Acetylation of H3 K56 Is Required for RNA Polymerase II Transcript Elongation through Heterochromatin in Yeast

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In Saccharomyces cerevisiae SIR proteins mediate transcriptional silencing, forming heterochromatin structures at repressed loci. Although recruitment of transcription initiation factors can occur even to promoters packed in heterochromatin, it is unclear whether heterochromatin inhibits RNA polymerase II (RNAPII) transcript elongation. To clarify this issue, we recruited SIR proteins to the coding region of an inducible gene and characterized the effects of the heterochromatic structure on transcription. Surprisingly, RNAPII is fully competent for transcription initiation and elongation at the locus, leading to significant loss of heterochromatin proteins from the region. A search for auxiliary factors required for transcript elongation through the heterochromatic locus revealed that two proteins involved in histone H3 lysine 56 acetylation, Rtt109 and Asf1, are needed for efficient transcript elongation by RNAPII. The efficiency of transcription through heterochromatin is also impaired in a strain carrying the K56R mutation in histone H3. Our results show that H3 K56 modification is required for efficient transcription of heterochromatic locus by RNAPII, and we propose that transcription-coupled incorporation of H3 acetylated K56 (acK56) into chromatin is needed for efficient opening of heterochromatic loci for transcription.

In order to produce mRNA, RNA polymerase II (RNAPII) has to contend with chromatin in regulatory and coding regions of genes. Different modifications of histone proteins, rearrangements of nucleosome positioning, and packaging of nucleosomes into higher-order chromatin structures are used to facilitate or repress the accessibility of cellular factors to DNA. Modifications of histone proteins by acetylation and methylation are the most common ways to initiate changes in chromatin structure. In addition to several transcription-coupled modifications of chromatin in active gene loci, nucleosomes can be removed from entire transcribed regions and replaced with new histones after shutdown of transcription (14, 18, 21, 42, 49, 54). A detailed study of nucleosome dynamics in budding yeast has revealed a remarkable replication-independent exchange of histones throughout the genome. While turnover of promoter nucleosomes is detectable regardless of transcriptional activity of the locus, exchange of histones in the coding regions is in good correlation with gene expression level, indicating that turnover of nucleosomes is a rather common feature of ongoing transcription, especially in highly transcribed loci (7).

Gene expression in eukaryotic cells is also controlled by the formation of repressive heterochromatic domains in loci of regulated genes. There are three main regions in the genome of Saccharomyces cerevisiae that are subject to silencing by heterochromatin: telomeres, ribosomal DNA (rDNA) locus, and silent mating type loci (HML and HMR). Heterochromatin at telomeres and HM loci is formed by the complex of silent information regulator (SIR) proteins Sir2, Sir3, and Sir4, which are also required for the maintenance of heterochromatin structure. Disruption of any of these SIR genes greatly reduces recruitment of other Sir proteins to heterochromatin loci and also leads to the loss of telomeric and HM silencing (12, 25, 38, 46). Upon initial recruitment, SIR proteins accumulate and spread along the chromatin. To avoid repression of euchromatic regions in the genome, cells have evolved several mechanisms to restrict the spreading of SIR complex. Those include acetylation and methylation of histones, incorporation of H2A.Z into nucleosomes, and maintenance of highly transcribed nucleosome-free regions in chromatin (37).

For the initiation of SIR protein recruitment and heterochromatin formation in HM loci, specific silencer regions E and I, flanking HMR and HML, are required. Although both E and I regions are involved in full silencing of HM loci, the HMR-E and HML-E silencers are crucial for the recruitment of SIR proteins to chromatin when inserted into different locations in the genome (24, 44).

The classical view of transcriptional silencing by heterochromatin states that the highly condensed structure of chromatin elicits its repressive effects by sterically hindering the access of sequence-specific regulatory factors, required for the binding of transcription machinery, and therefore blocking the whole process (17). Nevertheless, it has been established that repressive heterochromatin in Saccharomyces cerevisiae allows constitutive binding of transcription activators, preinitiation complex (PIC) components, and RNAPII to the promoter regions of repressed genes, indicating that steps other than initial recruitment of transcription factors might be repressed by heterochromatin (43, 44). Indeed, recent data argue that repression of transcription by silenced chromatin is mainly targeting the transition point between RNAPII initiation and elongation by diminishing the recruitment of 5'-capping enzymes and elongation factors (9).
Although the role of heterochromatin in the repression of gene promoters is well characterized, it has remained unclear whether RNAPII that has cleared the promoter and entered chromatic structures in the coding region. Addressing this question in the present study, we show that elongating RNAPII chromatic structures in the coding region of the gene.

