Differential regulation of repeated histone genes during the fission yeast cell cycle

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ABSTRACT

The histone genes are highly reiterated in a wide range of eukaryotic genomes. The fission yeast, *Schizosaccharomyces pombe*, has three pairs of histone H3-H4 genes: $hht1^+$-$hhf1^+$, $hht2^+$-$hhf2^+$ and $hht3^+$-$hhf3^+$. While the deduced amino acid sequences are identical, it remains unknown whether transcriptional regulation differs among the three pairs. Here, we report the transcriptional properties of each H3-H4 gene pair during the cell cycle. The levels of transcripts of $hht1^+$-$hhf1^+$ and $hht3^+$-$hhf3^+$ pairs and $hhf2^+$ are increased at S-phase, while that of $hht2^+$ remains constant throughout the cell cycle. We showed that the GATA-type transcription factor, Ams2, binds to the promoter regions of core histone genes in an AACCCT-box-dependent manner and is required for activation of S-phase-specific transcription. Furthermore, we found that Ams2-depletion stimulates feedback regulation of histone transcripts, mainly up-regulating the basal levels of $hht2^+$-$hhf2^+$ transcription, which are normally down-regulated by Hip1 and Slm9, homologs of the human histone chaperone, HIRA. These observations provide insight into the molecular mechanisms of differential regulation of transcripts from repeated histone genes in the fission yeast.

INTRODUCTION

The nucleosome is the most highly conserved fundamental repeating unit of eukaryotic chromatin. The nucleosome core is formed by an octamer comprised of two copies of each histone protein, H2A, H2B, H3 and H4, around which 145–147 bp of DNA is wrapped twice. Appropriate levels of histone expression are critical for transcription, chromosome segregation, repair, and other chromatin-mediated processes (1). Histone genes are highly reiterated and are often organized into clusters (2). For example,

the majority of human histone genes are located in major and minor clusters on chromosomes 6p21 and 1q21, respectively (3), while the budding yeast, *Saccharomyces cerevisiae*, contains two copies of each pair of H2A-H2B and H3-H4 histone genes (4,5). The fission yeast, *Schizosaccharomyces pombe*, investigated in the present study also has reiterated histone genes, the genomic organization of which consists of a single H2Aβ ($hta2^+$), a pair of H2Aα-H2B ($hta1^+$-$htb1^+$), and three pairs of H3-H4 ($hht1^+$-$hhf1^+$, $hht2^+$-$hhf2^+$ and $hht3^+$-$hhf3^+$) (6,7). The amino acid sequences deduced from three copies of histone H3 genes or those of histone H4 genes are identical, while the two predicted H2A proteins differ by three amino acids (6,7). Although the repetitive nature of the histone genes in the genome appears to be evolutionarily well conserved, its physiological significance remains obscure.

Histone production is up-regulated at S-phase coinciding with nucleosome assembly during DNA replication (8). In S-phase, DNA synthesis and histone synthesis are highly regulated in a coordinated and concerted manner. For example, in both yeast and higher eukaryotes, interference with DNA replication by genotoxic agents triggers repression of histone gene expression (1). Conversely, in human cells, the reduction of histone transcription by ectopic overproduction of a histone repressor leads to concerted blockage of DNA synthesis (9). In budding yeast, although DNA replication occurs in the absence of histone synthesis, passage through S-phase results in severe loss of cell viability (10,11). The increased histone synthesis during S-phase is due to regulation at both transcriptional and post-transcriptional levels. In higher eukaryotes, although such transcriptional regulation contributes to several-fold increases in histone synthesis during S-phase, the majority is likely due to post-transcriptional regulation of histone mRNA processing and stability (1). The up-regulation of histone synthesis in budding yeast is thought to be achieved through both repression in G1- and G2-phases and transcriptional activation just before entry into S-phase. Each of the budding yeast histone gene pairs is transcribed in divergent orientation from common

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regulatory elements (12,13), including upstream activator sequence repeats and a negative repressor element (14). Although transcriptional activation at S-phase is a common feature of the histone genes conserved among species, it remains unclear whether conserved molecules are involved in the activation.

HIRA proteins are members of an evolutionarily highly conserved family of histone chaperones that have been implicated in nucleosome assembly (1). HIRA proteins were originally discovered as repressors of histone gene expression outside S-phase or in response to HU, an inhibitor of DNA replication in budding yeast (15,16). Two HIRA proteins, Hir1 and Hir2, restrict the expression of six of the eight core histone genes at S-phase in budding yeast (15,17). The fission yeast also has two related HIRA proteins, Slm9 (18) and Hip1 (19), whereas higher eukaryotes have only a single protein. In higher eukaryotes, HIRA has been identified as a critical component of a replication-independent nucleosome deposition pathway as a chaperone for histone octamers containing the H3.3 variant (20). HIRA is also involved in transcriptional regulation, as over-expression of HIRA in human cells in tissue culture results in histone gene repression (9). It remains to be determined whether and how these two activities are functionally related.

Histone genes can be classified into three main subtypes based on their expression pattern and genomic organization: replication-dependent, replication- and cell cycle phase-independent, and tissue-specific histones (21). Higher eukaryotes, including humans, have three H3 histone variants, H3.1, H3.2 and H3.3. The major H3 histones, H3.1 and H3.2, have only a single amino acid difference, and are synthesized and deposited on DNA strictly during DNA replication (21,22). H3.3 differs from H3.2 in five amino acids in humans and is synthesized and deposited on DNA throughout the cell cycle (21,22). Interestingly, observations in flies and humans indicate that H3.3 is associated with transcriptionally active gene loci and is enriched in covalent modifications associated with gene activation (21,23). In contrast, H3.2 has been shown to be enriched in markers associated with gene silencing (22). The transcriptional state at the loci may be a critical determinant for H3.3 deposition (24). In contrast, yeasts have only a single H3 protein; the budding yeast genome possesses only H3.3-type histone genes, while the fission yeast H3 genes encode an identical hybrid protein exhibiting characteristics of both H3.3 and H3.2 (21).

In this study, we characterized the transcriptional properties of individual histone H3 and H4 genes throughout the cell cycle using the genetically tractable model organism, *S. pombe*. The DNA sequences of reiterated histone genes within the coding region in *S. pombe* show significant identity among the copies, while the 5′- and 3′-untranslated regions (UTRs) show divergence. Hereafter, we refer to the three pairs of H3-H4 genes ($hht1^+$-$hhf1^+$, $hht2^+$-$hhf2^+$ and $hht3^+$-$hhf3^+$) as copy-1, -2 and -3, respectively (Figures 2A and 3). A quantitative reverse transcription polymerase chain reaction (RT-PCR) method with allele-specific probes was used for precise comparison of the relative levels of transcripts derived from reiterated histone genes among wild-type, histone gene deletion mutants, and mutants in which histone transcription is compromised. We identified Ams2, a cell cycle-regulated GATA-type transcription factor (25,26), as an activator of core histone genes at S-phase. Ams2 protein has a zinc finger motif that is characteristic of the family of GATA-type transcription factors, some of which have been reported to bind to GATA-containing DNA consensus sequences (26). Indeed, Ams2 has been shown to exhibit DNA-binding activity to a GATA sequence *in vitro* (25). Ams2 accumulates on the nuclear chromatin when chromosomes are duplicated during S-phase, whereas little accumulation of this protein was detected in mid-to-late G2 or early M-phase (25). The results of the present study indicated the differential transcriptional regulation of three histone gene pairs, which is probably controlled by a combination of Ams2-dependent transcriptional activation at S-phase and Ams2-independent constitutive transcription throughout the cell cycle.

MATERIALS AND METHODS

General techniques, antibodies and strains

The general techniques and media used for manipulation of fission yeast were described previously (25). α-Ams2 polyclonal antibodies were generated by immunizing rabbits with GST-tagged Ams2 and affinity purified. Table 1 lists the genotypes of the strains used in this study. To construct an Ams2-depleted mutant strain, a linearized DNA fragment containing the Ams2-coding region, in which the 2.3-kb *Nhe*I-*Nco*I fragment was replaced with a KanMX marker, was integrated into the genome by selecting G418-resistant transformants. To construct a conditional *ams2*-shut-off strain, a linear DNA fragment containing the KanMX marker and *nmt1-81* promoter-driven $ams2^+$ gene (designated p^{nmt81}-$ams2$) was integrated into the genome. To construct mutant strains lacking one pair of histone H3 and H4, a PCR-based method (27) was used to replace copy-1, -2 and -3 with KanMX, hphMX and NAT markers, respectively ($Δ1$, $Δhht1^+$-$hhf1^+$; $Δ2$, $Δhht2^+$-$hhf2^+$; $Δ3$, $Δhht3^+$-$hhf3^+$, respectively). Strains retaining only one of the three pairs were generated by crossing strains with deletion of a single pair. $Δhip1$ and $Δslm9$ strains were constructed by replacing $hip1^+$ and $slm9^+$ with the KanMX marker, respectively, using the PCR-based method. Genomic Southern hybridization was performed to confirm disruption of the authentic genes.

