Functions of Protosilencers in the Formation and Maintenance of Heterochromatin in *Saccharomyces cerevisiae*

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Abstract

In *Saccharomyces cerevisiae*, transcriptionally silent heterochromatin at HML and HMR loci is established by silencers that recruit SIR complex and promote its propagation along chromatin. Silencers consist of various combinations of two or three binding sites for origin recognition complex (ORC), Abf1 and Rap1. A single ORC, Abf1 or Rap1 site cannot promote silencing, but can enhance silencing by a distant silencer, and is called a protosilencer. The mechanism of protosilencer function is not known. We examine the functions of ORC, Abf1 and Rap1 sites as components of the HMR-E silencer, and as protosilencers. We find that the Rap1 site makes a larger and unique contribution to HMR-E function compared to ORC and Abf1 sites. On the other hand, Rap1 site does not act as a protosilencer to assist HML-E silencer in forming heterochromatin, whereas ORC and Abf1 sites do. Therefore, different mechanisms may be involved in the roles of Rap1 site as a component of HMR-E and as a protosilencer. Heterochromatin formed by ORC or Abf1 site in collaboration with HML-E is not as stable as that formed by HMR-E and HML-E, but increasing the copy number of Abf1 site enhances heterochromatin stability. ORC and Abf1 sites acting as protosilencers do not modulate chromatin structure in the absence of SIR complex, which argues against the hypothesis that protosilencers serve to create a chromatin structure favorable for SIR complex propagation. We also investigate the function of ARS1 containing an ORC site and an Abf1 site as a protosilencer. We find that ARS1 inserted at HML enhances heterochromatin stability, and promotes de novo formation of a chromatin structure that partially resembles heterochromatin in an S phase dependent manner. Taken together, our results indicate that protosilencers aid in the formation and maintenance of heterochromatin structure.

Introduction

Transcriptional silencing in *Saccharomyces cerevisiae* is a form of region specific gene repression that exists at the HML and HMR loci and subtelomeric regions [1]. It is mediated by heterochromatin established via the association of the SIR silencing complex consisting of Sir2 through Sir4 with nucleosomes. Heterochromatin is a stable but dynamic structure [2]. It is relatively refractory to DNA modifying and repair enzymes as well as endonucleases [3–7]. On the other hand, it is permissive to homologous or site-directed recombination as well as transposon integration [8–10]. Nucleosomes in heterochromatin are generally regularly ordered and are hypoacetylated compared to those in euchromatin [11–14]. As a reflection of the special structure of heterochromatin, DNA in heterochromatin is more negatively supercoiled than that in euchromatin [8,9].

Formation of heterochromatin at the cryptic mating loci HML and HMR is promoted by small cis-acting elements called the E and S silencers flanking these loci [1]. Silencers each contain two or three recognition sites for ORC (origin recognition complex for DNA replication), Rap1 and Abf1. These silencer-binding proteins can interact with the Sir3 and Sir4 proteins in the SIR complex on their own or through Sir1 thereby recruiting them to the silencers. Sir2 is a histone deacetylase that is responsible for hypoacetylation of heterochromatin [15]. The SIR complex also binds to nucleosomes with a strong preference for unacetylated ones [16–21]. In addition, SIR complex self interacts and is able to form multisubunit chains. The current model for the de novo formation of heterochromatin proposes that SIR complexes recruited to a silencer deacetylate histones in adjacent nucleosomes. The newly deacetylated nucleosomes then bind additional SIR complexes. Through repeated cycles of histone deacetylation and SIR complex recruitment, SIR complexes are believed to spread along a continuous array of nucleosomes during which the primary chromatin structure pertaining to the distribution of nucleosomes along DNA is altered [1,11,12,22]. The spreading model for heterochromatin formation is supported by our finding that nucleosome-excluding structures can block the propagation of heterochromatin [23].

The function of a silencer is affected by other silencers or protosilencers present in its surroundings. Protosilencers are DNA
elements that can enhance the activity of a silencer at a distance without the ability to act as bona fide silencers on their own [24]. Single recognition sites for silencer-binding proteins have protosilencer activities [2,24–27]. Silencers and protosilencers are collectively referred to as silencing elements. There have been many documented examples of two silencing elements cooperating to promote stronger silencing, but the underlying mechanisms have not been resolved [2,24–27]. The fact that two silencers separated by up to several kb are able to cooperate to silence a reporter gene located between them can be explained by assuming that convergent spreading of Sir proteins emanating from the silencers is additive or synergistic, so that heterochromatin established between the silencers is stronger than that formed by either silencer alone [28]. However, this interpretation does not apply to silencer-protosilencer cooperation since a protosilencer is not able to initiate de novo silencing. It is possible that distant silencing elements cooperate by physically interacting with each other or with a common nuclear structure to create a stronger platform for recruiting Sir proteins [24,26]. Alternatively, or in addition, because a protosilencer is actually a binding site for ORC, Abf1 or Rap1 that has the potential of positioning nucleosomes [23,29–33], it is conceivable that a protosilencer modulates nucleosome positioning in the region between it and the silencer in a configuration that is more favorable for SIR complex spreading from the silencer [27]. However, definitive evidence for either hypothesis is lacking.

In this work, we examined the functions of the ORC, Rap1 and Abf1 binding sites as constituents of the HMR-E silencer, and as protosilencers. We found that the Rap1 site played a larger role than ORC or Abf1 site in HMR-E function, and imparted a unique property to the silencer. On the other hand, the ORC and Abf1 sites aided in the formation of heterochromatin by the HML-E silencer, but Rap1 site did not. ORC and Abf1 sites acting as protosilencers did not affect local chromatin structure in the absence of SIR complex, which argues against the hypothesis that a protosilencer assists the formation of heterochromatin by creating a chromatin structure favorable for the spread of SIR complex. We also examined the function of ARS1 containing an ORC site and an Abf1 site as a protosilencer. We found that ARS1 inserted at HML significantly enhanced the stability of HML heterochromatin, and had the ability to promote de novo formation of a SIR-dependent chromatin conformation that partially resembled heterochromatin.

