A Single Heterochromatin Boundary Element Imposes Position-Independent Antisilencing Activity in Saccharomyces cerevisiae Minichromosomes

Sangita A. Chakraborty1*, Robert T. Simpson2*, Sergei A. Grigoryev1*

1 Department of Biochemistry and Molecular Biology, College of Medicine, Pennsylvania State University, Milton S. Hershey Medical Center, Hershey, Pennsylvania, United States of America, 2 Department of Biochemistry and Molecular Biology, Eberly College of Science, Pennsylvania State University, University Park, Pennsylvania, United States of America

Abstract

Chromatin boundary elements serve as cis-acting regulatory DNA signals required to protect genes from the effects of the neighboring heterochromatin. In the yeast genome, boundary elements act by establishing barriers for heterochromatin spreading and are sufficient to protect a reporter gene from transcriptional silencing when inserted between the silencer and the reporter gene. Here we dissected functional topography of silencers and boundary elements within circular minichromosomes in Saccharomyces cerevisiae. We found that both HML-E and HML-I silencers can efficiently repress the URA3 reporter on a multi-copy yeast minichromosome and we further showed that two distinct heterochromatin boundary elements STAR and TEF2-UASrpg are able to limit the heterochromatin spreading in circular minichromosomes. In surprising contrast to what had been observed in the yeast genome, we found that in minichromosomes the heterochromatin boundary elements inhibit silencing of the reporter gene even when just one boundary element is positioned at the distal end of the URA3 reporter or upstream of the silencer elements. Thus the STAR and TEF2-UASrpg boundary elements inhibit chromatin silencing through an antisilencing activity independently of their position or orientation in S. cerevisiae minichromosomes rather than by creating a position-specific barrier as seen in the genome. We propose that the circular DNA topology facilitates interactions between the boundary and silencing elements in the minichromosomes.

Introduction

The DNA in the nuclei of eukaryotic cells is packed into nucleosomes, establishing the primary level of chromatin packing. The DNA transcription, replication, recombination, and repair processes occur in the context of DNA packed into nucleosome arrays [1,2]. The structural-functional relationship between chromatin packing and DNA transcription is manifested by segregation of nuclear chromatin into the open and active euchromatin and the condensed and repressed heterochromatin [3,4]. The question how the transcriptionally active euchromatin is functionally separated from the inactive heterochromatin has been of considerable interest.

Previous research has focused on identifying cis-acting genetic elements termed "boundary elements" that demarcate the heterochromatin from the euchromatin [5,6]. Such DNA elements were identified in evolutionary diverse organisms ranging from yeast to humans [7–9]. The heterochromatin boundary elements establish boundaries of chromatin domains by limiting the spread of silencing signals to the adjoining regions [10]. These elements are especially important when transcriptionally active genes are surrounded by condensed heterochromatin as they stop the incursion of silencing signals from the surrounding regions thereby protecting the genes from position-dependent variegation.

Similar to positional effects in higher eukaryotes, in the budding yeast Saccharomyces cerevisiae the telomeres and the silent mating-type loci (HML and HMR), all represent well-defined heterochromatin domains, where genes are transcriptionally silent. The transcriptionally silent copies of the mating-type genes are located at the HML and HMR silent loci near telomeres. The HML and HMR silent loci are flanked by “Essential” (E) and “Important” (I) silencer elements [11–14]. The cis-acting E and I elements are necessary and sufficient for initiating and mediating silencing in an orientation-dependent manner by interacting with a large number of trans-acting factors to repress transcription [11,13,15,16]. Transcriptional repression at HM loci is a gene-nonspecific event and the silencers can repress any reporter gene [17,18].

The silent chromatin structure does not extend indefinitely and is restricted within the HM loci and telomeres by the heterochromatin boundary elements that block silencer-mediated transcriptional repression.
repression of the gene in mating-type loci as well as shield repressive positional effects of telomeric heterochromatin [7,19–21]. It has been suggested that the 

HML-I silencer may itself establish a heterochromatin boundary by organizing heterochromatin in a uni-directional manner within the HML locus [22]. Furthermore, a tRNA gene surrounding the HMR locus in 

S. cerevisiae has been shown to have barrier activity and restrict the spread of silencing from the HMR locus [7,8]. In addition, two special heterochromatin boundary elements, STAR and TEF2-UASrpg, were shown to have boundary activity in 

S. cerevisiae genome [19,20].

In 

S. cerevisiae, the chromosomal ends contain the X and/or Y subtelomeric repeat elements abutting the telomeric repetitive DNA. Sequences within these X and Y subtelomeric repeats block silencing, exhibiting heterochromatin barrier activity and thus are named subtelomeric anti-silencing regions (STAR). The STAR boundary element has also been shown to counteract the silencer-driven repression of reporter genes at the HML locus in the genome, when interposed between the silencer and the reporter gene without transcriptional activation of the reporter [20,23].

The TEF2-UASrpg located on Chromosome II in 

S. cerevisiae is an example of heterochromatin boundary element. It was identified by the silencer-blocking assay by positioning the boundary element between the silencer and the reporter gene to test its ability to counteract the silencing mechanism. The TEF2 gene encodes the translational elongation factor-1alpha and the upstream activation sequence of the gene encodes the translational elongation factor-1alpha and the upstream activation sequence of the TEF2 is able to block the silencing activity and the spread of heterochromatin without transcriptional activation. In the genome, when the TEF2-UASrpg was placed at the HML locus it was able to resist transcriptional silencing of native or reporter genes in a position and orientation dependent manner [19,24].