Materials and Methods

Yeast strains. All Saccharomyces cerevisiae strains used were congenic with strain W303. The GAL-VPS13 (AKY210) strain (49) expressing H3 with a C-terminal E4 tag was used to create the strain AKY362 with a C-terminal E4 tag. To create the strain AKY362, GAL-VPS13-HMR-E (AKY354) and GAL-VPS13-HMR-E-0.1kb (AKY359) strains were created by insertion of the HMR-E silencer sequence (nucleotides 292008 to 292388 of chromosome III; Saccharomyces Genome Database) or 100 bp downstream of the VPS13 start codon, respectively (see Fig. 1A and 3A). In addition, Sir3 with a C-terminal nine-Myc tag and Rpb3 with a triple-E4 tag in their natural loci were introduced in strains AKY354 and AKY359. Deletion mutant strains with wild-type (wt) HMR-E silencer sequence at 6 kb downstream from the start codon of VPS13 were derived from AKY362. The H3 mutant K56R strain with wt GAL-VPS13 was created by replacing the HHT2 allele in strain AKY362 with a mutant one in its natural locus. The yeast strains and their genotypes are given in Table 1.

ChIP assay. Cells were grown overnight in yeast extract-peptone (YP) medium containing 2% glucose or galactose as a carbon source before fixation for the chromatin immunoprecipitation (ChIP) assay. For the detection of H3 K56 acetylation dynamics, cells were grown overnight in YP medium containing 2% raffinose, which was then replaced by 2% galactose and glucose for designated periods of time. ChIP assays were performed as described previously (19, 20).

Whole-cell extract from 1 × 10^7 cells was used for ChIP assays with antibodies directed against RNAPII (4H8; Upstate Biotechnology), anti-Myc tag (9E10; Abcam), anti-E2 tag (5E11; Icosagen), or anti-acetyl-histone H3 Lys56 (Upstate Biotechnology). Coprecipitated DNA was analyzed by quantitative real-time PCR using an ABI Prism 7900HT real-time PCR system under standard conditions (40 cycles; 95°C for 15 s and 60°C for 1 min). Absolute QPCR SYBR green reagents (ABgene) and 5 µl Hot FIREPol EvaGreen qPCR mix (Solis BioDyne) were used. PCRs were done with primer pairs covering coding region of VPS13, FBA1, the chromosome VI right arm telomere region, and the noncoding region on chromosome VIII. The VPS13 coding region primers were specific for the sequences at 0.1, 3.5, 5.5, 6.4, and 8.5 kb downstream from the start codon of GAL-VPS13. The length of all PCR products was about 160 bp. Exact sequences of all primers are available upon request. In SIR complex removal experiments, samples were normalized to the request. In SIR complex removal experiments, samples were normalized to the signal of Sir3 on the constitutively expressed TRP1 and Rpb3 on the noncoding region on chromosome VIII and the amount of RNAPII was normalized to the amount of histone H3 on the noncoding region on chromosome VIII and the amount of RNAPII was normalized to the amount of histone H3 on the noncoding region.

RT-PCR. For the detection of mRNA, cells were grown overnight in YP medium containing 2% galactose or raffinose. For transcription induction, galactose, with the final concentration of 2%, was added to cells grown in raffinose. Samples were collected before galactose addition and 30, 60, 120, and 180 min after transcription induction. For cell cycle arrest experiments, α-factor mating pheromone (Zymon Research) was used with the final concentration of 100 µM and cell cycle arrest in G1 phase during the experiment was confirmed by fluorescence-activated cell sorting (FACS) analysis. Cells (3 × 10^7) were collected, and RNA was extracted as described previously (5) with minor modifications. Cells washed with water were resuspended in 600 µl of RNA extraction

Table 1. Names and genotypes of yeast strains used in the study

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a SpHIS5, Schizosaccharomyces pombe HIS3.
buffer (10 mM EDTA, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5% sodium dodecyl sulfate [SDS]), mixed with equal volumes of acid phenol-chloroform (1:1), and lysed at room temperature for 6 min. Glass beads were added, and samples were vortexed at maximum speed for 4 min. Liquid was removed, and phenol-chloroform extraction was performed followed by chloroform extraction. RNA was precipitated with 1/10 volume of 3 M NaOAc and 3 volumes of ice-cold 96% ethanol. Precipitated RNA was washed with 70% ethanol and dissolved in 40 μl RNase-free water. DNase treatment was carried out, and 100 ng of RNA was used for one-step reverse transcriptase PCR (RT-PCR) (48°C for 30 min; 95°C for 5 min; and 20 cycles of 93°C for 3 min, 55°C for 30 s, and 72°C for 30 s). Primers specific for the coding regions of VPS13, FBA1, and GAL10 were used. RT-PCR products were separated on a 2.5% agarose gel stained with ethidium bromide.