Materials and Methods

Plasmids

Plasmid pYZ167-4 was made by replacing the EcoRI-HindIII fragment of pUC19 with an EcoRI-HindIII fragment corresponding to coordinates 290027 to 291560 of chromosome III that contains the HMR-E silencer (291245 to 291560). The KanMX cassette was inserted at the EcoRV site of pYZ167-4 to make pQY298. pQY299 was derived from pQY298 by replacing the Abf1-binding site (BS) (5’-TCATATAATACGAGC-3’) in HML-E with an MfeI restriction site (CAATTG) via site-directed mutagenesis. pQY300 and 301 were similarly made by replacing the ORC-BS (TAAATATANAA) and Rap1-BS (AAAGCCCATCAAAGGTTT) in HMR-E with SpeI sites (ACTAGT). The genomic fragment HindIII-HMR-I-HindIII (928927–294210) from chromosome III was inserted into pUC12, making pUC-HMR. The MfeI-HMR-I-XhoI fragment of pUC-HMR was replaced by an MfeI-HIS3-XhoI fragment to make pQY321. Plasmid pQY226 was made by first replacing the AatII-BamHI fragment of pUC12 with the AatII-BamHI fragment of chromosome III (12139–16269) containing the HML-I silencer, and then replacing the HpaI-HML-I-HindIII fragment with HindIII-HMR-E-HindIII fragment, followed by inserting the URA3 gene at the EcoRV site. The Rap1-BS and Abl1-BS in HMR-E in pQY226 were replaced by SpeI and MfeI sites, respectively, via site-directed mutagenesis to make plasmid pLO29. The ORC-BS and Rap1-BS in HMR-E in pQY226 were replaced by SpeI and MfeI sites, respectively, to make plasmid pLO28. The Orc-BS, Rap1-BS and Abl1-BS in HMR-E in pQY226 were replaced by KpnI, SpeI and MfeI sites, respectively, to make plasmid pLO30. The Rap1-BS from HMR-E was inserted at the MfeI site of pLO30 to make plasmid pLO40. The Abl1-BS from HMR-E was inserted at the SpeI site of pLO28 to make pLO33. Abl1-BS was inserted at the KpnI site of pLO33 to make pLO34. Plasmid pXB131-1 was made by inserting a BsrGI-ARS1-BsrGI sequence of chromosome IV (462460–262670) into plasmid pXB5 [8]. Plasmid pUC26 was made by inserting the BamHI-HML-BamHI fragment (9666 to 16269 of chromosome III) into pUC12. Plasmid pYZ121 was made by replacing the HpaI-HML-I-HindIII fragment of pUC26 with HindIII-HMR-E-HindIII fragment, and inserting URA3 gene at the BspHI site. The mutant alleles of HindIII-HMR-E-HindIII from plasmids pLO29, pLO28, pLO40, pLO30, pLO33 and pLO34 were used to replace the HindIII-HMR-E-HindIII fragment in pYZ121 to make pXZ31, pXZ33, pXZ34, pXZ35, pXZ37 and pXZ38, respectively.

Yeast strains

Strains 1, 3, 5 and 7 (Table S1) were made by transforming strain CCY101 to G418 (geneticin)-resistance with EcoRI and XbaI digested plasmids pQY298 through 301, respectively. The ΔS12 coding region in strains 1, 3, 5 and 7 was replaced by NatMX to make strains 2, 4, 6 and 8, respectively. Strains 1, 3, 5 and 7 were transformed to His+ by plasmid pQY321 digested with AatII and XbaI, making strains 9, 11, 13 and 15, respectively. Strains 10, 12, 14 and 16 were derived from 9, 11, 13 and 15, respectively, by replacing ΔS12 with NatMX. Strains 17, 18, 19, 20, 21, 22 and 23 were made by transforming strain YXB6 to Ura˚ with BspHI and NgoMIV digested plasmids pQY226, pLO29, pLO28, pLO40, pLO30, pLO33 and pLO34, respectively. The SIR3 gene in strains 17 through 21 were replaced with KanMX, making strains 17s through 21s, respectively. Strains 17n through 23n were made by transforming strain YXB6 to Ura˚ with BplI and NgoMIV digested plasmids pYZ121, pXZ31, pXZ33, pXZ34, pXZ35, pXZ37 and pXZ38, respectively. Strain 24 was made by transforming Y2047b to carbenicillin resistance with BamHI and NgoMIV digested plasmid pXB131-3. Strain 24s was derived from 24 by disrupting its SIR3 gene with BplI as described [34]. Strain 25 was from E. Xu and J.R. Broach (Princeton University). Strain 26 was made by transforming YXB5s to G418 resistance with Thr1111 digested plasmid pUC-SK [35]. Strain 27 was similarly derived from YXB125s.

Analysis of the supercoiling of DNA circles from yeast

Yeast cells were grown in YPR medium (1% yeast extract, 2% bacto-peptone and 2% raffinose). When needed, galactose was added to YPR cultures at a concentration of 2% 8-factor, hydroxyurea (HU), and nocodazole were used at 10 μg/ml, 0.2 M and 20 μg/ml, respectively. Nucleic acids were isolated from yeast cultures using the glass bead method and fractionated on agarose gels in 0.5 × TPE (45 mM Tris, 45 mM phosphate, 1 mM EDTA, pH 8.0) supplemented with chloroquine. DNA circles were detected by Southern blotting.
Chromatin mapping by micrococcal nuclease (MNase) digestion and indirect end-labeling

This was done as described before [23,36]. Briefly, about 2 x 10^6 permeabilized spheroplasts prepared from log phase cells were treated with MNase at 15 and 30 units/ml, respectively, at 37°C for 4 minutes, and the DNA was isolated. DNA in each sample was then digested with SnaBI and EcoNI, and run on a 1.0% agarose gel. Relevant DNA fragments were visualized by using a specific probe after Southern blotting.