Distinct models have been suggested for chromatin boundary formation. In one model, boundary elements act by creating nucleosomal gaps and establishing barriers for example when placed between the silencer and the regulated gene but not upstream of the silencer or downstream of the gene [24,25,26]. In the other model, the boundary element could form loops reaching out to and inhibiting silencers. Within this model the boundary elements may act independently of their position versus the silencer element and are assigned to have a desilencing or anti-silencing rather than barrier activity [27–31]. The exact molecular mechanism may vary between distinct boundary elements and different organisms and still remains largely unknown.

In order to understand the molecular mechanism of barrier formation by STAR and TEF2-UASrpg, two distinct heterochromatin boundary elements, we turned to the yeast minichromosome system. Yeast minichromosomes are multi-copy circular plasmids that assemble into chromatin in vivo [32] and have been used to study nucleosome positioning, chromatin remodeling, and interaction of trans-acting factors with cis-acting elements [33–36].

To dissect the topographic relationship of the silencers and boundary elements on a minichromosome, we generated a number of minichromosomal constructs containing different combinations of the “E” and “I” silencers from the HML locus with STAR and TEF2-UASrpg heterochromatin boundary elements. The URA3 has been used as the reporter gene and the TRP1 as the selection marker in all the minichromosome constructs examined. Identification of whether the URA3 reporter gene is ON/OFF has been tested using 5-FOA biochemical selection screen in addition to growth on Uracll-deficient media.

We report here that the URA3 reporter gene was efficiently silenced by the E and/or I silencers in the absence of heterochromatin boundary elements and the URA3 reporter was de-repressed in the presence of STAR and TEF2-UASrpg elements in circular minichromosomes similar to previous studies in the yeast genome [19,20]. However, our findings showed that the STAR and TEF2-UASrpg elements exhibit an antisilencing rather than boundary activity in 

S. cerevisiae minichromosomes. We propose that the topology of circular minichromosomes may help to bypass the strict positional requirements of chromatin boundaries that operate in linear chromosomes.

Results

Characterization of URA3-based reporter minichromosomes

The minichromosome constructs generated were tested for their functionality upon transformation into 

tp1- and ura3- deficient yeast strains. The dependence upon 

TRP1 selection marker has been used as a control for all experiments and the 

URA3 served as a reporter gene for transcriptional silencing in this study (Figure 1A). Cell growth on 5-FOA [37] and inability to grow on 

URA- media [38] indicates that the 

URA3 is repressed and therefore the cells are 5-FOA resistant, allowing us to identify whether the 

URA3 reporter is silenced in the presence of silencer elements and expressed in the presence of boundary elements. Recent reports point out to a potential problem with 5-FOA screenings, due to metabolic changes caused by the 5-FOA and suggest to check the reporter gene expression for epigenetic mechanisms or heterochromatic silencing studies [39,40]. However, in our study we have also directly assessed the 

URA3 reporter gene activity in medium lacking uracil for analysis of 

URA3 expression in the presence or absence of silencers and boundary elements independently of the 5-FOA assays.

We checked the 

S. cerevisiae strain YPH499 (a-cells) for its genotype. These cells were only able to grow on complete synthetic media (CSM) without any dropouts, but could not grow on any selective media such as TRP- or TRP-/URA- or TRP-/5-FOA media (Figure 2A). In the presence of the 

TRP1 marker, the cells were able to grow on CSM, TRP- and TRP-/5-FOA- selective media being 5-FOA resistant, but were unable to grow on 

TRP-/URA- due to the absence of 

URA3 gene product (Figure 2B). In the presence of both 

TRP1 marker and 

URA3 reporter, the cells were able to grow on CSM, TRP- and TRP-/ 

URA- selective media, but were unable to grow on TRP-/5-FOA+. Plates due to the presence of a functional 

URA3 gene product the cells exhibited sensitivity to 5-FOA (Figure 2C). Efficient ten-fold serial dilutions were established to cover the range of selection from ~2×10^5 cells/ml to ~2×10^7 cells/ml in the spotting assays. The copy numbers of the multi-copy 

S. cerevisiae minichromosomes were tested throughout this work and were found to be constant for different minichromosome constructs. The numbers of minichromosomes were determined to be ~20 copies compared to the genomic copy by southern hybridization with specific 

TRPI-ARS1 probe and (Figure 2M) and quantified using the ImageQuant software (Figure 2N).

Establishment of silencing in circular multi-copy minichromosomes

In order to study heterochromatin barrier function we had to establish robust silencing in the 

S. cerevisiae minichromosome system. Earlier reports indicate that silencing of a gene placed between the two silencer elements, 

HML – E and I, in the yeast genome is uniformly high and does not depend on the chromosomal context beyond the silenced locus [15,22,41].

The silencer minichromosome construct (Figure 1B) has been designed to study the effects of both the 

HML “E” and the “I”
marker gene located upstream of the _HML_ – E silencer was not silenced by either E or I silencer in the circular minichromosomes, apparently because both _HML_ – E and I silencers have directionality in the minichromosomes as seen in the genome and only silence the expression of genes positioned in between the silencers in an orientation-dependent manner [15,22,41]. As an additional control we also placed _HIS3_ marker gene upstream of the _HML_ – E silencer and downstream of the I silencer, and we found that neither E nor I silencer were able to silence the expression of the _HIS3_ gene (Figure 3A and 3B).

