Protosilencers in *Saccharomyces cerevisiae* Subtelomeric Regions

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ABSTRACT

Saccharomyces cerevisiae subtelomeric repeats contain silencing elements such as the core X sequence, which is present at all chromosome ends. When transplaced at *HML*, core X can enhance the action of a distant silencer without acting as a silencer on its own, thus fulfilling the functional definition of a protosilencer. Here we show that an ACS motif and an Abf1p-binding site participate in the silencing capacity of core X and that their effects are additive. In addition, in a variety of settings, core X was found to bring about substantial gene repression only when a low level of silencing was already detectable in its absence. Adjoining an X-STAR sequence, which naturally abuts core X in subtelomeric regions, did not improve the silencing capacity of core X. We propose that protosilencers play a major role in a variety of silencing phenomena, as is the case for core X, which acts as a silencing relay, prolonging silencing propagation away from telomeres.

SILENCING is a form of repression that is not promoter specific and can extend over large chromosomal regions. A classical example is the position effect variegation (PEV) of gene expression due to proximity to heterochromatin (reviewed in Henikoff 2000). In *Saccharomyces cerevisiae*, silencing is found at silent mating-type loci (*HM*) within rDNA repeats and in subtelomeric regions [telomere position effect (TPE)]. Key components of silent chromatin in *S. cerevisiae* include the complex of silent information regulators Sir2p, Sir3p, and Sir4p that is formed through homo- and heterotypic interactions, as well as histones H3 and H4 (for a review, see Gartenberg 2000). Both establishment and maintenance of silencing depend on *cis*-acting elements known as silencers that nucleate assembly of a SIR complex. *HM* loci are flanked by the E and I silencers, composed of specific combinations of binding sites for Abf1p, the origin replication complex (ORC), and Rap1p. These act as a "surface" for Sir interaction, either directly or through Sir1p, as in the case of Orc (Triolo and Sternglanz 1996). At telomeres, the SIR complex interacts with tandemly reiterated Rap1p molecules bound to telomere-repeat sequences. Silencing is thought to result from the subsequent "spreading" of the SIR complex across the silenced locus, involving interactions between H3/H4 and Sir3p/Sir4p. Isolated binding sites for silencer binding proteins, although unable to initiate silencing at a silencer-free *HM* locus, have been shown to cooperatively interact with intact, distant silencers to strengthen silencing (Boscheron *et al.* 1996). They have been called protosilencers by analogy with protoenhancers. Recently, protosilencers were shown to autonomously maintain silencing in G1-arrested cells but not in cycling cells (Cheng and Gartenberg 2000). This contrasts with *bona fide* silencers that stably maintain silencing during the cell cycle.

A new type of silencing element was recently described in *S. cerevisiae* subtelomeric X and Y′ repeats. It corresponds to the invariant part of X elements, called core X, and to an internal segment of Y′ (Louis *et al.* 1994; Louis 1995; Fourel *et al.* 1999; Pryde and Louis 1999). These elements act as relays prolonging the inward propagation of telomeric silencing. Albeit unable to induce silencing on their own when inserted at *HML*, they can reinforce the effect of a weak silencer, thus behaving like protosilencers (Fourel *et al.* 1999). Interestingly, these silencing elements may be involved in the SIR-dependent inhibition of replication initiation from subtelomeric ARS consensus sequences (ACSs; Stevenson and Gottschling 1999) and in the silencing of Ty5-1 retrotransposon at telomere III-L (Vega-Palas *et al.* 1997). Different sequence motifs that may play a role in silencing have been previously recognized in core X (Louis 1995). These are: (i) an ORC-binding site (ACS), also found in Y′; (ii) an Abf1p-binding site; (iii) a GC-rich sequence that is nearly identical to the meiosis specific regulatory sequence URS1, which allows tethering of the Rpd3p histone deacetylase through

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binding of the Ume6p factor (Kadosh and Struhl 1997). Subtelomeric silencing elements are separated from telomere repeats by insulator elements named STARs (for subtelomeric antisilencing region) that can protect a reporter gene from silencing when interposed between it and a silencer (Fourel *et al.* 1999). Within X elements, the STAR sequence (named X-STAR) coincides with the more variable STR region (Louis 1995; Fourel *et al.* 1999). Tbf1p and Reb1p proteins bind to multiple sites within STARS and likely contribute an essential role in their activity (Fourel *et al.* 1999, 2001; Koering *et al.* 2000; Figure 1A).

