of the Reb1p (or activator)-binding site (Fig. 3E and F). These results indicate nontargeted histone H4 acetylation at *GAL10* in the absence of NuA4 KAT, similar to the results for the ribosomal protein gene and other genes (18). Although picNuA4 is involved in global genome-wide histone H4 acetylation, its nontargeted association with chromatin is not generally observed by the ChIP assay (18, 19), possibly due to weak/transient interaction. Nontargeted histone H4 acetylation is not associated with transcription (19). For example, many genes/promoters (e.g., *ADH1*, *ACT1*, and *GAL1*) are globally acetylated at histone H4 by picNuA4, and such nontargeted histone modifica-

FIG 2 Analysis of NuA4 KAT recruitment to the 3′ end of the *GAL10* coding sequence. (A and B) Esa1p is associated with the 3′ end of the *GAL10* coding sequence in dextrose-containing growth medium. Immunoprecipitation (IP) was carried out using an anti-Myc antibody against Myc-tagged Esa1p. Immunoprecipitated DNA was analyzed by PCR using the primer pairs encompassing the 3′ end of the *GAL10* coding sequence (region O in Fig. 1A) and a region with 18S rDNA. The ratio of the immunoprecipitate over the input in the autoradiogram (termed a ChIP signal) was measured. The maximum ChIP signal was set to 100, and other ChIP signals relative to the maximum ChIP signal (represented as relative ChIP signal or relative occupancy) were plotted. (C, D, and E) ChIP analysis of Myc-tagged Esa1p at the 3′ and 5′ ends (regions O and M, respectively, in Fig. 1A) of the *GAL10* coding sequence, the *GAL1* core promoter (region P in Fig. 1A), the *RPS5* UAS, and an inactive region within chromosome V (Chr.-V). (F) ChIP analysis of Myc-tagged Esa1p at the 3′ end of the *GAL10* coding sequence and *RPS5* UAS in the presence and absence of a Reb1p-binding site at the 3′ end of the *GAL10* coding sequence. (G) ChIP analysis of Myc-tagged Eaf5p at the 3′ and 5′ ends of the *GAL10* coding sequence and Chr.-V in dextrose-containing growth medium. (H) ChIP analysis of Myc-tagged Eaf5p at the 3′ end of the *GAL10* coding sequence in the wild-type and Δ*eaf1* strains.

