Asymmetric Positioning of Nucleosomes and Directional Establishment of Transcriptionally Silent Chromatin by *Saccharomyces cerevisiae* Silencers

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In *Saccharomyces cerevisiae*, silencers flanking the HML and HMR loci consist of various combinations of binding sites for Abf1p, Rap1p, and the origin recognition complex (ORC) that serve to recruit the Sir silencing complex, thereby initiating the establishment of transcriptionally silent chromatin. There have been seemingly conflicting reports concerning whether silencers function in an orientation-dependent or -independent manner, and what determines the directionality of a silencer has not been explored. We demonstrate that chromatin plays a key role in determining the potency and directionality of silencers. We show that nucleosomes are asymmetrically distributed around the HML-I or HMR-E silencer so that a nucleosome is positioned close to the Abf1p side but not the ORC side of the silencer. This coincides with preferential association of Sir proteins and transcriptional silencing on the Abf1p side of the silencer. Elimination of the asymmetry in nucleosome positioning at a silencer leads to comparable silencing on both sides. Asymmetric nucleosome positioning in the immediate vicinity of a silencer is independent of its orientation and genomic context, indicating that it is the inherent property of the silencer. Moreover, it is also independent of the Sir complex and thus precedes the formation of silent chromatin. Finally, we demonstrate that asymmetric positioning of nucleosomes and directional silencing by a silencer depend on ORC and Abf1p. We conclude that the HML-I and HMR-E silencers promote asymmetric positioning of nucleosomes, leading to unequal potentials of transcriptional silencing on their sides and, hence, directional silencing.

The establishment of condensed and transcriptionally silent heterochromatin in the eukaryotic genome is achieved via an initiation process that recruits silencing/repressor complexes to specific nucleation sequences, followed by their propagation along the chromatin. A silencing complex usually contains a histone-modifying enzyme(s) responsible for yielding heterochromatin-specific “histone codes” and structural proteins that recognize these codes and bind the modified histones with high affinity (13). In the yeast *Saccharomyces cerevisiae*, transcriptional silencing of the HML and HMR loci as well as regions near the telomeres is mediated by a silent chromatin that shares many similarities with metazoan heterochromatin at the molecular level (13). Silent chromatin at the HM loci is established by combined actions of cis-acting DNA elements and trans-acting proteins (32). The cis-acting elements are small specialized sequences, known as the E and I silencers, that flank the HM loci (Fig. 1A). Each silencer is composed of a unique combination of binding sites for proteins Abf1p, Rap1p, and ORC (for “origin recognition complex for DNA replication”) (Fig. 1A), whose binding is required for silencing (32). The trans-acting factors include silencer-binding proteins, histones, and Sir1p through Sir4p. Sir2p is an NAD-dependent histone deacetylase that is believed to be responsible for the reduction of histone acetylation in silent chromatin (25, 32). Sir2p through Sir4p interact with each other and form the Sir complex that can interact with the silencer-binding proteins, as well as with histones H3 and H4. Moreover, the Sir complex has a higher affinity for histones H3 and H4 with reduced acetylation, which is important for the establishment and maintenance of silent chromatin (8, 19, 32).

The silencer-binding proteins are believed to promote the initiation of silencing by recruiting the Sir complex through physical interactions (32). Rap1p interacts with Sir3p and Sir4p, whereas ORC interacts with Sir1p, which binds Sir4p. The Sir complex associated with the silencer deacetylates histones in adjacent nucleosomes that then bind additional Sir complexes. The nucleosome-bound Sir complex then deacetylates the neighboring nucleosome, which in turn binds a new Sir complex. Interactions between Sir complexes also help recruit the incoming Sir complex (19, 31). In this manner, the Sir complex is thought to promote its own stepwise (nucleosome-by-nucleosome) propagation along a continuous array of nucleosomes, thereby establishing an extended region of silent chromatin (13). This model is supported by our finding that disrupting the regularity of nucleosomes by nucleosome-excluding structures hinders the spread of silent chromatin (3).

Although yeast silencers promote silencing by a common mechanism, they exhibit different efficiencies of silencing. The HML-E, HML-I, and HMR-E silencers have the ability to initiate silencing on their own, whereas HMR-I only plays an auxiliary role in silencing at the HMR locus (5, 22, 30, 32). There is evidence that HMR-E is stronger than HML-E, which is stronger than HML-I, in silencing (34). This likely reflects the structural differences among the silencers. For instance, the binding sites for Abf1p, Rap1p, or ORC in the silencers are actually distinct variants of a consensus sequence (see the...
legends to Fig. 1A) that may have different affinities for the corresponding protein (4, 7). Moreover, the arrangement of the factor-binding sites in each silencer concerning the distance and intervening sequence between any pair of adjacent sites is unique. These factors may collectively determine the efficiency of a specific silencer in recruiting Sir proteins, thereby determining its strength of silencing.

The model for silencer function discussed above implies that initiation of silencing is bidirectional. This is consistent with an early report that HMR-E could function in either orientation to silence the a1 gene at the HMR locus (5). However, equal silencing on the two sides of HMR-E was observed in the presence of HMR-I that could also contribute to silencing at HMR (5, 30). In the absence of HMR-I, silencing by HMR-E was detectably stronger on its Abf1p side than the ORC side (5). Moreover, HMR-E ectopically inserted at the MAT locus or the structurally similar HML-I in its native location also preferentially silences genes on its Abf1p side (2, 34). Therefore, the HMR-E or HML-I silencer per se seems more active in promoting silencing on its Abf1p side. However, such a directionality may be masked by other silencing elements in the genomic context (5, 43). Moreover, the observed directionality in a specific experiment is also dependent on the sensitivity/resolution of the assay used (43).

In this report, we have further examined the directionality of silencing by HMR-E both in its native HMR location and in the context of HML, using three different reporter genes, URA3, a1, and a1, respectively. Our results provide further support for the notion that for both HMR-E and HML-I silencing is generally robust on the Abf1p side but diminished on the ORC side. Importantly, we then show that this is correlated with asymmetric positioning of nucleosomes on the two sides of the silencer. Specifically, a nucleosome is stably positioned close to the Abf1p site but not the ORC site of the silencer. Elimination of the asymmetry in nucleosome positioning at a silencer leads to comparable silencing on both sides. Asymmetric positioning of nucleosomes in the immediate vicinity of a silencer is not dependent on its orientation or genomic context and is therefore the inherent property of the silencer. It is also independent of Sir proteins and therefore precedes the formation of silent chromatin. We also present evidence that ORC and Abf1p play key roles in asymmetric chromatin organization and directional silencing by HMR-E.