Cell cycle experiments

YTP91 cells carrying the $ams2^+$ gene under the control of a repressible *nmt81* promoter were pre-cultured in EMM2. After addition of $2\,\mu M$ thiamine to EMM2 (this treatment results in silencing the promoter and the Ams2 protein level becomes undetectable within 30 min), early G2 cells were collected immediately by centrifugal elutriation (Beckman Avanti HP-20XP, JE-5.0 elutriation rotor; Beckman Coulter, Fullerton, CA, USA), and then cultured in EMM2 with thiamine at 33°C. Wild-type

Table 1. The fission yeast strains used in this study

Name	Genotype	Reference
HM123	h^- leu1-32	
SP91	h^-leu1-32 ura4-D18 lys1	
SP2	h^+ his2 cdc25-22	(29)
SP45	h^- leu1-32 ura4-D18 Δams2:: ura4$^+$	(25)
SP148	h^+ leu1-32 ura4-D18 Δcnp1::ura4$^+$ lys1$^+$:: cnp1-1	(39)
YTP11	h^- leu1-32 ura4-D18 [pRep41]	This study
YTP12	h^- leu1-32 ura4-D18 [pRep41-ams2HAHis6]	This study
YTP91	h^- leu1-32 ura4-D18 lys1 p^{nmt81}ams2-Kan^R	This study
YTP155	h^- leu1-32 ura4-D18 lys1 Δams2:: Kan^R	This study
YTP779	h^- leu1-32 ura4-D18 lys1$^+$:: GFP	This study
YTP705	h^- leu1-32 ura4-D18 lys1$^+$:: Pro31-GFP	This study
YTP706	h^- leu1-32 ura4-D18 lys1$^+$:: Pro41-GFP	This study
YTP761	h^- leu1-32 ura4-D18 lys1$^+$:: ΔMP-GFP	This study
YTP759	h^- leu1-32 ura4-D18 lys1$^+$:: ΔM-GFP	This study
YTP760	h^- leu1-32 ura4-D18 lys1$^+$:: M-GFP	This study
YTP780	h^- leu1-32 ura4-D18 lys1$^+$:: MP-GFP	This study
YTP784	h^- leu1-32 ura4-D18 lys1$^+$:: ΔC-GFP	This study
YTP545 (Δ1)	h^+ leu1-32 ura4-D18 lys1 his5 Δhht1-hhf1:: Kan^R	This study
YTP547 (Δ2)	h^+ leu1-32 ura4-D18 lys1 his5 Δhht2-hhf2:: Hygro^R	This study
YTP549 (Δ3)	h^+ leu1-32 ura4-D18 lys1 his5 Δhht3-hhf3:: NAT^R	This study
YTP556 (1 only)	h^+ leu1-32 ura4-D18 lys1 his5 Δhht2-hhf2:: Hygro^R Δhht3-hhf3:: NAT^R	This study
YTP554 (2 only)	h^+ leu1-32 ura4-D18 lys1 his5 Δhht1-hhf1:: Kan^R Δhht3-hhf3:: NAT^R	This study
YTP551 (3 only)	h^+ leu1-32 ura4-D18 lys1 his5 Δhht1-hhf1:: Kan^R Δhht2-hhf2:: Hygro^R	This study
SP1500	h^- leu1-32 ura4-D18 lys1 Δhip1:: Kan^R	This study
SP1503	h^-leu1-32 ura4-D18 lys1 Δslm9:: Kan^R	This study

early G2 cells were also prepared and released synchronously at 33°C in EMM2 or YES for the experiments shown in Figure 1 (SP91) and in Figure 4 (HM123), respectively. To monitor the septation index (i.e. the percentage of cells with a septum), cells were fixed with 10% glutaraldehyde every 15 min after elutriation and stained with 5 μg/ml Fluostain I (Sigma, St. Louis, MO, USA). To estimate the DNA contents, cells were fixed in 70% ethanol (28), stained with 1 μM SYTOX Green (Molecular Probes, Eugene, OR, USA), and examined by flow cytometry (FACScalibur; Becton Dickinson, Mountain View, CA, USA). For the experiments shown in Figure 2B, cdc25-22 mutant cells (SP2) precultured in YES at 26°C were shifted to 36°C for 3 h to induce arrest at G2 (29). Cells were released into mitosis synchronously by shifting to 26°C and harvested at 30-min intervals. For the experiments shown in Figure 7, cells were grown in YES at 26°C and synchronized in S-phase by the addition of 12 mM hydroxyurea (HU) for 5 h for wild-type and 5.5 h for the other strains to enrich G2 cells on release into fresh YES for 60–75 min.

RNA preparation and northern blotting analysis

Schizosaccharomyces pombe total RNA was extracted from aysnchronously or synchronously cultured cells by the acid-phenol method described previously (30). Aliquots of 5 μg of total RNA were separated by electrophoresis on 1.25% denaturing agarose gels or 4% polyacrylamide gel containing 7 M urea (Figure 3B), and transferred onto Hybond N$^+$ nylon membranes (GE Healthcare Bio-Sciences, NJ, USA) for northern analysis. Probes labeled by incorporation of [α-^{32}P]dCTP using a random labeling kit (TaKaRa, Otsu, Japan) were added to the prehybridized membranes and hybridized at 65°C overnight in hybridization buffer consisting of 100 mM Na-Phosphate (pH 7.0), 5 mM EDTA, 1% SDS, 10% dextran sulfate and 0.4 M NaCl. For the hht2$^+$ probe, hybridization buffer containing 0.8 M NaCl was used. The membranes were washed twice with 2 × SSC/0.1% SDS at 65°C for 20 min, and exposed to imaging plates. The signals on northern blots were detected with a Typhoon 9410 imager (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The sequences of the DNA probes used are available upon request.

### Preparation of cell extracts for western blotting analysis

Protein extracts for western blotting analysis were prepared from trichloroacetic acid (TCA)-treated cells. Briefly, cells were collected and suspended in 1 ml of ice-cold double-distilled water. Then, 150 μl of ice-cold YEX lysis buffer (1.85 M NaOH, 7.5% β-mercaptoethanol) was added, and the suspension was kept on ice for 10 min. TCA was then added at a final concentration of 25%, and the suspension was kept on ice for a further 10 min. The proteins were precipitated by centrifugation at $18\,000 \times g$ for 5 min at 4°C, and then suspended in $1 \times$ gel loading buffer containing 50 mM Tris–HCl (pH 8.0).