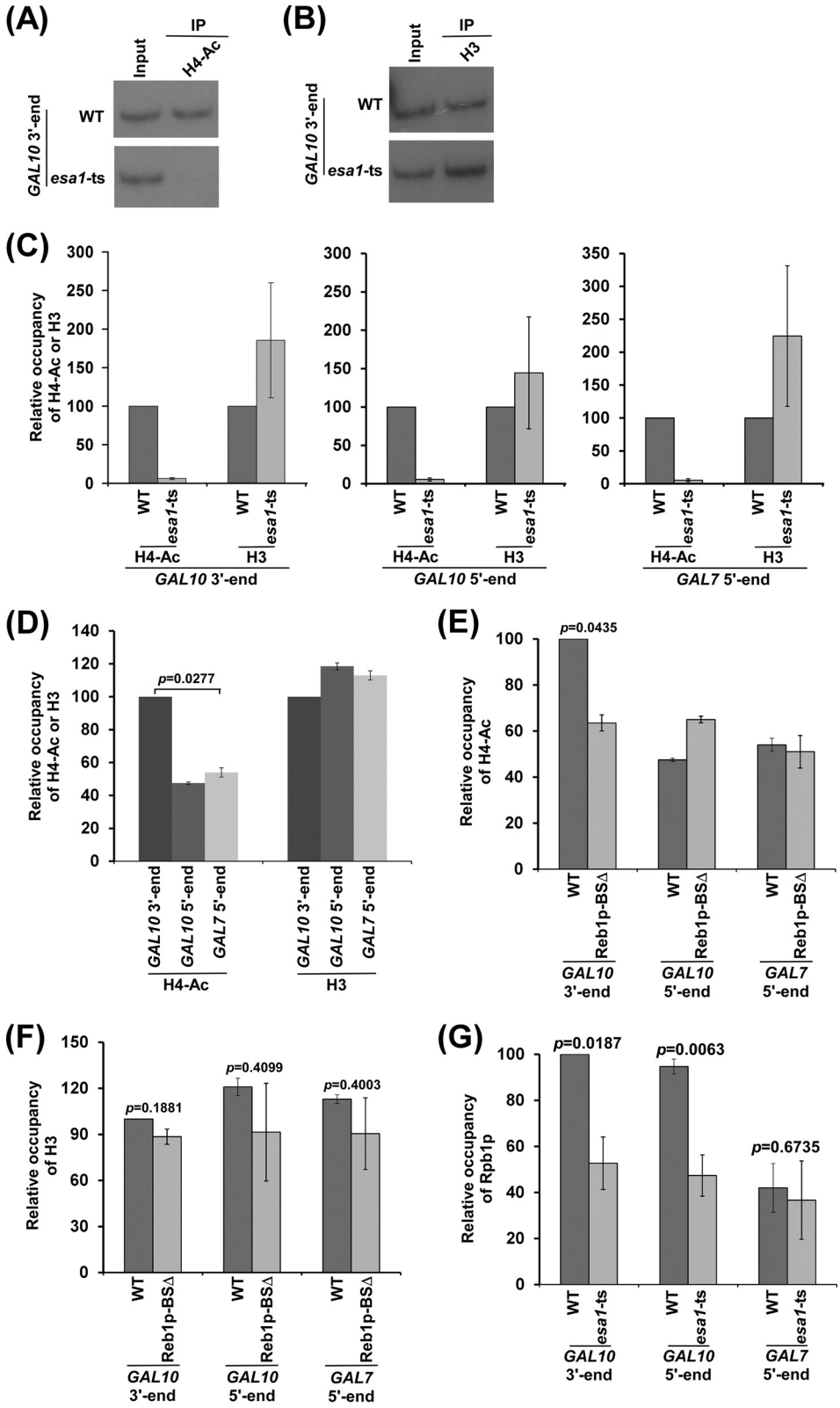


FIG 3 Analysis of histone H4 acetylation at the 3′ end of the *GAL10* coding sequence. (A to C) Analysis of the levels of histone H4 acetylation and histone H3/H4 tetramer at the 3′ and 5′ ends of the *GAL10* coding sequence and *GAL7* ORF (or *GAL7* 5′ end) in the *esa1*-ts mutant and wild-type strains. Immunoprecipitation was carried out using an anti-histone H4 acetylation antibody against acetylated histone H4 or an anti-histone H3 antibody against histone H3 of the histone H3/H4 tetramer. (D) Analysis of histone H4 acetylation and histone H3 at the 5′ and 3′ ends of the *GAL10* coding sequence and 5′ end of the *GAL7* coding sequence. (E and F) The Reb1p-binding site regulates histone H4 acetylation at the 3′ end of the *GAL10* coding sequence. (G) Analysis of Rpb1p association with *GAL10* in dextrose-containing growth medium in the *esa1*-ts mutant and wild-type strains.

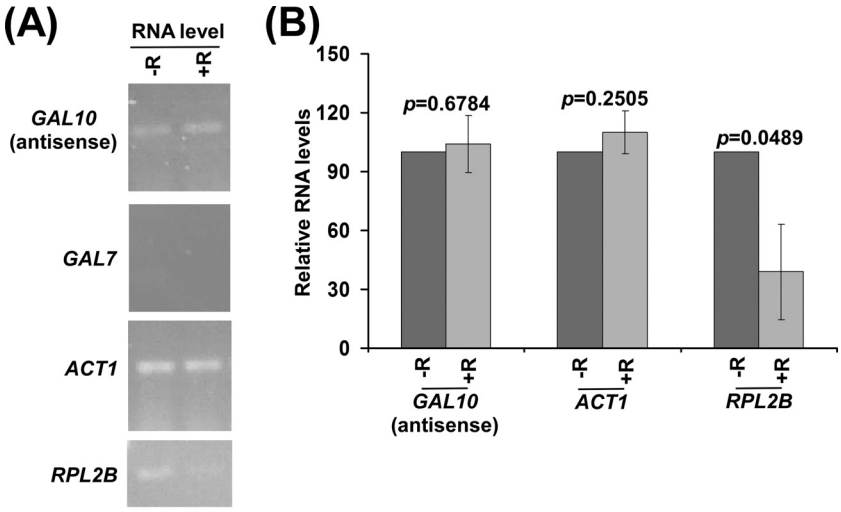


FIG 4 NuA4 KAT-dependent *GAL10* antisense transcription is not altered in response to rapamycin treatment. (A) RT-PCR analysis of the *GAL10* antisense transcript with (+R) or without (−R) rapamycin treatment. (B) The transcription data shown in panel A were plotted.

tion does not modulate their transcription (19). However, targeted histone H4 acetylation by NuA4 KAT regulates transcription. For example, the promoters of the ribosomal protein genes are acetylated at histone H4 by targeted recruitment of NuA4 KAT for stimulation of transcription (17–19). Likewise, targeted histone H4 acetylation at the 3′ end of the *GAL10* coding sequence facilitates antisense transcription (Fig. 1B and C; see Fig. S1A and B in the supplemental material) via enhanced recruitment of RNA polymerase II (Fig. 3G).

NuA4 KAT-dependent *GAL10* antisense transcription is not regulated by TOR, while NuA4 KAT-mediated sense transcription of the ribosomal protein genes is impaired following inhibition of TOR. Previous studies (17, 40) demonstrated that the association of NuA4 KAT with the ribosomal protein genes is impaired by rapamycin-mediated inhibition of the TOR pathway. Consequently, sense transcription of the ribosomal protein genes is decreased following rapamycin treatment (40). To determine whether TOR similarly regulates antisense transcription, we analyzed the levels of *GAL10* antisense transcripts with or without rapamycin treatment. We found that *GAL10* antisense transcription is not altered in response to rapamycin treatment (Fig. 4). As a control, we showed that sense transcription of the NuA4-independent *ACT1* gene (17, 19) is not altered following rapamycin treatment (Fig. 4). However, sense transcription of a ribosomal protein gene, *RPL2B*, is impaired in response to rapamycin treatment (Fig. 4), consistent with previous studies (17, 40). Thus, rapamycin (or the TOR pathway) differentially regulates NuA4 KAT-dependent sense transcription at the ribosomal protein genes (e.g., *RPL2B*) and antisense transcription at *GAL10*.