MATERIALS AND METHODS

Plasmids and strains. The sequences of “full-length” HML-I and HMR-E shown in Fig. 1 to 7 were those defined in the original deletion analyses of HML and HMR (1, 11). Specifically, HML-I and HMR-E correspond to coordinates 14555 to 14839 and 291425 to 291559 of chromosome III, respectively. Every silencer contains a core element consisting of binding sites for the silencer-binding proteins and flanking sequences, as illustrated in Fig. 1A and 4. HMR-E’ (291409 to 291559) and HMR-E’” (291245 to 291409) are shorter versions of HMR-E deleted for the 3′ flanking sequences of the autonomous replication sequence (ARS) consensus sequence (ACS) and the Abf1p site, respectively (Fig. 6B and 7A). Plasmid pUC26 was made by inserting the BamHI-HML-BamHI fragment (coordinates 9666 to 16263 of chromosome III) into pUC19. A 1.1-kb URA3 sequence (2) was inserted at the EcoRV site (15411) of pUC26 to make pYZ1. The HpaI-HML-I-HindIII sequence (coordinates 14555 to 14839) in pYZ1 was replaced by a HindIII site to make pYZ1m. HML-I was inserted at the HindIII site of pYZ1m in the opposite direction (compared to that in pYZ1), resulting in pYZ2. The HMR-E sequence in either direction was inserted at the HindIII site of pYZ1m, resulting in pYZ3 and pY2A. HMR-E’ (Fig. 6B) was inserted in either orientation at the HindIII site of pYZ1m, resulting in pYZ13 and pYZ16. HMR-E’m (Fig. 6B) was generated by PCR and inserted in either orientation at the HindIII site of pYZ1m, resulting in pYZ14 and pYZ17. ACS in pYZ13/16 was replaced by the Spel site, resulting in pYZ13/18. HMR-E’ (Fig. 7A) was inserted in either orientation at the HindIII site of pYZ1m, resulting in pYZ19 and pYZ22. HMR-E’a (Fig. 7A) was inserted in either orientation at the HindIII site of pYZ1m, resulting in pYZ20 and pYZ23. HMR-E’b (Fig. 7A) was inserted in either orientation at the HindIII site of pYZ1m, resulting in pYZ21 and pYZ24. Plasmid pRS416-HMR was made by inserting HindIII-HMR-HindIII(HMR)298227-294210 into pRS416pV5 was made by inserting URA3 at the SpeI site (29110) of pRS416-HMR. HMR-E in pYZ5 was replaced by a BamHI site, resulting in pYZ5m. An inserted HMR-E was inserted at the BamHI site of pYZ5m, making pYZ5m. URA3 was inserted at the XbaI site (291731) of pRS416-HMR, making plasmid pYZ7m. The HMR-I silencer was deleted from pYZ7m, resulting in pYZ7. HMR-E in pYZ7 was replaced by a BamHI site, resulting in pYZ8m. An inserted HMR-E was inserted at the BamHI site of pYZ8m, making pYZ8. Plasmids pYZ9 and pYZ10 were derived from pYZ5 and pYZ6, respectively, by deleting HMR-I.
Plasmids pYZ11 and pYZ12 were derived from pYZ3 and pYZ4, respectively, by deleting HML-E. pMB21 is an integration plasmid containing SIR3 open reading frame. The relevant genotypes of yeast strains are listed in Table 1. Strains 1 to 8 and 11 to 24 were made by transforming W303 in the genome, resulting in strains 1 to 8 and 11 to 24. Strains 9 and 10 were made by transforming Y729 to Ura⁻/His⁺. Strains 5s to 8s were made by transforming strains 3 and 4 to geneticin resistant with a PCR-generated fragment encoding 9-myc linked to SIR3 as the reporter for silencing. Three independent experiments were performed, and the products were analyzed by agarose gel electrophoresis. Shown are results from one of these experiments. In each lane, the template concentration for URA3 or HMRal transcript was the same, but that for ACT1 was 10-fold lower (see Materials and Methods). The template concentration in the even-numbered lanes was one-fifth of that in the odd-numbered ones. The RNA template for the no-RT control was of strain 9s. (C) Quantification and analysis of qRT-PCR data. Each open or shaded bar represents the ratio of intensity of URA3 or HMRal product over that of the ACT1 product in a strain. The means of data from all three experiments together with standard deviations are presented. (D) Modified HML loci in strains 11 and 12. [Δ] indicates the deletion of HML-E. (E) Measurement of the abundance of URA3 and HMLal transcripts in strains 11, 12, 11s, and 12s by qRT-PCR. In each lane, the template concentration for URA3 and HMLal was the same but was 10-fold lower for ACT1. The RNA template for the no-RT control was of strain 11s. (F) Quantification of qRT-PCR data. The ratio of intensity of URA3 or HMLal product over that of the ACT1 product in each strain is listed in the table.

**RESULTS**

Directional silencing by *S. cerevisiae* silencers. To further examine the directionality of silencing by HMR-E, we tested its function in the context of HML-I. HMR-E in either orientation was used to replace HML-I and tested for its ability to silence a URA3 gene inserted nearby (Fig. 1B, strains 3 and 4). The URA3 product can convert 5-fluoroorotic acid (FOA) into a toxic metabolite, thus, its expression renders cells sensitive to FOA. Therefore, URA3 silencing could be measured by FOA resistance of the cell. URA3 inserted to the right (centromere-proximal) side of HML-I was not silenced (Fig. 1B, lack of growth of strain 1 on FOA medium). However, it was silenced when HML-I was inverted (Fig. 1B, strain 2), confirming that HML-I acts in a directional fashion (2). Strain 3 was sensitive, whereas strain 4 was resistant, to FOA (Fig. 1B, strains 3 and 4 on FOA). Therefore, like HML-I, HMR-E in the context of HML-I also preferentially initiates silencing on its Abt1p side.

We also reexamined the directionality of HMR-E in its native location using the URA3 gene as the reporter for silencing. URA3 was not silenced when inserted to the left (centromere proximal) of the resident HMR-E silencer (Fig. 1B, strain 5) but was silenced when the direction of HMR-E was inverted (Fig. 1B, compare strains 6 and 5). On the other hand, URA3 was silenced when inserted to the right of the resident HMR-E silencer in the absence of HMR-I but was not silenced when
HMR-E was inverted (Fig. 1B, compare strains 7 and 8). These results demonstrate that HMR-E also preferentially initiates silencing on its Abf1p side in its endogenous context when URA3 is used as the reporter. Note that silencing of URA3 expression in strains 1 to 8 and other strains used in this work was SIR dependent, as it was completely abolished by deleting SIR3 (Fig. 1B, compare strains 1s to 8s with 1 to 8, respectively, on FOA; data not shown).