Chromatin immunoprecipitation (ChIP) assay

Aysnchronously or synchronously cultured cells were fixed with 3% formaldehyde on ice for 30 min, quenched with 330 mM glycine on ice for 10 min, and washed with ice-cold $1\times$ PBS. The cells were resuspend in FA lysis buffer 1 consisting of 50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2% SDS, 0.1% Na-deoxycholate, 1 mM PMSF and $1 \times$ Complete Mini EDTA-free (Roche, Indianapolis, IN, USA), broken by vortexing with glass beads, and the lysates were cleared
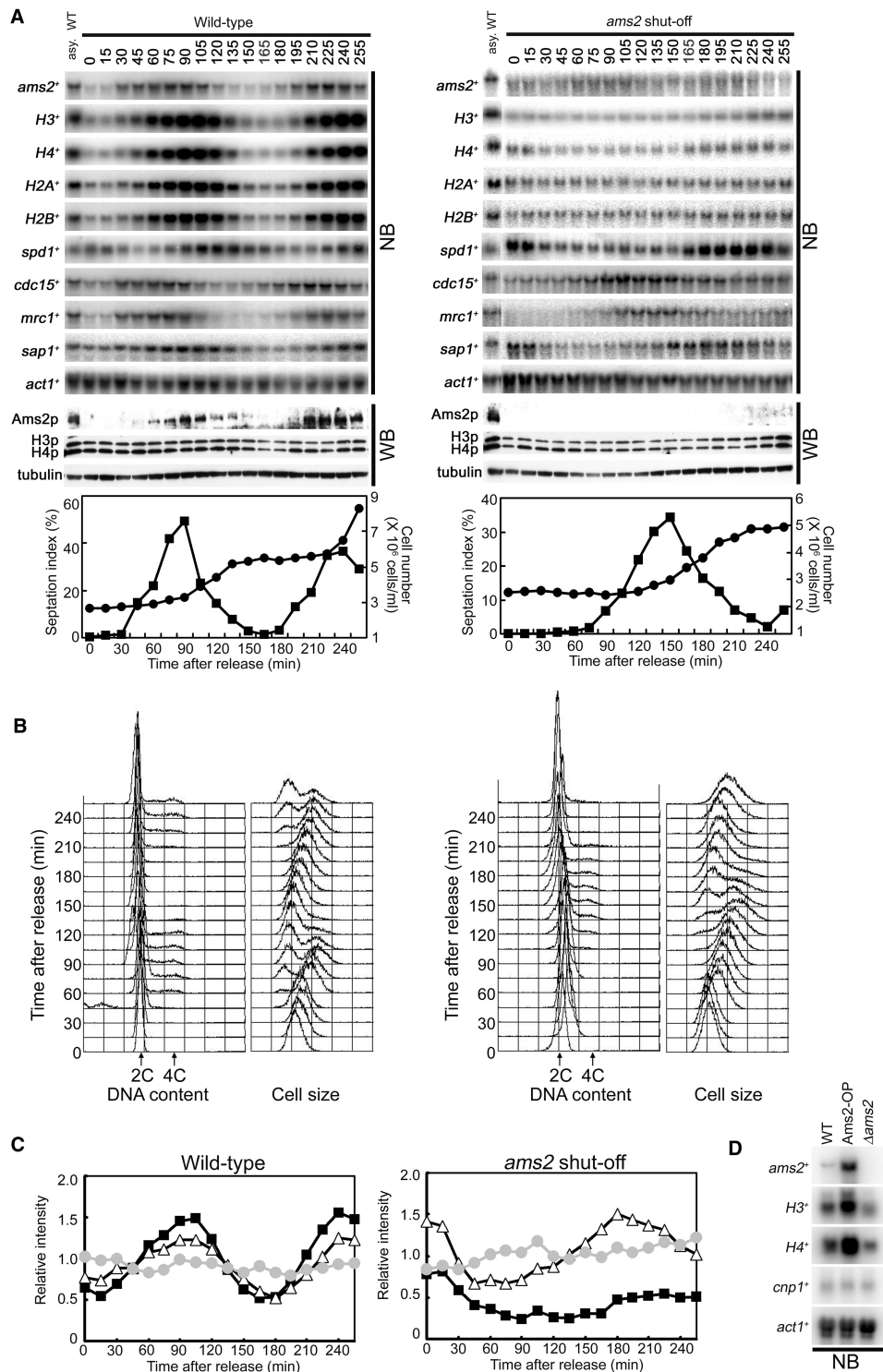
Figure 1. Ams2 is required for histone gene activation at S phase. (**A**) Early G2 cells of wild-type (SP91) and *ams2*-shut-off strain (YTP91) under conditions of Ams2 repression were inoculated synchronously at 33°C. The transcript levels of *ams2*[+], core histones, cell cycle markers (*spd1*[+] for G2, *cdc15*[+] for M, *mrc1*[+] for G1, *sap1*[+] for S) and actin (*act1*[+] as a loading control) were assessed by northern blotting (NB). Equal amounts of wild-type total RNA from asynchronous cultures (asy.WT) were blotted onto each membrane as exposure controls. Total protein levels of Ams2, H3, H4 and α-Tubulin (TAT1, loading control) were assessed by western blotting (WB). The cell number (circles) and septation index (square, percentage of cells with a septum) were monitored. (**B**) The DNA contents and the cell sizes estimated by flow cytometry are shown at 15-min intervals after release. 4C peaks were expected when DNA replication occurred in the binucleate/septated cells. (**C**) The ratios of northern signals for *H3*[+] (squares), *sap1*[+] (triangles), and *act1*[+] (circles) in synchronous wild-type or Ams2-deficient cells were quantified relative to the intensities of the corresponding signals in asynchronous wild-type cells (asy.WT). (**D**) The transcript levels of *ams2*[+], *H3*[+], *H4*[+], SpCENP-A *cnp1*[+] and *act1*[+] (loading control) were assessed by northern blotting in asynchronous wild-type (WT, YTP11), wild-type over-expressing Ams2 (Ams2-OP, YTP12), or Ams2-null (*Δams2*, SP45) cells.
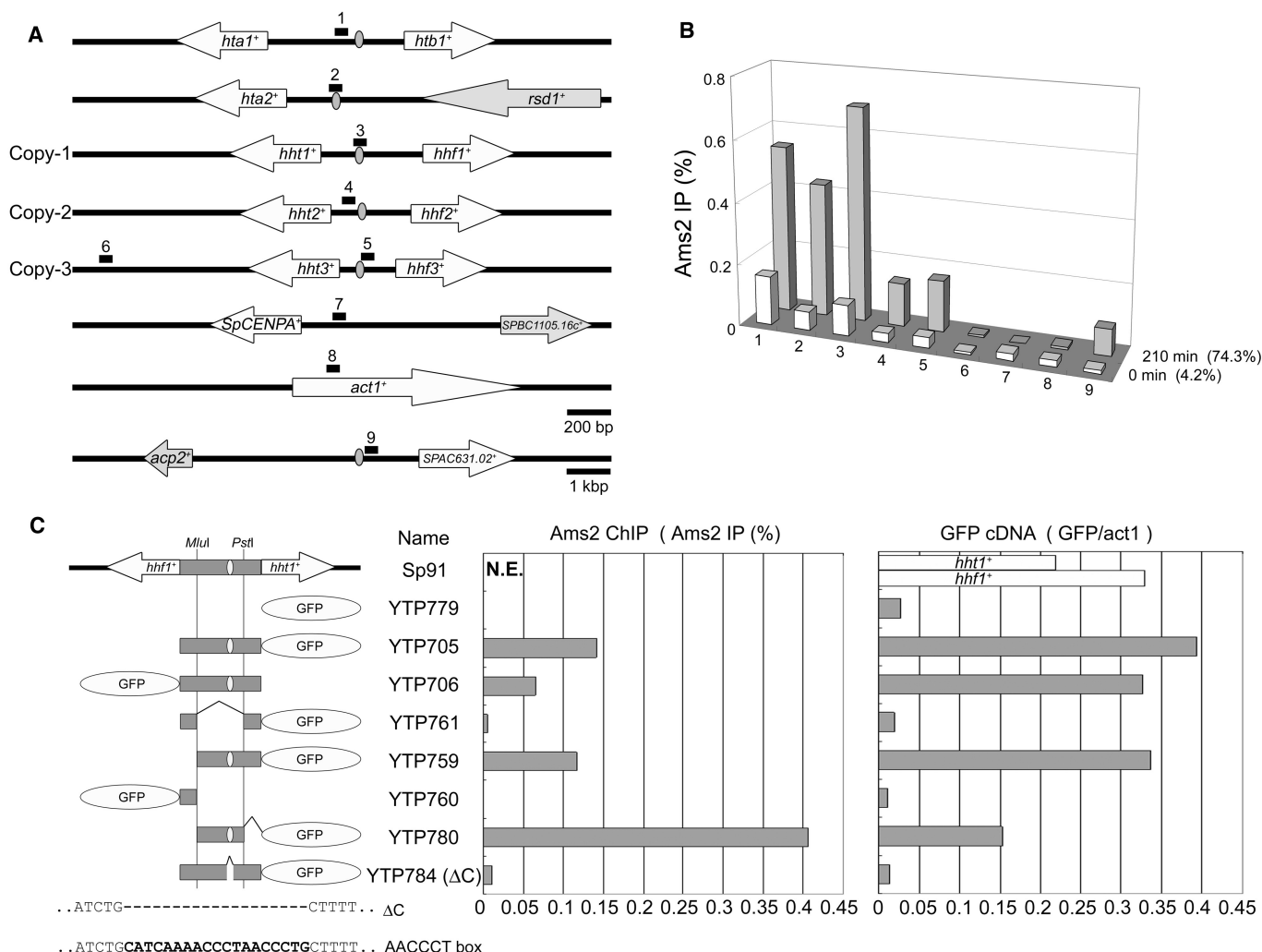
Figure 2. Histone gene activation requires AACCCT-box-dependent association of Ams2 with the upstream regions during S phase. (**A**) A schematic drawing showing the organization of genes encoding core histones, SpCENP-A, SPAC631.02 and Act1. Primer sets used for ChIP assay are indicated as numbered short bars and the AACCCT-box identified as a common motif in the intergenic regions among the three H3-H4 pairs (6,7) is indicated by an ellipse. (**B**) ChIP assay was performed to determine the Ams2-binding regions during S-phase. *cdc25-22* cells (SP2) arrested at late-G2-phase (septation index = 4.2%, time = 0 min) were prepared by inoculation in YES at 36°C for 3 h, and released synchronously into mitosis at 26°C. The septation index was monitored at 15-min intervals. The cell fraction showing a peak of the septation index (74.3%, time = 210 min) was used as the cell population at S-phase. DNAs co-precipitated with Ams2 using α-Ams2 polyclonal antibodies from cell extracts were quantified by real-time PCR using the probes indicated. The percentage of total DNA immunoprecipitated in ChIP fractions with respect to total input DNA is plotted. (**C**) Ams2 promotes histone gene activation through binding to a consensus 'AACCCT-box' sequence. Shown from left to right are diagrams of deleted or mutated constructs for *hht1⁺-hhf1⁺* promoter fused to the GFP gene (the AACCCT-box-motif is indicated by an ellipse), the percentage of ChIP enrichment with Ams2, and the amount of GFP transcript as determined by quantitative RT-PCR. White bars indicate the amounts of *hht1⁺* and *hhf1⁺* transcripts that were transcribed from authentic loci. Deleted sequences in the AACCCT-box-motif are shown on the bottom of the diagram. The relative amounts of the GFP, *hht1⁺* and *hhf1⁺* transcripts were calculated by normalizing to that of the *act1⁺* transcript. N.E., not examined.

by centrifugation at $12\,000 \times g$ for 20 min. The chromatin pellets were resuspended in FA lysis buffer 2 (FA lysis buffer 1 containing 0.1% SDS), and sonicated until the average DNA length was <500 bp. The sonicated lysates were cleared by centrifugation, followed by addition of 1/10 vol of 10% Triton X-100. The lysates were incubated with or without α-Ams2 polyclonal antibodies for 2 h at 4°C followed by addition of Dynabeads α-rabbit IgG (Dynal Biotech, Oslo, Norway). After overnight incubation at 4°C, the beads were washed twice with FA lysis buffer 2, washed once with wash buffer (50 mM HEPES-KOH, pH 7.5, 500 mM

NaCl, 1 mM EDTA, 0.1% SDS, 0.1% Na-deoxycholate, 0.1% Triton X-100, and 1 mM PMSF), washed once with chromatin immunoprecipitation (ChIP) buffer (10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, and 0.5% Na-deoxycholate, and 1 mM PMSF), and washed once with TE (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA). Protein–DNA complex was dissociated by incubation in TE with 1% SDS for 15 h at 65°C. The eluted DNA was treated with 250 μg/ml Proteinase K (Merck, Darmstadt, Germany) for 3 h at 50°C, subjected to phenol/chloroform extraction, and precipitated with ethanol. The purified DNA was analyzed

A

B
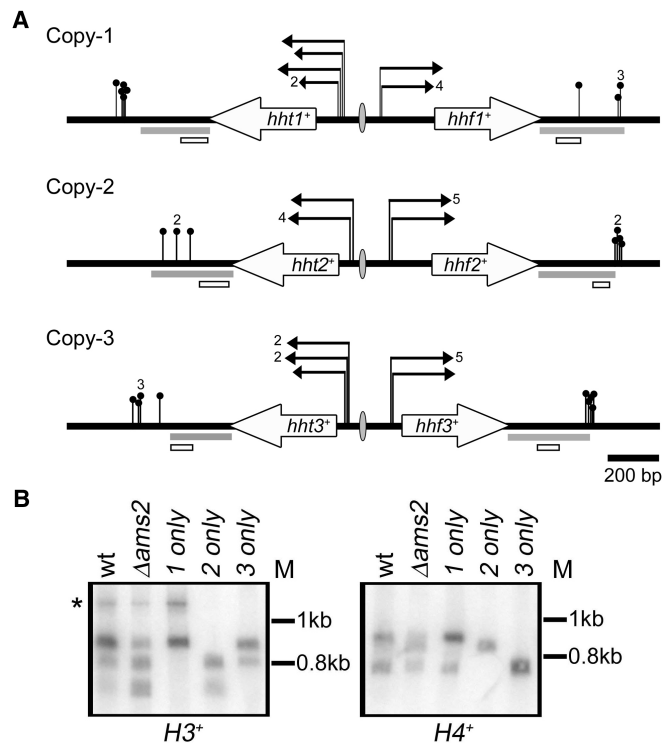
Figure 3. Identification of UTR regions for individual histone H3 and H4 gene pairs. (**A**) 3′- and 5′-RACE were performed using total RNA prepared from exponentially growing wild-type cells. The amplified PCR products were cloned and sequenced (>5 clones per RACE reaction). Arrows and lollipops indicate the locations of the 5′-ends of cDNAs obtained by 5′-RACE and the 3′-ends of cDNAs obtained by 3′-RACE, respectively. Ellipses indicate the locations of the AACCCT-box. Short gray and white bars indicate the allele-specific probes used for northern blotting (Figures 4A and 5A) and quantitative RT-PCR (Figures 4B, 5B, 6 and 7) analyses, respectively. (**B**) To determine whether the different-sized transcripts are actually produced from the histone alleles as predicted by the above RACE methods, the total RNA was separated on a denaturing polyacrylamide gel and blotted onto a Nylon membrane. The mixed H3- and H4-ORF probes that hybridized to the three histone H3 genes and three histone H4 genes, respectively, were used for northern analyses. The location of the transcript corresponding to the bands indicated by an asterisk could not be mapped on the *hht1⁺* locus by the RACE methods in the present study.

by real-time PCR using an ABI 7000 Sequence Detection System and *Power* SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of the primer sets are listed in Table S1 in the Supplementary Data.