Histone H3 K4 and K36 methyltransferases facilitate *GAL10* antisense transcription. Like histone H4 acetylation, histone H3 K4 trimethylation is also found at the 3′ end of the *GAL10* coding sequence in dextrose-containing growth medium (15, 16) (Fig. 5A). Such modification is targeted by Reb1p (15). However, Set1p, which methylates K4 of histone H3 (41), is not found to be associated with the 3′ end of the *GAL10* coding sequence in dextrose-containing growth medium (15). Likewise, we also did not observe the association of Set1p with the 3′ end of the *GAL10* coding sequence in dextrose-containing growth medium (Fig. 5B), while Set1p is associated with the 5′ end of the coding sequence of *ADH1*, which is constitutively engaged in sense transcription (Fig. 5B), consistent with previous studies (42). This could be due to weak/transient interaction (and/or cross-linking) of Set1p with the *GAL10* antisense transcription initiation site during less frequent antisense transcription, as also suggested in previous studies (15). Nonetheless, Set1p-mediated H3 K4 methylation is observed at the *GAL10* antisense transcription initiation site (15) (Fig. 5A). To determine the role of histone H3 K4 methylation in antisense transcription, we analyzed the levels of *GAL10* antisense transcripts in the wild-type and deletion mutant strains of *SET1*. We found that *GAL10* antisense transcription is impaired in the Δ*set1* strain in comparison to the wild-type equivalent (Fig. 5C and D). As a control, we demonstrated that sense transcription of the Set1p-independent *ACT1* gene (43, 44) is not altered in the absence of Set1p or histone H3 K4 methylation (Fig. 5C and D). Thus, our results support that histone H3 K4 methylation is associated with *GAL10* antisense transcription. Histone H3 K4 methylation has been previously implicated in controlling sense transcription via Rpd3S (histone deacetylase complex small or Rpd3 small) and the Set3p-containing histone deacetylase complex (15, 16, 45). However, we found that Set3p or Rpd3p does not regulate *GAL10* antisense transcription (Fig. 5E to G). Thus, Set1p regulates *GAL10* antisense transcription independently of Set3p and Rpd3p. Further, such function of Set1p is not mediated via histone H4 acetylation, as we did not observe an alteration of histone H4 acetylation at the *GAL10* antisense transcription initiation site in the Δ*set1* strain in comparison to the wild-type equivalent (Fig. 5H).

Previous studies (15) demonstrated Set2p-mediated histone H3 K36 trimethylation at *GAL10* under growth conditions permissive to *GAL10* antisense transcription. Such covalent modification is dependent on Reb1p-mediated *GAL10* antisense transcription (15). Histone H3 K36 methylation has been implicated in repression of sense transcription via the recruitment of Rpd3S (46, 47). In addition, histone H3 K36 methylation may also be involved in regulation of *GAL10* antisense transcription. To test

