Sir2 Silences Gene Transcription by Targeting the Transition between RNA Polymerase II Initiation and Elongation

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It is well accepted that for transcriptional silencing in budding yeast, the evolutionarily conserved lysine deacetylase Sir2, in concert with its partner proteins Sir3 and Sir4, establishes a chromatin structure that prevents RNA polymerase II (Pol II) transcription. However, the mechanism of repression remains controversial. Here, we show that the recruitment of Pol II, as well as that of the general initiation factors TBP and TFIIH, occurs unimpeded to the silent HMRa1 and HMLα1/HMLα2 mating promoters. This, together with the fact that Pol II is Ser5 phosphorylated, implies that Sir-mediated silencing is permissive to both preinitiation complex (PIC) assembly and transcription initiation. In contrast, the occupancy of factors critical to both mRNA capping and Pol II elongation, including Cet1, Abd1, Spt5, PaFc, and TFIIS, is virtually abolished. In agreement with this, efficiency of silencing correlates not with a restriction in Pol II promoter occupancy but with a restriction in capping enzyme recruitment. These observations pinpoint the transition between polymerase initiation and elongation as the step targeted by Sir2 and indicate that transcriptional silencing is achieved through the differential accessibility of initiation and capping/elongation factors to chromatin. We compare Sir2-mediated transcriptional silencing to a second repression mechanism, mediated by Tup1. In contrast to Sir2, Tup1 prevents TBP, Pol II, and TFIIH recruitment to the HMLα1 promoter, thereby abrogating PIC formation.

In eukaryotes, transcription occurs in the context of chromatin. A traditional view is that nucleosomes exert their regulatory role by impeding the access of proteins, both genespecific regulators and general transcription factors (GTFs), to the DNA (33). In support of this idea, when the TATA box is assembled into a nucleosome in vitro, its accessibility for TATA binding protein (TBP) is reduced by at least four orders of magnitude (27), while the accessibility of a high-affinity heat shock element for its cognate HSF activator is reduced by more than three orders of magnitude (66). However, it is now appreciated that in vivo, chromatin modification and remodeling complexes, in combination with histone variants and the intrinsically low affinity of many gene promoters for histones, collaborate in making the euchromatic template accessible to these and other regulatory factors (reviewed in references 36 and 71).

Heterochromatin, the cytologically condensed compartment of the euchromatic nucleus, likewise is a substrate of chromatin-remodeling complexes and other regulatory factors (reviewed in reference 16), yet genes residing in heterochromatin generally are transcriptionally silent. A key feature of heterochromatin is its ability to repress gene expression in a position-dependent but sequence-independent fashion. Thus, the position of a gene on the chromosome, rather than its associated enhancer, upstream activation sequence (UAS), and promoter elements, can dictate its expression state. The budding yeast Saccharomyces cerevisiae does not contain condensed chromatin at the cytological level; however, it does contain domains of silent chromatin that resemble, in both their molecular and epigenetic characteristics, the repressed heterochromatic domains of higher eukaryotes (46).

In S. cerevisiae, silent chromatin is found at the telomeres, the ribosomal DNA repeats, and the two cryptic mating-type loci, HMR and HML, located near the right and left telomeres of chromosome III, respectively (14, 51). The silent mating loci bear genes (α1 and a2 at HMR and α1 and α2 at HML) that encode transcriptional regulators. Their activation in a wild-type cell requires their transposition to a centromere-proximal euchromatic site, MAT, located on the same chromosome. This transposition, which occurs only in homothallic haploid cells, is initiated by the HO double-stranded DNA endonuclease that cuts a specific site within the MAT locus. The double-stranded break subsequently is repaired by nonreciprocal homologous recombination between the mating-type genes located in MAT and those of the opposite mating type found at either HMR or HML, which act as the donors of mating information. The directionality of mating-type interconversion is determined by a recombinational enhancer located proximal to HML and that, when active (as is the case in α cells), increases the probability that HML will serve as the donor of mating-type information. When the enhancer is repressed, as is the case in α cells, HMR serves as the donor (reviewed in references 64 and 72).

Silencing at the HM loci is controlled by cis-acting elements termed silencers. These contain binding sites for sequence-specific factors (ORC, Rap1, and Abf1) that trigger the formation of a specialized chromatin structure through the concerted recruitment of the Sir2, Sir3, and Sir4 silencing proteins (reviewed in references 18 and 43). The silencing complex
horizontally propagates along the chromatin fiber through iterative cycles of H4 K16 deacetylation catalyzed by Sir2, an evolutionarily conserved NAD\(^+\)-dependent lysine deacetylase (26, 63). The deacetylation of H4 K16 and the resultant production of O-acetyl-ADP-ribose are necessary for the formation of a trimeric complex between Sir2/Sir4 and Sir3 (25, 38). The resultant chromatin structure consists of positioned, hypoacetylated, and hypomethylated nucleosomes (7, 49, 52, 70).