Results

The ORC-, Abf1- and Rap1-binding sites in the HMR-E silencer differentially contribute to its silencing function

The HMR-E silencer is composed of one each of ORC-, Abf1- and Rap1-binding sites (abbreviated as -BSs hereafter). It was originally shown that deletion of any one of these sites did not affect the silencing of the resident HMRal gene at HMR, whereas deletion of any two sites abolished HMRal silencing [37]. This result suggests that ORC-, Abf1- and Rap1-BSs play similar and redundant roles in the function of HMR-E. Since the apparent efficiency of silencing by a silencer depends on the strength of the promoter of the reporter gene [30], the sensitivity/resolution of a silencing assay may depend on the reporter gene used. We attempted to further examine the contributions of the ORC-, Abf1- and Rap1-BSs to HMR-E function using an alternative reporter gene TRPI that is required for tryptophan biosynthesis. Strain 1 has its endogenous TRPI gene removed, and has TRPI with its own promoter inserted within HMR [Fig. 1A, left]. It also has the URA3 reporter gene inserted near the right telomere of chromosome V (Tel V-R) [Fig. 1A, left]. Therefore, strain 1 allows for simultaneous examination of both HMR and telomere silencing. Silencing of TRPI was robust as cells failed to grow on medium lacking tryptophan (-Trp) [Fig. 1A, -Trp panel, 1]. Deletion of ORC- or Abf1-BS from HMR-E had no effect on TRPI silencing [Fig. 1A, -Trp panel, 3 and 5], which is consistent with results from earlier studies on HMRal silencing [37]. However, removal of Rap1-BS from HMR-E significantly reduced TRPI silencing [Fig. 1A, -Trp panel, compare 7 with 1]. Therefore, using TRPI as a silencing reporter, we have revealed that Rap1-BS in HMR-E contributes more to HMR-E function than the ORC- or Abf1-BS. Removal of Rap1-BS prevents Rap1 from binding to HMR-E, which is likely the cause of the reduction in silencing. In the meantime, removal of Rap1-BS in strain 7 also decreases the distance between ORC- and Abf1-BSs in HMR-E, which may also affect the efficiency of silencing. URA3 near Tel V-R was silenced, which was not affected by the mutations of the HMR-E silencer, as expected [Fig. 1A, robust growth of strains 1, 3, 5 and 7 on FOA medium; note cells expressing URA3 are sensitive to killing by FOA, 5-fluoro-orotic acid].

The HMR-E silencer plays an auxiliary role in HMR silencing [39]. The roles of the ORC- and Abf1-BSs in HMR-E function might be better revealed in the absence of HMR-I. Along this line, we deleted HMR-I from strains 1, 3, 5 and 7 to make strains 9, 11, 13 and 15, respectively [Fig. 1B, left]. In the absence of HMR-I, TRPI silencing by HMR-E was moderately reduced [Fig. 1B, -Trp panel, compare 9 with 1]. TRPI silencing by HMR-EA1 (HMR-E lacking Abf1-BS) was slightly less efficient than that by HMR-E [Fig. 1B, -Trp panel, compare 11 with 9]. Therefore, Abf1-BS was mostly dispensable for TRPI silencing even in the absence of HMR-I. On the other hand, deletion of ORC-BS markedly reduced TRPI silencing [Fig. 1B, -Trp panel, compare 13 with 9], and Rap1p-BS deletion eliminated TRPI silencing [Fig. 1B, -Trp panel, compare 15 with 9]. Taken together, the above results demonstrate that Abf1-, ORC- and Rap1-BSs make increasingly larger contributions to silencing by the HMR-E silencer.

The positive regulation of HMR-E by SAS2 depends on the presence of the Rap1-BS, not ORC-BS or Abf1-BS of the silencer

SAS2 encoding a histone H4 acetyltransferase is required for telomeric silencing and full silencing by the HMR-E silencer [40–43]. However, SAS2 plays an inhibitory role in silencing by HMR-E with both its Rap1- and Abf1-BSs mutated [40,44]. It is possible that the positive role of SAS2 in HMR-E function depends on the presence of Rap1-BS and/or Abf1-BS in the silencer. We set out to determine whether it is Rap1- or Abf1-BS that is required for SAS2 to positively regulate HMR-E. To this end, we deleted SAS2 from strains 1, 3, 5, 7, 9, 11, 13 and 15, making strains 2, 4, 6, 8, 10, 12, 14 and 16, respectively [Fig. 1].

We showed that in the presence of HMR-I, sas2A significantly enhanced TRPI silencing by HMR-EA1 [Fig. 1, note that growth of strain 5 on –Trp medium was significantly less robust than that of strain 7], suggesting that SAS2 negatively regulates the function of HMR-EA1. On the other hand, TRPI silencing by HMR-E was also decreased by sas2A, albeit to lesser extents [Fig. 1B, note that growth of 12 and 14 was moderately more robust than 11 and 13, respectively, on –Trp medium]. HMR-EAR failed to silence TRPI, which was not affected by sas2A [Fig. 1B, -Trp, compare 2, 4 and 6 with 1, 3 and 5, respectively].

In the absence of HMR-I, sas2A markedly reduced TRPI silencing by HMR-E [Fig. 1B, compare 10 with 9]. TRPI silencing by HMR-EA1 or HMR-EAO was also decreased by sas2A, albeit to lesser extents [Fig. 1B, note that growth of 12 and 14 was moderately more robust than 11 and 13, respectively, on –Trp medium]. However, removal of Rap1-BS from HMR-I signifi cantly reduced TRPI silencing [Fig. 1A, -Trp panel, compare 11 with 9]. In the absence of HMR-I, sas2A abolished the silencing of URA3 near Tel V-R, which is independent of the status of TRPI silencing at HMR [Fig. 1, FOA panel].

The above results suggest that SAS2 positively regulates the function of HMR-I, as well as HMR-EA1 and HMR-EAO, but negatively regulates HMR-EAR. Therefore, the presence of Rap1-BS in HMR-E imparts a unique property to the silencer regarding regulation by SAS2.

The ORC-, Abf1- and Rap1-BSs from the HMR-E silencer have distinct protosilencer activities

The fact that individually deleting the ORC-, Abf1- and Rap1-BSs from HMR-E reduces the silencing function of the silencer to different extents demonstrates that these elements do not contribute equally to HMR-E function [Fig. 1]. It is not known whether the activities of these elements in the context of HMR-E silencer are related to their functions as protosilencers. To address this question, we set out to examine the ability of each element to facilitate the HML-E silencer in establishing heterochromatin. The structure of heterochromatin was examined by probing the topology of its DNA. This method is based on the fact that formation of a nucleosome constrains on average one negative supercoil on nucleosomal DNA, which is reduced by nucleosome acetylation, and hence the negative supercoiling of euchromatic DNA in a locus is mainly determined by nucleosome density and conformation [45,46]. Consistently, we and others have previously shown that DNA in heterochromatin at HML or HMR is characteristic ly more negatively supercoiled when the locus is silenced than when it is derepressed [8,9].