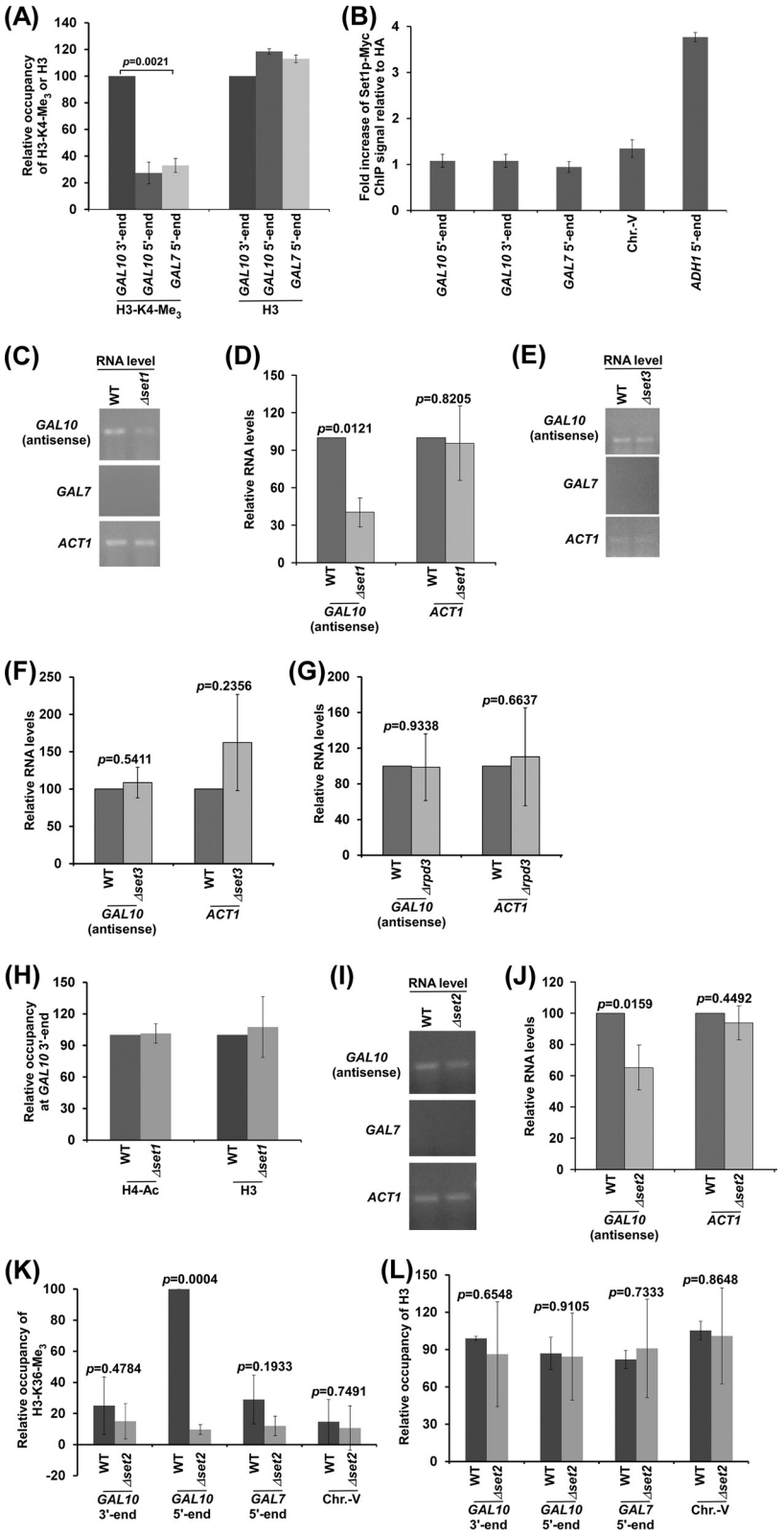


FIG 5 *GAL10* antisense transcription is regulated by histone H3 K4 and K36 methyltransferases. (A) Analysis of histone H3 K4 trimethylation (H3-K4-Me_3) and histone H3 levels at the 5′ and 3′ ends of the *GAL10* coding sequence and the 5′ end of the *GAL7* coding sequence. (B) ChIP analysis of Myc-tagged Set1p at the 5′ and 3′ ends of the *GAL10* coding sequence, Chr.-V, and 5′ ends of the *ADH1* and *GAL7* coding sequence. The fold increase of Set1p ChIP signal relative to HA is plotted. (C and D) RT-PCR analysis of *GAL10* antisense RNA in the Δ*set1* and wild-type strains. The transcription data shown in panel C were plotted in panel D. (E and F) RT-PCR analysis of *GAL10* antisense RNA in the Δ*set3* and wild-type strains. (G) RT-PCR analysis of *GAL10* antisense RNA in the Δ*rpd3* and wild-type strains. (H) ChIP analysis of histone H4 acetylation and histone H3 levels at *GAL10* in the wild-type and Δ*set1* strains. (I and J) RT-PCR analysis of *GAL10* antisense RNA in the Δ*set2* and wild-type strains. (K and L) ChIP analysis of histone H3 K36 trimethylation (H3 K36-Me_3) and histone H3 at *GAL10*, *GAL7*, and Chr.-V in dextrose-containing growth medium. The maximum ChIP signal was set to 100, and other ChIP signals relative to the maximum ChIP signal were plotted.

this, we analyzed the levels of *GAL10* antisense transcripts in the wild-type and deletion mutant strains of *SET2*. We found that the level of *GAL10* antisense transcript is decreased in the Δ*set2* strain in comparison to the wild-type equivalent (Fig. 5I and J). However, the level of *ACT1* sense transcript is not altered in the Δ*set2* strain (Fig. 5I and J), consistent with previous studies (48). Thus, our results support that histone H3 K36 methylation favors antisense transcription. Further, the deletion of Rpd3p does not alter *GAL10* antisense transcription (Fig. 5G). Thus, Set2p regulates *GAL10* antisense transcription independently of Rpd3p. Moreover, we found that the *GAL10* antisense coding sequence (i.e., the *GAL10* 5′ end) (Fig. 1A), but not the initiation site (i.e., *GAL10* 3′ end) (Fig. 1A), is methylated predominantly at K36 of histone H3 (Fig. 5K and L), consistent with previous studies (15), suggesting a role of histone H3 K36 methylation in regulation of *GAL10* antisense transcription elongation.

***GAL10* antisense transcription is facilitated by histone H2B ubiquitylation.** Histone H3 K4 methylation is promoted by histone H2B ubiquitylation during sense transcription (49). Further, histone H2B ubiquitylation has been shown to enhance sense transcription (50, 51). Similar to the regulation of sense transcription, histone H2B ubiquitylation may also control antisense transcription. To test this, we analyzed the levels of *GAL10* antisense transcripts in the *RAD6* (which has histone H2B ubiquitin conjugase activity for histone H2B ubiquitylation via Bre1p ubiquitin ligase [49]) wild-type and deletion mutant strains. We found that the level of *GAL10* antisense transcript was decreased in the Δ*rad6* strain in comparison to the wild-type equivalent (Fig. 6A and B). Likewise, Rad6p promoted sense transcription of *ACT1* (Fig. 6A and B), consistent with previous studies (26, 29). As a loading control, we showed that the 18S rRNA level was not altered in the Δ*rad6* strain (Fig. 6A and B). Thus, our results support that *GAL10* antisense transcription is facilitated by Rad6p (or histone H2B ubiquitylation). However, we could not detect Rad6p ubiquitin conjugase at *GAL10* in dextrose-containing growth medium (Fig. 6C). Similarly, Rad6p, which ubiquitylates histone H2B during sense transcription, was also not found to be associated with the active *ADH1* gene (Fig. 6C). This could be due to weak/transient association (or poor cross-linking) of Rad6p with the active gene, analogous to the fact that Mediator is not found to be associated with the promoters of the ribosomal protein genes which require the Mediator complex for their transcription (52). Nonetheless, we found histone H2B ubiquitylation at *GAL10*, and such modification was impaired in the absence of the RING domain (which is essential for histone H2B ubiquitylation) of Bre1p ubiquitin ligase (Fig. 6 to F), consistent with previous studies (53). Further, histone H2B ubiquitylation promotes *GAL10* antisense transcription (Fig. 6A and B). Such function of histone H2B ubiquitylation is not mediated via histone H4 acetylation, as we did not observe an alteration of histone H4 acetylation in the Δ*rad6* strain in comparison to the wild-type equivalent (Fig. 6G).