FIG. 4. Asymmetric positioning of nucleosomes around silencers. DNA isolated from MNase-treated chromatin in each strain was digested with AvaII and then subjected to gel electrophoresis and Southern blotting using a probe abutting the AvaII site (A). The positions of the silencer and URA3 in each strain are shown. Filled ellipse, inferred stably positioned nucleosome. Dashed ellipse, lack of stably positioned nucleosome. N1 to N4, MNase-treated naked genomic DNA from strains 1 to 4, respectively. See the text for detailed descriptions.
FIG. 5. Chromatin organization around the native HML-I and HMR-E silencers. DNA from chromatin of strain Y728 (Sir3*) or Y729 (Sir3−) treated with MNase was digested with AvaII (lanes 1 to 6), XbaI (7 to 12), and BsrGI (13 to 18), respectively. Relevant DNA fragments were revealed by probes a (lanes 1 to 6), b (7 to 12), and c (13 to 18), which are 200 bp in length, and they are indicated as black bars near the AvaII, XbaI, and BsrGI sites, respectively, in the diagrams of HML and HMR (top). The structures of the silencers are shown on the left of the three panels. N, naked DNA control. Other symbols are as described in the legend to Fig. 4.

To directly compare the directionality of HMR-E in silencing URA3 and that in silencing the native HMRα genes, we made strains 9 and 10, in which HMR-E was in opposite directions (Fig. 2A). These MATα strains both have URA3 inserted to the left of HMR and have HMR-I deleted (Fig. 2A) and would therefore allow simultaneous measurement of silencing of URA3 and HMRα by HMR-E alone. The abundance of the transcripts of URA3 and HMRα as well as the control ACT1 were measured by quantitative RT-PCR in strains 9 and 10, as well as their Sir3− derivatives 9s and 10s. Three independent experiments were performed, and a representative gel picture is shown in Fig. 2B. Data from all three experiments were quantified, and the mean of the relative abundance of URA3 or a1 in each strain (together with standard deviations) is graphed in Fig. 2C. The abundance of URA3 in the Sir3− strain 10 was only ~1/5 of that in the Sir3+ strain 10s, demonstrating that HMR-E silences URA3 inserted on its Abf1p side. On the other hand, the level of URA3 in strain 9 was not significantly different from that in strain 9s, demonstrating that HMR-E fails to silence URA3 inserted on its ORC side.

The HMRα1 message was barely detectable in strain 9 but was abundant in strain 9s (Fig. 2C, compare shaded bars 9 and 9s), demonstrating that HMRα is fully silenced by HMR-E. In strain 10, HMRα1 was detectable, and its abundance was ~1/3 of that in the Sir3+ strain 10s (Fig. 2C, compare shaded bars 10 and 10s). These data indicate that silencing of HMRα is partial in strain 10 compared to that in strain 9 (Fig. 2C, compare shaded bars 10 and 9), and therefore HMR-E preferentially silences HMRα on its Abf1p side. This is consistent with Brand et al.’s early S1 mapping result on a1 expression, showing that although HMR-E in either orientation could silence the a1 gene, the efficiency of silencing is nevertheless orientation dependent (a1 transcript was not detected when HMR-E was oriented toward a1 but was present, albeit with low abundance, when HMR-E was in the opposite direction) (5).

We also examined whether HMR-E ectopically placed at HML silenced HMLα in an orientation-dependent manner. The MATα strains 11 and 12 were deleted for HML-E, and they had HML-I replaced by HMR-E in opposite directions and URA3 inserted to the right of HML (Fig. 2D). We examined the expression of URA3 and HMLα1 in strains 11 and 12 as well as their Sir3− derivatives, 11s and 12s, simultaneously by qRT-PCR. Note that when HMLα is derepressed in a MATα strain (e.g., by disrupting the SIR genes), the a1 and a2 proteins form a complex that negatively regulates the expression of a1 and a2 (16, 26, 35). However, a1 mRNA is detectable in such a strain, especially when methods with high sensitivity like qRT-PCR are used (9, 23, 40). Mateeic et al. has demonstrated that the transcriptional state of HML could be examined quantitatively by measuring the abundance of HMLα1 message (23). As shown in Fig. 2E and F, URA3 expression in strain 12 was about one-fifth of that in 12s and was therefore silenced by HMR-E in strain 12. On the other hand, URA3 was not silenced by HMR-E in strain 11 (Fig. 2E and F, compare 11 and 11s). However, HMLα1 in strain 12 was about one-fifth of that in strain 12s, whereas it was not detectable in strain 11 (Fig. 2E and F, compare 12 to 12s and 11 to 11s). Therefore, while HMLα was completely silenced by HMR-E in strain 11, it was partially silenced in strain 12. These results support the notion that silencing by HMR-E is stronger on its Abf1p side and is therefore directional.

Directional establishment of transcriptionally silent chromatin by the HMR-E silencer. To test whether directional silencing by a silencer is correlated with directional formation of silent chromatin, we used chromatin immunoprecipitation (ChIP) to examine the association of Sir3p with HMR-E in either orientation as well as adjacent sequences. We tagged Sir3p with the myc epitope in strains 3 and 4 (Fig. 1B), resulting in 3‘ and 4‘, respectively (Fig. 3A, left). This had no detectable effect on the silencing of URA3 or the directionality of HMR-E (Fig. 1B and 3A, compare 3‘ and 4‘ to 3 and 4, respectively). ChIP was performed with a myc antibody (α-myc). The abundance of sequences a (corresponding specifically to HMR-E in the context of HML-I), b to d (Fig. 3A, left), a control sequence spanning the HMR-I silencer, and another control from the ACT1 locus in the immunoprecipitated chro-
FIG. 6. Role of ORC in asymmetric nucleosome positioning and directional silencing by HMR-E. (A) Configurations of chromatin at silencers and the replication origin ARS1. Illustrated is a summary of data presented in Fig. 4 and 5 as well as that presented by Lipford and Bell (20). Abf1p and Rap1p sites are drawn as stippled and filled arrows, and ACS is drawn as an open arrow. B1 and B2 elements in ARS1 are indicated. Filled ellipse, stably positioned nucleosome. Dashed ellipse, absence of stably positioned nucleosome. (B) Inverting or deleting ACS in HMR-E alters its directionality and strength in silencing. The modified HML in strains 3, 4, and 13 to 18 are shown on the left. The original and modified HMR-E silencers in these strains are highlighted. See the text for more description. Growth phenotypes of the strains are shown on the right. Deletion of ACS is marked with a cross. (C) Inverting ACS in HMR-E' leads to chromatin reorganization. Shown are results of chromatin mapping in strains 13, 13s, 14, 14s, 16, 16s, 17, and 17s. MNase digestion and indirect end labeling was done as described in the legend to Fig. 4A. (D) Direct comparison of chromatin at HMR-E'/H11032 in strains 14 and 14s to that at HMR-E' in strains 16 and 16s. (E and F) Effect of ACS deletion on chromatin structure at HMR-E. MNase digestion and indirect end labeling in strains 13, 15, 16, and 18 were carried out as described in the legend to Fig. 4A.
matin fragments were measured by PCR. All the sequences measured are between 250 to 350 bp in length. Three independent ChIP experiments were performed, and a representative gel picture was presented (Fig. 3B). The intensity of each band was quantified and normalized against input control. The relative abundance of Sir3p-myc associated with a sequence in a strain was estimated as the ratio of the intensity of the corresponding fragment over that of the \textit{HMR-I} control (taken as 1.0) in the same strain. The mean of data from all three experiments (together with the standard deviations) is graphed in Fig. 3C. For both strains 3' and 4', there was little or no Sir3p-myc associated with the \textit{ACT1} sequence (Fig. 3C, \textit{ACT1}), which was consistent with the fact that \textit{ACT1} is normally not subject to transcriptional silencing and not associated with Sir proteins. On the other hand, the abundance of Sir3p-myc at sequence a was high in both strains 3' and 4' (Fig. 3C, a), indicating that \textit{HMR-E} in either orientation is able to recruit Sir3p-myc. In strain 4', the Sir3p-myc abundance at b to d was also high (Fig. 3C, b to d, shaded bars), which was indicative of Sir3p-myc spreading from \textit{HMR-E} toward \textit{URA3}. On the other hand, Sir3p-myc abundance at b to d was significantly less abundant in 3' than in 4' (Fig. 3C, compare strain 3' and 4' at b to d), indicating that there was reduced spreading of Sir3p-myc from \textit{HMR-E}, which was in accord with diminished silencing of \textit{URA3} in strain 3'. These results demonstrate that \textit{HMR-E} initiates the spread of Sir proteins preferentially on its Abf1p side, which is consistent with previous data obtained by Loo and Rine indicating that Sir-dependent silent chromatin extends more efficiently on the Abf1p side than the ACS side of \textit{HMR-E} (21).