**STAR and TEF2-UASrpg inhibit silencing of the reporter on yeast minichromosomes irrespective of its orientation**

We examined if the _URA3_ reporter gene would be protected from silencing by two heterochromatin boundary elements, STAR and TEF2-UASrpg that are able to counteract the silencing in an orientation-dependent manner in the yeast genome [19,20]. Here, we found that either two STAR elements (Figure 1C, 2E–H) or two TEF2-UASrpg elements (Figure 2I–L) bracketing the _URA3_ reporter gene were able to inhibit the silencing by the _HML_ – E and _HML_ – I silencers in an orientation-independent manner in _S. cerevisiae_ minichromosomes (Figure 2E–L). There were no significant differences observed in the efficiency of the cell growth on TRP-/URA- selective media and growth inhibition on TRP-/5-FOA+ media. Thus in yeast minichromosome system, unlike the genomic studies [19,20], the two boundary elements STAR and TEF2-UASrpg can block both the _HML_ – E and _HML_ – I silencers in an orientation-independent manner.

**Combination of STAR and TEF2-UASrpg boundary elements on minichromosomes**

To determine if two identical boundary elements are required at both ends to protect the _URA3_ reporter from the silencing effects of the _HML_ – E and I silencers we have placed STAR on one end and TEF2-UASrpg heterochromatin boundary element at the other end. We found that all possible combinations of STAR and TEF2-UASrpg boundary elements on either ends of the _URA3_ in different orientations were able to protect the reporter gene from being silenced (Figure 3C–J). Thus in the context of minichromosomes two identical boundary elements at either end of the reporter gene are not required to counteract _HML_ – E and _HML_ – I driven silencing, and a combination of STAR and TEF2-UASrpg boundary elements are able to protect the _URA3_ from being silenced.

**A single boundary element is sufficient to block a single silencer**

We asked if a single silencer either E or I is sufficient to silence the expression of the _URA3_ reporter gene in our minichromosome system. This was important to examine since due to limiting Sir protein concentrations (amount of silencing factors) in the yeast cells we expected a weaker silencing activity for the multi-copy minichromosomes than a single-copy silenced domain in the genome [42]. We first tested a minichromosome where only the _HML_ – I silencer was inserted upstream of the _URA3_ reporter gene, so these cells were unable to grow on TRP-/URA- and are 5-FOA resistant (Figure 2D). Using serial dilution assay, we observed that _URA3_ gene was repressed strongly enough to mimic the URA-phenotype of the control plasmid indicating that the silencers repressed the reporter gene completely. In contrast, the _TRP1_ silencer elements on silencing the expression of the _URA3_ reporter gene. The differences in growth phenotypes and the silencing of the _URA3_ reporter gene were studied in the absence (Figure 2C) or presence of both the _HML_ – E and the _HML_ – I silencers placed on either side of the _URA3_ reporter gene (see schemes in Figures 2–6).

A single boundary element is sufficient to block a single silencer.
silence the URA3 reporter gene on a minichromosome as efficiently as a pair of two silencers.

In another series of experiments we observed similar results with a single HML–I silencer element has been inserted downstream of the URA3 reporter gene (Figure 4F). Thus the minichromosome-borne E and I silencers are both functional and efficient in silencing URA3 reporter gene on multi-copy circular minichromosomes. Each single silencer, either E or I, is capable and sufficient to silence the reporter gene even in the absence of the other silencer in the minichromosome constructs.
Next, we examined if a single heterochromatin boundary element, either STAR or TEF2-UASrpg, was sufficient to block silencing imposed by HML-E or HML-I on circular minichromosomes. The boundary elements STAR or TEF2-UASrpg were positioned between either the HML-E or HML-I silencer elements and the URA3 reporter. As observed in the genome, we found that a single boundary element STAR or TEF2-UASrpg was sufficient in blocking the silencing imposed by a single silencer either HML-E or HML-I in any orientation (Figure 4B–E and G–J).

A single boundary is sufficient to protect URA3 from silencing in presence of two silencer elements in circular minichromosomes

To determine if the STAR or TEF2-UASrpg elements behave as barriers in minichromosomes or they are able to overcome the silencing of both the E and I silencers in the presence of only one boundary element, we positioned a single boundary element downstream of either the E or the I silencer, leaving the other silencer upstream or downstream of the URA3 reporter intact (Figure 5A–D and E–H). We found that a single boundary element was able to overcome the silencing of both the E and I silencer elements on the URA3 reporter, even though the URA3 was protected only from one side and there was an equal opportunity for URA3 to be silenced by the other silencer. This finding is in striking contrast to the previous studies in linear chromosomes where the reporter gene had to be bracketed by two boundary elements to prevent the silencing in presence of both the silencers [19,20].

STAR and TEF2-UASrpg sequences are specific in blocking of silencing

To confirm the specificity of the STAR and TEF2-UASrpg sequences in inhibiting silencing in the context of minichromosomes we used DNA sequences of similar length from the Leu2 ORF in different orientations replacing the ~300 bp of STAR and ~150 bp of TEF2-UASrpg sequences. The control sequences...
inserted either between the HML-E or the HML-I silencer and the URA3 reporter gene were unable to block the silencing mediated by the silencers and the URA3 reporter was completely repressed by either E or I silencer elements (Figure 6A–D). Thus, blocking of the silencing activity is specific for the STAR and TEF2-UASrpg DNA sequences in the minichromosomes, as random sequences of similar length to STAR or TEF2-UASrpg were unable to protect URA3 reporter from being silenced.

