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In Vivo Evolution of an RNA-Based Transcriptional Silencing Domain in *S. cerevisiae*

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SUMMARY

Starting from a random RNA library expressed in yeast cells, we evolved an RNA-based transcriptional silencing domain with potency comparable to that observed when Sir1, a known silencing protein, is localized to a promoter. Using secondary-structure predictions and site-directed mutagenesis, we dissected the functional domains of the most active evolved RNA transcriptional silencer. Observed RNA-based silencing was general, rather than gene specific, and the origin recognition complex was required for full activity of the evolved RNA. Using genetic studies, we demonstrated that the RNA-based silencer acts through a Sir protein-dependent mechanism. Our results highlight the value of evolving RNA libraries as probes of biological processes and suggest the possible existence of natural RNA-based, RNAi-independent gene silencers.

INTRODUCTION

RNA has been shown to play a crucial role in essential biological processes such as splicing, tRNA processing, and peptide bond formation, in addition to serving as a transient carrier of genetic information [1, 2]. Noncoding RNAs have also emerged as important components in the control of gene expression [3, 4]. For example, riboswitches are a class of *cis*-regulatory RNAs in prokaryotes that undergo conformational changes in response to metabolite binding, influencing the expression of the corresponding gene [5]. The RNA interference (RNAi) pathway is a conserved mechanism for inhibiting gene expression [6–8] that uses small interfering RNAs (siRNAs) to target mRNAs for degradation or translational inhibition. Other gene regulatory processes such as genome purging in *Tetrahymena* [9, 10] and heterochromatin formation in *Saccharomyces pombe* [11] also involve siRNAs.

The functional versatility of RNA, combined with the powerful ways in which researchers can manipulate and characterize RNA, suggests its promise as a tool to probe cellular functions. Despite its limited chemical diversity, RNA can access diverse structure space mediated by a wide variety of base-pairing interactions [12]. Large RNA libraries can readily be expressed within populations of cells. The genes encoding RNAs that elicit desired cellular phenotypes can be amplified and diversified, allowing researchers to perform multiple rounds of directed evolution on RNA libraries in vivo. In addition, due to the modular nature of RNA domains, they can be engineered to exhibit different functional properties in the presence or absence of specific small molecules [13, 14], potentially enabling the precise temporal and dose-dependent control of cellular functions.

Previous efforts to engineer and evolve RNAs with desired intracellular properties support the potential of laboratory-created RNAs as probes of biological processes. Maher and coworkers successfully generated RNAs with a variety of novel functions, including the ability to bind spectinomycin, relieve transcriptional inhibition in *Escherichia coli*, and serve as a decoy for the transcription factor NFκB [15–18]. We previously reported the in vivo evolution of RNA-based transcriptional activation domains with potency comparable to that of the strongest known natural protein-based activation domains [19]. Subsequent engineering and evolution efforts yielded an RNA transcriptional activator that is 10-fold more active in the presence of the small molecule tetramethylrosamine (TMR) than in its absence [14].

In this work, we extend the use of RNA to probe biological functions by evolving an RNA-based transcriptional silencing domain in *Saccharomyces cerevisiae*. Gene silencing is a form of gene regulation that involves the formation of a specialized, long-range chromatin structure. In *S. cerevisiae*, silencing is observed at three classes of loci: the two cryptic mating-type cassettes *HML* and *HMR*, the rDNA repeats, and telomeres [20, 21]. At the mating-type loci, repression of gene expression is crucial for maintenance of the haploid state. Transcriptional repression is achieved by *cis*-acting DNA elements, known as the *E* and *I* silencers, that flank the *HMR* and *HML* loci, respectively. The *HMR-E* silencer consists of *A*, *E*, and *B* sites, recognized by the origin recognition complex (ORC), Rap1, and Abf1. Establishment of a transcriptionally silenced chromatin state requires the recruitment of Sir1, Sir2, Sir3, and Sir4 proteins by the silencer-bound proteins [20].

The deletion of any two of the *A*, *E*, or *B* sites at *HMR-E* results in the loss of silencing, which can be restored

by tethering a known silencing protein to the locus. Sternglanz and coworkers have also shown that this restoration of silencing can be achieved in a two-hybrid-like manner by replacing the endogenous silencing elements with Gal4 binding sites (UAS_G) and expressing a silencing protein as a fusion to the Gal4 DNA binding domain ($Gal4_{DBD}$) [22–26]. Such a targeted silencing system was used to identify novel proteins involved in transcriptional silencing at the *HMR* locus [22].

Small interfering RNAs have recently been shown to promote gene-specific transcriptional silencing in *S. pombe* in a process that requires components of the RNAi pathway [11]. However, RNAs that function as gene silencers in an RNAi-independent manner are not known to exist. Here we report the in vivo evolution of RNA-based silencing domains in *S. cerevisiae* from random libraries using a variant of the targeted silencing system [22]. The most potent evolved RNAs are capable of silencing transcription to an extent comparable to that observed when the known silencing protein Sir1 is localized to the *HMR-E* locus as a $Gal4_{DBD}$-Sir1 fusion. We used secondary-structure prediction and site-directed mutagenesis to dissect the functional domains of these RNA-based gene silencers. Furthermore, we gained insight into their mechanism of action by examining their dependence on known silencing proteins for their function. Our findings provide an RNA-based tool for studying gene silencing and further validate the use of evolved RNAs as powerful probes to perturb biological pathways.

RESULTS

Selection System and RNA Library Construction

Our approach to evolving an RNA-based gene silencer requires a selection method that enables cells to survive only if they express an active gene silencer. Transcriptional silencing at the *HMR-E* locus is established by the recruitment of Sir proteins by the ORC, Rap1, and Abf1, which recognize the *A*, *E*, and *B* sites, respectively (Figure 1A). Deletion of any two of these three silencer elements abolishes heterochromatin formation [22]. Andrulis and coworkers described a targeted silencing system [22] in which the *E* and *B* sites are replaced by Gal4 binding sites (referred to as *Aeb::G*) and a reporter gene is inserted in the *HMR* locus. In *S. cerevisiae* strains YEA76 [27] and YSB35 [23], this system places expression of the *URA3* and *TRP1* reporter genes, respectively, under control of gene silencing. YEA76 cells expressing the *URA3* gene cannot grow on medium containing 5-fluoroorotic acid (5-FOA), and therefore silencing of *URA3* expression enables survival on 5-FOA. YEA76 therefore can be used in a selection to link cell survival with gene silencing.