Unidirectional silencing by \textit{HML-I} or \textit{HMR-E} coincides with an asymmetric distribution of nucleosomes. The directional nature of \textit{HML-I} or \textit{HMR-E} function might result from the fact that it consists of an asymmetric DNA sequence, and perhaps more importantly that its factor-binding sites are arranged in a directional fashion: ORC-Rap1p-Abf1p (Fig. 1A). How might the sequence/structural asymmetry of a silencer be translated into functional asymmetry? Propagation of Sir complexes

FIG. 7. Abf1p is involved in chromatin organization and transcriptional silencing by \textit{HMR-E}. (A) Effects of inverting or mutating Abf1p sites in \textit{HMR-E} on silencing. The modified \textit{HML} in strains 3, 4, and 19 to 24 are shown on the left. The original and modified \textit{HMR-E} silencers in these strains are highlighted. See the text for descriptions. Growth phenotypes of the strains are shown on the right. (B) Chromatin mapping of strain 23 compared to that of strain 22. MNase digestion and indirect end labeling were carried out as described in the legend to Fig. 4A. (C) Chromatin mapping of strain 24 compared to that of strain 22. The mutated Abf1p site is marked with a cross.
along the chromatin depends on their association with nucleosomes and presumably requires a continuous array of closely positioned nucleosomes (3, 32). Given that Abf1p, Rap1p, and ORC all have the ability to modulate local chromatin structure upon binding to DNA (3, 20, 41, 42), we envisioned that nucleosomes might be organized differently (asymmetrically) on the flanks of HML-I and HMR-E, resulting in unequal potentials for the spread of Sir proteins on the two sides. To test this hypothesis, we mapped nucleosomes around HML-I and HMR-E using micrococcal nuclease (Mnase) digestion and indirect end labeling (33). Mnase introduces double-stranded breaks mainly in chromosomal DNA not assembled into nucleosomes (i.e., linker DNA), and therefore the presence of a nucleosome could be inferred from an Mnase-resistant region (33). MNase introduces double-stranded breaks mainly in chromosomal DNA not assembled into nucleosomes (i.e., linker DNA), and therefore the presence of a nucleosome could be inferred from an Mnase-resistant region (33).

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* The structures of HMR-E', HMR-E'm, HMR-E'n, HMR-E', HMR-E'a, and HMR-E'b are illustrated in Fig. 6 and 7.
putative positions of nucleosomes, the chromatin is subjected to partial digestion with MNase, yielding a series of fragments associated with various numbers of nucleosomes. DNAs from these fragments are then examined by indirect end labeling to reveal the positions of MNase cleavage sites relative to a reference restriction site, thereby allowing a tentative assignment of sequences protected by nucleosomes in the region of interest (33).

We first mapped chromatin around HML-I in its original orientation in strain 1. DNA purified from MNase-treated chromatin was digested with AvaII 300 bp downstream from HML-I and was then subjected to electrophoresis and Southern blotting using a probe corresponding to a 200-bp sequence abutting the AvaII site (Fig. 4A, bar labeled “probe”). We found that the HML-I core (consisting of the binding sites for Abf1p, Rap1p, and ORC) coincided with three MNase-sensitive sites that were likely caused by the binding of Abf1p, Rap1p, and ORC (Fig. 4A, lanes 1 to 3). Notably, the patterns of MNase cleavage on the two sides of the core sequence were different. Specifically, the region adjacent to ACS (ORC recognition site) contained an MNase-hypersensitive region (labeled by a dot) and two moderately sensitive sites that were indicative of the absence of a stably positioned nucleosome (Fig. 4A, compare lanes 1 to 3 with N1; the dashed ellipse indicates the lack of a stably positioned nucleosome). In contrast, on the Abf1p side there were two MNase-sensitive sites bracketing a region of ~150 bp that was relatively resistant to MNase cleavage, a pattern indicative of the presence of a positioned nucleosome (Fig. 4A, lanes 1 to 3, filled ellipse labeled 1’). Therefore, the organization of nucleosomes around HML-I in strain 1 was asymmetric, and the absence of a stably positioned nucleosome near ACS coincided with the lack of URA3 silencing on this side of HML-I, as shown in Fig. 1B. To examine if the Sir complex was involved in the asymmetric positioning of nucleosomes at HML-I, we disrupted SIR3 in strain 1, making 1s. The MNase cleavage pattern around HML-I in strain 1s was similar to that in 1 (Fig. 4A, compare lanes 4 to 6 with 1 to 3), indicating that an intact Sir complex is not required for asymmetric nucleosome positioning at HML-I.