The fact that histone H2B is ubiquitylated at the *GAL10* 5′ and 3′ ends in dextrose-containing growth medium (Fig. 6 to F) indicates that histone H2B ubiquitylation may regulate both *GAL10* antisense transcription initiation and elongation. To test this, we analyzed the association of RNA polymerase II with the *GAL10* antisense transcription initiation site (i.e., *GAL10* 3′ end) (Fig. 1A) and coding sequence (i.e., *GAL10* 5′ end) (Fig. 1A) in dextrose-containing growth medium in the wild-type and *bre1*Δ*500* strains. We found that the loss of histone H2B ubiquitylation in the *bre1*Δ*500* strain (Fig. 6 to F) did not significantly alter the association of RNA polymerase II with the *GAL10* antisense transcription initiation site (Fig. 6H). However, RNA polymerase II association with the *GAL10* antisense coding sequence was significantly decreased in the *bre1*Δ*500* strain (Fig. 6H). These observations indicate the role of histone H2B ubiquitylation in facilitation of *GAL10* antisense transcriptional elongation. Likewise, histone H2B ubiquitylation promotes sense transcriptional elongation (50, 51).

Histone H2B ubiquitylation is regulated by Paf1p or the Paf1p-containing complex (53, 54). Further, Paf1p interacts with RNA polymerase II and promotes sense transcription. We have recently demonstrated that Reb1p targets RNA polymerase II to *GAL10* for antisense transcription (14). These observations indicate that Paf1p may regulate *GAL10* antisense transcription. To test this, we analyzed the levels of *GAL10* antisense transcripts in the wild-type and Δ*paf1* strains. We found that *GAL10* antisense transcription is significantly decreased in the Δ*paf1* strain in comparison to the wild-type equivalent (Fig. 6I and J). Likewise, the loss of Paf1p impairs sense transcription of *ACT1* (Fig. 6I and J), consistent with previous studies (29). Thus, our results demonstrate that Paf1p facilitates *GAL10* antisense transcription. Similarly, Paf1p also regulates *GAL* sense transcription (29).

SAGA does not regulate *GAL10* antisense transcription but rather promotes sense transcription. We found that histone H2B ubiquitylation facilitates *GAL10* antisense transcription. Histone H2B ubiquitylation is regulated by the histone H2B deubiquitylation activity of SAGA (31, 49). Thus, SAGA may regulate *GAL10* antisense transcription. To test this, we analyzed *GAL10* antisense transcription in the *SPT20* (which impairs the structural and functional integrity of SAGA) wild-type and deletion mutant strains (31, 55). We found that *GAL10* antisense transcription is not altered in the absence of Spt20p (Fig. 7A and B). Likewise, transcription of the SAGA-independent *ACT1* gene (56, 57) but not the SAGA-dependent *ADH1* gene (31) was not altered in the absence of Spt20p (Fig. 7A and B). These results support that SAGA does not regulate *GAL10* antisense transcription. However, SAGA promotes *GAL1/10* sense transcription (58, 59). Hence, SAGA differentially regulates sense and antisense transcription of *GAL10*. The fact that SAGA or its histone H2B deubiquitylation activity is dispensable for *GAL10* antisense transcription indicates that SAGA may not be targeted to *GAL10* under the growth conditions (or dextrose-containing growth medium) permissive to *GAL10* antisense transcription. Indeed, SAGA is not targeted to *GAL1/10* in dextrose-containing growth medium (59) but is recruited to these genes in galactose-containing growth medium by an activator, Gal4p, for sense transcription (58, 59).

Swi/Snf does not regulate *GAL10* antisense transcription, while it promotes sense transcription. So far, we had found that *GAL10* antisense transcription is facilitated by NuA4 KAT, histone H3 K4 and K36 methyltransferases, and histone H2B ubiquitin conjugase, thus indicating the roles of different histone covalent modifications in regulation of antisense transcription. However, the functions of chromatin remodeling factors in regulation of antisense transcription remain largely unknown. In view of this, we analyzed the role of an important ATP-dependent chromatin remodeling factor, Swi/Snf, that is known to enhance *GAL1/10* sense transcription by remodeling chromatin and/or nucleosomal disassembly (23), in regulation of *GAL10* antisense transcription.