To localize RNA library members to the *HMR-E* locus, we used the high-affinity interaction ($K_d = 2 \times 10^{-10}$ M) between the MS2 coat protein and the 19 bp MS2 hairpin [28]. A plasmid expressing a fusion of the Gal4 DNA binding domain and the MS2 coat protein was introduced into the selection strain, enabling the localization of RNAs containing the MS2 hairpins to the promoter of the *URA3* reporter gene (Figure 1B).

RNA library diversity was provided by a random 40-base region (N_{40}) followed by two MS2 hairpins and was expressed from a plasmid-based RNA expression cassette described by Wickens and coworkers [29]. Transcription in this system is driven from the RNA polymerase III RNase P RNA (RPR) promoter [30], ensuring that RNAs are not capped, polyadenylated, or translated [28], and is terminated by an RPR terminator (Figure 1B). To enhance the intracellular stability of the RNA libraries, they were expressed between well-structured 3′ and 5′ ends. We cloned this RNA expression cassette into the yeast shuttle vector pRS424, generating the RNA expression vector pRNAIII. A synthetic N_{40} library was ligated into pRNAIII and amplified in *E. coli* to provide an estimated starting diversity of 2×10^7 sequences.

Selection of an RNA-Based Transcriptional Silencer

The amplified library was introduced into YEA76 yeast cells expressing the $Gal4_{DBD}$-MS2 fusion protein and transformants were isolated by growth on medium lacking histidine and tryptophan (resulting in 5×10^4 clones). Surviving colonies were harvested and replated on medium containing 1 mg/ml 5-FOA to select for clones capable of silencing the transcription of the *URA3* reporter gene. To enrich for active sequences over false positives, the plasmid DNA from surviving colonies was extracted and subjected to a second selection under identical conditions.

We individually characterized 24 clones capable of growing on medium containing 5-FOA by extracting their plasmid DNA, sequencing the variable region of the RNA construct, recloning the variable-region insert into pRNAIII, and retesting their ability to silence transcription phenotypically. As positive controls we used the known silencing proteins Esc2 and Sir1 fused to the Gal4 DNA binding domain. We observed that 13 of the 24 characterized clones were identical in sequence, suggesting that they corresponded to a clone expressing a highly enriched transcriptional silencing domain. Indeed, this highly represented clone (2SB1) exhibited robust *URA3* silencing activity at a potency comparable to that of the Esc2 positive control, and only modestly lower than that of the Sir1 positive control (Figure 2). None of the other 11 clones exhibited significant silencing activity as measured by the ability to grow on medium containing 5-FOA.

Evolution and Characterization of More Potent RNA-Based Silencers

To evolve more potent RNA-based transcriptional silencing domains, we used a synthetic oligonucleotide to introduce random mutations into the variable 40-base region of the round 1 clone 2SB1 at a 21% rate. The resulting library was amplified in *E. coli* (8×10^6 clones), introduced into yeast cells (7.5×10^4 clones), and subjected to selections as described above. An analysis of preselection library members revealed a total of 63 mutations within seven 40-base variable regions (22.5% mutation rate), in agreement with the designed mutagenesis rate.