We next mapped chromatin around the inverted HML-I in strain 2. The pattern of MNase digestion within the inverted HML-I sequence mirrored that of HML-I in its original direction, indicating that the structure of HML-I was not affected by its inversion (Fig. 4A, compare lanes 10 to 12 with 1 to 3). Strikingly, the pattern of nucleosome positioning on either side of the inverted HML-I in strain 2 was dramatically different from that of HML-I in strain 1 (Fig. 4A, compare lanes 10 to 12 with 1 to 3). On the Abf1p side, three consecutive nucleosomes could be inferred (Fig. 4A, right, filled ellipses labeled 1 to 3), which is consistent with data of chromatin mapping on the Abf1p side of native HML-I obtained by Weiss and Simpson (39). On the other hand, no stable nucleosomes were located immediately adjacent to ACS (Fig. 4A, right, the dashed ellipse). Therefore, flipping HML-I led to an apparent inversion of the pattern of nucleosome positioning around the silencer core (Fig. 4A, compare lanes 10 to 12 with 1 to 3; note the apparent twofold rotational symmetry between sites a and a’, between the two dashed ellipses, and between filled ellipses 1 and 1’). The presence of nucleosomes 1 to 3 on the Abf1p side of HML-I coincided with robust URA3 silencing on this side (Fig. 1B and 4A, strain 2). To examine if the Sir complex played any role in forming the asymmetric structure of chromatin around the inverted HML-I, we deleted SIR3 in strain 2, making 2s. In strain 2s, site b marking the border of nucleosome 3 disappeared, and certain sites within the region spanning nucleosomes 2 and 3 became sensitive to MNase (Fig. 4A, compare lanes 7 to 9 with 10 to 12). Therefore, it could be inferred that nucleosomes 2 and 3 were no longer stably positioned there in strain 2s. This suggested that the positioning of nucleosomes 2 and 3 was the result of the spread of the Sir complex in strain 2. On the other hand, nucleosome 1 remained positioned there in 2s (Fig. 4A, compare lanes 7 to 9 to 10 to 12; note that band a, marking the border of the putative nucleosome 1, was retained). Similar results regarding the effect of SIR3 deletion on nucleosomes near the Abf1p side of HML-I was obtained by Weiss and Simpson (39). In summary, we conclude that asymmetric positioning of nucleosomes on the Abf1p and ORC sides of HML-I is independent of the direction of the silencer and of intact Sir complexes.

HMR-E is similar to HML-I in that it also contains binding sites for Abf1p, Rap1p, and ORC in the same order (Fig. 1A). We examined if HMR-E-organized chromatin in a similar way as HML-I by mapping chromatin around HMR-E in strains 3 and 4 as well as their sir3 derivatives, 3s and 4s. The distribution of MNase-sensitive sites around HMR-E in strain 3 was reminiscent of that around HML-I in strain 1 (compare lanes 1 to 3 in Fig. 4B with 1 to 3 in A). A stable nucleosome was inferred to be positioned near the Abf1p site but not the ORC site of HMR-E (Fig. 4B, left, filled ellipse 1’ and dashed ellipse). The pattern of nucleosome positioning was largely unchanged by the deletion of SIR3 (Fig. 4B, compare lanes 4 to 6 with 1 to 3), which was similar to the situation regarding HML-I (Fig. 4A, lanes 1 to 6). Chromatin configuration around inverted HMR-E in strain 4 was again very similar to that around inverted HML-I in strain 2 (compare Fig. 4A to B). Three consecutive nucleosomes (labeled 1 to 3) were positioned near the Abf1p sites (Fig. 4B, lanes 10 to 12), a pattern similar to chromatin mapping data of native HMR-E obtained by Ravindra et al. (28). There were no stably positioned nucleosomes adjacent to ACS (lanes 10 to 12 in Fig. 4B). SIR3 deletion had no significant effect on the positioning of nucleosome 1 (Fig. 4B, compare lanes 7 to 9 with 10 to 12; note the presence of site a, inferred as the border of nucleosome 1 in lanes 7 to 9). On the other hand, site b, inferred as the border of nucleosome 3, became blurred, indicating that nucleosomes 3 and 2 were no longer as stably positioned in strain 4s as in 4 (Fig. 4B, lanes 7 to 9), which was again similar to the situation concerning HML-I (Fig. 4A, lanes 7 to 9). Nucleosomes 2 and 3 were likely positioned/stabilized by the Sir complex during the formation of silent chromatin on the Abf1p side of HMR-E in strain 4. In summary, these results demonstrate that, similar to HML-I, HMR-E also asymmetrically positions nucleosomes in an orientation- and Sir complex-independent fashion, and a stably positioned nucleosome coincides with the presence of Sir3p and robust silencing on the Abf1p side (Fig. 1B and 3C, strains 4 and 4’), whereas the lack of a positioned nucleosome adjacent to ACS coincides with the absence of silencing on the ACS side (Fig. 1B and 3C, strains 3 and 3’).
Nucleosome positioning in the immediate vicinity of a silencer is independent of the Sir proteins. We have shown above that asymmetric nucleosome positioning in the immediate vicinity of a silencer was not significantly affected by the deletion of SIR3 (Fig. 4A and B). However, the Sir2p/Sir4p complex can be recruited to silencers in a Sir3p-independent manner (15). This may be mediated by Rap1p-Sir4p interaction and/or Sir1p-Sir4p interaction (32). To test whether Sir2p/Sir4p as well as Sir1p plays a role in nucleosome positioning at silencers, we deleted SIR1, SIR2, and SIR4, respectively, from strain 4, resulting in strains 4sir1, 4sir2, and 4sir4 (Fig. 4C). We mapped nucleosomes near HMR-E in strains 4 (SIR⁺), 4sir1 (sir1⁻), 4sir2 (sir2⁻), 4s (sir3⁻), and 4sir4 (sir4⁻). The profile of MNase-sensitive sites in strain 4s indicated that deletion of SIR3 had no effect on the positioning of nucleosome 1 near the Abf1p site and the exclusion of a nucleosome adjacent to the ORC site of HMR-E (Fig. 4C, compare lanes 10 to 12 to 1 to 3; see also lanes 7 to 9 in B). The profiles of MNase-sensitive sites in strains 4sir2 (sir2⁻) and 4sir4 (sir4⁻) were both very similar to that in strain 4s (sir3⁻) (Fig. 4C, compare lanes 7 to 9 and 13 to 15 to lanes 10 to 12), which suggested that like Sir3p, Sir2p and Sir4p also did not play a significant role in nucleosome positioning on either side of HMR-E. The pattern of MNase-sensitive sites in strain 4sir1 (sir1⁻) was nearly identical to that in strain 4 (SIR⁺) (Fig. 4C, compare 4 to 6 to lanes 1 to 3), demonstrating that Sir1p was not involved in nucleosome positioning at HMR-E. In summary, the above data demonstrate that asymmetric nucleosome positioning in the immediate vicinity of HMR-E is generally independent of Sir proteins.

Asymmetric nucleosome positioning by a silencer is not dependent on its genomic context. In the above experiments on chromatin organization around the silencers, HMR-E was placed in the ectopic position of HML-I (Fig. 4B). While this enabled us to directly compare the properties of HML-I and HMR-E in the same genomic context, it also raised the question of whether the observed configuration of chromatin around HMR-E was the same as that in its native location. Moreover, URA3 inserted near HMR-E or HML-I (Fig. 4) might contribute to or alter the chromatin structure at the silencers. To address these issues, we mapped chromatin around HML-I and HMR-E in their unmodified native locations in strain Y728 (SIR⁺) and its sir3⁻ derivative, Y729 (the parent of all the strains used in this work). As shown in Fig. 5, the pattern of nucleosomes positioning around HML-I or HMR-E in its unmodified genomic context in strain Y728 (SIR⁺) resembled that around its counterpart in strain 1 or 3, respectively (compare lanes 1 to 3 in Fig. 5 to 1 to 3 in Fig. 4A, and 10 to 12 as well as 16 to 18 in Fig. 5 to 1 to 3 in Fig. 4B; note the filled and dashed ellipses). SIR3 deletion had little or no effect on the arrangement of nucleosomes in the vicinity of the native HML-I and HMR-E silencers (Fig. 5, compare lanes 4 to 6 to lanes 1 to 3, 10 to 12 to lanes 7 to 9, and 16 to 18 with 13 to 15, respectively). These results suggest that the ability of HML-I or HMR-E to asymmetrically organize chromatin in its immediate vicinity is an intrinsic property that does not depend on its genomic context.