We replaced the HML-I silencer at HML with the HMR-E silencer or its ORC-, Abf1- or Rap1-BS in a strain designed for measuring the supercoiling of HML DNA [Fig. 2A, top]. In this strain, a BstBI restriction fragment containing the promoters and
part of the coding regions of the HMLa genes was replaced by a sequence from the bacterial lacZ gene (designated b2) [8]. The modified HML locus (HML9) excluding the silencers was bracketed by two copies of FRT (Flp1 recombination target), recognition sites for the site-specific recombinase Flp1 (Fig. 2A, top). Induction by galactose of a PGAL-FLP1 fusion gene resident elsewhere in the genome would cause recombination between the FRTs resulting in the excision of HML9 as a minichromosome circle (Fig. 2C).

Upon deproteinization, the supercoiling of the DNA circle can be examined. In addition, this strain also bears a URA3 gene to the right of HML9 (Fig. 2A, top). Note that silencing by HMR-E is directional: robust silencing exists on its Abf1 side but not its ORC side [27,32]. As HMR-E is oriented away from HML9 in strain 17 (Fig. 2A), it would promote URA3 silencing as a silencer, and contribute to heterochromatin within HML9 mainly in the capacity of a protosilencer. Therefore, the set of strains shown on the left of Fig. 2A allow for the examination of both the abilities of HMR-E or its protosilencer constituents to silence the URA3 gene to the right of HML9 and to cooperate with HML-E silencer to establish heterochromatin structure within HML9.

We found that HMR-E in place of HML-I silenced URA3, but the ORC- Abf1- or Rap1-BS did not (Fig. 2A, right, note the minimum growth on –Ura medium and robust growth on FOA medium of strain 17, and robust growth on –Ura medium and lack of growth on FOA medium of strains 18 through 21). This confirms that HMR-E as a silencer can initiate silencing, whereas the ORC-, Abf1- or Rap1-BS as a protosilencer cannot.

We also examined the abilities of HMR-E and its protosilencer components to collaborate with HML-E to promote silencing within HML9 in strains 17n through 21n that were similar with strains 17 through 21, but had URA3 placed within HML9 (Fig. 2B, left). Robust URA3 silencing was found in strains 17n and 18n (Fig. 2B); URA3 silencing also existed in strain 19n albeit to a lesser extent than that in strains 17n and 18n (Fig. 2B). On the other hand, URA3 was not silenced in strain 20n or 21n (Fig. 2B). These results demonstrate that ORC- or Abf1-BS, but not Rap1-BS, can cooperate with HML-E to promote transcriptional silencing within the region they bracket. The order of the protosilencer activities of ORC-, Abf1- and Rap1-BSs is ORC-BS > Abf1-BS > Rap1-BS.

We next examined the topology of HML9 DNA as a proxy of chromatin structure in strains 17 through 21 as well as their sir2 (sir3Δ) derivatives (strain 17s through 21s, respectively). This was achieved by inducing the excision of HML9 circles in these strains, and subjecting them to agarose gel electrophoresis in the presence of the DNA intercalator chloroquine that resolves the topoisomers of a DNA circle according to their supercoiling (Fig. 2D, left; Fig. S1). Under the electrophoresis conditions used in this work, a more negatively supercoiled topoisomer migrated more slowly in...
the gel. The center of distribution of all the topoisomers of a circle is an indicator of the overall supercoiling of the circle (Fig. 2D, open dots).

As shown in Fig. 2D, the topoisomers of the HML' circle in strain 17 migrated markedly more slowly than those from strain 17s where HML' chromatin was derepressed (compare 17 and 17s). Therefore, HML' circle from strain 17 exhibited higher negative supercoiling than that from 17s, indicating that HMR-E together with HML-E promoted the formation of heterochromatin. The topology of HML' DNA in strain 17s was similar with that in strain 17s (Fig. S1), demonstrating that derepressed chromatin at HML' was not affected by the presence of any of the silencing elements in place of HML-I silencer.

The negative supercoiling of HML' circle in strain 21 was significantly reduced compared to that in strain 17, but was slightly higher than that from strain 17s (Fig. 2D, compare 21 with 17 and 17s). This suggests that in strain 21, HML-E alone cannot establish fully mature heterochromatin. The supercoiling of HML' DNA in strain 20 was similar to that in strain 21, suggesting that the Rap1-BS does not significantly enhance the ability of HML-E to form heterochromatin.

The topoisomers of HML' circle from strain 18 consisted of two distinct portions with one migrating similarly as HML' circles from strain 17 (designated SIR+) and the other as the HML' circles from the sir- strain 17s (designated sir-) (Fig. 2D, compare 18 with 17 and 17s). A similar result was obtained for strain 19. We have
previously shown that silent HML circles without silencers would gradually lose their high negative supercoiling and assume a topology similar to circles in sir− cells when the host cells progress in the cell cycle, suggesting that heterochromatin dissociated from silencers is subject to disruption during cell cycle progression (Fig. 2C) [8]. The HML circles excised from strains 18 and 19 (as well as strains 17, 20 and 21) all lack silencers. The sir− circles in strain 18 or 19 were therefore the result of disruption of heterochromatin on HML′ circle during the 2.5 hr galactose induction for circle excision in which cells continued to grow. The fact that sir− circles existed in strains 18 and 19 but not 17 suggests that heterochromatin formed at HML′ in the presence of ORC- or Abf1-BS is more susceptible to disruption than that formed in the presence of HMR-E. In other words, heterochromatin formed by ORC- or Abf1-BS together with HMLE-E silencer is less stable compared to that formed by HMR-E and HMLE-E silencers. Note that the relative abundance of sir− circles in strain 19 was detectably more than that in strain 18 (Fig. 2D, compare 19 with 18), suggesting that heterochromatin formed by Abf1-BS is moderately less stable than that formed by ORC-BS.

Taken together, results from the above analyses of HML′ DNA topology suggest that ORC-, Abf1- and Rap1-BSs from the HMR-E silencer have distinct abilities to cooperate with HML′-E to form heterochromatin structure. ORC-BS has the strongest ability, and Rap1-BS the weakest.