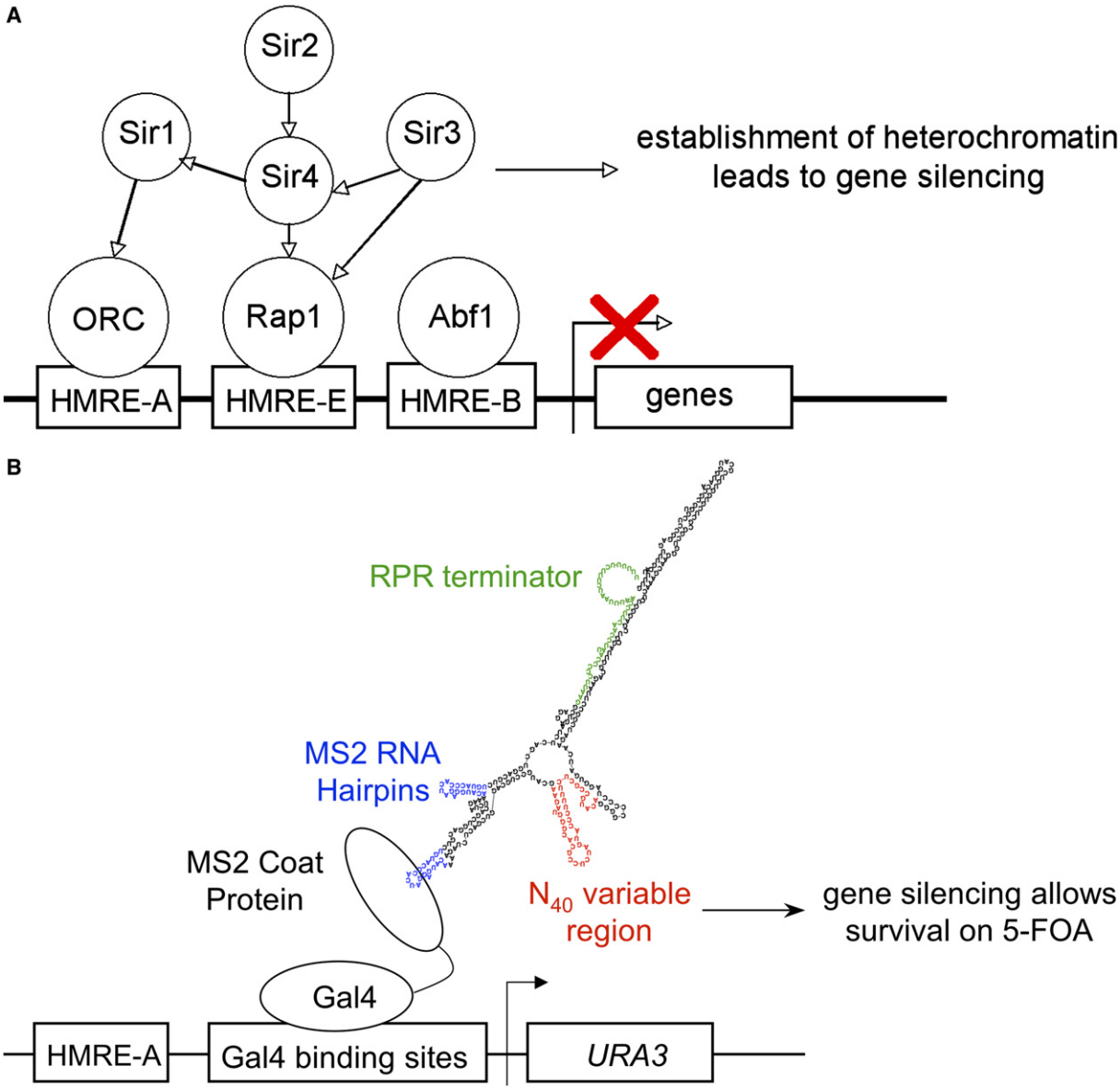


Figure 1. Selection System Design

(A) Transcriptional silencing at the *HMR-E* locus. DNA binding proteins ORC, Rap1, and Abf1 recruit the Sir proteins, leading to establishment of a heterochromatic state and subsequent gene silencing.

(B) Selection system for the evolution of RNA-based transcriptional silencing domains. RNAs are transcribed from a PolIII promoter and contain a 5′ leader sequence, an N_{40} variable region, two MS2 hairpins, and an RPR terminator. The selection strain has the *E* and *B* sites of the *HMR-E* locus replaced by Gal4 binding sites and a *URA3* reporter gene. RNA library members are localized to the *URA3* promoter region via recruitment by a fusion of the MS2 coat protein to the Gal4 DNA binding domain. RNAs capable of silencing the expression of the *URA3* gene enable survival on media containing 5-FOA.

We phenotypically characterized 22 surviving clones from round 2, of which 8 were capable of silencing transcription more potently than the parental clone 2SB1 (Figure 3). The most potent RNA-based silencer, m2SB1-1, is significantly more potent than the Esc2 positive control and of comparable potency to the Sir1 positive control (Figure 3).

Sequence alignment of characterized round 2 clones identified two main regions of sequence conservation (Figure 4). The predicted secondary structure of m2SB1-1, generated using the mfold program [31], suggests that the regions of conserved sequence are involved in the formation of two well-structured stems (Figure 5A). Bases 7–11 are predicted to interact with five nucleotides from the 5′ constant region, while bases 12–19 are predicted to pair with bases 33–41 to form a strong stem structure (Figure 5A). The loop region at the end of the second stem corresponds to the nonconserved bases

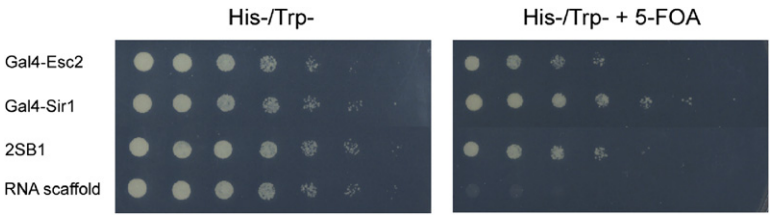


Figure 2. Silencing Activity of 2SB1, an RNA-Based Silencing Domain Emerging after One Round of Selection

Growth on media containing 5-FOA indicates silencing activity. Fusions of $Gal4_{DBD}$ to the known silencing proteins Esc2 and Sir1 were used as positive controls. A plasmid expressing only the flanking RNA scaffold without the active 40-base region was used as a negative control. From left to right, each clone is spotted in 5-fold serial dilutions on the growth media specified supplemented with 100 mg/l adenine.

22–30, implying that this loop is dispensable for silencer activity. Consistent with these predictions, clones m2SB1-4 and m2SB1-16, found to lack silencing activity upon secondary screening, both contain mutations in one or both of the highly conserved regions (Figure 4).

Structure-Activity Analysis of the Most Potent Evolved Silencer

RNA-based probes of biological processes are amenable to the elucidation of basic structure-activity relationships by combining secondary-structure predictions with site-directed mutagenesis. Based on the sequence alignment of the most evolved clones and on the predicted secondary structure of the highly active m2SB1-1 (Figures 4 and 5A), we hypothesized that the two highly conserved regions predicted to form strong stem structures were required for activity. We also expected the loop formed by the nonconserved bases 22–30 to be dispensable. To test these hypotheses and to gain further insight into the role of the conserved regions, we introduced 18 mutations within the variable N_{40} region of the most potent round 2 clone, m2SB1-1 (Figure 5A).