RESULTS

Elongating RNAPII displaces SIR proteins from the coding region of the gene. To study the influence of heterochromatic structures on transcription elongation, we used *Saccharomyces cerevisiae* strains containing a modified VPS13 gene as our model system. The *GAL-VPS13* fusion gene contains the galactose-inducible GAL10 promoter in front of the 9.5-kb-long VPS13 open reading frame, allowing transcription of the gene to be induced or repressed by growing cells in medium containing galactose or glucose, respectively (18). In order to promote SIR complex recruitment and formation of heterochromatic structures in the coding region of the model gene, we inserted an HMR-E silencer into the *GAL-VPS13* gene 6 kb downstream of the transcription start site (Fig. 1A). Using chromatin immunoprecipitation (ChIP) and quantitative PCR methods, we then monitored distribution of RNAPII, nucleosomes, and Sir3 protein at different points through the *GAL-VPS13-HMR-E* gene before and after transcription induction.

In the repressed state, SIR proteins were efficiently recruited to the *HMR-E* sequence on the *GAL-VPS13-HMR-E* gene. When bound to *HMR-E*, SIR complexes spread bidirectionally upstream and downstream from the silencer sequence in the wild-type strain where all SIR proteins were present but not in the corresponding sir4Δ strain (Fig. 1B). Spreading of SIR complexes was more prominent toward the 5' end of the gene, covering at least 3 kb to this side but leaving the promoter and the beginning of the gene free of heterochromatin. The 3' end of the gene was also essentially free of SIR proteins. This uneven distribution of SIR complex was not unexpected, as the *HMR-E* silencer also works in an orientation-dependent manner in its native locus (26, 55). Remarkably, upon induction of *GAL-VPS13-HMR-E* transcription, SIR complexes were removed from the entire locus (Fig. 1B), suggesting that transcriptional activity in the locus was not abolished by heterochromatic structures and indicating that SIR complexes can be removed as a consequence of transcription.

Although the majority of Sir3 protein was found in the open reading frame (ORF) region of the transcriptionally inactive *GAL-VPS13-HMR-E* locus, small amounts of SIR proteins were also detected in the proximity of the promoter (Fig. 1B). To test whether the *GAL-VPS13* promoter can be silenced when large amounts of SIR proteins were recruited directly to the region, we inserted HMR-E sequence into the *GAL-VPS13* coding region 100 bp downstream of the beginning of the ORF (see Fig. 3A). As expected, SIR proteins were recruited to the site under repressing conditions, and they were efficiently removed upon activation of the gene (see Fig. 3B). These results show that the *GAL-VPS13* promoter is insensitive to SIR-mediated repression, making the *GAL-VPS13-HMR-E* model gene a suitable experimental system for studies of how heterochromatin affects transcription elongation. These results are also in concordance with previous reports indicating that the GAL1-10 promoter is competent for initiation of transcription of shorter mRNAs when inserted into telomeric heterochromatin (4, 39).

The GAL10 promoter provides a very high-level induction of *GAL-VPS13*, leading to the removal of nucleosomes from the promoter and coding region of the gene (18, 49). In order to determine whether SIR complexes, as expected, reduce the level of gene induction, we analyzed the density of RNAPII and nucleosomes in *GAL-VPS13-HMR-E* compared to the unmodified *GAL-VPS13* locus. Surprisingly, even though a high level of Sir3p was detected in the *GAL-VPS13-HMR-E* locus prior to activation, this had no effect on RNAPII density in the region after gene induction. The levels of RNAPII and nucleosomes were similar in the tested strains upstream from the *HMR-E* insertion site, indicating no major difference in the levels of transcriptional initiation or elongation through this heterochromatic domain. However, neither RNAPII recruitment nor loss of nucleosomes was detected downstream of the *HMR-E* sequence in the *GAL-VPS13-HMR-E* strain (Fig. 2A and B). Similar results were observed also in the strain where