Transcription is a multistep process, and each step is highly regulated. Initially, sequence-specific activators bind to UAS elements (enhancers); these, in turn, recruit polymerase II (Pol II) and GTFs to the core promoter, leading to the formation of the preinitiation complex (PIC) (schematically summarized in Fig. 1). Transcription initiation requires the general factor TFIIH, the ATPase subunit of which unwinds the DNA, leading to the formation of an open complex. Also, the kinase subunit of TFIIH phosphorylates Ser5 residues of the carboxy-terminal domain (CTD) of Rbp1, the large Pol II subunit (53). Early elongation often is accompanied by a pause, during which the pre-mRNA is capped at its 5' end. Following this step, which generally takes place when the nascent mRNA chain is 25 to 35 nucleotides long (48), Pol II engages in productive elongation concomitant with the phosphorylation of Ser2 residues with the CTD and the recruitment of elongation factors, including TFIIIS, DSIF (Spt4/Spt5), and the Paf1 complex (Paf1C) (53). (As indicated in Fig. 1, Spt4/Spt5 may play an additional role in instigating the Pol II pause following early elongation [53].)

How silent chromatin represses gene transcription remains poorly understood, but theoretically it could act at any one of the above steps. Early observations suggested that silent chromatin is resistant to the activity of endogenous and exogenous nucleases, as well as DNA repair and modification enzymes (reviewed in references 15 and 51). This contributed to the idea that silent chromatin represses transcription through its ability to sterically hinder the access of sequence-specific proteins. This model is intuitively appealing, because it is consistent with the in vitro reconstitution studies discussed above that demonstrate the reduced accessibility of DNA sequences when assembled into stable nucleosomes. However, such a model cannot account for how the silent HM loci remain fully permissive to the binding of other sequence-specific factors, including enzymes that mediate homologous recombination, site-specific recombination, and retrotransposition (9, 24, 29, 75).

Investigations of an ectopically silenced heat shock transgene cast additional doubt on the steric hindrance mechanism. These studies showed that despite efficient, Sir-dependent silencing, the hsp82 promoter remained accessible, as measured by nuclease hypersensitivity (35). Consistently with this, UAS and TATA genomic footprints were retained (57), and essentially normal levels of the activator HSF, the initiation factor TBP, and Pol II itself were present (56). An analysis of the naturally silenced HMRa1 promoter supported these conclusions, as both TBP and Pol II were detected in the Sir-repressed state (56). These findings gave rise to the notion that Sir acts at a point downstream of both activator binding and PIC recruitment to silence transcription. More recently, a third model has been proposed: PIC interference. This model posits that Sir is permissive to activator binding, yet transcription is abolished because of a failure to recruit RNA polymerase. In support of this, at several Sir-silenced URA3 transgenes as well as at both the HML and HMR mating loci, Pol II, along with the general initiation factors TFIIH and TFIIA, could not be detected (8).

Here, we use chromatin immunoprecipitation (ChIP) to quantitatively measure the abundance of initiation, capping, and elongation factors at the naturally silenced HMRa1 and HMLa1/HMLa2 promoters. We employ two genetic backgrounds and rigorous controls for both nonspecific immunoprecipitation (IP) and spurious PCR amplification. We find, consistent with predictions of the downstream inhibition model, that three components of the PIC, namely, TBP, Pol II, and TFIIH, are present within the silent HMR and HML promoters. Furthermore, Pol II is efficiently phosphorylated at Ser5 within its CTD, indicating that polymerase is not only present but also has initiated transcription. In striking contrast, the occupancy of 5'-capping enzymes and elongation factors is virtually eliminated, and the recruitment of Mediator is restricted. Our results pinpoint the transition between Pol II initiation and elongation as the step targeted by Sir and provide important insight into how silent chromatin can abrogate gene expression.

MATERIALS AND METHODS

Yeast strains. Strains used in this study are derived from the S288C and SLY101 genetic backgrounds (Table 1). SLY101 is congenic to W303 (35). MATa strains of the SLY101 background used here bear hsp82 alleles flanked by HMR-E silencers (57). sir2Δ strains were generated using one-step transplacement of the SIR2 open reading frame (ORF) with a PCR-amplified DNA fragment bearing the URA3 marker and gene-specific flanking sequences (21) and were confirmed by genomic PCR in conjunction with mating-type assays (cells bearing sir2Δ lose the ability to mate with cells of the opposite identity). To excise the KANMX marker, cells were transformed with the plasmid pSH47 that bears a URA3+ marker and a Cre recombinase regulated under a GAL1 pro-
TABLE 1. Yeast strains