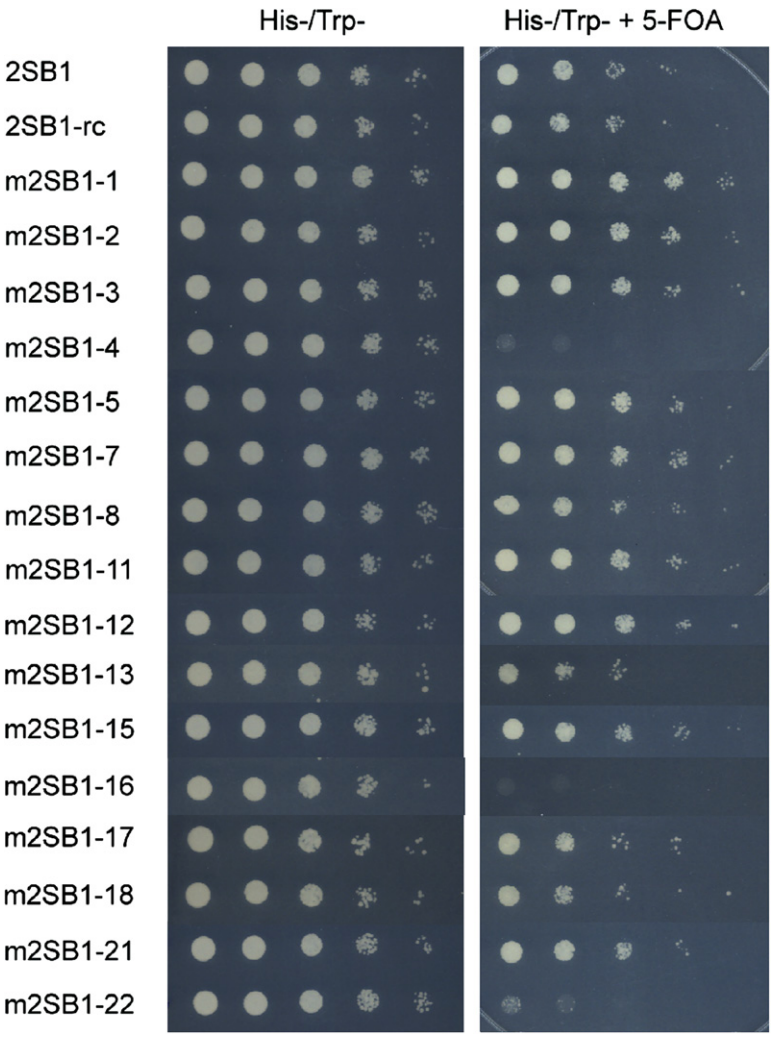


Figure 3. Activity of RNA-Based Transcriptional Silencers after Two Rounds of Evolution

2SB1-rc is the active first-round sequence 2SB1 after recloning into fresh vector and retransformation into fresh yeast cells. From left to right, each clone is spotted in 10-fold serial dilutions on the growth media specified.

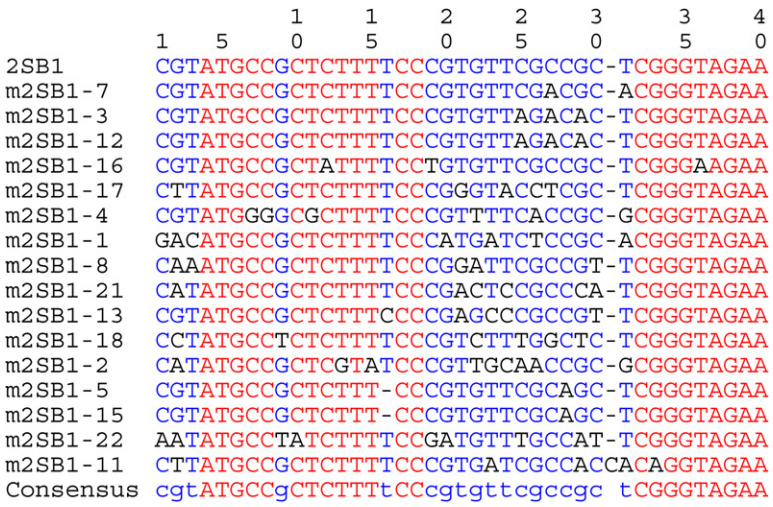


Figure 4. Sequence Alignment of RNA-Based Transcriptional Silencing Domains Identified after Two Rounds of Evolution

Red and blue indicate high and low consensus, respectively.

As expected, deleting predicted loop bases 22–30 (mutant M9) has no effect on silencing. Bases 17–19 (CCC) form the beginning of a strong stem structure by base pairing with bases 33–35 (GGG). We mutated G33, G34, or G35 to A (M7, M10, and M11, respectively), and also combined each of these three mutations with the corresponding complementary (potentially rescuing) mutations of C19, C18, or C17 to a U (M7res, M10res, and M11res, respectively), which are predicted to restore stem structure. M7 caused an incomplete reduction in silencing efficiency, while M10 induced a complete loss of silencing. Both mutations were rescued by the corresponding covariance mutations M7res and M10res (Figure 5B). Mutating G35 to an A (M11) did not have a significant effect on silencing activity (see M11, Figure 5B). These results imply that bases 17–18 and 33–34 form a stem in the active structure of m2SB1-1, and further that this stem is not involved in base-specific contacts.

Next we dissected the functional importance of bases 12–16 and 36–40, which are predicted to continue the stem formed by bases 17–19 and 33–35. The deletion of U36 (M5) abolished silencing although the base is predicted to be an extrahelical bulge. Mutation of bases 37 and 38 (AG) to CC (M1) resulted in a loss of activity that could not be rescued by the corresponding mutation of bases 15 and 16 (UU) to GG (M1res, Figure 5). The point mutation of A39 to U (M8) destroyed silencing activity and was not restored by the corresponding mutation M8res (Figure 5). These results suggest that bases 12–16 and 36–40 may play more than a secondary-structural role and are most likely involved in base-specific contacts necessary for the tertiary structure of the active RNA or for interactions with cellular targets.

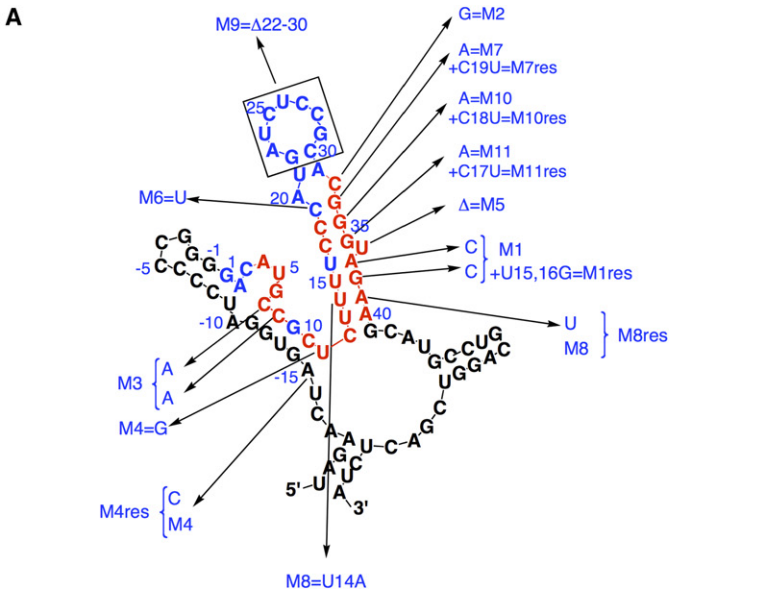
Conserved bases 7–11 are expected to participate in pairing with a portion of the 5′ constant region to form a stable stem structure. Indeed, mutating bases 7–8 (CC) to AA (M3) or U11 to G (M4) resulted in the loss of silencing activity (Figure 5). Changing A(−15) to C (M4res) may restore the base-pairing interaction destroyed by M4. This complementing mutation, however, did not rescue RNA-dependent transcriptional silencing (Figure 5), suggesting that both the paired structure and base-specific sequences of this region may be required for activity.