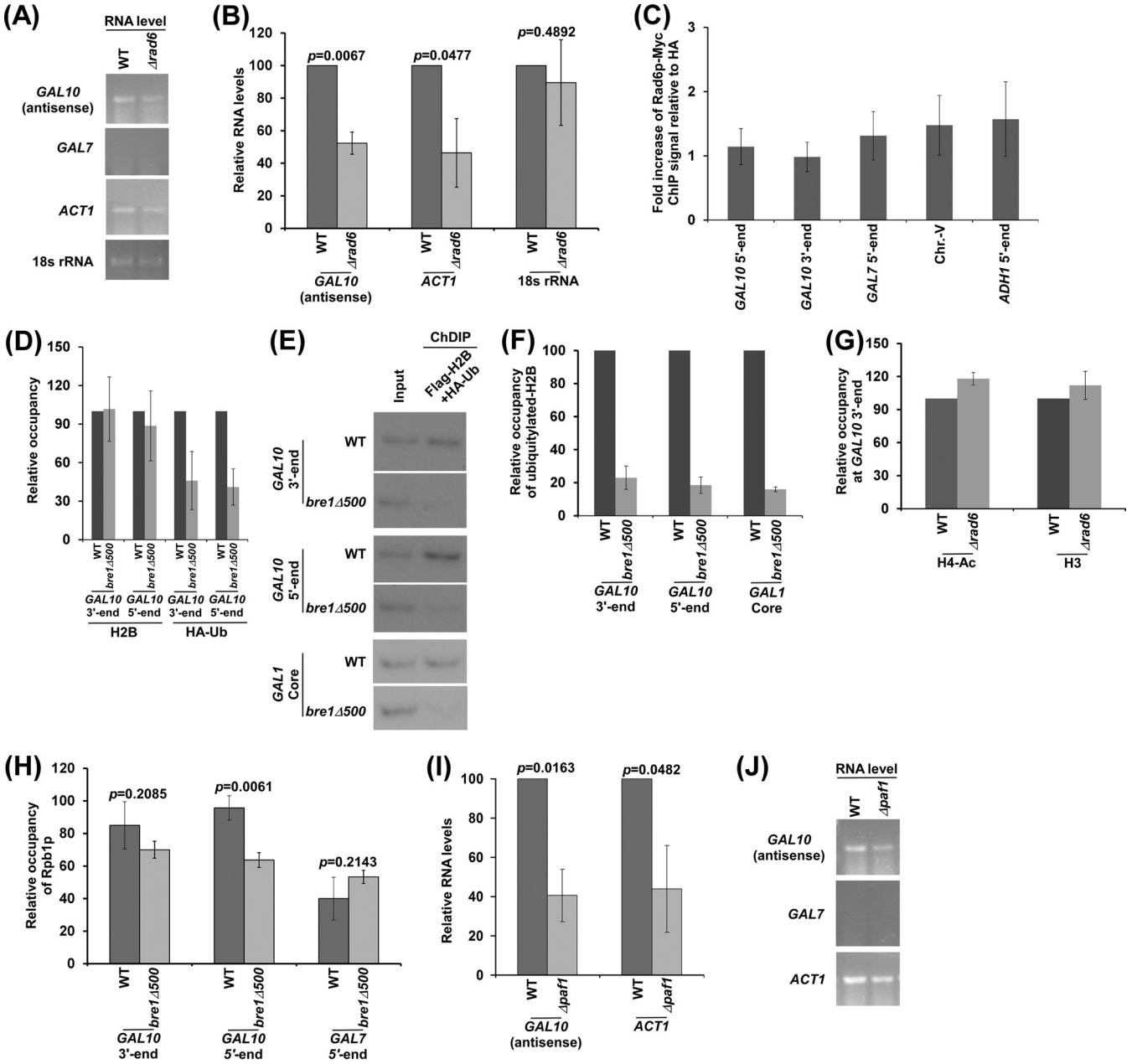


FIG 6 *GAL10* antisense transcription is regulated by histone H2B ubiquitin conjugase and Paf1p. (A and B) Analysis of *GAL10* antisense RNA in the Δ*rad6* and wild-type strains. (C) ChIP analysis of Myc-tagged Rad6p at the 5′ and 3′ ends of the *GAL10* coding sequence, Chr.-V, and 5′ ends of the *ADH1* and *GAL7* coding sequences. (D) ChIP analysis of Flag-tagged histone H2B and HA-tagged ubiquitin at the 5′ and 3′ ends of the *GAL10* coding sequence in the absence of the RING domain of Bre1p (the *bre1Δ500* strain without 500 amino acids at Bre1p's C terminus that contain the RING domain). Immunoprecipitation was carried out using anti-Flag and anti-HA antibodies against Flag-tagged histone H2B and HA-tagged ubiquitin as described previously (31). (E and F) ChDIP analysis for the levels of ubiquitylated histone H2B in the wild-type (YKH045) and *bre1Δ500* strains expressing Flag-tagged H2B and HA-tagged ubiquitin. (G) Analysis of histone H4 acetylation and histone H3 levels at *GAL10* in the wild-type and Δ*rad6* strains. (H) Analysis of Rpb1p association with the 3′ and 5′ ends of the *GAL10* coding sequence in the wild-type and *bre1Δ500* strains. The maximum ChIP signal was set to 100, and the other ChIP signals relative to maximum ChIP signal were plotted. (I and J) Analysis of *GAL10* antisense RNA in the Δ*paf1* and wild-type strains.