The efficiency of silencers upon ectopic chromosomal insertion decreases as the distance from telomeres increases (Maillet *et al.* 1996). However, their silencing capacity can be restored at a long distance away from telomeres either by overexpressing Sir proteins or by decreasing the ability of Sir proteins to bind telomeres. This substantiates the view that telomeres play the role of reservoirs of silent chromatin components (Maillet *et al.* 1996; Marcand *et al.* 1996; Martin *et al.* 1999). Core X seemingly functions as a silencer in the vicinity of a telomere but not at *HML*, *i.e.*, 13 kb away from telomere III-L. Core X was therefore speculated to be a weak silencer that is potentiated in its natural telomeric environment by the specific enrichment in Sir proteins. Here we addressed this hypothesis by dissecting core X silencing activity and by asking whether core X might behave as a *bona fide* silencer under other circumstances.

TABLE 1

Oligonucleotides used in plasmid constructions

Primer	Sequence
19A1	5′-GCGCAGATCTGTATGACTATAGAGTA CAG
19A2	5′-GCGCGGATCCTATATCTCATTCGGCGG CCC
19B1	5′-GCGCAGATCTAGATATTAAAATGTGGA TAATCG
19B2	5′-CGCGGATCCATCGTAAATAATACATAC ATAC
19C1	5′-GCGCAGATCTTAACGTTTCAATATGG AGG
19C2	5′-GCGGGATCCACACACCCTAACACAAT CCTAAC
STRD-Ba	5′-TCCTGGATCCTTTGTTAACG
STRD-RAP	5′-TCCTGGATCCCATCCCAAACAAAACCC ACACATCATTTGTTAACG
STRA-BgBis	5′-TCCTAGATCTACACCCACTACTCTAA CCC
ACS−	5′-GATCGGATCCAATACCTAAATATAAA AAATGTTATTGTTTAAAC
ACS+	5′-GATCGTTTAAACAATAACATTTTTTAT ATTTAGGTATTGGATCC
ABF−	5′-GATCGGATCCTCAATATATCGTTATTA ACGATATATGTTTAAAC
ABF+	5′-GATCGTTTAAACATATATCGTTAATAA CGATATATTGAGGATCC
HML-E	5′-TCCTGGTCTCTGGCCGCCCACTGTTT TTTCCGCCTCC
HML-I	5′-TCCTGGTCTCTGGCCGCGAGATCGAA AGAAAGCCCC

MATERIALS AND METHODS

Plasmid constructions: Molecular biology techniques were performed as described in Sambrook *et al.* (1989). The Expand HiFi system (Boehringer Mannheim) was employed to carry out PCRs according to the manufacturer's recommendations.