Mutagenesis

Site-directed mutagenesis using a PCR-based method was performed as described previously (31) to introduce deletions in the AACCCT-box motifs in the promoter regions of *hht1⁺-hhf1⁺* genes. The PCR products were cloned and sequenced to confirm introduction of the expected mutations. The appropriately mutated promoters were cloned upstream of the GFP gene in an integration vector for the *lys1* locus, and then used to transform *lys1⁻ S. pombe* cells. The resultant *lys1⁺*

transformants were used for the experiments shown in Figure 2C.

5′- (3′-) Rapid amplification of cDNA ends (RACE)

Rapid amplification of cDNA ends was performed using a SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. The allele-specific primers used for 5′ and 3′ RACE are listed in Table S2 in the Supplementary Data. The PCR product obtained from each RACE reaction was cloned into the pGEM-T vector (Promega, Madison, WI, USA), and representative clones were sequenced. In 3′-RACE experiments, we carried out the reverse transcription using an oligo-dT containing primer (3′-RACE CDS PrimerA), and thus the histone transcripts identified in Figure 3 are expected to have polyA tail.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Prior to cDNA synthesis, aliquots of 5 μg of total RNA samples prepared as described above were treated with 2.5 U of Deoxyribonuclease I (TaKaRa) at 37°C for 1 h. First-strand cDNA synthesis was performed with 0.5 μg of DNase I-treated RNA in 10-μl reaction mixtures containing 0.5 μM oligo(dT)20 primer, 1 μM dNTP and 5 U of ReverTra Ace (Toyobo, Osaka, Japan) at 42°C for 20 min. The reaction was stopped by incubating at 100°C for 5 min. The amounts of synthesized cDNAs were quantified by real-time PCR using an ABI 7000 Sequence Detection System and *Power* SYBR Green PCR master mix (Applied Biosystems). Overall efficiencies of quantitative RT-PCR were calculated from the slopes of the standard curves of serial dilutions of *S. pombe* genomic DNA in steps of 4 and found to be nearly identical for each primer set. Expression profiles for individual histone cDNAs and GFP cDNA across the cell cycle were normalized by comparing that for Act1 cDNA. The nucleotide sequences of the primer sets are listed in Table S3 in the Supplementary Data.

RESULTS

Ams2 is a key regulator of histone gene activation at S-phase

Previously, we reported that the cell cycle-regulated GATA-type transcription factor, Ams2, promotes the centromeric localization of the centromere-specific histone H3 variant CENP-A in *S. pombe* (Sp) (25). To obtain insight into the action of Ams2 in SpCENP-A localization at the molecular level, we examined the transcriptional targets of Ams2. The transcription factor Ams2 was originally identified as one of four multicopy suppressors in the SpCENP-A ts mutant *cnp1-1* (25). Ams2 is dispensable for cell viability, but Ams2-depletion results in growth retardation with a high frequency of chromosome missegregation (25). In addition to *ams2⁺*, three genomic histone H4 genes act as suppressor genes for *cnp1-1*, and ectopic over-expression of H4, but not other histones, results in partial amelioration of the growth
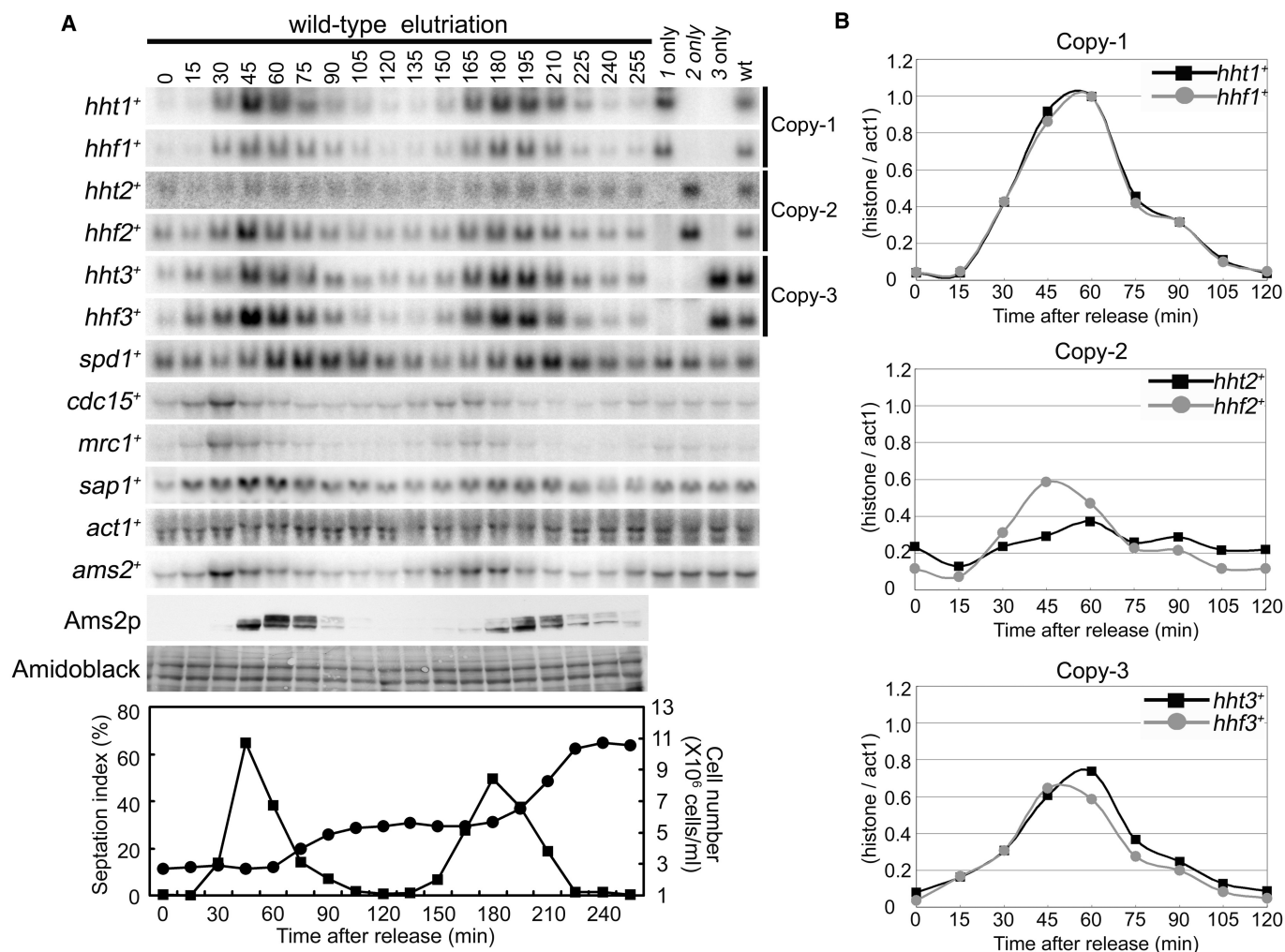
Figure 4. The transcriptional properties of individual histone H3 and H4 genes during the cell cycle. (**A**) Wild-type cells (HM123) were synchronized by centrifugal elutriation. Total RNAs collected at the indicated time points were assayed by northern blotting analysis to determine transcript levels of $ams2^+$, histones ($hht1^+$, $hht2^+$, $hht3^+$, $hhf1^+$, $hhf2^+$ and $hhf3^+$), cell cycle markers ($spd1^+$ for G2, $cdc15^+$ for M, $mrc1^+$ for G1, $sap1^+$ for S) and $act1^+$ (loading control). Total RNA samples from exponentially growing $\Delta pair-2\ \Delta pair-3$ cells (*1 only*, YTP556), $\Delta pair-1\ \Delta pair-3$ cells (*2 only*, YTP554), or $\Delta pair-1\ \Delta pair-2$ cells (*3 only*, YTP551) were used to determine the specificity of the allele-specific probes. The total amount of Ams2 protein was also examined by western blotting analysis using α-Ams2 polyclonal antibodies. A portion of the membrane was stained with amido black as a protein loading control. The cell number (black circles) and the septation index (black boxes) are also shown. (**B**) Quantification of the levels of individual histone H3 and H4 transcripts in wild-type cells across the cell cycle. Total RNA samples during the first cell cycle were extracted from elutriated wild-type cells (HM123 in Figure 4A, from 0 to 120 min after release) and used for cDNA synthesis. The levels of transcripts of individual histone H3 and H4 genes were determined by quantitative RT-PCR. The relative levels of each transcript (H3, black squares; H4, gray circles) were calculated by normalizing to that of $act1^+$ transcript.

retardation seen in Ams2-null cells (see Figure S1A in the Supplementary Data) (25,32). Thus, as demonstrated in budding yeast (33), the availability of H4-SpCENP-A dimers may cause defective SpCENP-A incorporation, leading to growth defects in $\Delta ams2$ cells. Therefore, in the present study, we examined whether Ams2 regulates histone transcription (Figure 1).