STAR and TEF2-UASrpg boundary elements exhibit antisilencing activity in S. cerevisiae minichromosomes

To further dissect the mechanism of inhibition of silencing, we conducted a genetic test showing whether STAR and TEF2-UASrpg act as barriers or exhibit antisilencing activity in minichromosomes. The anti-silencing activity is defined as an ability of a boundary element to block the silencing independently of its position in relation to the silencer or the reporter distinct from the desilencing activity [28,30]. We therefore positioned the STAR and TEF2-UASrpg elements downstream of the HML-E or the HML-I silencers (Figure 6E–H). We found that either STAR or TEF2-UASrpg was able to protect the URA3 from being silenced even though they were not interposed between the silencer and the reporter, but was instead placed downstream of the silencers (Figure 6E–H).

Unlike the position downstream of the silencer elements, at which there is a possibility of competition between the boundary and the silencer to either activate or repress URA3, we next placed the STAR and TEF2-UASrpg upstream of the HML-E or the HML-I silencers and the URA3 reporter gene where the silencer is in closer proximity to the reporter than the boundary. We found that in this setting the STAR and TEF2-UASrpg displayed a strong antisilencing activity (Figure 6I–L) as effectively as it had

---

**Figure 5.** A single boundary efficiently blocks the silencing of the URA3 reporter from both the E and I silencers. Single BE (shown by arrows) either STAR (S) or TEF2-UASrpg (T) were examined in the presence of both the HML– E and I silencer elements, placing the BE between E and URA3 reporter (A–D) or between I and URA3 reporter (E–H) in different orientations and the reporter gene on/off was determined by the growth phenotypes of the yeast cells tested in different selective media.

doi:10.1371/journal.pone.0024835.g005

---

**Figure 6.** STAR or TEF2-UASrpg activity is sequence-specific and acts by imposing antisilencing. **A–D:** Minichromosome constructs with ~300 bp or ~150 bp of Leu2 ORF sequences replacing the STAR or TEF2-UASrpg boundary element were positioned in between the E silencer or the I silencer and the URA3 reporter. The E or I silencers was capable of silencing the expression of the URA3 reporter gene. The cells were able to grow on TRP- and TRP-/5-FOA+ media being 5-FOA resistant, but unable to grow on TRP-/URA- selective media. **E–H:** Boundary elements positioned downstream of the silencer and the URA3 reporter gene are able to block the silencing of the URA3 reporter independent of its position unlike in the genome. These cells are able to grow on TRP- and TRP-/URA- media, but are unable to grow on TRP-/5-FOA+, as the URA3 gene is not repressed and exhibits sensitivity to 5-FOA. **I–L** STAR and TEF2-UASrpg are positioned upstream of the HML-E and I silencer and the URA3 reporter gene. Unlike in the genome where the BE has to be positioned in between the reporter and the silencer, in minichromosomes the upstream BE is able to counteract silencing exhibiting antisilencing mechanism. These cells are able to grow on TRP- and TRP-/URA- media, but are unable to grow on TRP-/5-FOA+, as the URA3 gene is not repressed and exhibits sensitivity to 5-FOA.

doi:10.1371/journal.pone.0024835.g006
already been observed with positioning STAR and TEF2-UAStpg downstream of the silencers. Only one copy of either STAR or TEF2-UAStpg irrespective of its position in relation to the silencers or the reporter was sufficient to stop the silencing of the URA3 reporter. Thus, in contrast to linear chromosomes where the STAR or TEF2-UAStpg boundary elements show a position- and orientation-dependent barrier function [19,20], in the S. cerevisiae minichromosomes these elements restrict silencing in a position and orientation independent manner and exhibit an antisilencing rather than barrier activity.

Discussion

The objective of this study is creating a minichromosome model system recapitulating functional and spatial relationships between genetic elements controlling heterochromatin in yeast and facilitating its topographic analysis. We conducted a detailed characterization of STAR and TEF2-UAStpg heterochromatin boundary elements in minichromosomes in S. cerevisiae to determine if these elements act as barriers separating an active locus from silenced locus or are they able to inhibit silencing where their topology is not essential. We found that S. cerevisiae can maintain and pack episomal DNA into chromatin and minichromosomes at a stable copy number and thus provide a robust model for studying relationships between the heterochromatin elements and gene regulation independently of the chromosomal context. Our newly established minichromosome system can be employed as a screen for testing other candidate barriers elements such as tRNA genes and positioned nucleosomes.

We used the HML-E and HML-I silencer elements for our study, since both the E and I silencers at the HML locus are equally capable in silencing, unlike the silencers at the HMR locus [11,17] and as the HML silencers have been used in previous experiments with STAR and TEF2-UAStpg barriers [19,20]. In the genomic HM loci the E and I silencers are ~3.5 Kb apart and the silencing is known to work if that distance is increased only up to a certain extent (~6–7 Kb), after which the silencing activity decreases [7,17,29,44,45] as it is limited by the silencing propagating factors such as Sir3 [45]. In this study, in the minichromosome context, the silencing-initiating HML- E and I elements are ~2 Kb apart but with ~20 copies of the minichromosome the total DNA length through which silencing is propagated in the minichromosomes exceeds ~10-fold the effective spreading length limit between the HML- E and I silencer elements in the genome. We found that the URA3 reporter gene was completely repressed in all the ~20 minichromosomal copies in the yeast cells, since expression of only one gene copy was sufficient for growth on URA- media as well as for inhibiting growth on 5-FOA. As the plasmid-borne silencers are less constrained than those in the genome, the silencers on minichromosomes can effectively silence the reporter genes and efficiently maintain a total length of ~40 Kb silenced loci on minichromosomes. Similar to earlier reports, the silencing in the minichromosomes is much more robust and multi-fold higher than seen in the genome [46]. Furthermore, consistent with earlier reports [11,47], stating that a single silencer element is capable of acting alone in the genome, we have shown for the first time that a single silencer element (either HML – E or I) is sufficient in silencing the URA3 reporter gene even in circular multi-copy minichromosomes. Thus we were able to construct a circular minichromosome model system where both HML- E and I silencers were functional and efficient in silencing the URA3 reporter gene in multi-copy minichromosomes in the yeast cells.