Plasmids used in end replacements at telomere VII-L or to modify the *HML* locus were obtained by inserting various fragments at the *Bam*HI site of pURTEL or at the *Bcl*I site or pE-i, respectively, as described in Fourel *et al.* (1999). GF1, GF3, GF6, GF19, GF21, GF25, GF31, GF48, GF53, GF62, GF63, GF64, GF65, and GF72 were previously obtained and analyzed in Fourel *et al.* (1999). PCR amplification of natural subtelomeric regions was carried out using plasmids p19X10 and p89H9 [containing, respectively, chromosome II-R and XI-L end (Louis and Borts 1995), kindly provided by E. Louis] as templates and oligonucleotides listed in Table 1. The PCR amplification reactions used to generate the various constructs were performed with the following pairs of primers: STR(IIR), 19C1 + 19C2; X1(IIR), 19A1 + 19A2; X2(IIR), 19B1 + 19B2; coreX(IIR), 19A1 + 19B2; X(IIR), 19A1 + 19C2; X2-STR(IIR), 19B1 + 19C2; STR(XI-L), STRD-Ba + STRA-BgBis; Rap1p (RAP)/ STR(XI-L), STRD-RAP + STRA-BgBis. Note that the limits of the core X fragment were defined as in Louis *et al.* (1994), *i.e.*, as corresponding to the highly conserved portion of 450–475 bp (Pryde *et al.* 1995) plus a telomere distal sequence up to 600 bp. The construct used to generate GF76 strain was linearized with *Bam*HI and the STR(IIR) fragment was inserted to produce GF77 strain. An ACS motif (ACS) and a binding site for Abf1p (ABF) were obtained through annealing of ACS− and ACS+ and ABF− and ABF+ oligonucleotides, respectively, and inserted at the *Bam*HI site of pURTEL, and of the construct used to generate GF3. The Rap1p-binding site in RAP corresponds to that found in UASα (Giesman *et al.* 1991). The oligonucleotide ACS contains a perfect ARS consensus sequence and the indicated flanking region is identical to the flanking sequence of the ACS in *HMR*-E. The oligonucleotide ABF contains a perfect Abf1p-binding site consensus and the flanking sequence is identical to that used in the synthetic silencer (McNally and Rine 1991).

Replicative plasmids harboring a modified *HML* locus were obtained through PCR amplification of this locus from the genomic DNA of the corresponding strains using the HML-E and HML-I primers and insertion at the *Not*I site of pRS315 (Sikorski and Hieter 1989). These primers contain a terminal *Bsa*I site and adjacent sequence engineered to create *Not*I compatible ends after *Bsa*I digestion of the PCR products.

p2μASir3His and the corresponding control p2μAHis were obtained from p2μASir3 and pAAH5 (Maillet *et al.* 1996) by inserting a *HIS3* fragment at the unique *Bgl*II and *Pvu*II sites, respectively. These plasmids allow both leucine and histidine prototrophy.

Yeast strains, media, and methods: Genetic manipulations and growth of yeast are as described in Fourel *et al.* (1999). Yeast strains described in this article harboring a modified VII-L telomere are derivatives of W303-1a (*MAT***a** *ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*) and those with a modified *HML* locus have a S150-2B background (*MAT***a** *leu2-3,112*

ura3-52 trp1-289 his3D gal2 gal4:LEU2). Deletion in the *HDF1* gene was obtained by replacement of the full-length open reading frames (ORFs) by the KanMX4 cassette as described in Pryde and Louis (1999). A large number of control strains was previously described in Fourel *et al.* (1999). All telomere fragmentation and loci replacement were confirmed by Southern blot analysis.

For overexpressing Sir3p and/or Sir4p, the appropriate strains were transformed with the high-copy plasmids p2μASir3His (see above) and pFP320 (Maillet *et al.* 1996) or the corresponding control plasmids p2μAHis and pRS424 and grown on medium lacking histidine and tryptophane. Yeast strains harboring *HML*-related replicative plasmids were obtained through transformation of W303-1A with the corresponding plasmids and selection on SC− leucine medium.

Analysis of *URA3* expression: The variegated expression of *URA3* was monitored essentially as described in Fourel *et al.* (1999) by spotting 10 μl of serial dilutions of overnight culture onto SC, SC − uracil (SC-U), and SC + 5-FOA (1 g/liter) and allowing growth for 3 days at 30°. In situations in which particularly low silencing levels were considered (Figures 4 and 5), 1-ml cultures were plated on 10-cm 5-fluoroorotic acid (5-FOA)-containing dishes of appropriate selective medium and assayed classically by spotting 10-μl dilutions on corresponding 5-FOA-lacking medium to estimate the total cell number. After growth for 4 days at 30°, three representative colonies were picked from dishes harboring colonies, which were sometimes of varying size, and serial 10-μl dilutions were spotted on SC and SC-U to address possible mutation of the reporter *URA3* gene. A total of 12–24 cultures seeded with independent transformants were assayed for each strain, and the average value indicated by the histogram bar was calculated as the cumulation of 5-FOA-resistant colonies harboring a functional *URA3* reporter among the estimated total number of colonies for all 12–24 cultures.