Synchronous cultures of wild-type and Ams2-deficient cells were prepared by centrifugal elutriation, and the cell extracts were analyzed by northern and western blotting (Figure 1A). Ams2 transcription is regulated in a cell cycle-dependent manner, which is similar to G1-specific Mrc1 transcription in wild-type cells (34); the promoter of the $ams2^+$ gene has an *Mlu*I cell-cycle box 1 (MCB1)

motif, genes carrying which are known to be regulated by MCB binding factor (MBF), a conserved transcription complex (Cdc10-Res1-Res2-Rep2 in *S. pombe*) that functions in G1-S progression (34,35). Many of the genes required for S-phase functions, such as $mrc1^+$ (which encodes a protein required for DNA replication checkpoint), $cdc18^+$ (which encodes a protein that couples cell cycle signals to the DNA replication machinery and induces replication), and $cdc22^+$ (which encodes a ribonucleoside diphosphate reductase large chain), contain an MBF1 motif in their promoters and are regulated simultaneously at G1-phase (34). Ams2 protein level also oscillates through the cell cycle (Figure 1A) (25), reaching a peak concomitant with S-phase-specific

Sap1 transcription and histone gene activation in S-phase (34,36). In Ams2-deficient cells, although the basal level of transcription of histone genes was the same as that in wild-type controls (Figure 1C), timely accumulation of core histone mRNAs (H2A, H2B, H3 and H4) during DNA synthesis was markedly suppressed. The division cycle in Ams2-null cells is longer than that in wild-type cells; maximal septation index is seen at 90 min in wild-type cells, but does not peak until ~150 min in the Ams2-null mutant. Given the lack of available core histones with which to form nucleosomes, the longer generation time of Ams2-null cells could be due to a significant delay of DNA replication. However, flow cytometric analysis indicated that Ams2-null cells duplicated their DNA when they formed septa, similarly to the wild-type control cells (Figure 1B). No significant delay was observed in DNA replication in Ams2-depleted cells in which S-phase-specific activation of histone genes was blocked. The observation that DNA replication in *Δams2* cells, which were released from S-phase-arrest caused by addition of HU, proceeds without a significant delay (Figure 7) also supported this conclusion. These results indicated that although Ams2-depletion affects the duration of the cell cycle, the reason for which remains unclear, blockade of histone gene activation has no marked effect on DNA replication. The S-phase-specific transcription of *sap1*$^+$, as well as other phase-specific cell cycle markers (34), was preserved in Ams2-depleted cells. However, it should be noted that the progression of S-phase (the beginning of the expression of at least a portion of S-phase-specific genes) may be delayed to some extent in the *ams2* shut-off strain, although the extension of the cell cycle duration is still much longer than this delay; in Ams2-depleted cells, the peak of expression of the S-phase-specific marker, Sap1 (180–195 min after release), occurs 30–45 min later than the peak of the septation index (150 min after release), while these peaks are roughly coincident with each other in wild-type cells at 90 min. In both wild-type and Ams2-depleted cells, the timing of the end of expression of the G1 specific-marker, Mrc1 and the beginning of the expression of the G2 specific-marker, Spd1, are coincident with the peak of the septation index (Figure 1A), suggesting that cell cycle events other than expression of some S-phase-specific genes occur normally in Ams2-depleted cells.

The total levels of the canonical histone mRNAs prepared from *Δams2* cells and from cells over-expressing Ams2 were decreased and increased, respectively, as compared with asynchronous cultures of wild-type cells (Figure 1D). No changes were observed in SpCENP-A mRNA level under conditions of Ams2-depletion or overproduction (Figure 1D). The histone H2A.Z variant *pht1* mRNA level in *Δams2* was comparable to that in wild-type (data not shown). Therefore, in the absence of Ams2, the cell specifically loses one of the main periodicities of the cell cycle, i.e. transcriptional oscillation of canonical histone genes (34), although the intracellular levels of histone proteins (H3 and H4) are not markedly affected (Figure 1A).

Ams2 binds to the upstream regions of core histone genes *in vivo*

If Ams2 directly promotes expression of core histone genes at S-phase as a transcriptional regulator, it should bind to the upstream regions of core histone genes. Therefore, ChIP assay was performed to examine whether Ams2 associates with DNA close to the promoter regions of histone genes *in vivo* (Figure 2). The *cdc25-22* mutant strain was used to synchronize *S. pombe* cells; G2-arrested cells were prepared by inactivating Cdc25 phosphatase, an inducer of mitosis, at the restrictive temperature of 36°C for 3 h. Cells were then released synchronously into mitosis at 26°C. As most fission yeast cells with a septum are in S-phase, the septation index (i.e. the percentage of cells with a septum) is a good hallmark for estimating of cell synchronization. We prepared cell extracts for ChIP experiments from cell populations in late-G2 and S-phase (time 0 and 210 min; septation indexes of 4.2 and 74.3%, respectively). Cells were treated with 3% formaldehyde for cross-link of DNAs with the binding proteins. After sonication, DNAs co-immunoprecipitated with α-Ams2 polyclonal antibodies were purified and quantified by real-time PCR using the probes indicated in Figure 2A. In S-phase cells, the ChIP signals were detected using primers derived from the intergenic regions between *H3*$^+$-*H4*$^+$ pairs (probes 3, 4 and 5), the intergenic region between the *hta1*$^+$-*htb1*$^+$ pair (probe 1), and the upstream region of the *hta2*$^+$ gene (probe 2) (Figure 2B). The ChIP signals were amplified reproducibly in the assay. In contrast, DNAs derived from the downstream region of the *hht3*$^+$ gene (probe 6), the upstream region of the *cnp1*$^+$ gene encoding the centromere-specific histone H3 variant, SpCENP-A (probe 7) and the *act1*$^+$ coding region for actin (probe 8) were not co-immunoprecipitated with Ams2. The upstream region of the *pht1*$^+$ gene encoding the histone H2A.Z variant was not co-immunoprecipitated with Ams2 (data not shown). The ChIP signal was not detected in *Δams2* cells using α-Ams2 polyclonal antibodies. Ams2 was enriched to a greater extent in the upstream regions of histone genes in S-phase (time 210 min) than in late-G2-phase (time 0 min). These results indicate that Ams2 activates core histone transcription directly as an S-phase specific GATA factor (25).

Identification of Ams2-binding sites upstream of histone genes

To further dissect the DNA sequence required for Ams2 binding, the intergenic region between *hht1*$^+$ and *hhf1*$^+$ genes was divided into three sections—the N-terminus, Middle and C-terminus—and the combined constructs tagged with the open reading frame (ORF) of the GFP gene followed by the *nmt1* terminator sequence were integrated into the *lys1* locus. The Ams2-binding activities of the truncated promoter regions were then tested by ChIP assay *in vivo*. GFP-specific primers were used for real-time PCR amplification to estimate the amounts of DNA co-immunoprecipitated using α-Ams2 polyclonal antibodies (Figure 2C, center panel). The *Mlu*I-*Pst*I 281 bp DNA fragment in the middle of the intergenic region was shown to be essential for Ams2 binding. This region contains a so-called 'AACCCT-box'

consensus sequence, which consists of a 17-bp sequence (5′-ATCA(C/A)AACCCTAACCCT-3′) commonly present upstream of histone genes (6,7) (Figure 2C, left). To examine whether this consensus sequence is required for Ams2 binding, this motif was deleted from the intergenic region between *hht1*[+] and *hhf1*[+] genes (ΔC, YTP784), and used for ChIP assay. The promoter region lacking the AACCCT-box (ΔC) showed complete loss of Ams2-binding activity, indicating that Ams2 binds upstream of the core histone genes during S-phase in an AACCCT-box-dependent manner. To determine whether Ams2 binding actually activates transcription of the histone genes, we next examined expression of the GFP gene under the control of the *hht1*[+] promoter by quantitative RT-PCR. As shown in the panel on the right in Figure 2C, the promoter constructs to which Ams2 cannot bind (YTP761 and YTP760) lost the ability to promote transcription of the GFP reporter gene. Importantly, the transcriptional level of the GFP gene in the ΔC construct was comparable to that of the control GFP gene without the promoter (YTP779). These observations strongly suggest that S-phase-specific histone activation is promoted by direct association of Ams2 with the histone promoters through the AACCCT-box.

Next, a BLAST homology search was performed using the fission yeast chromosome and mitochondrial genome database (http://www.genedb.org/genedb/pombe/blast.jsp) to find the sequences matching 100% to a 17-bp AACCCT-box-motif sequence (5′-ATCA(C/A)AACCCT AACCCT-3′). Only one additional sequence that shows 100% identical to the consensus was found ∼0.9 kb upstream of the SPAC631.02 gene besides those on histone promoters. Intriguingly, this ORF is reported to be transcribed at S/G2-phase (34) and encodes a protein containing two bromodomains, which recognize acetylated lysine residues on the N-termini of histones. The protein encoded by SPAC631.02 is one of two homologs showing low levels of similarity to *S. cerevisiae* Bdf1p, which interacts with acetylated histones H3 and H4 (37,38). We performed ChIP assay (Figure 2A and B) and quantitative RT-PCR analysis of the SPAC631.02 gene to determine whether Ams2 binds to the upstream region and regulates transcription of this gene. The amplified ChIP signal obtained using a primer set derived from the genomic region adjacent to the AACCCT-box located upstream of the SPAC631.02 gene (probe 9) (Figure 2A) was detected reproducibly in the DNAs co-immunoprecipitated with Ams2 (Figure 2B). The ChIP signal was increased in S-phase samples, implying that the SPAC631.02 gene may be a physiological transcriptional target of Ams2 in addition to the core histone genes. The physiological significance of the binding of Ams2 to the upstream region of the SPAC631.02 gene at S-phase remains unclear.