In this direction, we determined the levels of *GAL10* antisense transcripts in the *SWI2* (which has an ATPase activity) wild-type and deletion mutant strains. We found that the level of the *GAL10* antisense transcript was not altered in the Δ*swi2* strain in comparison to the wild-type equivalent (Fig. 7C and D). Likewise, Swi/Snf did not regulate the sense transcription of *ACT1* (Fig. 7C and D), consistent with previous studies (60). However, sense transcription of *PYK1* was impaired in the Δ*swi2* strain (Fig. 7C and D), consistent with previous studies (23). Therefore, our results support that *GAL10* antisense transcription is not regulated by Swi/Snf. On the other hand, *GAL1/10* sense transcription is promoted by Swi/Snf (61). Thus, *GAL10* sense and antisense transcriptions are differentially controlled by Swi/Snf. However, another ATP-dependent chromatin remod-

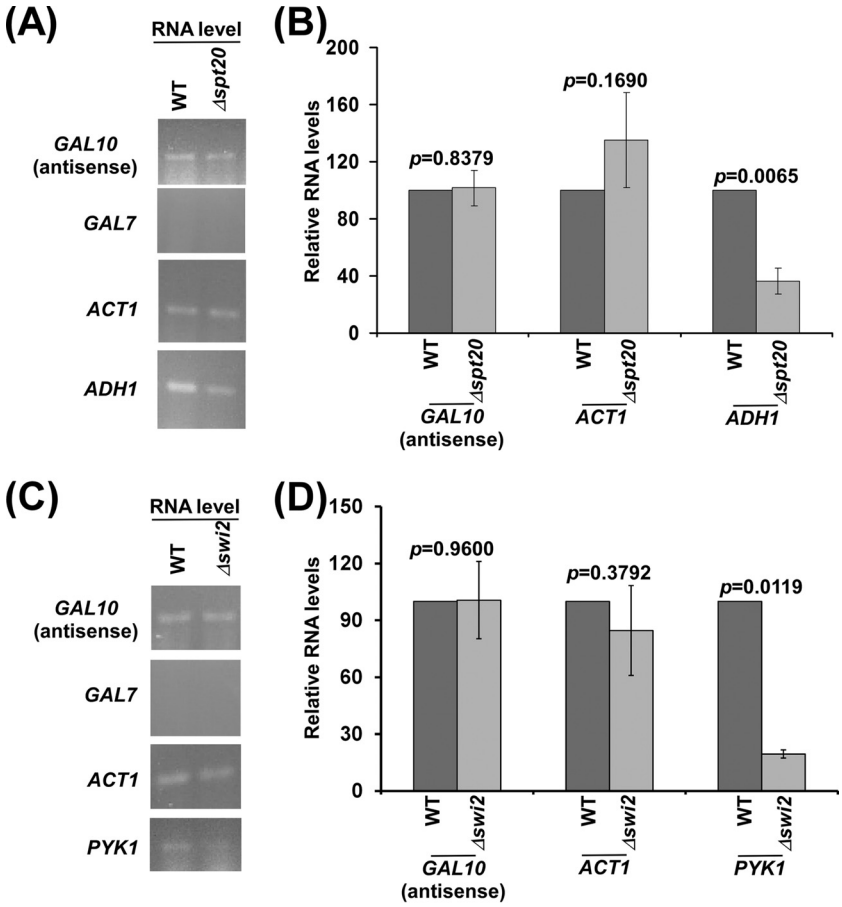


FIG 7 *GAL10* antisense transcription is not regulated by SAGA and Swi/Snf. (A and B) Analysis of *GAL10* antisense RNA in the Δ*spt20* and wild-type strains. (C and D) Analysis of *GAL10* antisense RNA in the Δ*swi2* and wild-type strains.

eling factor may regulate *GAL10* antisense transcription, which remains to be further elucidated.

DISCUSSION

Although antisense transcripts/transcription is involved in regulation of sense transcription and cellular functions, it is not clearly understood how antisense transcription is initiated. We have recently demonstrated that an activator (Reb1p) and a set of GTFs target RNA polymerase II to initiate antisense transcription from the 3′ end of the *GAL10* coding sequence (14). Here, our results reveal that NuA4 KAT is targeted to the 3′ end of the *GAL10* coding sequence by Reb1p for histone H4 acetylation (Fig. 2 and 3). The loss of NuA4 KAT-mediated histone H4 acetylation is associated with impaired *GAL10* antisense transcription (Fig. 1, 2, and 3). Thus, our results support the role of NuA4 KAT in facilitation of *GAL10* antisense transcription.

Like its role in stimulation of *GAL10* antisense transcription, Reb1p has been shown to promote sense transcription of many housekeeping genes (15, 62–64). Further, intergenic Reb1p-binding sites have been detected in genome-wide ChIP-on-chip studies (65). Moreover, 215 perfect matches to the Reb1p-binding sequences within coding sequences in *Saccharomyces cerevisiae* have been identified by BLAST analysis (15). Thus, Reb1p is likely to act in these regions to initiate antisense transcription, similar to the results for *GAL10*. Further, it is yet unknown how Reb1p promotes transcriptional initiation. The results presented here demonstrate that Reb1p targets NuA4 KAT for histone H4 acetylation at the *GAL10* antisense transcription initiation site for recruitment of RNA polymerase II and hence *GAL10* antisense transcription (Fig. 1, 2, and 3). Reb1p might similarly regulate antisense transcription and/or sense transcription at other genes via NuA4 KAT, which remains to be further elucidated.

Like its recruitment at the *GAL10* antisense transcription initiation site, NuA4 KAT is targeted to the promoters of the TAF-dependent ribosomal protein genes by an activator, Rap1p, to enhance their sense transcription (17–19). Transcription of the ribosomal protein genes depends on the TOR pathway via a mechanism that acts on Ifh1p and Fhl1p, which are recruited to the ribosomal protein genes by Rap1p. Although both sense transcription of the ribosomal protein genes and *GAL10* antisense transcription depend on NuA4 KAT, the latter requires Reb1p rather than Rap1p and thus may not be regulated by the TOR pathway. We tested this by using rapamycin to inhibit the TOR pathway and indeed found that *GAL10* antisense transcription was not affected (Fig. 4).