Probing the Mechanism of Evolved RNA-Based Gene Silencers

We hypothesized that the mechanism of action of the evolved RNA-based transcriptional silencers involves the RNA-mediated recruitment of silencing proteins such as Rap1, Abf1, or the Sir proteins to the *HMR-E* locus. However, the evolved RNA silencers in principle could act directly on the *URA3* mRNA, inhibiting translation in a *URA3*-specific manner. To test this possibility, we used yeast strain YSB35 in which silencing represses *TRP1* gene expression rather than *URA3* expression [27]. In the absence of silencing, YSB35 cells survive on medium lacking tryptophan. Upon establishment of a silenced chromatin state at the *HMR-E* locus, YSB35 can no longer grow on minimal media lacking Trp. Consistent with our envisioned mechanism of action, the most active RNA sequence m2SB1-1 showed strong levels of silencing in YSB35, as evidenced by the lack of growth on Trp-deficient media (Figure 6A). These results indicate that the observed silencing phenomenon is a general, rather than gene-specific, phenomenon.

In selection strain YEA76, two of the three silencer elements are replaced by Gal4 recognition sequences. The remaining *A* site is bound by the ORC. To test whether RNA-based silencing requires the ORC for activity, we evaluated the activity of m2SB1-1 in a *TRP1* reporter strain in which all three silencer elements were deleted (*aeb::G*). Clone m2SB1-1 was capable of silencing transcription to a much lower extent than in a strain with an intact *A* site, indicating that the ORC is required for full activity of the evolved RNA-based silencing domain, although a lower level of silencing is possible even in the absence of all three elements (Figure 6A).

Recently, Sutton and coworkers reported an alternative form of Sir-independent transcriptional silencing at *HMR-E* that requires the dominant mutation *SUM1-1* [32]. In the



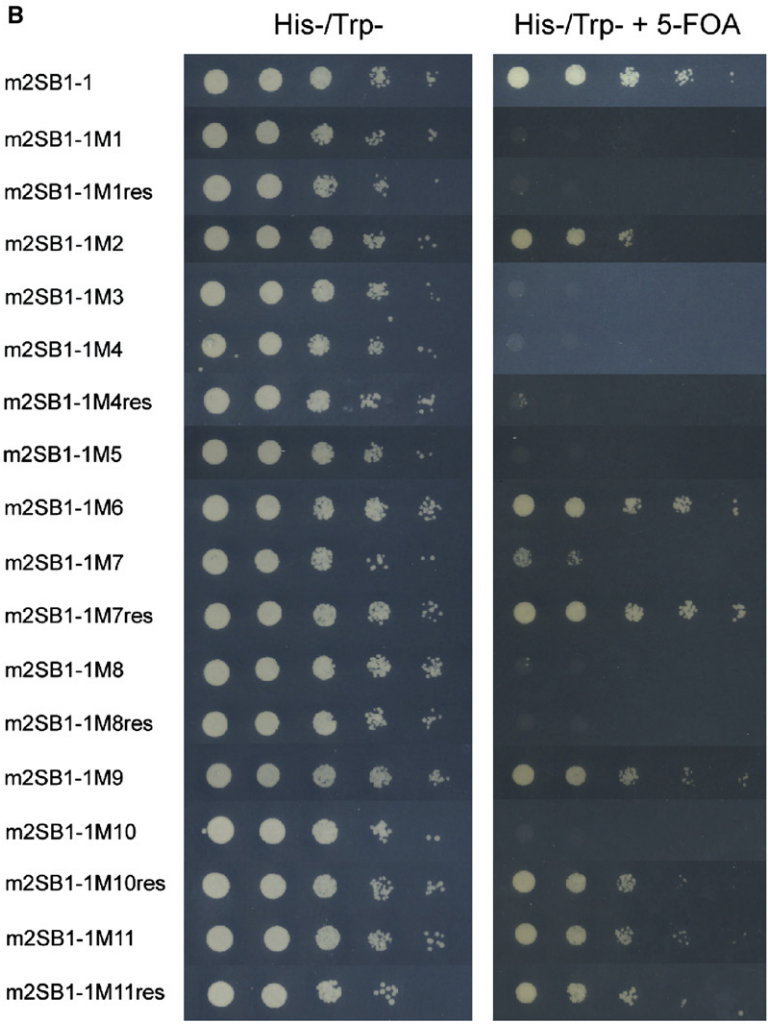


Figure 5. Structure-Function Analysis of Clone m2SB1-1

(A) Mutagenesis of m2SB1-1. The variable 40-base region is shown in color, with red and blue indicating positions of high and low consensus, respectively.

(B) Activity of m2SB1-1 mutants shown in (A). See the main text for details. From left to right, each clone is spotted in 10-fold serial dilutions on the growth media specified.