Properties of transcriptional oscillation of three pairs of histone H3 and H4 genes

The fission yeast genome has three pairs of histone H3-H4 genes (copy-1, -2 and -3; Figures 2A and 3A).

Although the total amounts of histone H3- and H4-transcripts have been reported to be increased in S-phase during the cell cycle (34,39), the transcriptional profiles of each of the histone H3 and H4 genes have yet to be defined. To determine the individual transcriptional patterns, allele-specific probes for each of the three copies of the H3 and H4 genes were designed for northern blotting analysis. As the three histone H3- or H4-ORFs show 88–93% identities at the nucleotide sequence level, the DNA probes derived from the ORF regions could not distinguish individual H3 or H4 transcripts on northern blotting analysis. Therefore, we designed DNA probes based on the sequences outside the ORFs that exhibit lower levels of similarity to each other. We identified UTRs of the three histone H3 and H4 genes using a combination of 5′- and 3′-RACE methods (Figures 3A and 2S, see Materials and Methods section). We verified that the transcripts of different sizes from each locus determined by the RACE methods were actually transcribed in wild-type cells; the sizes of the bands identified by northern blotting using H3 or H4 probes were approximately coincident with the sizes of the predicted transcripts taking into consideration the subsequent addition of a poly(A) tail (40,41) (Figure 3B). The individual histone probes were designed based on the sequence information derived from the 3′ UTRs (Figure 3A, gray bars). Total RNAs from cells retaining only a single pair (*1 only*, Δcopy-2 Δcopy-3; *2 only*, Δcopy-1 Δcopy-3; *3 only*, Δcopy-1 Δcopy-2) were used to verify the specificity of the probes for northern blotting analysis. The individual histone H3 and H4 transcripts were recognized exclusively by these 3′UTR probes (Figure 4A, *hht1*[+], *hhf1*[+], *hht2*[+], *hhf2*[+], *hht3*[+] and *hhf3*[+]). Synchronized wild-type cells at early G2 by centrifugal elutriation were released into fresh medium at 33°C, and were collected at 15-min intervals to prepare total RNAs for northern blotting analysis using 3′ UTR histone probes and cell cycle markers. The transcriptional levels of *hht1*[+], *hhf1*[+], *hhf2*[+], *hht3*[+] and *hhf3*[+] genes oscillate through the cell cycle, reaching a peak roughly concomitant with S-phase-specific Sap1 transcription (34), while the *hht2*[+] gene was transcribed constitutively throughout the cell cycle (Figure 4A).

Quantitative comparison of the band intensities on northern blots is difficult. To compare the levels of transcription of individual histone H3 and H4 genes during the cell cycle, quantitative RT-PCR was performed; cDNA was synthesized using total RNA extracted from synchronized wild-type cells during the first cell cycle (Figure 4A, from 0 to 120 min), and the relative amounts of individual histone H3 or H4 cDNA to the Act1 cDNA were calculated across the cell cycle with primer pairs specific to the unique 3′ UTR of histone genes. Primer pairs were tested against dilutions of genomic DNA to verify that the amplification efficiencies were similar (Figure 3A, white bars, see Materials and Methods section). The levels of *hht1*[+], *hhf1*[+], *hhf2*[+], *hht3*[+] and *hhf3*[+] transcripts peaked in S-phase, while that of the *hht2*[+] transcript did not exhibit a clear peak throughout the cell cycle (Figure 4B), consistent with the results of northern blotting analyses described above; the levels of

transcription of copy-1 genes in S-phase (45 and 60 min after release) were 20- to 25-fold greater than those in G2-phase (0 min after release). The amplitudes of the peaks of $hht3^+$ and $hhf3^+$ gene expression in S-phase were 12- to 14-fold and 17- to 19-fold greater than those in G2-phase, respectively. The $hhf2^+$ gene was still expressed periodically but with lower amplitude (4- to 5-fold) as compared to copy-1 genes.

Both over-expression and depletion of Ams2 up-regulate the transcriptional amounts of copy-2 genes

To determine whether Ams2 protein is required for transcriptional activation of each copy of the reiterated H3 and H4 genes at S-phase, the levels of the individual H3 and H4 transcripts in Ams2-depleted or Ams2-over-expressing cells were compared with those in wild-type controls (Figure 5A–C). Northern analysis (Figure 5A) and quantitative RT-PCR using the allele-specific 3′UTR histone probes (Figure 5B and C) indicated that the levels of each copy-1 or copy-3 transcript were increased by over-expression of Ams2 and decreased by depletion of Ams2, consistent with our conclusion that Ams2 is required for S-phase activation of histone genes (Figures 1 and 2). Surprisingly, the transcripts deduced from copy-2 prepared from asynchronous cells were increased reproducibly in both Ams2-over-expressing and -depleted cells in comparison with wild-type controls (Figure 5A–C). This observation suggested the existence of a feedback control mechanism(s) to up-regulate at least $hht2^+$ and $hhf2^+$ transcript expression levels under conditions where the activation of core histone genes in S-phase is compromised. This feedback control appears to function in an Ams2-independent manner. As shown in Figure 5D, the up-regulation of histone H3 and H4 genes was also observed in asynchronous cells with deletion of two of the three H3-H4 gene pairs. In these cases, the increases in the levels of transcripts from copy-2 and copy-3 were prominent as compared with those from copy-1. The physiology of feedback regulation may differ between Ams2 null mutants and histone deletion mutants.