Further, we have recently demonstrated that the 19S regulatory particle (RP) facilitates the targeting of NuA4 KAT to the promoters of the ribosomal protein genes to enhance sense transcription (17). Consistently, the 19S RP has been shown to promote sense transcription of the NuA4 KAT-dependent ribosomal protein

genes (17). It is not clearly known whether other genes that are regulated by NuA4 KAT also require the 19S RP. Our results reveal that NuA4 KAT-regulated *GAL10* antisense transcription is independent of the 19S RP (14). Thus, NuA4 KAT-mediated transcription is differentially regulated by the 19S RP. Such a differential requirement of the 19S RP could be dictated by activators, as sense transcription of the ribosomal protein genes and *GAL10* antisense transcription are activated by the Rap1p and Reb1p activators, respectively.

Like NuA4 KAT, histone H3 K4 methyltransferase (Set1p) also favors *GAL10* antisense transcription (Fig. 5A to D). Histone H3 K4 methylation associated with *GAL10* antisense transcription has been implicated in repression of sense transcription (15, 16), thus deciphering the role of antisense transcription in regulation of sense transcription. Our results demonstrate that, in addition to repressing sense transcription, histone H3 K4 methylation also promotes antisense transcription. Like histone H3 K4 methylation (or Set1p), histone H3 K36 methylation or methyltransferase (Set2p) facilitates *GAL10* antisense transcription (Fig. 5I and J). Histone H3 K36 methylation has been previously shown to repress cryptic transcription via the recruitment of the Rpd3S complex at the coding sequence during sense transcription (46, 47). Further, previous studies (15) at the *GAL10* locus revealed a high level of histone H3 K36 methylation under the growth conditions permissive to *GAL10* antisense transcription. Such methylation of histone H3 at the *GAL10* locus has been implicated in repression of sense transcription via Rpd3S and hence provided a mechanism for regulation of sense transcription by antisense transcription. However, the role of histone H3 K36 methylation in antisense transcription was not clear. Here, we show that Set2p (or histone H3 K36 methylation) promotes antisense transcription (Fig. 5I and J). Thus, in addition to repressing sense/cryptic transcription, histone H3 K36 methylation also promotes antisense transcription.

Like histone H3 K4/36 methylation, histone H2B ubiquitylation also facilitates *GAL10* antisense transcription (Fig. 6A, B, D to F and H). Since the level of histone H2B ubiquitylation is regulated by ubiquitin protease (or deubiquitylation), histone H2B ubiquitin protease or deubiquitylation activity of SAGA is likely to alter the level of *GAL10* antisense transcription. To test this, we analyzed the role of SAGA in regulation of *GAL10* antisense transcription. SAGA has histone H3 acetylation activity in addition to its histone H2B deubiquitylation activity. Moreover, SAGA functions to stimulate the preinitiation complex formation during sense transcription (58, 59). We found here that SAGA is dispensable for *GAL10* antisense transcription (Fig. 7A and B). However, SAGA is essential for *GAL1/10* sense transcription (58, 59). Thus, SAGA is differentially required for *GAL10* sense and antisense transcription. Like SAGA, the activator Gal4p (which is essential for *GAL10* sense transcription) is dispensable for *GAL10* antisense transcription (14). These results support that both *GAL10* sense and antisense transcriptions are regulated independently and differently. Further, the fact that we did not observe the effect of SAGA on *GAL10* antisense transcription is due to the failure of SAGA association with *GAL1/10* in the absence of active Gal4p in dextrose-containing growth medium (58, 59). However, some other ubiquitin protease(s) may be involved in regulation of histone H2B ubiquitylation associated with antisense transcription.

Previous studies (23, 66) have demonstrated that histones are evicted during sense transcription. Such nucleosomal disassembly promotes transcription. The Swi/Snf chromatin remodeling complex participates in histone eviction during sense transcription and facilitates transcription (23). We found here that Swi/Snf is not required for *GAL10* antisense transcription (Fig. 7C and D), while it promotes *GAL1/10* sense transcription (23, 61). Thus, another factor may be involved in altering chromatin structure/disassembly during antisense transcription, which needs to be further elucidated.

In summary, we demonstrate here that NuA4 KAT is associated with the 3′ end of the *GAL10* coding sequence for histone H4 acetylation and antisense transcription initiation. However, such a function of NuA4 KAT in stimulation of *GAL10* antisense transcription is independent of the 19S RP and TOR pathway. On the other hand, the 19S RP and TOR play important roles in recruiting NuA4 KAT to the promoters of the ribosomal protein genes during sense transcription (17, 40). Thus, NuA4 KAT recruitment is differentially regulated at the Rap1p-dependent ribosomal protein genes and Reb1p-dependent antisense *GAL10* to control sense and antisense transcription. Like NuA4 KAT, histone H3 K4 and K36 methyltransferases also facilitate antisense transcription. Similarly, histone H2B ubiquitylation promotes antisense transcription. Collectively, our results illuminate for the first time the chromatin regulation of antisense transcription. Since the aforementioned histone covalent modifications or associated factors are conserved among eukaryotes, similar chromatin regulation of antisense transcription is likely to exist in humans and other eukaryotes.

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