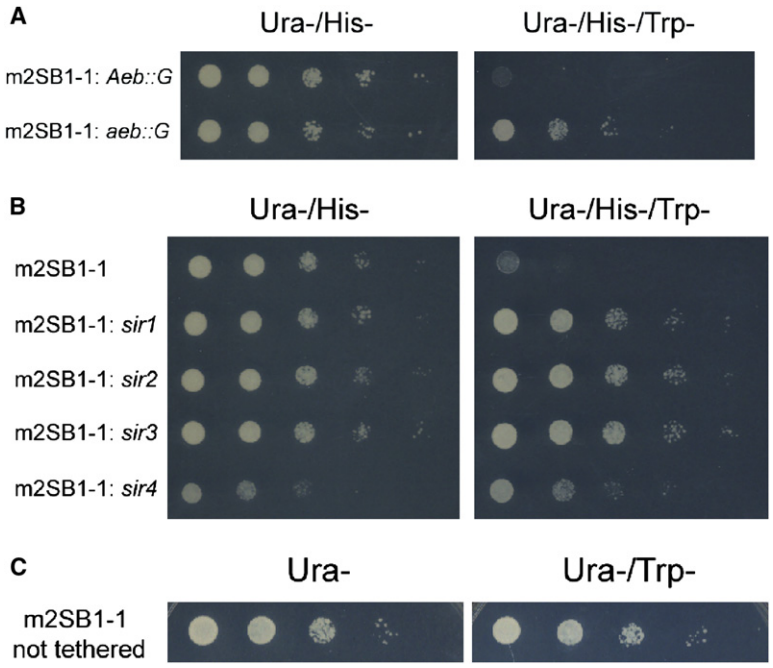


Figure 6. Probing the Mechanism of Action of an RNA-Based Transcriptional Silencer

(A) Ability of the most evolved clone m2SB1-1 to silence transcription of the *TRP1* reporter in a strain with two (Aeb) or all three (aeb) of the *HMR-E* silencing elements deleted. The lack of growth on media lacking tryptophan indicates silencing activity.

(B) Dependence of RNA-based transcriptional silencing on Sir proteins.

(C) Requirement of localization to the *HMR-E* locus for RNA-based transcriptional silencing. The m2SB1-1 was untethered from the *HMR-E* locus by using a yeast strain that did not express the Gal4-MS2 fusion protein. From left to right, each clone is spotted in 10-fold serial dilutions on the growth media specified.

proposed mechanism, the ORC interacts with the Sum1-1 protein, which in turn recruits the Sir2 homolog Hst1. Hst1 is an NAD^+-dependent histone deacetylase believed to deacetylate histones in the *HMR-E* locus and lead to heterochromatin formation and silencing without the need for the deacetylation activity of Sir2 [32]. We tested the Sir dependence of the RNA-induced silencing using the *TRP1* reporter strain deleted for Sir1, Sir2, Sir3, or Sir4. Deletion of any of these four Sir proteins abolished the silencing activity of m2SB1-1, indicating that the evolved RNA-based silencer acts through the traditional Sir-dependent mechanism (Figure 6B). We have no evidence, however, that the RNA directly recruits the Sir proteins rather than effecting their localization to the *HMR-E* locus indirectly through an interaction with another cellular protein. In addition, these results further support a model in which silencing occurs at the level of transcription.

We believe the MS2-mediated localization of the evolved RNAs to the HMR-E promoter to be crucial for activity, as it increases the effective molarity of the active RNAs with respect to the silenced locus. We tested this RNA localization requirement by introducing the most active silencer, m2SB1-1, into the selection strain YEA76 which lacks the plasmid expressing the Gal4 DNA binding domain-MS2 fusion protein. As expected, no silencing was observed (Figure 6C), indicating that localization to the promoter of interest is essential for the activity of the RNA-based gene silencers.

DISCUSSION

We applied in vivo directed evolution methods to generate RNA sequences capable of silencing transcription when tethered to the *HMR-E* locus in *S. cerevisiae*. After only two rounds of evolution, the most potent RNAs were capable of silencing transcription at levels comparable to the silencing observed when a $Gal4_{DBD}$-Sir1 fusion is localized to the *HMR-E* locus, indicating RNA might be well suited for acting as a transcriptional silencer. Indeed, in *S. pombe*, heterochromatic structure is established via an RNAi mechanism that requires small RNAs [11]. *S. cerevisiae* lacks the components of the RNAi machinery [33], and although natural RNAs that participate in transcriptional silencing have not yet been discovered, our results are consistent with the possibility that such RNAs might exist.

We used secondary-structure analysis and site-directed mutagenesis to identify regions of one of the most evolved RNAs, m2SB1-1, that are necessary for activity. Our findings suggest that sequences conserved among all active RNAs are involved in forming the secondary structures crucial for RNA-based transcriptional silencing. Covariance experiments strongly support the structural importance of one of these paired regions (bases 17–19 interacting with bases 33–35). We also identified sequences that may be involved in base-specific tertiary interactions or contacts with cellular targets (bases 12–16 and 36–40). A part of the conserved variable region formed essential base pairs with the 5′ invariable sequences, suggesting an important role for the flanking RNA scaffold sequences in the activity of these transcriptional silencers, as was previously observed in our evolution of RNA-based transcriptional activators [19].

Our evolved RNAs are general silencers of transcription and require the ORC for full activity. The dependence on Sir1, Sir2, Sir3, and Sir4 supports our model that the observed silencing is caused by establishment of a heterochromatic state in the *HMR-E* locus. We hypothesize that the RNAs function by a simple recruitment mechanism in

which they localize one or more silencing proteins to the promoter of interest.

In contrast to in vitro selections for RNA aptamers for a specific protein, in which the rate of active RNAs among random library members is approximately 1 in 10^{10}–10^{14} [34], we were capable of selecting a potent RNA silencer from a library of only 5×10^4 library members. We believe there are at least two reasons for the surprisingly high fraction of active RNAs. First, in the above approach, we do not target a specific protein but rather an entire biological process, increasing the number of proteins that an active RNA could target. Second, we believe that RNA might be especially well suited for perturbing processes involving other nucleic acids, such as transcriptional activation or silencing. We previously reported the in vivo evolution of RNA-based transcriptional activation domains. A surprisingly large fraction of random N_{40} RNAs (0.2%) was capable of activating transcription when localized to a reporter gene [19], suggesting that RNA is well suited to act as a transcriptional activator. A possible reason for the high rate of identifying RNAs capable of activating or silencing transcription is the nature of the proteins involved in such processes. In order to interact with negatively charged DNAs and RNAs, proteins that participate in the control of gene regulation commonly have positively charged patches [35, 36]. It is tempting to speculate that RNA, by virtue of its polyanionic character and structural diversity, is an especially potent biopolymer for the evolution of transcriptional regulators that recruit the positively charged portions of such proteins.