| Strain       | Genotype                          | Source or reference   |
|--------------|-----------------------------------|-----------------------|
| SLY101       | MAt10 ade2 can1-100 cyh2 his3-11,15 leu2-3,112 trp1-1 ura3 | 35                    |
| EAS2011      | SLY101; hisp82-2001               | 57                    |
| EAS2001      | SLY101; str42::HIS3               | 57                    |
| LG1001       | EAS2001; hmrα2::KANMX             | This study            |
| LG1101       | SLY101; MAt10                     | This study            |
| LG1102       | LG1101; str2Δ::KANMX              | This study            |
| LG1103       | LG1101; str2Δ::hmlα2::KANMX       | This study            |
| JHD10        | EAS2011; KIN28-9Myr               | This study            |
| JHD11        | EAS2001; KIN28-9Myr               | This study            |
| SBFK701      | LG1101; KIN28-9Myr                | This study            |
| LG11         | SBFK701; str2Δ::KANMX             | This study            |
| S28Ca        | MAt10 his3Δ1 leu2Δ2 met15Δ0       | Research Genetics     |
| S28CaΔ       | MAt10 his3Δ2 leu2Δ2 met15Δ0       | Research Genetics     |
| LG2881       | S28CaX; str2Δ::KANMX              | This study            |
| LG2882       | S28CaX; str2Δ::hmrα2::KANMX       | This study            |
| LG2883       | S28CaX; str2Δ::KANMX              | This study            |
| LG2884       | S28CaX; str2Δ::hmlα2::KANMX       | This study            |
| TAP-SPT5     | S28CaX; SPT5-TAP                  | Open Biosystems       |
| LG101        | TAP-SPT5; str2Δ::KANMX            | Open Biosystems       |
| TAP-DST1     | S28CaX; DST1-TAP                  | Open Biosystems       |
| TAP-RTFI     | S28CaX; RTFI-TAP                  | Open Biosystems       |
| TAP-GAL11    | S28CaX; GAL11-TAP                 | Open Biosystems       |
| LG104        | GAL11-TAP; str2Δ::KANMX           | This study            |
| TAP-SRB4     | S28CaX; SRB4-TAP                  | Open Biosystems       |
| LG105        | TAP-SRB4; str2Δ::KANMX            | This study            |
| TAP-TFB1     | S28CaX; TFB1-TAP                  | Open Biosystems       |
| LG106        | TAP-TFB1; str2Δ::KANMX            | This study            |
FIG. 2. *SIR* extinguishes transcription at the *HM* loci yet is fully permissive to Pol II recruitment. (A) Physical maps of *HMLα* and *HMRa*. These loci are located near the left and right ends of chromosome III, respectively. Locations of mating-type genes, flanking silencer elements (termed E and I), and PCR amplicons (black rectangles) are shown. Coordinates are provided relative to the ATG codons (+1) of the *HMLα1* and *HMRa1*.
removed prior to the addition of formaldehyde) and purified, electrophoresed, and blotted to a Gene Screen as described previously (57). Blots were hybridized overnight to a1-, a2-, or a2-specific probes at 55, 65, and 52.5°C, respectively, to visualize mating-type transcripts or the HS992 probe at 45°C to detect the HS992 transcript, washed, exposed to a PhosphorImager, and then rehybridized (without stripping) to the ACT1 probe at 55°C. We note that the a2 probe shares sequence similarity with the a2 gene and therefore cross-hyibridizes to the a2 mRNA; nonetheless, the two transcripts can be distinguished by size and individually quantified. Gel-purified templates were generated by PCR from yeast genomic DNA. All hybridization probes were synthesized by 25 cycles of linear PCR in the presence of 5 mM MgCl2; 300 μM each of dCTP, dGTP, dTTP, 3 μM of dATP; 100 μCi of [α-32P]dATP; and 1.25 U of Taq DNA polymerase. The following probes were used (coordinates relative to ATG): a1, +156 to +326; a2, +356 to +562; a2, +101 to +496; a2, +63 to +322; HS992, +2167 to +2228; and ACT1, +606 to +1000.

RESULTS

Pol II and TBP are efficiently recruited to hyperrepressed HM promoters. To investigate the mechanism by which Sir proteins silence transcription, we used ChIP to measure factor abundance at the HM mating-type loci, the relevant features of which are schematically illustrated in Fig. 2A. We analyzed SirR parental strains and isogenic sir2Δ or sir4Δ deletion strains in parallel, allowing a direct comparison between heterochromatic and euchromatic gene states. In SirR strains, Sir3 is recruited to both HM loci, yet it appears to be more abundant at HML than at HMR (Fig. 2B). This might reflect the presence of two functionally autonomous silenceds at this locus (HML-E and HML-I) as opposed to HMR, which has only one, HMR-E (6, 50, 58). The efficiency of Sir2/3/4 recruitment has been shown to correlate with both the dosage and arrangement of silencers (56) (L. Gao and D. S. Gross, unpublished data). As expected, in either a sir2Δ or sir4Δ mutant, Sir3 recruitment is abolished (Fig. 2B). Concomitantly, the HM loci are transcriptionally derepressed (Fig. 2C, lanes 2 and 5). The exception to this is the a1 gene, which fails to express in either sir2Δ strain, as it is the target of Tup1 repression in sir mutants (22). The role played by Tup1 is further considered below.

We first investigated the occupancy of Pol II. As expected, polymerase is present at the derepressed HMLa1/a2 promoters (sir2Δ background). This is most clearly seen in MATa strains that bear a1 and a2 genes only at HML (Fig. 2D). Strikingly, Pol II occupancy remains high in a SirR context (black arrows) when HMLa1 and HMLa2 transcription is extinguished (Fig. 2C, lane 4), Sir3 occupancy is high (Fig. 2B), and local nucleosomes are both hypoacetylated (7) and H3 K4 hypomethylated (52). Importantly, Pol II occupancy at the silent a1/a2 promoters is well above that seen at a nontranscribed euchromatic region (AR504) (Fig. 2D) and is observed in two distinct genetic backgrounds (S288C and SLY101). A virtually identical pattern of Pol II occupancy is seen at HMRa1 in MATa cells, and these data, along with those for HMLa1/HMLa2, are quantified in Fig. 2D. To confirm that the PCR amplification was specific, we constructed strains individually deleted for either HML or HMR and subjected these to ChIP as described above. As expected, neither deletion strain evinced a significant PCR product (see Fig. 5A, lane 5 of each gel). Taken together, these findings argue that Sir is permissive to the recruitment of Pol II at stably silenced target genes.