FIG. 1. Recruitment and displacement of the SIR complex at the coding region of *GAL-VPS13-HMR-E*. (A) Schematic representation of the *GAL-VPS13* locus with HMR-E silencer sequence (black rectangle) inserted at 6 kb from the VPS13 promoter. Horizontal lines beneath the gene region indicate the positions of coding region (0.1-, 2.6-, 3.5-, 5.5-, 6.4-, and 8.5-kb) PCR probes used in ChIP and RT-PCR assays. (B) ChIP assay was used to determine the density of Sir3 protein on the coding region of *GAL-VPS13* in *GAL-VPS13-HMR-E* (AKY354) and *GAL-VPS13-HMR-E* sir4Δ (AKY355) strains before and after overnight transcription induction. The amount of Sir3 on the right arm of the chromosome VI telomere region is shown as the indicator of SIR complex occupancy on its natural locus. The signal from the FBA1 locus was used to normalize fluctuations in sample handling, and the amount of Sir3 as background signal in the sir4Δ strain was set to 1.

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FIG. 2. Transcription-coupled nucleosome removal and RNAPII recruitment at the GAL-VPS13-HMR-E locus. (A and B) ChIP assay was used to determine the density of RNAPII (A) and histone H3 (B) on GAL-VPS13 (AKY210) and GAL-VPS13-HMR-E (AKY354) after transcription induction. (C) Density of RNAPII and nucleosomes in GAL-VPS13-HMR-E sir4/H9004 strain (AKY355). The bars representing H3 and RNAPII indicate the level of respective proteins in galactose medium relative to the level in cells grown in glucose-containing medium (set as 1).

FIG. 3. Dynamics of the SIR complex, RNAPII, and nucleosomes at the promoter proximal region of GAL-VPS13-HMR-E-0.1kb. (A) Schematic representation of the GAL-VPS13 locus with HMR-E silencer sequence inserted at 0.1 kb from the VPS13 start codon. Horizontal lines beneath the gene region indicate the approximate positions of coding region (0.05-, 3.5-, 5.5-, 6.4-, and 8.5-kb) PCR probes used in ChIP assay. (B to D) ChIP assay was used to determine the density of Sir3 protein (B), RNAPII (C), and histone H3 (D) on the coding region of VPS13 in the GAL-VPS13-HMR-E-0.1kb strain (AKY359). Recruitment of Sir3 is shown before and after overnight transcription induction. The amount of Sir3 on the right arm of the chromosome VI telomere region is shown as the indicator of SIR complex occupancy on its natural locus. The signal from the FBA1 locus was used to normalize fluctuations in sample handling, and the amount of Sir3 as background signal in the sir4 strain was set to 1. The bars representing H3 and RNAPII indicate the levels of the respective proteins in galactose-containing medium relative to the levels in cells grown in glucose-containing medium. FBA1 (for RNAPII) and the noncoding region on chromosome VIII (for H3) were used as internal controls.
**HMR-E** sequence was inserted at 0.1 kb of the **GAL-VPS13** gene (Fig. 3C and D). Reverse transcriptase PCR (RT-PCR) experiments also failed to detect **VPS13** transcripts beyond the **HMR-E** sequence, further indicating that although polymerase can traverse the heterochromatin formed upstream of the silencer, it is unable to get through the **HMR-E** region itself (see Fig. 5; 6.4 kb in the **GAL-VPS13-HMR-E** strain). Nevertheless, upon galactose induction SIR complexes were largely removed both up- and downstream of the silencer sequence, making us question whether termination of transcription was caused by heterochromatin structure or whether other mechanisms might be responsible. To investigate this further, we carried out similar experiments in a **sir4Δ** strain which is deficient in heterochromatin and SIR complex formation (12, 25, 32, 35, 38, 46). As shown in Fig. 2C, RNAPII remained unable to transcribe through the **HMR-E** silencer even in the absence of SIR complexes, strongly suggesting that **HMR-E** itself contains a signal, for example, an efficient transcription terminator sequence, which causes polymerase to dissociate from the gene. In further support of this idea, nucleosomes were not removed downstream of **HMR-E** in the **sir4Δ** strain, indicating that no transcription took place beyond the **HMR-E** sequence (Fig. 2C). This is in concordance with our previous results showing that the insertion of a strong transcription terminator into the coding region of **GAL-VPS13** leads both to dissociation of RNAPII and to lack of nucleosome removal from the region downstream of the terminator sequence (49). The observation that the **HMR-E** sequence itself is a strong terminator of transcription is interesting but was not the subject of this study and was therefore not pursued further.