ORC plays a critical role in asymmetric chromatin organization and unidirectional silencing by the HMR-E silencer. The above results demonstrated that a stably positioned nucleosome on the ACS side in HMR-E or HML-I was lacking, which was correlated with a deficiency in silencing on this side of the silencer (summarized in Fig. 6A). It was possible that ORC bound to the silencer prevented nucleosome positioning near the 3' end of ACS (the direction of ACS is taken as 5'-WTTTAYRRTTW-3'). ORC is also involved in the initiation of DNA replication at ARSs (autonomous replication sequences) that are composed of the conserved ACS and Abf1p site as well as the poorly conserved B1 and B2 elements (Fig. 6A, ARS1). Interestingly, a unique chromatin configuration is maintained around ARS1, with two nucleosomes stably positioned near both the Abf1p-binding site and the ACS sequence, respectively (Fig. 6A, ARS1) (20). This configuration is important for ARS function, and Abf1p and ORC are responsible for its creation/maintenance (20, 37). Taken together, the features of chromatin organization at silencers and ARS1 (Fig. 6A) suggest that ORC allows the positioning of a nucleosome near the 5' end, but hinders nucleosome positioning near the 3' end, of ACS. If this were the case, then inverting ACS in HMR-E should lead to the positioning of a nucleosome near the ACS side of the silencer, which is predicted to allow URA3 silencing to occur on this side.

To test this idea, we set out to precisely invert ACS in HMR-E in strains 3 and 4 (Fig. 1B). For the convenience of cloning, we employed a shortened version of HMR-E, named HMR-E', that lacked the flanking sequence of ACS in the HMR-E silencer (Fig. 6B, left, HMR-E' versus HMR-E). We first made strains 13 and 16 that were identical to 3 and 4, respectively, except having HMR-E' in place of HMR-E in opposite directions (Fig. 6B, left). URA3 in strain 13, like that in 3, was not silenced by HMR-E' (Fig. 6B, compare 13 and 3), while silencing in strain 16 was comparable to that in 4 (Fig. 6B, compare 16 to 4). Therefore, deleting the flanking sequence of ACS from HMR-E had no detectable effect on its strength and directionality of silencing. Moreover, although the deletion caused some subtle changes in chromatin, it did not alter the overall pattern of asymmetric nucleosome positioning around the silencer (Fig. 6C and 4B, compare strains 3 and 13s to 13 and 13s, as well as 4 and 16s, respectively). For example, the MNase-hypersensitive site indicated by a dot in strain 13, 16s, or 16s (Fig. 6C) was closer to ACS in strain 13, was able to significantly silence URA3 in strain 13, like that in 3, was not silenced by HMR-E' (Fig. 6B, compare 13 and 3), while silencing in strain 16 was comparable to that in 4 (Fig. 6B, compare 16 to 4). Therefore, deleting the flanking sequence of ACS from HMR-E had no detectable effect on its directionality of silencing. Moreover, although the deletion caused some subtle changes in chromatin, it did not alter the overall pattern of asymmetric nucleosome positioning around the silencer (Fig. 6C and 4B, compare strains 3 and 13s to 13 and 13s, as well as 4 and 16s, respectively). For example, the MNase-hypersensitive site indicated by a dot in strain 13, 13s, 16, or 16s (Fig. 6C) was closer to ACS in HMR-E' compared to that in 3, 3s, 4, or 4s (Fig. 4B), but this did not affect the asymmetric nature of nucleosome positioning (Fig. 6C, 13, 13s, 16, and 16s). Therefore, HMR-E' was to a large extent structurally and functionally equivalent to the “full length” HMR-E.

We then precisely inverted ACS in HMR-E' in strain 13, resulting in HMR-E'm in strain 14 (Fig. 6B, left). Remarkably, we now observed relatively robust growth of strain 14 on FOA (Fig. 6B), indicating that HMR-E'm, in contrast to HMR-E' in strain 13, was able to significantly silence URA3. Therefore, the direction of ACS in HMR-E' is important for silencing. We demonstrated that nucleosome positioning around HMR-E'm in strain 14 was markedly different from that around HMR-E' in 13 (Fig. 6C, compare 14 and 13). Notably, there was a sharp decrease in the intensity of the band denoted by a dot in lanes 7 and 8 relative to that in lanes 1 and 2 in Fig. 6C. Moreover, sites a and b, inferred as the boundaries of nucleosomes 2 and 3 in strain 16, were also present in strain 14, albeit with reduced intensity (Fig. 6C and D, compare lanes 7 and 8 to 15 and 16).
As a consequence, the distribution of MNase-sensitive sites on the ACS side of HMR-E' in strain 14 started to resemble that on the Abf1p side of HMR-E' in strain 16 (Fig. 6D, compare 14 and 16). We interpreted these data as indicative of the presence of nucleosomes 1 to 3 near ACS of HMR-E' in strain 14 (Fig. 6C and D, lanes 7 and 8, filled ellipses 1 to 3) that were less stably positioned than those in strain 16 (Fig. 6C and D, lanes 15 and 16, filled ellipses 1 to 3). This might be the reason why URA3 silencing in strain 14 was weaker than that in 16 (Fig. 6B, compare 14 and 16), in light of the correlation between nucleosome positioning near a particular side of a silencer and preferential silencing on this side (see Fig. 6A). We noted that in strain 14s, the sir3- derivative of 14, the MNase cleavage site denoted by a dot was weaker than that in 13 and 13s but stronger than that in 14 (Fig. 6C, compare lanes 5 and 6 to 1 to 4 as well as 7 and 8). This, together with the fact that the site a, inferred as the border of nucleosome 1, was present in strain 14s (Fig. 6C, lanes 5 and 6), indicated that nucleosome 1 was positioned in strain 14s, albeit with reduced stability compared to that of 14. In other words, the Sir complex further stabilized nucleosome 1 (and induced the positioning of nucleosomes 2 and 3) in strain 14 (Fig. 6C, lanes 7 and 8), which was similar to the Sir-dependent stabilization/positioning of nucleosomes 1 to 3 in 16 (Fig. 6C, compare lanes 15 and 16 to 13 and 14). The above results demonstrated that inverting ACS in HMR-E' led to the positioning of a nucleosome near the ACS site (Fig. 6C, nucleosome 1 in strains 14 and 14s), which coincided with the occurrence of significant silencing on the ACS side of HMR-E' (Fig. 6B, strain 14).