We next investigated whether TBP was present at the silent mating promoters. While TBP is typically the first PIC component recruited to the core promoter (Fig. 1), exceptions exist (2), and the absence of TBP at the HM loci could explain silencing. However, TBP can be readily detected, irrespective of the strain background (Fig. 3A), at a level that is at least equivalent to its occupancy in the euchromatic state. The occupancy of Pol II and TBP at the hyperrepressed a1 and a1/a2 promoters is consistent with the downstream inhibition model of transcriptional silencing while simultaneously arguing against both steric hindrance and PIC interference models.

Silent chromatin is permissive to TFIIH recruitment. TFIIH is typically the last component assembled within the PIC during the activation of Pol II genes (45). Although its presence is not required for the stable association of TBP (34), Pol II transcription is critically dependent on TFIIH. There-
fore, given that both TBP and Pol II are present at the hyper-repressed HM loci, transcriptional silencing might arise from the impaired recruitment of this nine-subunit, 438-kDa complex. However, as shown in the gel analysis of Fig. 3B, the essential TFIIH subunit, Kin28 (TFIIH kinase), is efficiently recruited to the heterochromatic HMRa1 and HMLα1/HMLα2 promoters, and its abundance at each locus equals or exceeds that seen in the sir2Δ or sir4Δ euchromatic state. To rule out the possibility that Kin28, as part of the TFIIK kinase subcomplex, is recruited independently of core TFIIH, we also examined the occupancy of Tfb1, an essential core subunit of TFIIH. As shown in Fig. 3C, the Tfb1 occupancy of the
HMLα1/HMLα2 promoter is significant in the SIR-induced heterochromatic state and actually exceeds its occupancy in the sir2Δ-induced euchromatic state. We conclude that TFIH is present, and abundant, within the SIR-silenced HM promoters. The paradoxical enhanced occupancy of TFIH at α1/α2 in the SIR strain parallels findings for Pol II and TBP (Fig. 2D, 3A) and is further considered below.

Silent chromatin is permissive to Ser5 phosphorylation of the Pol II CTD, yet Pol II arrests at or near the promoter. A critical function of the TFIH kinase is to phosphorylate the Pol II large subunit at Ser5 within the CTD heptad repeat (23). The Ser5-phosphorylated isoform of Pol II is characteristic of the polymerase that has initiated transcription (53). Therefore, a potential way that SIR could act is by inhibiting the phosphorylation of Ser5 residues within the Pol II CTD, thereby aborting transcriptional initiation. However, the Ser5-phosphorylated isoform of Pol II is present at the silent HM promoters, and its abundance is comparable to that seen in the sir2Δ euchromatic state (Fig. 4A). This result provides additional evidence for the presence of Pol II at the silent α1 and α1/α2 genes and further suggests that Pol II has initiated transcription.

We next investigated whether phosphorylated Pol II is capable of productive elongation by testing its presence at the 3′ end of HMRα2 in a Sir2+ strain. We focused on α2, since the small sizes of the α1, α1, and α2 ORFs (378, 525, and 630 bp, respectively), along with the presence of closely abutting (and similarly regulated) genes 3′ of both HMRα1 and HMLα1 (see Fig. 8), prevent a definitive analysis of Pol II localization at these genes. Figure 4B reveals that Pol II is virtually undetectable within the 3′-flanking region of HMRα2, a site located ~1 kb downstream of the α2 promoter. This contrasts with Pol II abundance at the HMRα1 promoter in Sir2+ cells as well as its abundance within the 3′ flank of HMRα2 in sir2Δ cells and is consistent with the stalling of Ser5-phosphorylated Pol II at the 5′ end of HMRα2. We extended this analysis to a previously characterized hsp82 transgene, ectopically silenced by integrated, flanking HMRE silencer elements (57). Consistent with the above results, Pol II occupancy at the hsp82-2001 promoter is undiminished by Sir2, in contrast to its significantly reduced occupancy within the gene’s ORF and 3′-UTR (Fig. 4C).

SIR restricts the recruitment of both 5′-capping enzymes and elongation factors. The inability of Pol IαSer5 to recover from a stalled state at the 5′ end of silent genes could reflect the presence of a number of obstacles. For example, SIR-stabilized nucleosomes could physically block the transit of RNA polymerase despite the normal recruitment of factors critical to productive elongation. Alternatively, SIR could impair Pol II processivity by preventing the access of these factors. To distinguish between these possibilities, we initially tested for the presence of capping enzyme, given that capping takes place early following initiation (Fig. 1). Indeed, Celt/Ceg1’s recruitment to the transcription elongation complex (TEC) is triggered by the Ser5 phosphorylation of the CTD (32, 54). Strikingly, Celt is present only at background levels within the silent α1 and α1/α2 gene promoters (Fig. 5A), although it is abundant under derepressing conditions. Quantification of four biological replicates indicates that Celt occupancy is reduced 16- to 20-fold by SIR repression (Fig. 5A).

This impairment in Cet1 recruitment stands in stark contrast to the essentially complete accessibility of TBP, Pol II, and TFIH to silent chromatin.

We next addressed whether the occupancy of the 5′ mRNA cap methylase, Abd1, also is influenced by SIR. Abd1 binds the CTD independently of Celt/Ceg1, and its interaction requires both Ser5 phosphorylation and the TFIH kinase (54). Given this, it was possible that despite the absence of Celt at the silent α1 and α1/α2 genes, Abd1 recruitment occurred unpaired. However, as shown in Fig. 5B, the recruitment of Abd1, like that of Celt, is highly restricted (~10-fold). This restriction has important mechanistic implications, given that Abd1 has been functionally linked to subsequent elongation in both S. cerevisiae and the fission yeast Schizosaccharomyces pombe (20, 55). Taken together, the data argue that silent chromatin is highly restrictive to the recruitment of capping machinery.