There are no known natural RNA-based transcriptional silencing or activating domains in *S. cerevisiae*, and a BLASTA search [37] failed to identify regions in the yeast genome with sequence similarities to our evolved RNA-based silencer. The ease of evolving RNA sequences with such properties suggests the intriguing possibility that such RNAs might exist in the nontranslated region of the budding yeast genome, but have not yet been discovered.

SIGNIFICANCE

We describe the in vivo evolution of a potent RNA-based transcriptional silencing domain in *S. cerevisiae*. Starting with a relatively small, random library expressed in yeast cells, we identified a silencing domain comparable in potency to a $Gal4_{DBD}$-Sir1 fusion localized to the *HMR-E* locus after only two rounds of evolution. The high frequency of active clones in our study contrasts with the traditionally low rates of finding RNA-based binders to proteins using in vitro selections. We speculate that the polyanionic character combined with the great structural and functional diversity of RNA makes it especially well suited to mediate processes that involve proteins with cationic patches such as transcriptional silencing. Secondary-structure predictions and site-directed mutagenesis identified the important functional domains of the evolved RNA-based transcriptional silencers. Genetic studies suggest that our evolved RNAs establish silencing via a traditional Sir-based mechanism. Our results further demonstrate the value of RNA as a tool to perturb biological functions and also suggest that natural RNA-based silencing or activating domains may exist in *S. cerevisiae*.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

Media consisted of yeast nitrogen base (Sigma), 2% dextrose, and synthetic dropout supplements (Bio101). Yeast were cultured either in liquid media or on agar plates at 30°C. Plates were supplemented with 1 g/l 5-fluoroorotic acid (5-FOA) to select for lack of expression of *URA3*. *S. cerevisiae* strains YSB1 [23] (*HML*α, *MAT*α, *HMR***a**, *ade2-1*, *ura3-1*, *his3-11,15*, *leu2-3,112*, *trp1-1*, *can1-100*, *aeB hmr::TRP1*, *gal4::LEU2*), YSB35 (YSB1 except *Aeb::3xUAS*$_G$), YSB41 (YSB1 except *aeb::3xUAS*$_G$), and YEA76 [27] (YSB1 *Aeb::UAS*$_G$*::hmr::URA3*) were kindly provided by Professor Rolf Sternglanz. Full open reading frame (ORF) deletions of *Sir1*, *Sir2*, *Sir3*, and *Sir4*, replaced by the *kanMX4* gene, were generated using a PCR-based deletion strategy [38, 39]. All gene disruptions were confirmed by PCR and automated DNA sequencing.

Plasmid and RNA Library Construction

Plasmids encoding the RNA library were constructed by subcloning the fragment encoding the *Ade2* gene and the RNA expression cassette from pIIIa/MS2 [28] into the yeast shuttle vector pRS424 using unique NotI and KpnI sites. The resulting plasmid (pRNAIII) carries a *His3* marker as well as the *Ade2* gene that can be used to screen for false positives. Random single-stranded N_{40} library sequences were generated on an Applied Biosystems Expedite 8909 DNA synthesizer and extended with the Klenow fragment of *E. coli* DNA polymerase I to give double-stranded, blunt-ended library inserts. After digestion with XmaI and SphI, the N_{40} library was ligated into pRNAIII and amplified by transformation into electrocompetent DH10B *E. coli* cells (Invitrogen).

A fusion of the Gal4 DNA binding domain ($Gal4_{DBD}$) and the MS2 coat protein was expressed from the ADH1 promoter on p423Gal4MS2. $Gal4_{DBD}$ was amplified from pGBKT7 (Clontech) using primers 5′-CCGCCGCTGCAGATGAAGCTACTGTCTTCTATCGAAC-3′ and 5′-AGCCATACCCGGGAGGTCCTCCTCTGAGATCAGC-3′, digested with PstI and XmaI, and cloned into the PstI- and XmaI-digested vector pADH1LexAMS2term. The resulting Gal4-MS2 region was excised together with the ADH1 promoter using NgoMI and SacII and subcloned into the NgoI- and SacII-digested pRS423. All constructs were verified by automated DNA sequencing. Molecular biology enzymes were purchased from New England Biolabs. Plasmids pRS423 and pRS424 were gifts of Professor Andrew Murray.

Selection and Screening Procedures

Yeast strain YEA76, carrying p423Gal4MS2, was transformed with the RNA expression plasmid using a standard lithium acetate procedure. Transformants were selected on media lacking tryptophan and histidine, and then harvested and replated on selective media supplemented with 5-FOA. Survivors were pooled and their plasmid DNA was extracted using a Plasmid Mini-Prep kit (Bio-Rad) with an initial step of glass-bead lysis of the yeast cells in resuspension buffer (Bio-Rad). Plasmid p423Gal4MS2 was digested with BglII to preclude further propagation and the RNA expression plasmid was amplified in *E. coli*. The selected library members were retransformed in YEA76 strains carrying p423Gal4MS2 and passed through a second round of selection. Plasmid DNA from individual surviving clones was extracted, and the variable N_{40} region was sequenced and recloned in pRNAIII as well as in pIIIa/MS2 for use in the secondary screen in yeast strain YSB35.

Retransformed clones were assayed for the ability to silence the expression of *URA3* by spotting 10- or 5-fold serial dilutions of cells grown to mid-log phase on media lacking tryptophan and histidine, in the presence or absence of 5-FOA. The most concentrated spot corresponds to 10 μl of undiluted yeast culture at an OD_{600} of 1–1.5. As a secondary screen of activity, RNA sequences were cloned into pIIIa/MS2 and assayed for their ability to silence the expression of *TRP1* in YEA35 yeast cells. All assays were performed at least three independent times and figures shown reflect representative results. Secondary structures of selected RNA sequences were predicted with the mfold program [31].