We were also interested in whether ACS inversion had any effect on silencing on the Abf1p side of HMR-E'. We precisely inverted ACS in HMR-E' in strain 16, resulting in 17 (Fig. 6B, left). Silencing of URA3 in 17 was weaker than that in strain 16 (Fig. 6B, compare 17 to 16). Therefore, inverting ACS reduced silencing on the Abf1p side of HMR-E'. We also mapped chromatin around HMR-E'm in strain 17 and its sir3- derivative, 17s. The distribution of MNase-sensitive sites on the Abf1p side of HMR-E'm in strain 17 or 17s was similar to that in 16 or 16s (Fig. 6C, compare 9 and 10 to 15 and 16, as well as 11 and 12 to 13 and 14). This was consistent with the fact that significant silencing remained on the Abf1p side of HMR-E'm in strain 17 (Fig. 6B, 17).

On the basis that URA3 was silenced in strain 14 but not 13, one could conclude that ACS in its native orientation apparently serves as an obstacle to the initiation of silencing, presumably promoted by the rest of the HMR-E silencer consisting of the Abf1p and Rap1p-binding sites. If this were the case, then deleting ACS from HMR-E' in strain 13 should lead to URA3 silencing. To test this proposal, we replaced the 11-bp ACS consensus sequence in HMR-E' with the 6-bp restriction site for SpeI in strains 13 and 16, resulting in HMR-E'n in strains 15 and 18, respectively (Fig. 6B). HMR-E'n in strain 15 promoted significant silencing of URA3 (Fig. 6B, compare strain 15 to 13). This is consistent with the notion that ACS in HMR-E' serves as an inhibitor of the silencing function of the rest of the silencer consisting of the Abf1p and Rap1p sites (Fig. 6B, lack of silencing in strain 13). Moreover, we showed that deleting ACS allowed a nucleosome (labeled 1) to be positioned close to HMR-E'n in strain 15 (Fig. 6E, compare lanes 17 and 18 to 1 and 2; note the disappearance of a salient MNase site, labeled by a dot in lanes 17 and 18). The positioning of nucleosomes 2 and 3 in 15 likely reflected the spreading of Sir proteins (Fig. 6E). ACS deletion in the inverted HMR-E'n in strain 18 led to a moderate decrease in URA3 silencing (Fig. 6B, compare 18 to 16) but did not significantly affect nucleosome positioning on the Abf1p side of the silencer (Fig. 6B, compare lanes 19 and 20 to 15 and 16). Therefore, ORC at HMR-E' in strain 16 helps silencing on the Abf1p side (Fig. 6B) likely by increasing the local concentration of Sir proteins, as it can recruit Sir2p/Sir4p via Sir1p.

The above results provided direct evidence for a role of ORC in the establishment of asymmetric nucleosome positioning and directional silencing by HMR-E. We think that at HMR-E, ORC helps the initiation of silencing on the Abf1p side by recruiting Sir proteins but at the same time hinders silencing on the ORC side by preventing the positioning of a nucleosome. When ACS is inverted or deleted from HMR-E', the resultant silencer (HMR-E'm or HMR-E'n) becomes bidirectional (Fig. 6B, compare 14 to 17 and 15 to 18). Therefore, ORC plays a critical role in making HMR-E' function in a unidirectional fashion.

Role of Abf1p in nucleosome positioning and transcriptional silencing. Of the three silencer-binding proteins, Abf1p is the least studied concerning its role in the initiation of silencing. A comparison of the configurations of chromatin in the vicinities of HMR-E, HML-1, and ARS1 revealed that a nucleosome was always positioned near the Abf1p site, which coincided with strong silencing on this side of the silencers (Fig. 6A). Like ACS, the sequence of the Abf1p-binding site is also asymmetric (see the legend to Fig. 1A), and its direction might also be important for its function in silencers. We therefore examined the effect of inverting the Abf1p site in HMR-E on nucleosome positioning and silencing. Again, for the convenience of cloning, we used a shortened version of HMR-E, HMR-E', that lacked the flanking sequence of the Abf1p site in HMR-E (Fig. 7A, compare HMR-E' to HMR-E). Strains 19 and 22 were identical to 3 and 4, respectively, except they bore HMR-E' in place of HMR-E in opposite directions (Fig. 7A, left). URA3 silencing in strains 19 and 22 was comparable to that in 3 and 4, respectively (Fig. 7A, compare 19 and 22 to 3 and 4, respectively). Therefore, HMR-E' was equivalent to HMR-E regarding the potency and directionality of silencing. The pattern of chromatin organization around HMR-E' was also similar to that around HMR-E, despite the fact that there were some changes in the distribution of MNase sites (Fig. 4B and 7B, compare 22 to 4; note the inferred presence of nucleosomes 1 to 3 in both strains 4 and 22). We then precisely inverted the Abf1p site in HMR-E' in strains 19 and 22, resulting in 20 and 23, respectively (Fig. 7A, left). URA3 silencing in strains 20 and 23 was comparable to that in 19 and 22, respectively (Fig. 7A, compare 19 and 22 to 3 and 4, respectively). Therefore, HMR-E' was equivalent to HMR-E regarding the potency and directionality of silencing. The pattern of chromatin organization around HMR-E' was also similar to that around HMR-E, despite the fact that there were some changes in the distribution of MNase sites (Fig. 4B, compare 22 to 4). In summary, the presence of ACS on the Abf1p side of HMR-E led to the formation of a nucleosome near this side of the silencers, which together with the correlation between nucleosome positioning and silencing, suggests that ACS is involved in the initiation of silencing on the Abf1p side.
Abf1p played no role in silencing. To test this hypothesis, we mutated the Abf1p site (ATCATaaaATG→ATgATaaaATG; the conserved nucleotides are capitalized, and mutated nucleotides are italicized) in HMR-E in strain 22, making 24 (Fig. 7A). Previous studies have demonstrated that such a mutant site would not bind Abf1p in vitro or in vivo (17). URA3 silencing in strain 24 was significantly reduced compared to that in 22 (Fig. 7A, compare 24 to 22), demonstrating that an intact Abf1p site was necessary for efficient silencing by HMR-E\(^{-}\). Chromatin configuration in the vicinity of HMR-E\(^{-}\) in strain 24 was markedly different from that in the vicinity of HMR-E\(^{-}\) in strain 22 (Fig. 7C, compare 24 and 22). For example, sites a and c present in strain 22 were diminished in strain 24, whereas a’ appeared in strain 24 (Fig. 7C). It could be inferred that putative nucleosomes 1’ to 3’ were positioned at or near the mutated Abf1p site in strain 24 (Fig. 7C). However, compared to nucleosomes 1 to 3 in strain 22, 1’ to 3’ were less closely spaced (Fig. 7C, compare 24 and 22; note that the inferred nucleosomes 1’ to 3’ spanned a longer sequence than 1 to 3). This was correlated with reduced silencing in strain 24 compared to that in strain 22 (Fig. 7A). Based on these results, we propose that Abf1p contributes to silencer function by, at least in part, positioning nucleosomes, which is independent of the orientation of its binding site. This is reminiscent of its role in the initiation of DNA replication at ARS1 (20).