The absence of capping enzymes raises the possibility that SIR additionally restricts other factors whose association with the TEC takes place either concomitantly with or subsequent to that of capping enzymes. We focused on three elongation factors: Spt5, TFIIS, and the Paf1 complex (Paf1C). The essential elongation factor Spt5, as part of the Spt4/Spt5 complex, has been implicated in the control of early transcription (Fig. 1) and physically and functionally interacts with both Ceg1/Cet1 and Abd1 (37, 41). As shown in Fig. 6A, SIR reduces the recruitment of Spt5 to the silent α1/α2 promoter to an undetectable level. The sir2Δ mutation alleviates this block, and Spt5 is efficiently recruited concomitantly with transcriptional activation.

TFIIS, an elongation factor that reactivates stalled Pol II, is localized principally at the 5′ end of genes (53). Given the role of TFIIS in releasing inappropriately paused Pol II, its absence from silent coding regions might further underlie the inability of Pol II to elongate within silent chromatin. Indeed, as shown in Fig. 6B, the occupancy of TFIIS (Dst1) at HMLα1/HMLα2 is reduced 12-fold by SIR.

To investigate the effect of SIR silencing on Paf1C recruitment, we examined the occupancy of its Rtf1 subunit. Paf1C associates with Ser5-phosphorylated Pol II and genetically and physically interacts with the Spt4/Spt5 complex (61), as well as with Spt6, FACT, and Chd1. Moreover, Paf1C plays a critical role in regulating transcription-associated histone modifications, including H2B ubiquitylation and H3 K4 methylation, and does this through mediating the interaction between Pol II and the enzymes responsible for these modifications. As shown in Fig. 6C, SIR strongly impedes the binding of Rtf1 and, by extension, of Paf1C to the silent HMLα promoters, thereby providing a basis for the absence of activating histone modifications within silent chromatin (reviewed in references 15 and 46) as well as further evidence accounting for the failure of Pol II to escape from the gene’s 5′ end.

Efficiency of silencing inversely correlates with the presence of capping enzyme. To investigate the functional link between capping enzyme recruitment and transcriptional silencing in more detail, we employed silencer-flanked hsp82 transgenes. As discussed above, these consist of the native HSP82 heat shock gene flanked by chromosomally integrated HMRE silencers. The efficiency of transcriptional silencing at these alleles correlates with Sir2 recruitment: hsp82-2001, flanked by four silencers, exhibits ~95% of the level of Sir2 observed at
FIG. 4. Silent chromatin is permissive to the Ser5 phosphorylation of the CTD, yet Pol II fails to elongate through the gene coding region. (A) Pol II<sup>Ser5</sup> ChIP analysis of the α1, α1/α2, and PMA1 promoters (prom) of the indicated S288C strains detected using the monoclonal antibody H14. M, mock IP (sheared chromatin precipitated with preblocked anti-IgM-agarose beads only). Black and white arrows indicate signals arising from silenced and derepressed states, respectively. In this experiment, the PMA1 promoter serves as a positive control for Ser5-phosphorylated Rpb1 occupancy; ARS504 serves as a negative control. On the right is a summary of four independent experiments (means ± standard errors of the means [SEM]). (B) In vivo cross-linking analysis of Pol II within the 3′ flank of the HMRα2 gene in strain S288C (left). Also evaluated are...
HMR and is repressed ~30-fold; hsp82-1001, flanked by two silencers and containing ~80% of the level of Sir2, is repressed 5- to 10-fold; and hsp82-201, bearing tandem upstream silencers and containing ~30% of Sir2, is silenced 2- to 4-fold (56, 57). If the reduction in recruitment of capping enzymes is linked to silencing, then the degree to which these factors are prevented from accessing the hsp82 transgenes should correlate with the degree to which they are silenced. This is in fact what is seen: despite Pol II being present at normal levels at the 5' ends of all three silenced hsp82 alleles, Cet1 recruitment is reduced by almost 10-fold at hsp82-2001, 5-fold at hsp82-1001, and 3-fold at hsp82-201 (Fig. 7). Therefore, restriction in Cet1 occupancy, unlike that of Pol II, directly correlates with SIR-mediated silencing.