Surprisingly, in sharp contrast to the genome, where the STAR or TEF2-UAStpg are known to block the spreading of silencing acting as barriers, i.e. only when interposed between a silencer and the reporter gene [19,20] with minichromosomes, we found that both STAR and TEF2-UAStpg were able to inhibit the silencing of URA3 irrespective of their orientations and positions in relation to the silencer or the reporter. In minichromosomes the STAR and TEF2-UAStpg exhibit position-independency and antisilencing activity where only one copy of either the STAR or TEF2-UAStpg is sufficient in inhibiting silencing of the URA3 reporter gene. Although the silencer elements exhibit efficient silencing of the reporter gene in a direction-dependent manner, similar to what is exhibited by silencer elements in the genome, we cannot rule out that the altered function of barrier elements on minichromosomes is (at least partially) due to the altered properties of the silencer.

To explain the antisilencing mechanism observed in the circular minichromosomes, we propose that within a minichromosome, its circular topology would promote interactions between the boundary element and the silencer bypassing the topographical constraints (Figure 7). This would allow the boundary element to block silencing of the URA3 reporter gene by the E silencer, irrespective of the position of the boundary element in relation to the silencer or the reporter. Thus unlike in the genome, the STAR or TEF2-UAStpg elements in the S. cerevisiae minichromosomes...
are capable of inhibiting the silencing of the URA3 reporter gene by the HML-E and HML-I silencers in a position independent manner.

Why do boundary elements act so differently on a minichromosome as compared to the genome? In the genome, the silent loci are known to have an altered chromatin organization that, in addition to spreading of the silencing complex and histone deacetylation, also involves chromatin higher-order structural transitions [44,48–50]. In the genome, the boundary elements need to be positioned between the silencer element and the promoter because otherwise they will be spatially hindered from the silenced region that forms a stable fold-back loop. Perhaps, this is not the case with the minichromosome where sliding of the interwound DNA helices of the covalently closed supercoiled DNA ring against each other “slithering” [51] facilitates DNA interaction at a distance and brings distal regulatory elements into close contact [52]. Furthermore, on a minichromosome associated with nucleosomes, the nucleosomal folding per se promotes contacts between the distal regulatory elements without supercoiling [53]. Looping of the telomeric heterochromatin has been shown to facilitate transcription by bringing Upstream Activating Sequences in a close contact with promoter [54] when the former was positioned downstream of the gene. We suggest that a facilitated looping may explain the more efficient functioning of the boundary elements in the minichromosome where they could find an easier access to the promoter (Figure 7) and out-compete silencers even when positioned downstream of the gene or upstream of the silencer. Based on the study of DNA minicircles excised from the yeast genome, it has been previously shown that DNA topology changes are associated with silencing [55]. Here we propose that DNA topology may regulate interactions between the boundary and silencing elements in the genome and its effect on silencing can be functionally dissected in the minichromosome system.

It is known that plasmid-borne silencers exhibit very strong silencing [46], similarly we show here that the STAR and TEF2-UASrpg may exhibit robust antisilencing in a minichromosomal environment. This “easy come – easy go” mode of silencing on a minichromosome implies that in this system the heterochromatin structure is relatively relaxed compared to the more rigid chromatin organization in the genome. The yeast minichromosome system that we have genetically characterized here is especially suited for isolating of the minichromosome in different functional states [32,56] for subsequent ultrastructural analysis that may finally clarify the actual 3D chromatin organization of the silent and active minichromosomes.

Materials and Methods

Minichromosome Constructs

A) Reporter constructs. The URA3 reporter minichromosome construct was generated by inserting ~1.3 kb FspI DNA fragment from the YIp5 plasmid containing ~800 bp URA3 reporter gene (GenBank accession number NC_001137.3, Chromosome V, 116167 to 116970). The URA3 reporter gene (FspI fragment) is cleaved from YIp5 plasmid and inserted into the multiple cloning site (SacI) of the ALT (ARS1, URA3, TRP1) plasmid. All minichromosome shuttle vectors contain an autonomously replicating sequence - ARS1 (GenBank accession number NC_001136.10, Chromosome IV, 462354 to 463192), and a selectable marker - TRP1 gene for selection in S. cerevisiae [56] (GenBank accession number NC_001136.10, Chromosome IV, 461842 to 462516) and a pBR322 vector-derived sequence with an AmpR gene for selection and ColE1 origin for propagation in E. coli. The URA3 reporter is adjacent to the TRP1 marker gene and in the same orientation in all the minichromosomal constructs (Figure 1A).