Although the displacement of SIR complex from **GAL-VPS13-HMR-E** was concurrent with the appearance of elongating RNAPII in the region, it remained possible that the process of transcriptional activation at the **GAL-VPS13-HMR-E** promoter might in itself lead to the disassembly of heterochromatic structure, independently of elongating RNAPII. To clarify this issue, we inserted a powerful transcription terminator sequence into **GAL-VPS13-HMR-E** 3 kb downstream from the promoter, creating a model gene on which transcription by RNAPII was terminated before it reached the heterochromatic region in the locus (Fig. 4A). As expected (49), RNAPII was detected only upstream from the terminator sequence upon activation (Fig. 4B), showing that the terminator was efficient. Strikingly, SIR complexes located downstream from the terminator were not removed from the locus (Fig. 4C), confirming that elongation through the heterochromatic region is required for displacement of SIR complexes.

**The efficiency of RNAPII elongation is not affected by heterochromatin.** Although our results suggested that the presence of the heterochromatic structure did not in itself abolish transcription of the locus, it remained possible that the kinetics of transcription through this region might be significantly slower on a heterochromatic template. For example, DNA replication-dependent removal of SIR complexes might be responsible for the initial displacement of SIR proteins, and only after that, constantly active transcription through the locus might preclude SIR complex re-formation. Therefore, we monitored the accumulation of **VPS13** mRNA immediately after the induction of the **GAL-VPS13-HMR-E** gene. RNA samples were collected 30, 60, 120, and 180 min after galactose induction, and the presence of mRNA was detected by RT-PCR. We observed nearly identical kinetics of **VPS13** mRNA accumulation in **GAL-VPS13** and **GAL-VPS13-HMR-E** strains (Fig. 5), indicating that, surprisingly, the SIR complexes in the coding region of the gene did not change the kinetics of gene induction and transcript elongation through the region.

**Acetylation of H3 K56 is required for RNAPII elongation through heterochromatin.** Given that histone modifications play an important role in silent chromatin formation, mainte-
However, severe impairment of a role in the control of heterochromatin spreading (16, 20, 47). Earlier results that both histone acetyltransferases (HATs) play a role in the control of heterochromatin spreading (16, 20, 47). Nevertheless, severe impairment of a role in the control of heterochromatin spreading (16, 20, 47). Accordingly, severe impairment of a role in the control of heterochromatin spreading (16, 20, 47). Nevertheless, severe impairment of a role in the control of heterochromatin spreading (16, 20, 47). Nevertheless, severe impairment of a role in the control of heterochromatin spreading (16, 20, 47). Nevertheless, severe impairment of a role in the control of heterochromatin spreading (16, 20, 47). Nevertheless, severe impairment of a role in the control of heterochromatin spreading (16, 20, 47). Nevertheless, severe impairment of a role in the control of heterochromatin spreading (16, 20, 47).

Although obvious defects in GAL-VPS13-HMR-E induction were detected in rtt109Δ, asf1Δ, and htz1Δ strains, it remained unclear whether the delay of induction was specific for the heterochromatin-covered GAL-VPS13-HMR-E coding region (controlled by the GAL10 promoter) or whether it was due to general defects in the induction of GAL10 promoter-driven transcription in these strains. To clarify the issue, we determined the induction efficiency of the endogenous heterochromatin-free GAL10 gene as an internal control in the same samples. As shown in Fig. 6B, induction of GAL10 was severely delayed in the htz1Δ strain, suggesting that Htz1 deletion leads to a general delay in the induction of GAL10 promoter-driven genes. Although slightly slower induction of GAL10 was also observed in rtt109Δ and asf1Δ strains, this delay was significantly stronger in the GAL-VPS13-HMR-E locus (compare wt, asf1, and rtt109 rows in Fig. 6A and B), suggesting that Rtt109 and Asf1 become crucial for transcription of a heterochromatic locus.