**Genes abutting HMLa1 and HMRa1 are silenced by SIR but permissive to Pol II recruitment.** To extend the generality of our observations, we tested whether Pol II, TBP, or Cet1 is recruited to two additional silenced genes, YCL065W and YCR097W-A. These cryptic genes are located immediately downstream of HMLa1 and HMRa1, respectively (Fig. 8A), and they are expressed in a sir2 mutant but not in the SIR parental strain (data not shown). As illustrated in Fig. 8B and C, Pol II and TBP are in fact present and abundant at the YCL065W and YCR097W-A promoters in the SIR strain. We the a1/a2 promoter and ARS504, which serve as positive and negative ChIP controls, respectively. PI, immunoprecipitation with preimmune serum. On the right is a comparison of Pol II occupancy at the HMRa1 promoter region and the HMRa2 3’ flank, obtained from independent PCRs of the same chromatin DNA templates (n = 3; results are given as means ± SEM). The occupancy for each locus is normalized to that seen in the sir2 strain, which is set at 1.0. (C) Pol II stalls at the SIR-silenced hsp82 promoter. The upper graphic is a schematic of the HMR-E silencer element, illustrating binding sites for ORC, Rap1, and Abf1, and the location of integrated silencers flanking the hsp82-2001 gene (silencers are symbolized by arrows) (57). Also illustrated are PCR amplicons (cross-hatched rectangles) centered at coordinates ~170, ~1400, and ~2100 relative to the ATG (+1) of hsp82-2001. The lower graphic is a summary of Pol II occupancy at hsp82-2001 in SIR” and sir4Δ strains (means ± SEM; n = 3). Note that although there is virtually no difference in Pol II promoter occupancy in SIR” versus sir4Δ cells, there are significant differences in Pol II occupancy within the ORF and 3’-UTR (P < 0.05; two-tailed t test).
then asked whether either Pol IIoSer5 or Cet1 is present, given our findings with HML/H92511/HML/H92512 and HMRa1. Strikingly, Pol IIoSer5 is present at these genes (Fig. 8D), while Cet1 is not (Fig. 8E). These results recapitulate findings for the HML/H92511/HML/H92512 and HMRa1 promoters and, intriguingly, suggest the presence of two additional SIR-regulated genes within the HM loci.

SIR partially restricts recruitment of Mediator. Finally, we examined the recruitment of Mediator, a transcriptional co-regulator thought to bridge sequence-specific activators with RNA Pol II. Consistent with a general role in transcription, Mediator has been detected at the 5′ ends of many, if not most, genes (3). In light of this, it has been proposed that Mediator should be considered a general transcription factor that is equivalent to components of the PIC (65). An alternative view is that Mediator primarily acts as a gene-specific coactivator (13). Mediator has been detected in holoenzyme preparations of Pol II (31) but is not corecruited with polymerase at certain genes (5, 10). As shown in Fig. 6D, the occupancy at HML/H92511/HML/H92512 of both head (Srb4) and tail (Gal11) Mediator subunits is reduced 50 to 75% by SIR. This observation argues that Mediator is recruited to HML independently of Pol II and that its access, unlike that of Pol II, is restricted.

DISCUSSION

SIR is permissive to GTF recruitment and PIC assembly. Here, we have investigated the mechanism by which SIR regulates transcription in Saccharomyces cerevisiae. We demonstrate that at SIR-repressed genes, steps in the transcription cascade that occur upstream of PIC recruitment are fully permitted, while those occurring downstream are essentially abolished. This conclusion arises from a quantitative assessment of factor occupancy at two naturally silenced targets of SIR, HMRa1 and HML/H92511/HML/H92512, and in two distinct genetic backgrounds. At these genes transcription is extinguished, yet three GTFs—TBP, Pol II, and TFIIH—are present and abundant within their promoters. The presence of TFIIH, the last factor typically recruited to the PIC, implies that the initiation complex is fully assembled. Additionally, the presence of Ser5-phosphorylated Pol II suggests that Kin28 is functional and that Pol II has initiated transcription. Nonetheless, the recruitment of downstream factors involved in mRNA capping and transcript elongation is virtually abolished (considered more fully below). Importantly, PIC assembly in silent chromatin is not a peculiarity of the mating-type promoters. We observed that two other genes, YCL065W and YCR097W-A, are similarly regulated: Pol II and TBP are present at normal levels, and Pol II is Ser5 phosphorylated, yet there is no detectable transcription in a sir2Δ background. Both, however, are expressed in a sir2Δ mutant.

Our detection of Pol II at silent HMRa1 and HML/H92511/HML/H92512 is at odds with a recent, quantitative ChIP analysis that gave rise to the PIC interference model (8). That study employed strains of the S288C background, as done here, and
investigated the presence of Pol II at the silent HM promoters using both a CTD-specific antibody (8WG16) and a phospho-
Ser5-CTD-specific antibody (H14, as employed here [Fig. 4A]). However, the authors of that study failed to detect the presence of Pol II using either antibody; likewise, they were unable to detect either TFIIB or TFIIE (8). While the reason for this is unclear, three lines of evidence argue against the PIC interference model. First, as discussed above, we detected TBP and TFIIH, in addition to Pol II, at the hyperrepressed promoters. Normal levels of Pol II are consistent with the unimpaired function of Kin28, given that Kin28 thermal inactivation strongly reduces Pol II promoter occupancy (42, 54). Second, the ChIP methodology we employed is sufficiently sensitive to detect differences in factor occupancy at these genes, as dem-