To complement the DNA topology-based assay of chromatin state of HML′ in strains 17 through 21, we also mapped HML′ chromatin with micrococcal nuclease (MNase) digestion and indirect end-labeling [36]. Results from this experiment revealed that HML′ chromatin in strains 17 through 21 exhibits heterochromatic (SIR-dependent) characteristics to various degrees, with strain 17 having the most heterochromatic characteristics, and strains 20 and 21 having the least, and strain 18 having more heterochromatic characteristics than 19 (Fig. S2). This suggests that the order of the abilities of ORC-, Abf1- and Rap1-BSs to contribute to the formation of heterochromatin structure is ORC-BS > Abf1-BS > Rap1-BS, which is consistent with our conclusion on the order of activities of these protosilencers based on data from analyzing gene silencing and DNA topology at HML′ (Fig. 2B and 2D). This further validates the use of DNA supercoiling as an indicator of chromatin state.

In summary, results from our studies of gene silencing, DNA topology and primary chromatin structure at HML′ in strains 17n to 21n and 17 to 21 demonstrate that the HMR-E silencer is able to cooperate with HML′-E to form robust, stable heterochromatin, whereas the ORC- or Abf1-BS can work with HML′-E to form a heterochromatin structure with reduced stability. On the other hand, the Rap1-BS is not able to assist HML′-E in establishing heterochromatin. This is in contrast to the fact Rap1-BS as part of HMR-E makes a greater contribution to silencer function than ORC- and Abf1-BS (Fig. 1).

Additive effects of multiple copies of Abf1-BS on the maintenance of heterochromatin

The fact that heterochromatin formed by Abf1-BS was not as stable as that formed by HMR-E together with HML′-E (Fig. 2D) prompted us to ask whether increasing the copy number of Abf1-BS could make heterochromatin more stable. To this end, we made strains 22 and 23 that were identical with 19 except having two and three Abf1-BSs in place of HML′-I, respectively (Fig. 3A, left). URA3 silencing was silenced in strain 23 (Fig. 3A, growth phenotypes of 23), suggesting that three tandem Abf1-BSs in the context of HML′-I have silencing function similar to HMR-E (Fig. 3A, compare 23 with 17). However, URA3 silencing in strain 23, but not in strain 17, was lost when the HML′-E silencer was deleted (data not shown). Therefore, URA3 silencing by three tandem Abf1-BSs is dependent on HML′-E, whereas that by HMR-E is not, indicating that the Abf1-BSs are not a bona fide silencer like HMR-E, but are a protosilencer with enhanced activity that is sufficient to cooperate with HML′-E to silence URA3 in strain 23.

To examine the effect of increasing the copy number of Abf1-BS on silencing within the HML′ locus, we made strains 22n and 23n that were similar with 22 and 23, respectively, but had URA3 placed within HML′ (Fig. 3B, left). URA3 silencing in strains 22n or 23n was significantly higher than that in strain 19n (Fig. 3B), indicating that increasing the copy number of Abf1-BS enhances transcriptional silencing within HML′. The HML′ circle excised from strain 22 or 23 lacked sir− topoisoformers, which was similar with HML′ circle from strain 17 (Fig. 3C, compare 22 and 23 with 17). Therefore, compared with HML′ heterochromatin in strain 19, heterochromatin in strain 22 or 23 is more stable. This result suggests that multiple Abf1-BSs have additive effects on the stability of heterochromatin structure, which correlates with the additive effects of Abf1-BSs on transcriptional silencing.

Protosilencers ORC-BS and Abf1-BS from HMR-E do not facilitate heterochromatin formation by modulating chromatin structure in preparation for SIR complex spreading

How protosilencers act to assist the formation of heterochromatin has been speculated before, but direct experimental tests of the models are lacking [24,26,27]. We have shown previously that the structure of chromatin in the path of SIR complex spreading affects the formation of heterochromatin [23,32]. It is possible that a protosilencer serves to modulate chromatin prior to heterochromatin formation in a way that favors the spread of SIR complex. This model is consistent with the fact that the association of ORC, Abf1 or Rap1 with DNA often influences the positioning of nucleosomes [23,29–33].

The above hypothesis implies that ORC-, Abf1- and Rap1-BSs as protosilencers affect chromatin before (or in the absence of) the association of SIR complex with chromatin. To test this prediction, we examined chromatin at the HML′ locus in strains 17s through 21s that are the sir3Δ derivatives of strains 17 through 21, respectively (Fig. 2A, left). The primary chromatin structure was mapped by MNase digestion and indirect end labeling. Chromatin in each strain was subjected to limited MNase digestion, and the DNAs from the chromatin fragments were then isolated and digested with SmaI restriction enzyme at a site 200 bp to the right of the HMR-E silencer or protosilencer and EcoNI within HML′ (Fig. 4, top). The DNA fragments were then fractionated, and those ending at the SmaI site to the right of HML′ were detected with a probe corresponding to a 200 bp sequence indicated by a bar at the top of Fig. 4.

As shown in Fig. 4, the profiles of MNase cleavage at HML′ in strains 17s though 20s were not significantly different from each other, or from that of 21s (compare 17s through 20s with 21s), despite the existence of some subtle differences, such as the slight reduction in MNase sensitivity of a site marked by an arrowhead in strain 18s compared with that in the other strains (Fig. 4). This is consistent with the fact that HML′ DNA in strains 17s through 21s assumed a similar topology (Fig. S1). Therefore, the presence of protosilencer ORC-BS, Abf1-BS or Rap1-BS did not affect the overall structure of derepressed chromatin, which argues against the idea that a protosilencer helps rearrange chromatin in preparation for SIR complex spreading.
ARS1 can counteract cell cycle-dependent disruption of heterochromatin

The fact that all the silencers flanking the HML and HMR loci are composed of two or three binding sites for ORC, Abf1 and Rap1 raises the question of whether other naturally occurring combinations of these sites could also promote the formation of heterochromatin. The autonomous replicating sequence 1 (ARS1) contains an ORC-BS (also named ACS, ARS consensus sequence) and an Abf1-BS. ARS1 located on chromosome IV is a well-studied replication origin that fires early in S phase. We investigated if ARS1 could act to maintain heterochromatin when ectopically placed at the HML locus.