Alternatively, inefficient transcription of the GAL-VPS13-HMR-E locus in rtt109Δ, asf1Δ, and htz1Δ strains might indicate their inability to transcribe long genes, for example, due to general defects in transcription elongation. To test this possibility, we introduced the same deletions into strains carrying the GAL-VPS13 gene without the HMR-E sequence inserted. Induction of GAL-VPS13 in the asf1Δ strain was indistinguishable from that in the wild type, while deletion of Rtt109 led to a slight delay in GAL-VPS13 induction (Fig. 6C). However, the induction defect was considerably stronger in the GAL-VPS13-HMR-E strain, where heterochromatic structures covered the GAL-VPS13 locus (compare rtt109 rows in Fig. 6A and C). Again, a very low level of GAL-VPS13 mRNA was induced in the htz1Δ strain, confirming that the lack of GAL-VPS13-HMR-E induction was not caused specifically by the heterochromatic structures in the locus. The effect of Htz1 observed here was expected, as previous studies have reported slow induction of inducible genes in the htz1Δ strain (1, 40). Interestingly, after overnight growth in galactose-containing medium, GAL-VPS13-HMR-E mRNA was induced in all deletion strains (Fig. 6A, lane O/N gal), indicating that Rtt109, Asf1, and Htz1 were required only for the normal, rapid induction of the heterochromatin-covered gene. It is likely that during the long-term growth under inducing conditions DNA replication-dependent displacement of SIR proteins occurs, and once the SIR proteins are removed, RNAPII gains access to the locus and inhibits re-formation of SIR complexes.

Rtt109 and Asf1 are both involved in acetylation of lysine 56 on histone H3 (8, 10, 34, 41, 48). To investigate the importance of H3 K56 acetylation for efficient transcription of the heterochromatic locus, we also generated a derivative of the GAL-VPS13-HMR-E strain carrying the K56R mutation in histone H3 that mimics the deacetylated state of the residue. Again, transcription in the heterochromatic GAL-VPS13-HMR-E
cus was impaired, while induction of the endogenous \( \text{GAL10} \) or heterochromatin-free \( \text{GAL-VPS13} \) loci was essentially unchanged compared to the wild-type strain (Fig. 6D and C). To explore if constitutive acetylation of H3 K56 also might have an effect on \( \text{GAL-VPS13-HMR-E} \) transcription, we made two additional strains: one with the deletion of \( \text{HST3} \) and \( \text{HST4} \) genes, whose products are the major deacetylases of H3 K56 \textit{in vivo} \( (3, 27, 52) \), and another one carrying the K56Q mutant of H3 to mimic the acetylated state of the residue. As expected, \( \text{GAL-VPS13-HMR-E} \) transcription was not inhibited in these
strains (Fig. 6E), and importantly, when the H3 K56Q mutation was combined with the RTT109 deletion, the inhibitory effect of rtt109/H9004 was reversed (Fig. 6E, compare rtt109 and rtt109/H3K56Q rows). Together, these results indicate that acetylation of lysine 56 on histone H3 plays a key role in efficient transcription through heterochromatic loci.

Acetylation of H3 K56 occurs predominantly in S phase of the cell cycle, where newly synthesized K56-acetylated histones are incorporated into chromatin (28). As acetylation of K56 is required for efficient transcription of a heterochromatic gene, it might be possible that progression through the S phase is also needed for GAL-VPS13-HMR-E transcription. To test this, we compared the induction kinetics of GAL-VPS13 and GAL-VPS13-HMR-E loci in cells arrested in G1 phase by rtt109-factor. As shown in Fig. 7A and B, the two strains had very similar induction patterns of VPS13 mRNA and the overall kinetics of the test gene induction was essentially the same as that in asynchronous cells (Fig. 5). Again, induction of GAL-VPS13-HMR-E in the H3 K56R background was strongly inhibited also in cell cycle-arrested cells (Fig. 7C). These results indicate that cells can transcribe a heterochromatic gene in an H3 K56 acetylation-dependent manner without progressing through the S phase.

To test whether acetylation of H3 K56 actually occurs in the GAL-VPS13-HMR-E locus during transcription, we compared the presence of H3 acetylated K56 (acK56) in GAL-VPS13 and GAL-VPS13-HMR-E loci before, during, and after transcriptional induction. Acetylated K56 was detected in both GAL-VPS13 and GAL-VPS13-HMR-E strains under induced conditions (Fig. 8, “galactose 120 min”) and also shortly after repression of transcription (Fig. 8, “glucose 10 min”). While the GAL-VPS13 strain contained H3 K56 acetylation at both 5.5-kb and 6.4-kb regions of the locus, the GAL-VPS13-HMR-E strain gained acK56 only at the 5.5-kb region (Fig. 8). These results confirm that H3 acK56 was brought to the locus in a transcription-dependent manner, as the 6.4-kb region of
VPS13 was not transcribed in the GAL-VPS13-HMR-E strain (Fig. 2A and 5).