FIG. 7. Efficiency of SIR-dependent silencing strongly correlates with a restriction in recruitment of the Cet1 capping enzyme. Illustrated is a summary of Cet1 and Pol II (Rpb1) occupancy of SIR-silenced hsp82 transgenes (top and bottom histograms). Factor occupancy at the euchromatic (derepressed) hsp82-1001 allele also is depicted (sir4Δ background). These transgenes differ in the dosage and arrangement of integrated silencers that flank the hsp82 promoter and coding region (symbolized by arrows; see the legend to Fig. 4C). Cet1 abundance is quantified for the promoter, ORF, and 3′-UTR (the location of amplicons and shading key are indicated at the top); Pol II abundance is quantified for the promoter only. For both analyses, bars represent means ± standard errors of the means of three biological replicates. A representative Northern analysis of each strain also is displayed, with hsp82 transcript levels, normalized to ACT1, indicated (values represent the means of two independent experiments).
onstrated by the fact that we observed large differences in the occupancy of downstream factors at euchromatic versus heterochromatic promoters. Third, a recent high-resolution genome-wide analysis of Pol II density supports the notion that Pol II is present at SIR-silenced promoters (62). In this ChIP-chip analysis, the Rpb3 subunit of Pol II was detected at genome-average levels within the HML-I and HMR-I silencers. Also shown are PCR amplicons used for this analysis (black rectangles). Coordinates are numbered relative to the ATG codons of the αI and αl ORFs. (A) Physical maps of HMLαI/YCL065W and HMRαI/YCR097W-A showing the location and orientation of ORFs and the location of the HML-I and HMR-I silencers. Also shown are PCR amplicons used for this analysis (black rectangles). Coordinates are numbered relative to the ATG codons of the αI and αl ORFs. (B) Summary of Pol II CTD ChIP assays (S288C background) for YCL065W and YCR097W-A. ChIP analysis and the quantification of factor occupancy were performed as described in the legend to Fig. 2D. Bars represent means ± standard errors of the means (SEM) for four independent biological replicates. (C to E) TBP, Pol IIoSer5, and Cet1 abundance at YCL065W and YCR097W-A. ChIP analysis and quantification were performed as described above (depicted are means ± SEM; n = 4).

FIG. 8. The SIR-regulated YCL065W and YCR097W-A genes are fully permissive to Pol II and TBP recruitment and to Ser5 CTD phosphorylation, but they are restrictive to the recruitment of capping enzyme. (A) Physical maps of HMLαI/YCL065W and HMRαI/YCR097W-A showing the location and orientation of ORFs and the location of the HML-I and HMR-I silencers. Also shown are PCR amplicons used for this analysis (black rectangles). Coordinates are numbered relative to the ATG codons of the αI and αl ORFs. (B) Summary of Pol II CTD ChIP assays (S288C background) for YCL065W and YCR097W-A. ChIP analysis and the quantification of factor occupancy were performed as described in the legend to Fig. 2D. Bars represent means ± standard errors of the means (SEM) for four independent biological replicates. (C to E) TBP, Pol IIoSer5, and Cet1 abundance at YCL065W and YCR097W-A. ChIP analysis and quantification were performed as described above (depicted are means ± SEM; n = 4).
SIR imposes a 5’ arrest on Pol II by restricting recruitment of capping and elongation factors. In contrast to the unhindered access of Pol II and GTFs to silent promoters, the occupancy of the capping enzymes Cet1/Ceg1 and Abd1 and elongation factors Spt5, TFIIH, and Paf1C is highly restricted. Of particular significance is the tight inverse correlation between silencing efficiency and Cet1 occupancy (Fig. 7). This suggests a mechanistic link between a block in capping enzyme recruitment and silencing. Abd1 harbors a transcription elongation activity independent of cap methylation, and the inactivation of this function reduces Pol II occupancy at the 5’ end and/or Pol II processivity (55). Therefore, SIR, by impeding capping enzyme recruitment, may contribute to Pol II stalling. Such stalling takes place at or near the gene’s 5’ end, given that Pol II abundance is considerably reduced (relative to that of the sir2Δ-derepressed state) at points downstream (Fig. 4B, C). We have tested for the presence of short 5’ transcripts associated with the silenced hsp82-2001 gene. None could be found with primer extension assays (56). Therefore, the silencing of stably repressed loci such as HML, HMR, and hsp82-2001 is unlikely to involve posttranscriptional processing events, as recently shown for Sir2-regulated telomeric reporter genes and the nontranscribed spacer regions within the ribosomal DNA array (68).
of the Spt4/Spt5 complex. We speculate that the Sir2/3/4 complex plays that role through its deacetylation and stable positioning of nucleosomes.

What underlies the differential accessibility of initiation and capping/elongation factors to silent chromatin? An important implication of our findings is that SirR-mediated silent chromatin is differentially accessible to initiation and capping/elongation factors. Although our work does not address how this might be achieved, there are several possibilities. One way is via molecular sieving. This idea is appealing, given the long-standing view that chromatin can repress gene expression by excluding factors from accessing their target DNA sequences (see Introduction). However, the proteins with the most severely restricted access, Cet1/Ceg1, Abd1, TFIIS, and Spt4/5, tend to be relatively small, with molecular masses of 115, 50, 35, and 127 kDa, respectively. In contrast, the accessible factors Pol II and TFIIH have respective masses of 550 and 438 kDa. As TBP occupancy may signify the presence of TFIID, with a mass of ~1.2 MDa, it is unlikely that SirR-silenced chromatin acts by molecular sieving.

Alternatively, SirR may prevent polymerase elongation as a consequence of the structural features of silent chromatin itself. Hypoacetylated nucleosomes are very stable, with adjacent nucleosomes possessing the potential to interact with each other through ionic bonding (39) and arrays of H4 K16 hypoacetylated nucleosomes capable of forming 30-nm-like fibers (59). Thus, Pol II, although able to gain access to silent promoters, may be unable to elongate through hypoacetylated nucleosomes complexed with Sir2/3/4. The inability of polymerase to elongate through stabilized silent nucleosomes may prevent the stable association of capping and elongation factors with the TEC. Additionally, it is possible that Sir2 inhibits the recruitment of one or more capping/elongation factors by virtue of its intrinsic lysine deacetylase activity. This could occur by direct deacetylation of an elongation factor or via the deacetylation of H4 AcK16, which may serve as a binding site for one or more downstream factors. Although our data indicate that at least five factors are excluded from silent chromatin, it is conceivable that a subset of them, or an as-yet-unidentified factor, actually is targeted, abrogating subsequent steps in the transcriptional cascade.