B) Silencer constructs. The silencer constructs were generated by inserting HML-E and HML-I silencer elements into the minichromosome backbone. An ~500 bp fragment containing the “E silencer” with ~200 bp upstream and downstream flanking sequences (GenBank accession number NC_001135.5, chromosome III, 10966 to 11499), were PCR amplified from the HML locus using genomic DNA and primers with unique SacI and XhoI restriction enzyme sites for integrating into the minichromosome (Table S1). Similarly an ~500 bp fragment containing the “I silencer” with ~200 bp upstream and downstream flanking sequences (GenBank accession number NC_001135.3, Chromosome III, 14364 to 14912), were PCR amplified from the HML locus using genomic DNA and primers with unique SacI and XhoI restriction enzyme sites (Table S1) for integrating into the minichromosome and were verified by DNA sequencing. The PCR products were cloned into pGEMT vectors. The silencer elements were excised from the pGEMT vectors and ligated into the minichromosome constructs. The SacI site in the minichromosome vector was too close to the XhoI site, this problem was overcome by adding a short DNA linker containing a SacI site. The HML-E and I silencer elements have the same directionality in the minichromosome as in the genome (Figure 1B). The control HIS3 gene (GenBank accession number NC_001147.6, Chromosome XV, 721946 to 722608) was PCR amplified from pRS413 vector (ATCC pRS series) and inserted at the SacI restriction site upstream of the E silencer or at the BandI restriction site upstream of the I silencer. The HIS3 is in the same orientation as the TRP1 and the URA3 in the minichromosome constructs.

C) Boundary constructs. The heterochromatin boundary element constructs were generated by inserting TEF2-UASrpg and STAR boundary elements in the minichromosome. The ~150 bp TEF2-UASrpg (GenBank accession number NC_001134.8, Chromosome II, 477109 to 477257) and ~300 bp STAR (GenBank accession number NC_001143.9, Chromosome XI, 70 to 345) were PCR amplified from genomic DNA using specific primer sets with unique restriction enzyme sites (Table S1) for integrating into the minichromosome and were verified by DNA sequencing. The PCR products were cloned into pGEMT vectors. The boundary elements were excised from the pGEMT vectors and ligated into the minichromosome constructs. The TEF2-UASrpg and STAR boundary elements has XhoI ends inserted in between the E-silencer and the URA3-reporter and has XhoI ends inserted in between the I-silencer and URA3-reporter in the minichromosomes (Figure 1C). The STAR and TEF2-UASrpg were also positioned upstream of the E or the I silencer using SacI and BandI restriction sites. The TEF2-UASrpg and the STAR boundary elements have been inserted in both orientations and in different positions in the various minichromosomal constructs. The control sequences of ~300 bp (similar to STAR in length) and the ~150 bp (similar to TEF2-UASrpg in length) were PCR amplified from Leu2 ORF (GenBank accession number NC_001135.5) from pRS413 vector (ATCC pRS series) and inserted at the XhoI or NotI restriction sites between the E or I silencer and the URA3 reporter replacing STAR or TEF2-UASrpg boundary elements.

Yeast strains and media

All minichromosome constructs were transformed into E. coli DH5α competent cells and bacterial colonies were screened using restriction enzyme digests, PCR analysis and DNA sequencing. The minichromosome constructs isolated from bacteria were re-transformed into S. cerevisiae a-cells YPH499 strain [MATa, ade2–101, his3–D200, leu2–Δ1, his4–201Δ1, trp1–Δ63, ura3–52] [57].
Yeasts colonies grown on complete synthetic media lacking tryptophan (TRP-) were selected for all minichromosome constructs containing TRP1 marker gene in the construct backbone. The functionality of the regulatory elements in various minichromosome constructs and the expression of the URA3 reporter gene in the presence or absence of the HML-E and silencer elements and STAR or TEF2-UASrpg boundary elements were determined using different selective media. The yeast colonies were grown on CS (complete synthetic media, TRP-lacking tryptophan), TRP-/URA- (lacking both tryptophan and uracil), TRP-/5-FOA- (lacking tryptophan, but containing 5-fluoro-orotic acid) and HIS- (lacking histidine).

Southern Hybridization

The minichromosome DNA integrity and copy number were examined by Southern blotting. DNA was purified, linearized with XhoI restriction enzyme digestion, subjected to electrophoretic separation on 1% agarose gel, and then transferred to Hybond-NX membrane (Amer sham Biosciences), as per standard procedures [58]. The DNA was cross-linked to the membranes with UV light, and hybridized with TRP1-ARS1 specific minichromosome probe (~1.4 kb EcoR1 fragment) that was gel purified and random primer labeled with [32P]dATP. After hybridization and washing the membranes were exposed to imaging screen (BioRad) and the signal intensities were analyzed using Typhoon 9400 Phosphoimag er (A mer sham Biosciences) and quantified by the ImageQuant 5.2 software (Molecular Dynamics). The genomic hybridization signal was normalized to the size of the genomic TRP1/ARS1 fragment recognized by the probe to determine the copy numbers.