Next, we performed quantitative RT-PCR to evaluate the effects of Ams2-depletion on the transcriptional profiles of each histone gene in S-phase and G2-phase (Figure 6) using the elutriation samples prepared for the experiments shown in Figure 1. Cell samples were taken 90 min after release for the wild-type strain, which were expected to be enriched in cells at S-phase. Corresponding cell samples were taken at two time points, 150 and 180 min, for the *ams2* shut-off strain; under the conditions using Ams2-depleted cells in Figure 1, the boundary between G1- and G2-phase—and presumably DNA replication—seemed to occur at 150–165 min after release, while the expression of the S-phase marker, Sap1, peaked at around 180–195 min. The cell samples at 30 and 165 min for wild-type and at 60 and 240 min for the *ams2* shut-off strain were used as fractions at late-G2/M-phase because these time points lie at the boundary of expression of the G2-phase marker, Spd1, and the M-phase marker, Cdc15 (Figure 1A). The results of the quantitative RT-PCR experiments shown in Figure 6 clearly indicated that the
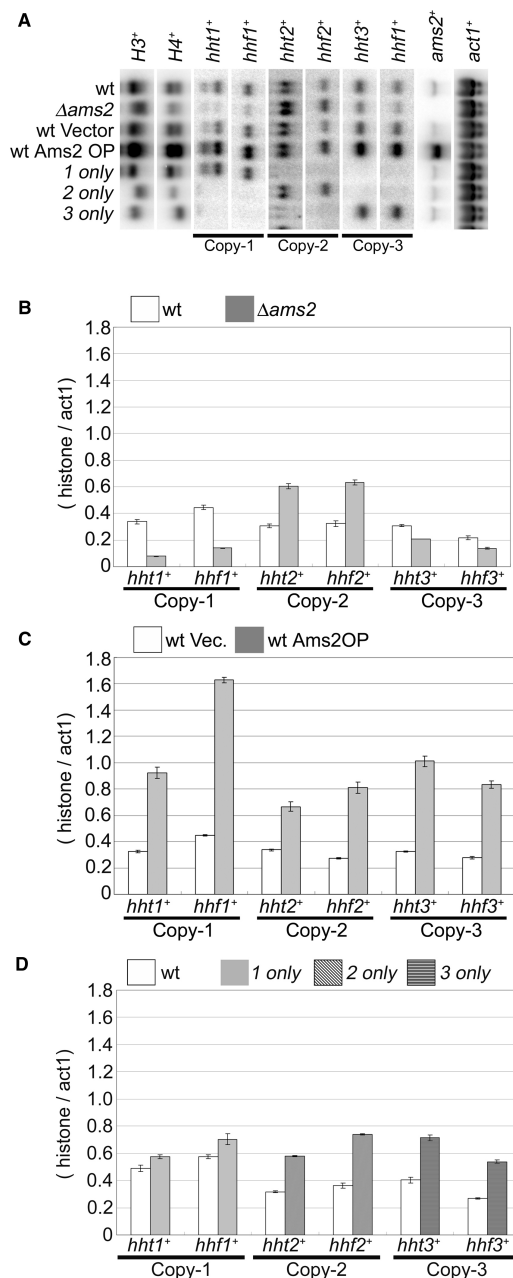
Figure 5. Depletion of Ams2 silences the transcription of copy-1 and -3 histone genes but promotes those of copy-2. The transcript levels of the total histones ($H3^+$ and $H4^+$), individual histones ($hht1^+$, $hht2^+$, $hht3^+$, $hhf1^+$, $hhf2^+$ and $hhf3^+$), $ams2^+$ and $act1^+$ (loading control) were assessed by northern analysis (A) and quantitative RT-PCR (B, C and D). For the experiments shown in (B) and (C), asynchronous wild-type cells (WT, SP91), Ams2-null cells (*Δams2*, SP45), wild-type cells carrying empty vector only (WT vector, YTP11), or wild-type cells over-expressing Ams2 (Ams2-OP, YTP12) were examined at 33°C. For the experiments shown in (D), asynchronous wild-type cells (WT, SP91), *Δpair-2 Δpair-3* cells (*1 only*, YTP556), *Δpair-1 Δpair-3* cells (*2 only*, YTP554), or *Δpair-1 Δpair-2* cells (*3 only*, YTP551) were examined at 26°C. Total RNA samples were prepared from cells inoculated in YES for (B) and (D) or in EMM2 for (C), and used for northern blotting and cDNA synthesis. In (B), (C) and (D), the levels of histone H3 and H4 gene transcripts were quantified individually by RT-PCR. The relative levels of each transcript (H3, $hht1^+$, $hht2^+$ and $hht3^+$; H4, $hhf1^+$, $hhf2^+$ and $hhf3^+$) were calculated by normalizing to that of $act1^+$ transcript. The error bars indicate the standard deviation from 4 independent PCR experiments.
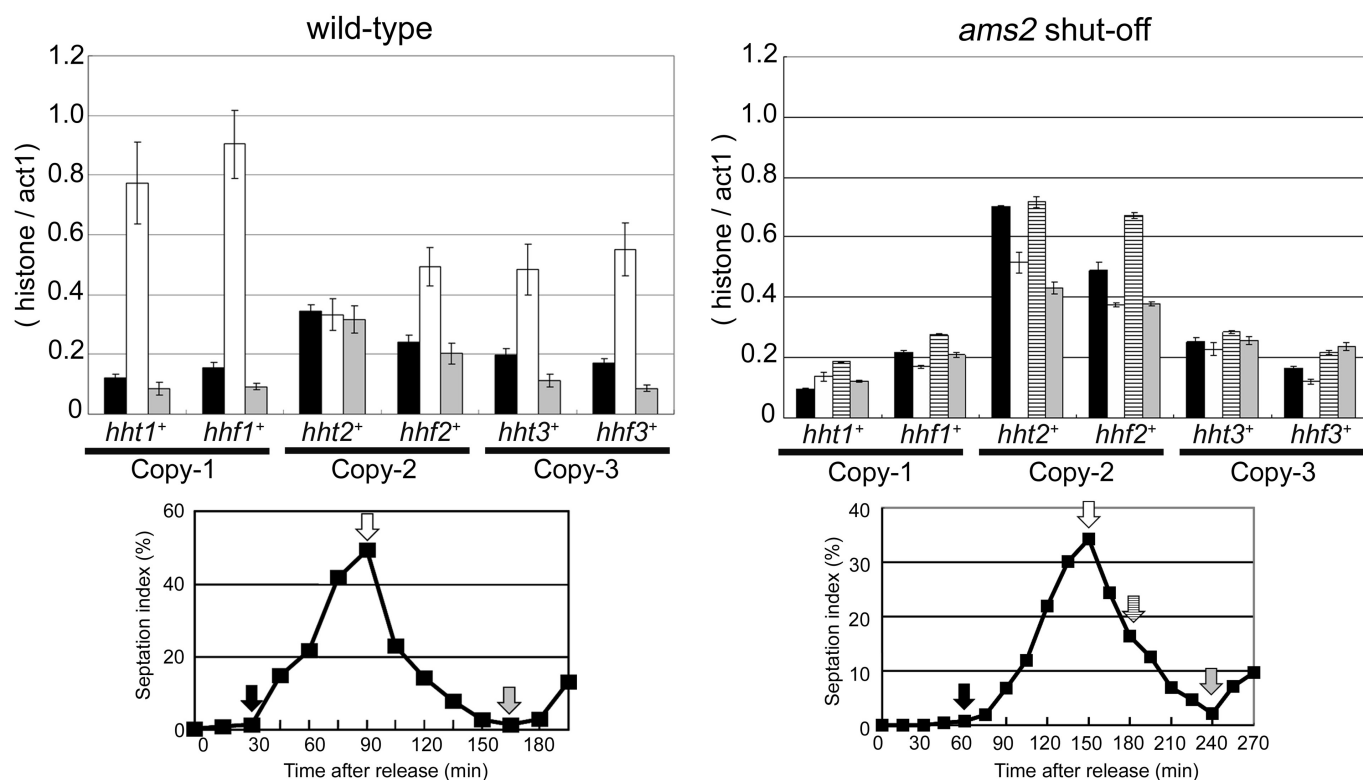
Figure 6. Feedback up-regulation of basal transcription of histone genes in copy-2 in *Δams2* cells. The levels of transcripts for individual histone H3 and H4 genes remained constant in Ams2-depleted cells (YTP91) across the cell cycle. Each histone cDNA was synthesized from total RNA extracted from cells synchronized in early G2 or S-phase by centrifugal elutriation. Late-G2/M samples (black and gray bars; time points 30 and 165 min for wild-type SP91, 60 and 240 min for *ams2*-shut-off YTP91) or potential S-phase samples (white bars, time point 90 min for wild-type; white and striped bars, time points 150 and 180 min for *ams2*-shut-off). The corresponding arrows (black, white, striped and gray) in the bottom graphs indicate the positions where the cell samples were collected. The levels of transcripts for histone genes were determined by quantitative RT-PCR. The error bars indicate the standard deviation of 4 independent PCR experiments. The relative levels of each transcript were calculated by normalizing to that of *act1*[+] transcript.

levels of expression of H3 and H4 genes in copy-1 and copy-3 during S-phase in Ams2-depleted cells were decreased compared to those seen in late-G2/M-phase in wild-type cells, while the basal levels of expression of histone H3 and H4 in copy-2 in late-G2/M-phase were increased by 1.4- to 2.0-fold in Ams2-depleted cells as compared with wild-type controls. Therefore, the feedback up-regulation of histone H3 and H4 genes under the conditions of Ams2-depletion appeared to be achieved mainly through up-regulation of the basal expression of *hht2*[+] and *hhf2*[+]. Consistent with this suggestion, tetrad analysis indicated that copy-2 null mutant (*Δ2*; YTP547), but not copy-1 null mutant (*Δ1*; YTP545) or copy-3 null mutant (*Δ3*; YTP549), was synthetic lethal with *Δams2* (SP45) (Figure S3 in the Supplementary Data).

Fission yeast HIRA homologs are required for repression of the basal expression of copy-2 genes

In fission yeast, it has been shown that loss of Hip1, one of two homologs of the human HIRA histone chaperone, leads to derepression of core histone gene expression outside of S-phase (19). Therefore, we performed quantitative RT-PCR to investigate how depletion of

Hip1 (or Slm9, another homolog of HIRA) may affect the expression patterns of each histone H3 and H4 gene during the cell cycle. For these experiments, we used synchronized cells released from arrest in S-phase by transient treatment with HU (Figure 7). The DNA contents estimated by flow cytometric analyses indicated that the cell samples 15 min after release were in S-phase in all strains tested (Figure 7, left). As the septation index began to increase from 105 min in *Δslm9* and 90 min in the other strains, we selected the fraction obtained at 60 min after release as cell samples in G2-phase. In accordance with our expectations, wild-type cells (Figure 7, upper middle) showed accumulation of each transcript of histone H3 and H4, with the exception of that of *hht2*[+], in S-phase, consistent with the results obtained using elutriation samples. In *Δams2* cells (Figure 7, upper right), S-phase activation of copy-1 genes was markedly suppressed, whereas the basal levels of transcription of copy-2 genes appeared to be up-regulated. However, *Δams2* cells arrested by HU seemed to retain the S-phase-specific activation of copy-3 genes, which was inconsistent with our conclusions based on the results of experiments using the elutriated *ams2* shut-off strain shown in Figure 6. The reason for this discrepancy remains unclear.
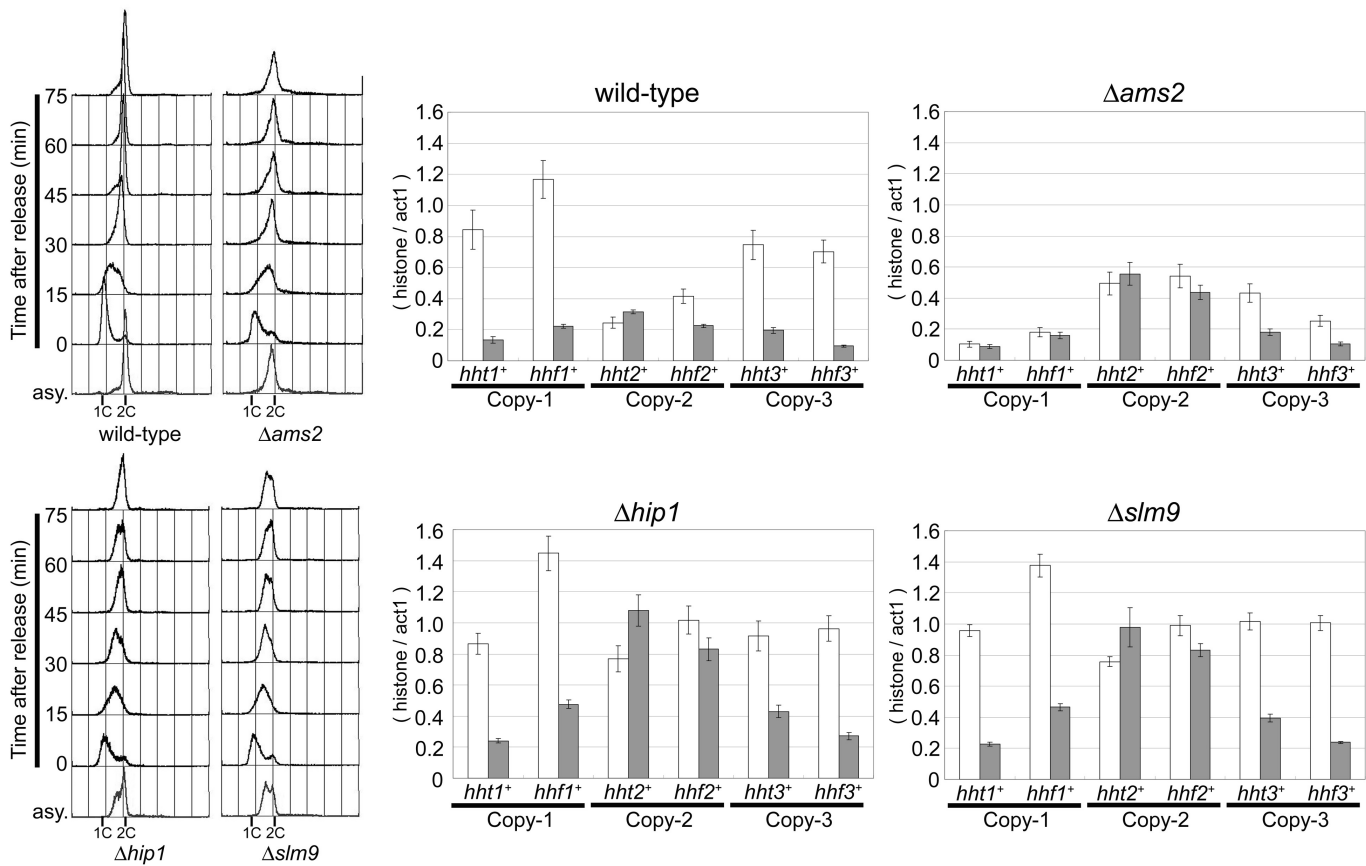
Figure 7. Homologs of the HIRA histone chaperone, Hip1 and Slm9, are involved in basal expression of histone H3 and H4 genes. Wild-type (SP91), *Δams2* (YTP155), *Δhip1* (SP1500), and *Δslm9* (SP1503) cells were synchronized in S-phase by the addition of HU and released into fresh YES at 26°C. DNA contents estimated by flow cytometry at 15-min intervals after release and the level of each histone transcript determined by quantitative RT-PCR in cells at S-phase (white bar; 15 min after release) or at G2 phase (gray bar; 60 min after release) are shown. The relative levels of each transcript were calculated by normalizing to that of *act1+* transcript. The error bars indicate the standard deviation of 4 independent PCR experiments.