Importantly, our data appear to rule out a third mechanism, elongational arrest via the proteolysis of stalled Pol II complexes. Inappropriately stalled Pol II is targeted for proteasome-mediated degradation by Rsp5-dependent ubiquitylation (4). However, Sir5 phosphorylation, a signature of SirR repression, strongly inhibits Pol II ubiquitylation (60). A schematic summarizing our findings at SirR-repressed HMLα1/HMLα2 is illustrated in Fig. 9A.

Silent yeast promoters and quiescent mammalian promoters are both characterized by the presence of stalled polymerase. Of relevance to our findings is a recent genome-wide analysis of human cells that demonstrated the presence of Pol II at the vast majority of promoters of protein-encoding genes, including >50% of inactive promoters (19). Interestingly, just as we have seen for silenced yeast promoters, Pol II association with inactive human promoters likely is due to transcription initiation without transcript accumulation. This phenomenon was seen in three different cell types (embryonic stem cells, hepatocytes, and B cells), so it likely represents the prevalent mechanism by which gene transcription is controlled in humans. While it is intriguing that this basic attribute, PIC assembly and Pol II initiation at an otherwise inactive gene promoter, is evolutionarily conserved, the mechanism by which these human genes are kept in a quiescent state is unknown. Our findings that SirR-silenced, heterochromatinic genes are repressed via the differential recruitment of initiation and capping/elongation factors provide a potential model by which this large and important class of genes is transcriptionally regulated.

Tup1/Ssn6 represses the α1 promoter by interfering with PIC recruitment. Our results indicate that TBP, Pol II, Kin28, and Pol IIαSer5 occupancy at the HMLα1/HMLα2 promoters paradoxically is more abundant in the context of SirR silencing than in the euchromatic (Sir2Δ or sir4Δ), derepressed state. This contrasts with the essentially equivalent occupancy of the same factors at the HMRα1 promoter and provides a stark contrast to the null occupancy of capping/elongation factors at either HML or HMR (summarized in Table 2). A probable explanation is that the α1 promoter is subject to a second form of negative regulation, conferred by the α1/α2 haploid genespecific repressor (22), present in sir haploids and wild-type diploids. This repressor negatively regulates the transcription of linked promoters by the recruitment of the Tup1/Ssn6 corepressor, which in turn recruits type I and type II histone deacetylases (reviewed in references 11 and 40). Previous stud-

### TABLE 2. In vivo occupancy of initiation, capping, and elongation factors at silent and derepressed HM loci

| Factor/strain background | Occupancy level at HML | Occupancy level at HMR |
|--------------------------|------------------------|------------------------|
|                          | SIR        | str2Δ    | P value | SIR        | str2Δ sir4Δ | P value |
| Rpb1/S288C               | 1.51 ± 0.19 | 1 ± 0.05 | 0.038   | 1.07 ± 0.09 | 1 ± 0.04 | 0.220   |
| TBP/S288C                | 1.53 ± 0.14 | 1 ± 0.12 | 0.031   | 1.19 ± 0.09 | 1 ± 0.09 | 0.110   |
| Kin28/SLY101             | 1.95 ± 0.14 | 1 ± 0.37 | 0.014   | 1.39 ± 0.34 | 1 ± 0.12 | 0.340   |
| Tbi1/S288C               | 1.51 ± 0.16 | 1 ± 0.32 | 0.039   |
| Serp-P-Rpb1/S288         | 1.28 ± 0.07 | 1 ± 0.09 | 0.045   | 1.29 ± 0.06 | 1 ± 0.11 | 0.060   |
| Cet1/S288C               | 0.04 ± 0.02 | 1 ± 0.11 | 0.001   | 0.06 ± 0.04 | 1 ± 0.09 | 0.002   |
| Abd1/S288C              | 0.11 ± 0.06 | 1 ± 0.15 | 0.005   | 0.11 ± 0.07 | 1 ± 0.19 | 0.009   |
| Spt5/S288C              | −0.09 ± 0.02 | 1 ± 0.38 | 0.003   |
| Dst1/S288C              | 0.08 ± 0.18 | 1 ± 0.47 | 0.012   |
| Rtf1/S288C              | 0.17 ± 0.05 | 1 ± 0.24 | 0.027   |

* Occupancy levels were quantified as described in Materials and Methods. Values represent means ± standard errors of the means (n = 3 or 4). P values were determined using a two-tailed t test. Statistically significant differences in factor occupancy (P < 0.05) are highlighted in boldface.
ies have demonstrated that Tup1 prevents the recruitment of TBP and Pol II to the promoters it regulates (34, 73). Therefore, our data, in combination with these previous observations, suggest that at the "HMLα" promoter, Tup1 and Sir2 use distinct mechanisms to repress transcription. Tup1 blocks the recruitment of TBP, Pol II, and TFIH, thereby aborting PIC assembly (illustrated in Fig. 9B), while the Sir2/3/4 complex permits the recruitment of these factors but abrogates transcription by restricting the access of capping and elongation factors, resulting in the irreversible stalling of Pol II at the gene's 5′ end.

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