Strain YXB10 has two FRTs flanking HML9 including the HML-E and I silencers (Fig. 5A, top), whereas strain YXB5 has FRTs flanking HML9 excluding the silencers (Fig. 5C, top). Each strain has a BstBI restriction fragment containing the promoters and part of the coding regions of the HMLx genes replaced by a sequence from the bacterial lacZ gene (designated lac[1] [8]. ARS1 was inserted in the middle of HML9 in strain YXB5 to make strain YXB125 (Fig. 5D, top). Cells of each strain were first arrested in G1 phase by α-factor treatment. The HML9 circle was then excised. Since the host cells were in G1 and not progressing in the cell cycle, the HML9 circle in each strain was stably maintained regardless of whether the silencers are present (Fig. 5B through 5D, lanes 0). The cells were then released from G1 arrest and allowed to grow in fresh YPD medium (without α-factor), and the topology of the HML9 circle was measured at a series of time points afterwards. Consistent with our earlier findings, HML9 circle containing the E and I silencers remained highly negatively supercoiled throughout the 20 hr of cells growth (Fig. 5B, compare lane 20 with lane 0), whereas HML9 circle lacking silencers gradually lost its high negative supercoiling (Fig. 5C, compare lanes 1, 2, 4, 6, 8, and 20 with lanes 0 and sir2). Regarding HML9 circles bearing ARS1 but not the E and I silencers, only a portion of them lost their high negative supercoiling, the rest retained their original topology during the 20 hours of cell growth (Fig. 5D, compare lane 20 with lane 0). Therefore, ARS1 is able to counteract, or slow down, cell cycle-dependent disruption of heterochromatin structure.
ARS1 can promote the formation of a putative partially heterochromatin structure in a S-phase dependent manner

How does ARS1 antagonize disruption of heterochromatin? One possibility is that it repairs damaged euchromatinized part of heterochromatin by promoting local de novo formation of heterochromatin. This hypothesis is reasonable since ARS1 contains an ORC-BS and an Abf1-BS that may cooperate to induce heterochromatin by promoting local cell cycle-dependent change in chromatin structure on the HML circle. On the other hand, during the 8 hr incubation in the presence of nocodazole, cells were able to progress from G1 (point of S-factor arrest) to early S phase (point of HU arrest). The topology of HML DNA in these cells was not significantly different from that in cells before the incubation (Fig. 6E and 6F, compare j with i). Therefore, blocking cells from progressing beyond early S phase eliminates the SIR3-dependent change in chromatin structure on the HML circle containing ARS1. On the other hand, during the 8 hr incubation in the presence of nocodazole, cells progressed from G1 to G2/M (point of nocodazole arrest) of the cell cycle. The HML circle in these cells was 1.5 supercoils more negatively supercoiled than that in cells before the incubation (Fig. 6E and 6F, compare j with i). Therefore, blocking cells from progressing beyond early S phase eliminates the SIR3-dependent change in chromatin structure on the HML circle containing ARS1. These results suggest that the putative role of ARS1 in promoting the formation of a SIR-dependent chromatin conformation requires the host to traverse through S phase of the cell cycle.

Discussion

Transcriptional silencing is a conserved mechanism of region-specific gene repression that may affect large regions of the
The locus-specificity of silencing in yeast is determined by cis-acting silencers and telomeres that serve to initiate the formation of a repressive heterochromatin structure. Silencers each consist of two or three of ORC-BS, Abf1-BS and Rap1-BS and serve as a recruitment center for the SIR complex. Individual ORC-BS, Abf1-BS and Rap1-BS do not have the ability to initiate de novo formation of heterochromatin, but can facilitate silencing by a bona fide silencer at a distance, and are called protosilencers. However, intriguingly, ORC-BSs also exist at all replication origins, or autonomous replication sequences (ARSs), and bind ORC involved in the initiation of DNA replication. Abf1-BSs and Rap1-BSs are also found at many gene promoter regions and associate with Abf1 and Rap1, respectively, as general regulatory factors involved in gene activation [53–55]. Therefore, whether these binding sites function in silencing, replication initiation, or gene activation is likely dependent on the genomic environment. Moreover, how efficiently a silencer or protosilencer functions also depends on its context [27].

The HMR-E silencer is the strongest among all the silencers in promoting transcriptional silencing [27]. Of the ORC-BS, Abf1-BS and Rap1-BS components of HMR-E, we found Rap1-BS to be especially important for its function (Fig. 1). A recent analysis of a synthetic minimum HML-E silencer consisting of an ORC-BS, a Rap1-BS and a Sum1-BS also suggests that Rap1-BS plays a more important role than the other two elements [56]. Both ORC and Rap1 are believed to contribute to silencer function by recruiting Sir3 and/or Sir4 proteins [1]. Rap1 directly binds Sir3 and Sir4, whereas ORC binds Sir1 which in turn binds Sir4. How Abf1 participates in the initiation of silencing has not been resolved, although there has been anecdotal information that Abf1 interacts with Sir3. Why the Rap1-BS is particularly important for HMR-E function is not clear. One possibility is that because Rap1-BS is located in the middle of HMR-E (flanked by ORC- and Abf1-BSs) (Fig. 1A), it is critical for the cooperation of the three silencer binding proteins. Loss of Rap1-BS may severely hinder the collaboration between ORC and Abf1 in recruiting SIR complex, due to the relatively large distance between ORC-BS and Abf1-BS. On the other hand, loss of Abf1-BS may not affect the cooperation between ORC and Rap1, and loss of ORC-BS may not affect the cooperation between Rap1 and Abf1.