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REFERENCES

1. Doudna, J.A., and Cech, T.R. (2002). The chemical repertoire of natural ribozymes. Nature *418*, 222–228.
2. Moore, P.B., and Steitz, T.A. (2002). The involvement of RNA in ribosome function. Nature *418*, 229–235.
3. Gottesman, S. (2004). The small RNA regulators of *Escherichia coli*: roles and mechanisms. Annu. Rev. Microbiol. *58*, 303–328.
4. Mandal, M., and Breaker, R.R. (2004). Gene regulation by riboswitches. Nat. Rev. Mol. Cell Biol. *5*, 451–463.
5. Winkler, W.C., and Breaker, R.R. (2005). Regulation of bacterial gene expression by riboswitches. Annu. Rev. Microbiol. *59*, 487–517.
6. Baulcombe, D. (2004). RNA silencing in plants. Nature *431*, 356–363.
7. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806–811.
8. Mello, C.C., and Conte, D., Jr. (2004). Revealing the world of RNA interference. Nature *431*, 338–342.
9. Mochizuki, K., Fine, N.A., Fujisawa, T., and Gorovsky, M.A. (2002). Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in Tetrahymena. Cell *110*, 689–699.
10. Yao, M.C., Fuller, P., and Xi, X. (2003). Programmed DNA deletion as an RNA-guided system of genome defense. Science *300*, 1581–1584.
11. Grewal, S.I., and Moazed, D. (2003). Heterochromatin and epigenetic control of gene expression. Science *301*, 798–802.
12. Keefe, A.D., and Szostak, J.W. (2001). Functional proteins from a random-sequence library. Nature *410*, 715–718.
13. Soukup, G.A., and Breaker, R.R. (2000). Allosteric nucleic acid catalysts. Curr. Opin. Struct. Biol. *10*, 318–325.
14. Buskirk, A.R., Landrigan, A., and Liu, D.R. (2004). Engineering a ligand-dependent RNA transcriptional activator. Chem. Biol. *11*, 1157–1163.
15. Ferber, M.J., and Maher, L.J., III. (1998). Combinatorial selection of a small RNA that induces amplification of IncFII plasmids in *Escherichia coli*. J. Mol. Biol. *279*, 565–576.
16. Lebruska, L.L., and Maher, L.J., III. (1999). Selection and characterization of an RNA decoy for transcription factor NF-κ B. Biochemistry *38*, 3168–3174.
17. Soukup, G.A., and Maher, J.J., III. (1998). Selection and characterization of RNAs that relieve transcriptional interference in *Escherichia coli*. Nucleic Acids Res. *26*, 2715–2722.
18. Zimmerman, J.M., and Maher, L.J., III. (2002). In vivo selection of spectinomycin-binding RNAs. Nucleic Acids Res. *30*, 5425–5435.
19. Buskirk, A.R., Kehayova, P.D., Landrigan, A., and Liu, D.R. (2003). In vivo evolution of an RNA-based transcriptional activator. Chem. Biol. *10*, 533–540.
20. Rusche, L.N., Kirchmaier, A.L., and Rine, J. (2002). Ordered nucleation and spreading of silenced chromatin in *Saccharomyces cerevisiae*. Mol. Biol. Cell *13*, 2207–2222.
21. Tanny, J.C., Kirkpatrick, D.S., Gerber, S.A., Gygi, S.P., and Moazed, D. (2004). Budding yeast silencing complexes and regulation of Sir2 activity by protein-protein interactions. Mol. Cell. Biol. *24*, 6931–6946.
22. Andrulis, E.D., Zappulla, D.C., Alexieva-Botcheva, K., Evangelista, C., and Sternglanz, R. (2004). One-hybrid screens at the *Saccharomyces cerevisiae* HMR locus identify novel transcriptional silencing factors. Genetics *166*, 631–635.
23. Chien, C.T., Buck, S., Sternglanz, R., and Shore, D. (1993). Targeting of SIR1 protein establishes transcriptional silencing at HM loci and telomeres in yeast. Cell *75*, 531–541.
24. Triolo, T., and Sternglanz, R. (1996). Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. Nature *381*, 251–253.
25. Buck, S.W., and Shore, D. (1995). Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between HMR and telomeres in yeast. Genes Dev. *9*, 370–384.
26. Lustig, A.J., Liu, C., Zhang, C., and Hanish, J.P. (1996). Tethered Sir3p nucleates silencing at telomeres and internal loci in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *16*, 2483–2495.
27. Andrulis, E.D., Zappulla, D.C., Ansari, A., Perrod, S., Laiosa, C.V., Gartenberg, M.R., and Sternglanz, R. (2002). Esc1, a nuclear periphery protein required for Sir4-based plasmid anchoring and partitioning. Mol. Cell. Biol. *22*, 8292–8301.
28. Bernstein, D.S., Buter, N., Stumpf, C., and Wickens, M. (2002). Analyzing mRNA-protein complexes using a yeast three-hybrid system. Methods *26*, 123–141.
29. Zhang, B., Kraemer, B., SenGupta, D., Fields, S., and Wickens, M. (2000). Yeast three-hybrid system to detect and analyze RNA-protein interactions. Methods Enzymol. *318*, 399–419.
30. Good, P.D., and Engelke, D.R. (1994). Yeast expression vectors using RNA polymerase III promoters. Gene *151*, 209–214.
31. Mathews, D.H., Sabina, J., Zuker, M., and Turner, D.H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. *288*, 911–940.
32. Sutton, A., Heller, R.C., Landry, J., Choy, J.S., Sirko, A., and Sternglanz, R. (2001). A novel form of transcriptional silencing by Sum1-1 requires Hst1 and the origin recognition complex. Mol. Cell. Biol. *21*, 3514–3522.
33. Perrod, S., and Gasser, S.M. (2003). Long-range silencing and position effects at telomeres and centromeres: parallels and differences. Cell. Mol. Life Sci. *60*, 2303–2318.

34. Wilson, D.S., and Szostak, J.W. (1999). In vitro selection of functional nucleic acids. Annu. Rev. Biochem. *68*, 611–647.

35. Boggon, T.J., Shan, W.S., Santagata, S., Myers, S.C., and Shapiro, L. (1999). Implication of tubby proteins as transcription factors by structure-based functional analysis. Science *286*, 2119–2125.

36. Jones, S., Shanahan, H.P., Berman, H.M., and Thornton, J.M. (2003). Using electrostatic potentials to predict DNA-binding sites on DNA-binding proteins. Nucleic Acids Res. *31*, 7189–7198.

37. Gish, W. (1996–2004). http://blast.wustl.edu.

38. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993). A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucleic Acids Res. *21*, 3329–3330.

39. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast *10*, 1793–1808.