**DISCUSSION**

Our results reveal that, surprisingly, heterochromatic structures in the coding region of an RNAPII-transcribed gene are permissive for transcript elongation and do not diminish RNAPII transcriptional efficiency. We also show that disruption of histone H3 K56 acetylation, either by mutation of the residue or by the deletion of the H3 K56-specific histone acetyltransferase (HAT) Rtt109 or histone chaperone Asf1, severely impairs the efficiency of RNAPII transcription through heterochromatin.

SIR complexes are removed from the coding region of the gene during RNAPII transcription elongation. It has been long established that relocation of silencers to other ectopic loci results in the silencing of the adjacent genes (22, 45). The exact mechanisms of transcriptional silencing in yeast are not fully understood; however, it has been shown that promoters embedded into heterochromatin are accessible for binding by basal transcription factors and that those genes are competent for transcription initiation but not for mRNA production (43, 44). These observations were further supported by recent data, suggesting that RNAPII does not switch from transcription initiation to elongation in silenced loci (9), leaving open the question whether RNAPII is capable of elongation on a heterochromatin template at all. To clarify this issue, we induced SIR protein recruitment and heterochromatin formation in the coding region of an inducible model gene and studied how the elongating polymerase can cope with heterochromatic structures in the middle of the gene. We found that the heterochromatic region was efficiently transcribed by RNAPII and that SIR proteins were removed from the gene in a transcription-dependent manner (Fig. 1B, 2, and 4). Surprisingly, transcription was induced with the same kinetics in the heterochromatic locus as in the euchromatic locus, indicating that heterochromatin poses no obvious barrier to transcript elongation by RNAPII (Fig. 5 and 7).

Interestingly, SIR complexes were removed from the entire induced GAL-VPS13-HMR-E locus, despite the fact that transcription of the gene was terminated at the HMR-E sequence and no polymerases were detected beyond the HMR-E site. The HMR-E silencer consists of binding sites for three essential factors—origin recognition complex, Rap1, and Abf1 proteins. All these proteins have affinity for one or more SIR proteins, and all of them are required for the recruitment of the SIR complex to HMR-E (37). We surmise that some or all of these factors might be displaced from DNA by transcribing RNAPII, which in turn disables the efficient recruitment and subsequent spreading of SIR proteins in the locus.

Histone H3 K56 acetylation is needed for effective heterochromatin disruption in the coding regions of genes. In order to determine which factors are needed for transcription of heterochromatric structures, we analyzed the efficiency of GAL-VPS13-HMR-E mRNA induction in 12 strains with deletions of genes required for different transcription-coupled chromatin modifications. We observed an impairment of transcription induction in three deletion strains. Of those, HTZ1 deletion led to a general delay in galactose induction, while deletions of RTT109 and ASF1 caused slow induction primarily in the heterochromatic locus (Fig. 6). As both Rtt109 and Asf1 are required for H3 K56 acetylation in vivo and in vitro (8, 10, 34, 41, 48), we assumed that this modification was crucial for efficient RNAPII transcription in the presence of SIR proteins. To test it directly, we made a yeast strain carrying the K56R mutation in histone H3 and observed a heterochromatin-specific delay of gene induction similar to those in rtt109 and asf1 strains (Fig. 6D). Moreover, when the H3 K56Q mutation (mimicking the constitutively acetylated state of the residue) was introduced into the test strain, the deletion of RTT109 did not cause the impairment of transcription in the heterochromatin locus (Fig. 6E).