Transcriptional silencing is subject to regulation by many factors including SAS2 encoding a histone H4 acetyltransferase. SAS2 plays a positive role in silencing as sas2Δ reduces HML silencing as well as telomeric silencing [40–43]. Consistently, we...
Figure 6. ARS1 can promote the establishment of a SIR-dependent chromatin on extra-chromosomal circles in a S-phase dependent fashion. (A) Method for investigating the de novo establishment of heterochromatin on HML circle. The HML locus including the E and I silencers is flanked by two FRTs in a sir3-8 strain. HML circle is excised in cells grown at 30°C. Cells are then shifted to fresh YPD medium and grown further at 23°C, which activates sir3-8 and allows the formation of heterochromatin on the HML circle. Shaded and filled circles represent nucleosomes in derepressed chromatin and heterochromatin, respectively. Cells of strain YXB141 (B), strain 25 (C) or strain 26 (D) were grown to log phase and then further incubated for 2.5 hr in the presence of 2% galactose. Cells were pelleted and resuspended in fresh YPD medium, and were incubated for 20 hr at 23°C. Samples were taken for DNA isolation at the indicated time points. DNA isolated from cells was fractionated by agarose gel electrophoresis in the presence of 17 μg/ml chloroquine. (E) Top, modified HML allele in strain 27. The ARS1 sequence inserted at HML is indicated. Cells of strain 27 were initially grown at 30°C. An aliquot of the culture was incubated for 2.5 hr in the presence of 2% galactose to induce the excision of the HML’ circle. Cells were then pelleted and resuspended in fresh YPD medium and further grown for 20 hr at 23°C. Samples were taken for DNA isolation after the indicated times (samples d through h). Another aliquot was treated with α-factor for 2.5 hr at 30°C to arrest cells in G1 phase of the cell cycle. Galactose was then added to this culture that was incubated at 30°C for another 2.5 hr. A third of this culture was used to isolate DNA (sample i). The rest was shifted to fresh YPD medium, and half of it was grown in the presence of 0.2 M HU (sample j) and the other in the presence of 20 μg/ml nocodazole (sample k), for 8 hr at 23°C. DNA isolated from each sample of cells was fractionated by agarose gel electrophoresis in the presence of 17 μg/ml of chloroquine. Dots indicate the Gaussian center of the distribution of HML’ circles. (F) The distribution of topoisomers in each sample examined in (E) was determined using the NIH image software. The centers of distribution are marked by dots. Note that all the strains examined here have a BstBI restriction fragment containing the promoters and part of the coding regions of the HMLα genes replaced by a sequence from the bacterial lacZ gene (designated β1) [8].

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showed in this report that silencing of TRP1 by intact HMR-E, or HMR-E lacking ORC-BS or Abf1-BS is reduced by sa2a (Fig. 1). Given that the SIR complex preferentially binds deacetylated nucleosomes, acetylation of histone H4-K16 by Sas2 in euchromatin has been proposed to hinder ectopic spreading of SIR complex from heterochromatin, thereby helping restricting SIR complexes to silent loci [57,58]. Global reduction in H4-K16 acetylation as a result of sa2a may allow a subset of Sir proteins to leave HM loci and associate with euchromatin regions, thereby reducing HM silencing. Consistent with this model, we have shown that in sa2a cells HML heterochromatin adopts an intermediate state between fully silent and derepressed structures [43]. As we have also shown that sa2a and orc2-1 have a synthetic effect on HMR silencing, we envisioned that Sas2 might regulate silencing by affecting ORC function at the HMR-E silencer [43]. However, the positive role of Sas2 in HMR-E silencing does not seem to depend on the presence ORC-BS in the silencer (Fig. 1). Therefore, it is unlikely that Sas2 contributes to HMR-E function via regulating ORC.

Intriguingly, sa2a enhances silencing by HMR-E deleted for Rap1-BS (HMR EAIR) (Fig. 1). In other words, Sas2 plays an inhibitory role in the function of HMR-EAR. It seems that Rap1-BS helps determine the mode (positive vs. negative) of function of Sas2 in silencing by HMR-E. Given that HMR-E function is affected by its chromatin context [32], it is possible that sa2a induced reduction in H4-K16 acetylation affects chromatin around HMR-E in a manner that is conducive to HMR-EAR, but inhibitory to HMR-E, HMR EA0 and HMR EAA. Rap1 interacts with Rif1 and Rif2 proteins, in addition to the SIR complex, and Rif1 and Rif2 are required for full silencing at HMR [59,60]. It would be interesting to explore whether Rif1 and/or Rif2 are involved in determining the regulation of HMR-E by Sas2.

It is interesting that although Rap1-BS plays a larger and unique role in HMR-E silencer function compared to ORC-BS or Abf1-BS, it does not serve as a protosilencer for HML-E as does ORC-BS or Abf1-BS (Fig. 2). Therefore, the functions of Rap1-BS as part of HMR-E silencer and as a protosilencer may be mechanistically different. It is noteworthy that the Rap1-BS in HMR-E (5'-AAACCCCATCAGCC-3') is a variant of a consensus sequence (5'-ACACCCRCYACAYM-3'; M, A or C; R, A or G; Y, C or T) for Rap1 recognition [55,61]. Other Rap1-BSs existing elsewhere in the genome are distinct variants of the consensus. Since Rap1-BSs exhibit considerable sequence heterogeneity, they are likely to have different affinities for Rap1, which may affect their functions [55,61,62]. This may be the reason why unlike Rap1-BS from HMR-E, the Rap1-BS from HML-E silencer and UAS2 (a Rap1-BS) both exhibit protosilencer functions [2,24,27].

Given the fact that each silencer consists of a combination of two or three protosilencers, it would be reasonable to think that multiple copies of the same protosilencer should have a stronger protosilencer activity. We found this to be the case for the protosilencer Abf1-BS from HMR-E: three tandem Abf1-BSs display a greater activity in enhancing silencing and the stability of heterochromatin (Fig. 3). However, we have previously shown that two or three tandem Rap1-BSs could serve as a barrier to the propagation of heterochromatin instead of a stronger protosilencer [63]. It would be interesting to investigate what determines if Rap1-BSs act as protosilencers or heterochromatin barrier elements.

A protosilencer can enhance the action of a silencer or telomere located at a distance of up to several kb. The mechanism underlying this functional interaction has not been elucidated. As the establishment of silencing is mediated by the binding of SIR complexes to an array of nucleosomes, the primary chromatin structure may play a role in determining the efficiency of SIR complex association. This notion is supported by our finding that disrupting the regularity of nucleosomes by nucleosome-excluding structures blocks the spread of heterochromatin [23]. Since protosilencers ORC-BS, Abf1-BS and Rap1-BS all have the potential of modulating nucleosome positioning upon associating with their corresponding proteins [25,29–33], it is possible that a protosilencer assists SIR complex propagation from a silencer by altering chromatin structure in a way that favors SIR-chromatin interaction [27]. However, we found that protosilencer ORC-BS or Abf1-BS in place of the HML-I silencer does not affect the primary chromatin structure between it and the HML-E silencer in a silencer background (Fig. 4), which argues against the model involving chromatin structure. An alternative hypothesis proposes that the silencer and protosilencer physically contact persistently or transiently to establish a stronger silencing center that can better recruit the SIR complex [26]. However, there has not been direct evidence supporting such a physical interaction model.