Spotting Assay

All yeast strains containing different minichromosomal constructs were grown to mid-log phase (A600 of ~1.0) in liquid TRP-media with 2% dextrose at 30°C with aeration by shaking at 250 RPM. The a-cells not containing any minichromosome construct were grown in CSM. The optical density of all yeast cultures were adjusted to absorbance 1 at 600 nm wavelength containing ~2×10^9 cells/ml. Ten-fold serial dilution up to ~2×10^3 cells/ml of each strain was made for the spotting assay to assess URA3 expression for assaying the silencing and insulating efficiency of the structures under different growth conditions [20,46]. For each strain at least 6 independent transformants were verified by Southern blot analysis. Transformed cells from isolated colonies were inoculated and grown in TRP-liquid medium and spotted on to different selective media CSM, TRP-, TRP-/URA- (to check if 5-FOA resistance is due to silencing and not due to URA3 mutation), and TRP-/5-FOA+ [Toronto Research Chemicals]. Cells with repressed URA3 are able to form colonies in the presence of 5-FOA compound known to be toxic for cells expressing a functional URA3 gene [38]. The selective media plates were spotted with 5 μl cells per spot and grown for 2 days at 30°C prior to imaging the plates to study the differences in growth phenotypes.

Supporting Information

Table S1 List of primers used in this study.

(DOCX)

Acknowledgments

We thank the Department of Biochemistry and Molecular Biology (Penn State, University Park) for equipment support, Drs. John Diller, Abid Kazi, Ralph Keil, and Laura Carrel for their technical advice, reagents and helpful discussions.

Author Contributions

Conceived and designed the experiments: SAC RTS SAG. Performed the experiments: SAC. Analyzed the data: SAC RTS SAG. Contributed reagents/materials/analysis tools: SAC RTS SAG. Wrote the paper: SAC. Edited the paper: SAG.