In *Δhip1* and *Δslm9* cells, we showed that the levels of transcription of histone H3 and H4 genes are up-regulated at both S- and G2-phases (Figure 7, lower panels). Although Hip1 and Slm9 form a large protein complex and share common functions in pericentric heterochromatin formation and faithful mitotic chromosome segregation (19), the two proteins are not functionally equivalent; Hip1 represses the expression of core histone genes outside S-phase (19), while the pattern of H2A expression during the cell cycle remains unaffected in Slm9-depleted cells (18). However, we found the transcriptional derepression of all H3 and H4 genes in *Δslm9* cells and the resultant expression profile resembled that in *Δhip1* cells (Figure 7, lower panels), suggesting that both Hip1 and Slm9 play common roles at least in repressing the expression of H3 and H4 genes. Hip1- or Slm9-deletion mutants showed marked transcriptional derepression of copy-2 genes, suggesting that the complex comprised of HIRA homologs mainly silences the transcription and/or destabilizes the transcripts of copy-2 genes. Further studies are required to determine how this complex contributes to the repression of histone gene transcription. The feedback up-regulation of copy-2 genes observed in *Δams2* cells may be achieved through down-regulation of the level and/or activity of the Hip1–Slm9 complex.

DISCUSSION

Ams2 GATA factor and HIRA homologs function for the periodic histone transcription

The results of the present study demonstrated that Ams2 GATA factor is an essential component of a transcriptional activator for all core histone genes during S-phase. We found that Ams2 binds directly to the promoter regions of all the core histone genes *in vivo* in an AACCCT-box-dependent manner (Figure 2C). The 17-bp AACCCT-box [5'-ATCA(C/A)AACCCTAACC CT-3'] was identified previously as a common DNA sequence located upstream of core histone genes (6,7). Although the AACCCT-box does not show a perfect match to the GATA consensus sequence, similar sequences (5'-GATn-3' and 5'-GtTA-3') were found within the box in the reverse strand and are potential candidates for Ams2-binding sites. Alternatively, the AACCCT-box may be a binding site for proteins required for association of Ams2 with the promoter.

Elimination of the AACCCT-box from the $hht1^+$ and $hhf1^+$ upstream region results in a marked reduction of promoter activity to the background level, suggesting that the AACCCT-box is essential for the promotion of histone gene transcription.

Ams2 is the first GATA factor reported to be involved in histone transcription. GATA factors are present in all eukaryotes, but it is not yet known whether similar GATA-type transcription factors are commonly utilized in histone gene regulation in different species. Some developmentally regulated GATA factors, such as GATA-4, are referred to as pioneer or decisive factors, because they function as determinants of tissue identity by opening compacted chromatin on tissue-specific promoters to aid access to the promoters by other factors (42). To control developmentally programmed transcription, the expression of these molecules is known to be highly restricted during development. Similarly, Ams2 protein level is tightly regulated during the cell cycle, peaking at S-phase. Thus, Ams2 may be involved in the first remodeling step of nucleosomes on the histone promoters.

We also found that the fission yeast HIRA proteins, Hip1 and Slm9, down-regulate the basal levels of H3 and H4 gene expression. The effect on derepression of the histone transcription by HIRA-depletion was greater in cells at G2-phase than at S-phase (Figure 7), consistent with the previous report that HIRA functions in repressing histone transcription outside S-phase (19). As the levels of transcription of core histones were increased reproducibly at S-phase in both Hip1-null and Slm9-null cells, the cell cycle-dependent expression seems to be mediated through not only repression of the expression outside S-phase but also by positive activation at S-phase. In addition, it should be noted that, in Ams2-deficient cells, the levels of histone transcripts became constant across the cell cycle including S-phase. These observations might be explained by regulation of histone gene transcription by a combination of Ams2-dependent up-regulation at S-phase and non-periodic basal transcription down-regulated by the HIRA complex throughout the cell cycle. HIRA-depletion predominantly affects the repression of $hht2^+$ and $hhf2^+$ genes in copy-2, which show periodic expression with low amplitude.

Feedback regulation of reiterated histone H3-H4 genes

In this study, we clarified the transcriptional properties of individual histone H3 and H4 genes during the cell cycle. Copy-1 ($hht1^+$ and $hhf1^+$) and copy-3 ($hht3^+$ and $hhf3^+$) genes are predominantly transcribed in S-phase in wild-type cells. In contrast, copy-2 ($hht2^+$ and $hhf2^+$) genes are transcribed throughout the cell cycle with relatively high levels of basal expression. We also demonstrated the existence of a feedback mechanism(s) differently up-regulating the reiterated histone H3-H4 gene pairs in Ams2-deficient cells. Ams2 is dispensable for cell viability, although S-phase-specific histone activation is severely compromised in such cells, presumably because non-periodic, basal histone transcription remains and is up-regulated in Ams2-null cells (Figures 1 and 4).

The majority of Ams2-independent, non-periodic transcripts of histone H3 and H4 genes are likely supplied by $hht2^+$ and $hhf2^+$ genes in copy-2; copy-2 genes are expressed outside S-phase at relatively high levels in wild-type cells and are up-regulated in $\Delta ams2$ cells (Figures 5 and 6). These observations are consistent with the finding that Ams2-deletion mutants require copy-2 genes for cell viability (Figure S3). It has been reported that cells retaining only copy-1 genes (*1 only*) are notably sensitive to thiabendazole, a spindle poison, and cells retaining only copy-3 genes (*3 only*) show a temperature sensitive growth defect at 36°C. In contrast, cells retaining only copy-2 genes (*2 only*) showed no such sensitivities to thiabendazole or elevated temperature (43). Non-periodic basal transcription of H3 and H4 genes, which is mainly from the copy-2 loci, may be an important component of the feedback regulation when cells encounter stresses that somehow perturb the normal progression of the cell cycle.

Among the three copies, copy-2 genes were the most up-regulated responded to the elimination of histone activation in S-phase (Figure 5B). The feedback up-regulation of copy-2 and copy-3 but not copy-1 genes were detected responded to the reduction in copy number of H3-H4 pairs by gene disruption (Figure 5D). During G2-phase in the normal cell cycle, the basal levels of the copy-3 gene transcripts were up-regulated in Ams2 shut-off cells as well as those of copy-1 and copy-2 genes (Figure 6). However, following release of Ams2-null cells from HU block, up-regulation of copy-3 genes was not detected in G2-phase (Figure 7). The feedback response of histone transcripts may be regulated differently according to changes in rates of DNA synthesis. These observations may indicate the existence of a variety of feedback modes for histone gene regulations. In budding yeast, deletion of one pair of H3-H4 genes does not affect the expression of other pairs of H3-H4 genes (44), whereas the levels of histone H2A-H2B gene transcripts (HTA1-HTB1 and HTA2-HTB2 gene pairs) are controlled by either up-regulation of HTA1-HTB1 transcription (45) or direct amplification of HTA2-HTB2 genes by circular chromosome formation (46). For the maintenance of genomic integrity, eukaryotic cells probably utilize several different mechanisms to compensate for histone dosage effects when perturbed.

One intriguing property of the divergent transcription of H3-H4 gene pairs is the asymmetric production of H3-H4 transcripts from copy-2 genes. The $hht2^+$ transcript is present throughout the cell cycle, while the $hhf2^+$ transcript is cell cycle regulated. As both $hht2^+$ and $hhf2^+$ genes are up-regulated by overproduction of Ams2, transcription of the $hht2^+$ gene as well as the $hhf2^+$ gene is probably promoted during S-phase. Thus, the transcript of $hht2^+$ may be destabilized post-transcriptionally or may be suppressed by transcriptional repressors that abrogate Ams2-dependent activation. Further studies are required to determine how this asymmetric transcription of copy-2 genes is converted into meaningful physiological output.

Despite the identical amino acid sequences deduced from the three H3-H4 gene pairs, their differential transcription profiles may result in differences in timing of deposition of the histones encoded by these genes into

the nucleosomes. Although recent experiments in higher eukaryotes have demonstrated that differences in a few amino acids between H3 and H3.3 can specify which nucleosome assembly pathway is used (21,23), the timing of the expression of H3 may be a more critical determinant for the timing of deposition into the nucleosomes in lower eukaryotes. Recently, it has been reported that, although remarkably similar in amino acid sequence, H3.1, H3.2 and H3.3 differ in their patterns of expression and the post-transcriptional modification in human cells (22). It will be intriguing to investigate whether three histone H3 and H4 proteins in fission yeast are also differentially modified and whether the timing of their expression can influence their deposition.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

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