ORC-BS, or ACS (ARS consequence sequence), is the core component of an ARS, as well as a silencer. ORC-BSs also exist at subtelomeric regions where they act as protosilencers aiding in telomeric silencing [25,64]. A single ORC-BS in place of the HML-E or HML-I silencer also acts as a protosilencer to enhance the function of the other HML silencer [24] (Fig. 2). ARS1 contains an ORC-BS and an Abf1-BS required for replication origin function. Under a special circumstance (high copy expression of FKH1) ARS1 in place of HML-E silencer has been shown to mediate HMR silencing (together with HML-I silencer) [65]. In this report, we showed that ARS1 inserted at HML locus makes HML heterochromatin more resistant to cell cycle-dependent disruption (Fig. 5). Importantly, we obtained evidence suggesting that ARS1 on an HML circle lacking silencers has the ability to promote the transformation of derepressed chromatin structure into an intermediate or altered structure that is between heterochromatin and derepressed chromatin (Fig. 6). Moreover, such a transformation is dependent on S-phase progression of the host, which is similar to the S-phase (but not DNA replication) requirement for de novo formation of heterochromatin mediated by bona fide silencers [49–52]. Based on these results, it is possible that ARS1 repairs damages to heterochromatin (e.g., partial loss of SIR complex association) inflicted by cell cycle progression by promoting de novo formation of heterochromatin in limited regions.

Supporting Information

Figure S1 Protosilencers ORC-BS, Abf1-BS and Rap1-BS do not affect derepressed HML chromatin. Cells of each of the strains 17s through 21s were grown in YPR to late log phase, and were then treated with 2% galactose for 2.5 hr. Nucleic acids were isolated and fractionated in the presence of 26 μg/ml chloroquine. The topoisomerases were labeled ∼1–2. The relevant silencing element in each strain is shown at the top. The nicked and linear forms of HML circle are marked N and L, respectively.

Figure S2 Contributions of protosilencers to heterochromatin structure. Top, the modified HML locus in strains 17 and 17s. The black bar indicates the sequence corresponding to the probe used in indirect end labeling. Bottom, chromatin mapping in strains 17 through 21, as well as 17s by MNase digestion and indirect end labeling. MNase treated chromatin in each strain was digested with SnaBI and EcoNI and fractionated on an agarose gel. After Southern-blotting, DNA fragments ending at the SnaBI site were detected by hybridization with the probe shown at the top. The positions of the HMR-E silencer and FRT
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site are shown on the left of the blot. M, DNA markers. N, naked genomic DNA from strain 17s treated with MNase. The profile of MNase cleavage at \textit{HML} in strain 17 (\textit{SIR}) was clearly distinct from that in 17s (\textit{air}) (note the strain 17-specific bands indicated by diamonds and 17s-specific bands labeled by filled circles), which is consistent with the marked difference in \textit{HML} DNA topology between strains 17 and 17s (Fig. 2D). This confirms the formation of heterochromatin at \textit{HML} in strain 17 with a primary structure different from derepressed chromatin in strain 17s. As shown in Fig. 4, MNase digestion pattern in strains 18s to 21s was not significantly different from that in strain 17s, suggesting that the presence of protosilencer ORC-BS, Abf1-BS or Rap1-BS did not affect the overall structure of derepressed chromatin at \textit{HML}. As such, \textit{HML} chromatin in strain 17s can represent derepressed \textit{HML} chromatin in strains 18s to 21s. MNase digestion pattern in strain 21 shares several characteristics with that of 17s (bands indicated by filled circles in both lanes 21 and 17s), and also share some features with that of strain 17 (bands indicated by diamonds in lane 21). Therefore, \textit{HML} chromatin in strain 21 has features of both derepressed chromatin (as in strain 17s) and heterochromatin (as in strain 17). In addition, there were two MNase sensitive sites (indicated by open circles) that existed only in strain 21. These results support the notion that an intermediate chromatin structure different from both heterochromatin and derepressed chromatin is formed in strain 21 by the \textit{HML-E} silencer alone. This notion was also supported by the fact that the negative supercoiling of \textit{HML} DNA in strain 21 was lower than that in strain 17, but higher than that in strain 17s (Fig. 2D). Strain 20 was identical with 21 regarding MNase digestion of \textit{HML} chromatin, which is in line with the fact these two strains were also identical with respect to the supercoiling of \textit{HML} DNA (Fig. 2D). This further demonstrates the inability of the Rap1p site in strain 20 to assist \textit{HML-E} silencer in establishing mature heterochromatin. MNase digestion at \textit{HML} in strain 18 was similar but not identical with that of strain 17 (note 18 and 17 share bands indicated by diamonds, but 18 has an extra band denoted by an open dot). This suggests that heterochromatin formed at \textit{HML} in strain 18 has a conformation that is similar, but not identical, with that in strain 17. Compared with strain 18, strain 19 lost a heterochromatin-specific MNase site denoted by a diamond), and gained two derepressed chromatin-specific sites (denoted by filled dots) at \textit{HML}. Taken together, the above results demonstrate that \textit{HML} in strains 17 though 20 exhibit less and less heterochromatric features, and more and more derepressed chromatin-specific features. This suggests that the ORC-, Abf1- and Rap1-BSs from the \textit{HMR-E} silencer have different abilities to contribute to the structure of heterochromatin, with the order of their activities being ORC-BS>Abf1-BS>Rap1-BS.

Table S1 Yeast strains. (TIF)

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Author Contributions

Conceived and designed the experiments: XB. Performed the experiments: NZ. QY LO. Analyzed the data: XB NZ. QY. Contributed reagents/materials/analysis tools: NZ QY LO XB. Wrote the paper: XB.

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