**DISCUSSION**

The silencers in *S. cerevisiae* present an excellent model system for studying the mechanisms of the establishment of transcriptionally silent chromatin in eukaryotes (13, 32). In this work we have investigated the directionality of silencer function and how it is determined. For both HML-I and HMR-E, which are structurally similar, silencing was found to preferentially occur on the Abf1p sides of the silencers (Fig. 6A). This is in accord with data from previous studies demonstrating unequal silencing on the two sides of HML-I or HMR-E (2, 34). A comparison of data obtained here and in previous studies indicates that the difference in silencing efficiency between the two sides of HMR-E may vary depending on the assay employed (2, 5, 34, 43). This is because the apparent strength of silencing on either side of a silencer may be affected by non-silencer factors, including its genomic location (43). Different genomic loci may contain distinct sets of elements that could influence silencer function (e.g., other silencers and protosilencers) (12, 27, 43). We have recently shown that a protosilencer located on the ORC side of HMR-E could enhance the efficiency of silencing on the ORC side to a level that is comparable to that on the Abf1p side, thereby masking the directionality of the silencer (43). Along this line, the presence of HMR-I at HMR likely masked the directionality of HMR-E in silencing HMRa observed in an early experiment done by Brand et al. (5). Consistently, silencing of a1 by HMR-E alone was stronger on the Abf1p side than the ORC side (5) (Fig. 2). The efficiency of silencing on either side of a silencer is also dependent on the promoter strength of the reporter gene (36). For example, we found that on the ORC side of HMR-E, URA3 was completely depressed, whereas a1 or a1 was still silenced, albeit partially (Fig. 2).

Chromatin plays an important role in the regulation of cellular transactions involving DNA. It has been well established that correct nucleosome positioning at promoters and replication origins is crucial for the initiation of transcription and replication, respectively. We have demonstrated in this work that chromatin also plays a key role in determining the directionality of a silencer. We found that nucleosomes are asymmetrically positioned around the HMR-E and HML-I silencers. Specifically, a nucleosome is stably positioned close to the Abf1p site but not the ORC recognition site of the silencers, which coincides with robust silencing on the Abf1p side and reduced silencing on the ORC side (Fig. 6A). Importantly, asymmetric nucleosome positioning at a silencer is largely independent of its orientation and genomic location. It is also independent of the Sir proteins and therefore happens before the establishment of silent chromatin. We propose that the asymmetry in chromatin structure at a silencer determines its directional nature of silencing. Specifically, we envision that the nucleosome positioned close to the Abf1p side of the silencer allows or facilitates the Sir complex to start spreading on this side, whereas the lack of a stably positioned nucleosome adjacent to the ORC site reduces the efficiency of initiation of Sir propagation on this side. This hypothesis is based on the fact that nucleosomes are both the substrates for the histone deacetylase activity of the Sir complex and also the platform for Sir recruitment, according to the current view of the nucleosome-by-nucleosome propagation of Sir complexes along chromatin (3, 13, 32). In support of this contention, we have demonstrated that Sir3p preferentially associates with sequences on the Abf1p side of the HMR-E silencer (Fig. 3).

How is asymmetric nucleosome positioning established at silencers? To answer this question, we turned to known examples of chromatin organization at gene promoters and replication origins for clues. At promoters, transcriptional activators can induce changes in chromatin structure via their own nucleosome disrupting/positioning activities and/or by recruiting chromatin remodeling complexes. Of particular interest is chromatin reorganization by Rap1p and Abf1p at the upstream activating sequences of the genes they regulate (18, 24). Rap1p or Abf1p is believed to facilitate the access of primary transcriptional activators such as Gcn4p to their binding sites located nearby by disturbing nucleosomes bearing its binding sites, thereby “opening” chromatin (41, 42). Note that by preventing nucleosome formation on the sequence containing its binding site, Abf1p/Rap1p may help define the boundary of an array of nucleosomes, thereby serving to position nucleosomes. Chromatin also plays an important role in the initiation of DNA replication. The ARS sequences consisting of ACS, B1, B2, and Abf1p sites are free of nucleosomes, whereas two nucleosomes are stably positioned near the Abf1p and ORC sites of ARS (Fig. 6A) (20). This pattern of nucleosome arrangement is accomplished by combined actions of Abf1p and ORC, and its perturbation affects the formation of the prereplication complex and disrupts replication initiation (20).

In light of the roles of Abf1p, Rap1p, and ORC in chromatin organization at gene promoters and replication origins, these factors may also be responsible for the establishment of asymmetric nucleosome positioning at silencers. A comparison of chromatin configurations around silencers and ARS suggests that ORC allows the positioning of a nucleosome near the 3’ end of ACS but excludes one near the 5’ end (Fig. 6A). In
accompany with this, we showed that inverting ACS in HMR-E so that it was orientated toward the inside of the silencer led to the positioning of a nucleosome near the ACS side of the silencer and a concomitant significant increase in silencing on this side. As a result, there appeared to be symmetric positioning of nucleosomes and comparable silencing on the two sides of the modified HMR-E. On the other hand, inversion of ACS in an ARS disrupts ARS function (14), likely because it disrupts the nucleosome normally positioned near ACS outside of ARS (see Fig. 6A) that is important for ARS function. These results suggest that ACS (via ORC) asymmetrically positions nucleosomes on its sides, which is likely the reason why its direction is important for its function. We have also found that removing ACS from HMR-E led to symmetric positioning of nucleosomes and comparable silencing on both sides, which further demonstrated that ORC in its native orientation serves as an obstacle to silencing on the ACS side.

The fact that the Abf1p site in a silencer or ARS is associated with a stably positioned nucleosome nearby led us to propose that Abf1p binding is responsible for nucleosome positioning. Consistent with this hypothesis, we demonstrated that mutating the Abf1p-binding site in HMR-E caused clear changes in nucleosome positioning as well as a significant reduction in silencing on the Abf1p side of the silencer. However, inverting the direction of the Abf1p site in HMR-E caused little or no change in chromatin configuration or silencing. Therefore, we think that Abf1p can position nucleosomes on both sides of its site. We conclude that both ORC and Abf1p play major roles in promoting asymmetric positioning of nucleosomes on the two sides of HMR-E or HML-I, which determines the directional nature of silencing by the silencer.

In summary, results presented in this work demonstrate that a yeast silencer (via the silencer-binding proteins) promotes asymmetric positioning of nucleosomes, which is independent of its orientation or genomic location. We think such a structural asymmetry results in distinct potentials for the initiation of transcriptional silencing on the two sides of the silencer.

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