References

1. Felsenfeld G, Groudine M (2003) Controlling the double helix. Nature 421: 448–453.
2. Kornberg R, Lorch Y (2007) Chromatin rules. Nature Structural and Molecular Biology 14: 986–989.
3. Rusche LN, Lynch PJ (2009) Assembling heterochromatin in the appropriate places: A boost is needed. Journal of cellular physiology 219: 525–528.
4. Oliver B, Parisi M, Clark D (2002) Gene expression neighborhoods. Journal of biology 1: 4.
5. Donze D, Kamakaka RT (2002) Braking the silence: how heterochromatic gene repression is stopped in its tracks. BioEssays 24: 344–349.
6. Giles K, Gowher H, Ghirlando R, Jin C, Felsenfeld G (2010) Chromat in boundaries, Insulators, and Long-Range Interactions in the Nucleus. Cold Spring Harbor symposia on quantitative biology 75: 006.
7. Donze D, Adams CR, Rine J, Kamakaka RT (1999) The boundaries of the silenced HMR domain in Saccharomyces cerevisiae. Genes Dev 13: 698–708.
8. Donze D, Kamakaka RT (2001) RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in Saccharomyces cerevisiae. Embo J 20: 520–531.
9. Gur adanta B, Corces VG (2009) Chromatin insulators: lessons from the fly. Briefings in functional genomics & proteomics 8: 276–282.
10. Gaszner M, Felsenfeld G (2006) Insulators: exploiting transcriptional and epigenetic mechanisms. Nat Rev Genet 7: 703–713.
11. Mahoney D, Broach JR (1989) The HML mating-type cassette of Saccharomyces cerevisiae defines a heterochromatin domain boundary by directional establishment of transcriptional repression. Genes & development 3: 605–615.
12. Bi X, Broach JR (1999) UASrpg can function as a heterochromatin boundary element in yeast. Genes Dev 13: 1089–1101.
13. Fourel G, Revardel E, Lebrun E, Hu YF, et al. (2001) An activation-independent role of transcription factors in insulator function. EMBO Rev 2: 124–132.
14. Bi X, Broach JR (1999) Silencers and focus control regions: opposite sides of the same coin. Trends Biochem Sci 22: 124–128.
15. Shui GJ, Broach JR (1995) Yeast silencers can act as orientation-dependent gene inactivation centers that respond to environmental signals. Mol Cell Biol 15: 3496–3506.
16. Mahoney DJ, Marquardt R, Shui GJ, Rose AB, Broach JR (1991) Mutations in the HML E silencer of Saccharomyces cerevisiae yield metastable inheritance of transcriptional repression. Genes & development 5: 605–615.
17. Haber JE (1998) Mating-type gene switching in Saccharomyces cerevisiae. Annu Rev Genet 32: 561–599.
18. Holmes SG, Broach JR (1996) Silencers are required for inheritance of the repressed state in yeast. Genes & development 10: 1021–1032.
19. Bi X, Broach JR (1999) UASrpg can function as a heterochromatin boundary element in yeast. Genes Dev 13: 1089–1101.
20. Fourel G, Revardel E, Koe ring CE, Gilson E (1999) Cohabitation of insulators and silencing elements in yeast subtelomeric regions. Embo J 18: 2522–2537.
21. Kamakaka RT (1997) Silencers and locus control regions: opposite sides of the same coin. Trends Biochem Sci 22: 124–128.
22. Bi X, Braunstein M, Shui GJ, Broach JR (1999) The yeast HML E silencer defines a heterochromatin domain boundary by directional establishment of silencing. Proc Natl Acad Sci U S A 96: 11354–11359.
23. Fourel G, Boscheron C, Revardel E, Lebrun E, Hu YF, et al. (2001) An activation-independent role of transcription factors in insulator function. EMBO Rep 2: 124–132.
24. Bi X, Broach JR (2001) Chromosomal boundaries in S. cerevisiae. Curr Opin Genet Dev 11: 199–204.
25. Fourel G, Magdeliner F, Gilson E (2004) Insulator dynamics and the setting of chromatin domains. BioEssays : news and reviews in molecular, cellular and developmental biology 26: 529–532.
26. Bi X, Yu Q, Sandmeyer JJ, Zou Y (2004) Formation of boundaries of transcriptionally silent chromatin by nucleosome-excluding structures. Mol Cell Biol 24: 2118–2131.
27. Ishi K, Arib G, Lin C, Van Houwe G, Laemmli UK (2002) Chromatin boundaries in budding yeast: the nuclear pore connection. Cell 109: 551–562.
28. Ishii K, Laemmli UK (2003) Structural and dynamic functions establish chromatin domains. Mol Cell 11: 237–248.
29. Kamakaka RT (2002) Chromatin: a connection between loops and barriers? Curr Biol 12: R35–337.
30. Stover DM, Zehner ZK (1992) Identification of a cis-acting DNA antisilencer element which modulates vimentin gene expression. Molecular and cellular biology 12: 2230–2240.
31. Schedl P, Broach JR (2003) Making good neighbors: the right fence for the right job. Nature structural biology 10: 241–243.
32. Simpson RT, Ducker CE, Diller JD, Ruan C (2004) Purification of native, defined chromatin segments. Methods Enzymol 375: 158–170.
33. Simpson RT (1999) Nucleosome positioning can affect the function of a cis-acting DNA element in vivo. Nature 343: 387–389.
34. Patterson HG, Simpson RT (1994) Nucleosomal location of the STG6 TATA box and Mat alpha 2p-mediated repression. Mol Cell Biol 14: 4002–4010.
35. Morse RH (1993) Nucleosome disruption by transcription factor binding in yeast. Science 262: 1563–1566.
36. Kim Y, Shen CH, Clark DJ (2004) Purification and nucleosome mapping analysis of native yeast plasmid chromatin. Methods 33: 59–67.
37. Boeke JD, Trueheart J, Natsoulis G, Fink GR (2001) 5-Fluoro-orotic acid as a selective agent in yeast molecular genetics. Methods 154: 164–175.
38. Boscheron C, Maillet L, Marcand S, Tsai-Plugfelder M, Gasser SM, et al. (1996) Cooperation at a distance between silencers and proto-silencers at the yeast HML locus. The EMBO journal 15: 2104–2105.
39. Lebrun E, Fourlet G, Defossez PA, Gibson E (2003) A methyltransferase targeting assay reveals silencer-telomere interactions in budding yeast. Mol Cell Biol 23: 1498–1508.
40. Rusche LN, Kirchmaier AL, Rine J (2003) The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu Rev Biochem 72: 481–516.
41. Shaulsky G, Hecht A, Lao K, Grunstein K (1997) SIR2 and SIR4 interactions differ in core extended telomeric heterochromatin in yeast. Genes & Devel 11: 55–93.
42. Maillet L, Boscheron C, Gotta M, Marcand S, Tsai-Plugfelder M, Gasser SM, et al. (1996) Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression. Genes & development 10: 1796–1811.
43. Rusche LN, Kirchmaier AL, Rine J (2002) Ordered nucleation and spreading of silenced chromatin in Saccharomyces cerevisiae. Mol Biol Cell 13: 2207–2222.
44. Loo S, Rine J (1994) Silencers and domains of generalized repression. Science 264: 1768–1771.
45. Boeke JD, Maillet L, Marcand S, Tsai-Plugfelder M, Gasser SM, et al. (1996) Cooperation at a distance between silencers and proto-silencers at the yeast HML locus. The EMBO journal 15: 2104–2105.
46. Lebrun E, Fourlet G, Defossez PA, Gibson E (2003) A methyltransferase targeting assay reveals silencer-telomere interactions in budding yeast. Mol Cell Biol 23: 1498–1508.
47. Rusche LN, Kirchmaier AL, Rine J (2003) The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu Rev Biochem 72: 481–516.
48. Strahl-Bolsinger S, Hecht A, Lao K, Grunstein K (1997) SIR2 and SIR4 interactions differ in core extended telomeric heterochromatin in yeast. Genes & development 11: 83–93.
49. de Bruin D, Kantrow SM, Liberatore RA, Zakian VA (2000) Telomere folding is required for the stable maintenance of telomere position effects in yeast. Mol Cell Biol 20: 7991–8000.
50. Valenzuela L, Dhillon N, Dubey RN, Gartenberg MR, Kamakaka RT (2008) Long-range communication between the silencers of HMR. Mol Cell Biol 28: 1924–1935.
51. Huang J, Schlick T, Vologodskii A (2001) Dynamics of site juxtaposition in supercoiled DNA. Proc Natl Acad Sci U S A 98: 968–973.
52. de Bruin D, Kantrow SM, Liberatore RA, Zakian VA (2000) Telomere folding is required for the stable maintenance of telomere position effects in yeast. Mol Cell Biol 20: 7991–8000.
53. Anari A, Gartenberg MR (1999) Persistence of an alternate chromatin structure at silenced loci in vitro. Proceedings of the National Academy of Sciences of the United States of America 96: 543–548.
54. Ducker CE, Simpson RT (2000) The organized chromatin domain of the repressed yeast a cell-specific gene STE6 contains two molecules of the corepressor Tup1p per nucleosome. Embry J 19: 400–409.
55. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122: 19–27.
56. Ausubel F (1999) Current protocols in molecular biology. Brooklyn, NY, Media, Pa.: J. Wiley & Sons Inc. pp 2.9.1–2.9-20.