Interestingly, mutation of H3 K56 to either glycine, glutamine, or arginine leads to partial derepression of telomeric and HM mating loci without affecting the binding of Sir proteins to these sites (51). We confirmed that also in our strain background the H3 K56R mutation led to partial derepression of HM silencing, as the K56R strain had about a 10-fold-lower mating efficiency than did its wt counterparts (data not shown). However, the K56R mutation had the opposite effect on gene expression in the VPS13-HMR-E locus. We propose that as the H3 K56 mutations increase chromatin accessibility in telomeric regions (51), this might offer a chance for telomeric promoters to initiate transcription more frequently than in wt cells. However, in the case of GAL-VPS13-HMR-E strains, the GAL10 promoter in front of the model gene remains functional regardless of SIR complex recruitment to the very proximity of the promoter (Fig. 3), and therefore, the possible changes of chromatin structure in the H3 K56R strain cannot make the GAL10 promoter more accessible than it already is. Therefore, the reduced ability of RNAPII to transcribe a heterochromatic locus in rtt109Δ, asf1Δ, and K56R strains most likely reflects the difficulties of RNAPII elongation during transcription. We would also like to emphasize that, in all mutant strains where the slow induction of the GAL-VPS13-HMR-E model gene was detected, the effects were observable only at early time points of gene induction. After overnight induction, all mutants were able to transcribe the model gene as efficiently as did the wt strain (lanes “O/N gal” in Fig. 6A, D, and E). Therefore, the initial activation of the repressed locus might occur during S phase, when SIR proteins are temporarily removed from chromatin, and later on the transcriptional state of the locus might be maintained by RNAPII. This assumption was also supported by the fact that the induction of GAL-VPS13-HMR-E was notably slow in the H3 K56R mutant strain when cells were arrested in G1 (Fig. 7C).

We also studied whether mutations abolishing acetylation of H3 K56 might lead to excessive loading of SIR complexes to the GAL-VPS13-HMR-E locus. However, we did not detect increased recruitment of SIR complexes in rtt109Δ, asf1Δ, and H3 K56R strains compared to wt cells (data not shown). Therefore, the slower induction of the GAL-VPS13-HMR-E gene in these strains was not caused by the larger amount of SIR complexes in the locus.

H3 K56 acetylation is characteristic for newly synthesized histones incorporated into chromatin during S phase, where it is required for proper functioning of the DNA damage checkpoint (28). When DNA synthesis is completed and cells go through G2 and M phases, K56 acetylation is removed by
Hst3/4 deacetylases (3, 27, 52). Apparently, acetylation of K56 occurs only before incorporation of H3 into chromatin and histone chaperone Asf1 is required for proper exposition of H3 to Rtt109 acetyltransferase (11, 34, 48). Outside S phase, replication-independent incorporation of H3 acK56 has been reported mainly at promoter nucleosomes but also in the coding regions of highly expressed genes. As H3 K56 acetylation occurs on newly synthesized histones but not on nucleosomes in chromatin, the appearance of acK56 in transcription sites reflects mainly transcription-coupled incorporation of new histones into loci (15, 28, 36, 41). Our results also show that K56-acetylated H3 can be detected in the transcribed but not in the nontranscribed part of the GAL-VPS13-HMR-E gene (Fig. 8), confirming transcription-dependent incorporation of the H3 acK56 into chromatin.

What could be the role of K56 acetylation in transcription-coupled displacement of SIR proteins? Taking into account the results from the current and previous studies, we propose that K56 acetylation might either facilitate removal of nucleosomes during transcription elongation or inhibit reassociation of SIR complex with the transcribed locus. K56 lies in the globular domain of histone H3 extending toward the DNA major groove at the entry and exit points of the nucleosome core particle (13, 33), making modification of this residue a very attractive candidate for regulation of the properties of DNA-nucleosome or nucleosome-nucleosome interactions. Once the elongating RNAPII enters the locus, it will displace nucleosomes from its path and new histones, with K56 acetylation, will be loaded to chromatin as RNAPII moves away (15, 28, 36, 41). This might create a more favorable environment for the next polymerase, as nucleosomes are bound to DNA more loosely due to K56 acetylation and can be removed more easily than in the absence of the modification.

Alternatively, acetylation of H3 K56 might directly inhibit SIR complex binding to the locus and therefore help to open heterochromatic structures. Recent studies have shown that telomeric silencing is disrupted by K56 acetylation and that H3 K56 is maintained in a hypoacetylated state in silenced loci (31, 51, 52). Although a silenced locus might be generally inaccessible due to chromatin-bound SIR proteins, the border of a heterochromatic region is not strictly defined but is likely in a constantly changing equilibrium between SIR protein binding and “free” chromatin. As the original nucleosomes will be replaced by new ones during transcription-coupled reassembly of chromatin (7, 14, 49), they will contain acK56 in histone H3 (15, 36), repelling SIR proteins from the locus. As a result, the border between euchromatin and heterochromatin might be shifted toward the origin of SIR complex spreading and a wider area in the locus will be free of SIR complexes. If acetylation of H3 K56 is abolished by deletion of either ASFI or RTTI09 or by H3 K56R mutation, nucleosomes loaded to the locus do not restrict SIR complex spreading and the opening of the locus is considerably slower than that in wt cells.

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