RESULTS

Core X silencing activity involves redundant elements: Insertion of an X-STAR sequence (Figure 1A) between telomere repeats and a *URA3* reporter gene strikingly impedes its silencing, as indicated both by a decrease in the frequency of 5-FOA-resistant colonies—a compound toxic for cells expressing a functional *URA3* gene product (Boeke *et al.* 1984)—and an increase of the ratio of colonies able to grow to a normal size on a medium lacking uracil (SC-U; Figure 1B, compare lines 1 and 3, 2 and 4). This decrease in silencing is due to the insulator activity of X-STAR, also called STAR activity, which is itself counteracted by the silencing activity of core X upon insertion of a full-length X element, thus restoring predominant *URA3* silencing (Figure 1B, lines 7–8) (Fourel *et al.* 1999).

In an attempt to identify elements responsible for core X silencing capacity, the core X sequence of II-R telomere was arbitrarily dissected into two 0.3-kb fragments (X1 and X2, Figure 1A). Strikingly, the combination of either X1 or X2 with the X-STAR region restored substantial silencing as compared to the insertion of the X-STAR region by itself, thus qualitatively recapitulating the activity of the whole core X element (Figure 1B, compare lines 3, 7, 11, and 15 with lines 2, 4, 8, 12, and 16). Silencing levels were nevertheless not quite as high as when full-length core X sequences were provided, suggesting that each of X1 and X2 do not recapitulate all the silencing capacities of full-length core X elements. This conclusion is strengthened by the fact that the insertion of full-length core X displaces *URA3* 0.3 kb farther away from the telomere and would thus theoretically decrease the influence of TPE on *URA3* expression as compared with the insertion of either X1 or X2. Of note, the X2 fragment clearly strengthens telomeric silencing upon insertion between *URA3* and telomere repeats. This does not hold true for X1 and core X fragments, as better visualized on SC-U (Figure 1B, compare lines 2, 6, 10, and 14). Similar results were obtained using subtelomeric sequences derived from chromosome XI-L end (data not shown). We conclude that core X consists of at least two independent functional elements, which cooperate to yield full core X silencing activity.

Core X silencing activity is recapitulated by elementary protosilencers—single binding sites for Rap1p, Abf1p, or the ORC complex: The presence of putative ORC- and Abf1p-binding sites was previously noted through sequence inspection of the X1 and X2 fragments, respectively (Louis 1995; Figure 1A). These elements, together with Rap1-binding sites, constitute the building blocks of silencers at the silent mating-type cassettes. In addition, silencing at *HM* loci and at telomeres harboring an X element were found to share many properties, such as a partial requirement for Sir1p and only a weak dependence on the *HDF1* gene product (Fourel *et al.* 1999). The ORC- and Abf1p-binding sites therefore emerged as strong candidates in mediating core X silencing activity.

Oligonucleotides, including either an ACS or an Abf1p-binding site (named ABF), were found to substitute for core X in restoring significant silencing levels (Figure 2A, compare GF78 and GF80 to GF1, GF3, and GF6). However, the silencing effect was not as strong as that of core X and was actually slightly weaker than that of the corresponding X2 and X1 fragments. Significantly, whereas insertion of the X2 fragment by itself resulted in enhanced TPE (see Figure 1B, GF74), this was not the case with the ABF oligonucleotide (Figure 2A, GF81). Conversely, abutting an ACS oligonucleotide to telomere repeats improved TPE, as better visualized on SC-U (Figure 2A, compare lines 2 and 8). Core X silencing activity was also recapitulated by targeting Gal4 chimera containing either Orc1p N terminus or Abf1p transcription activation domain, to UASg binding sites placed in subtelomeric position (data not shown). These findings suggest that full core X activity involves the additive cooperation of several protosilencers among which are the Abf1p- and ORC-binding sites. This conclusion is also supported by the impairment of subtelomeric silencing upon mutation of the ACS or the Abf1p-binding site (Pryde and Louis 1999). We

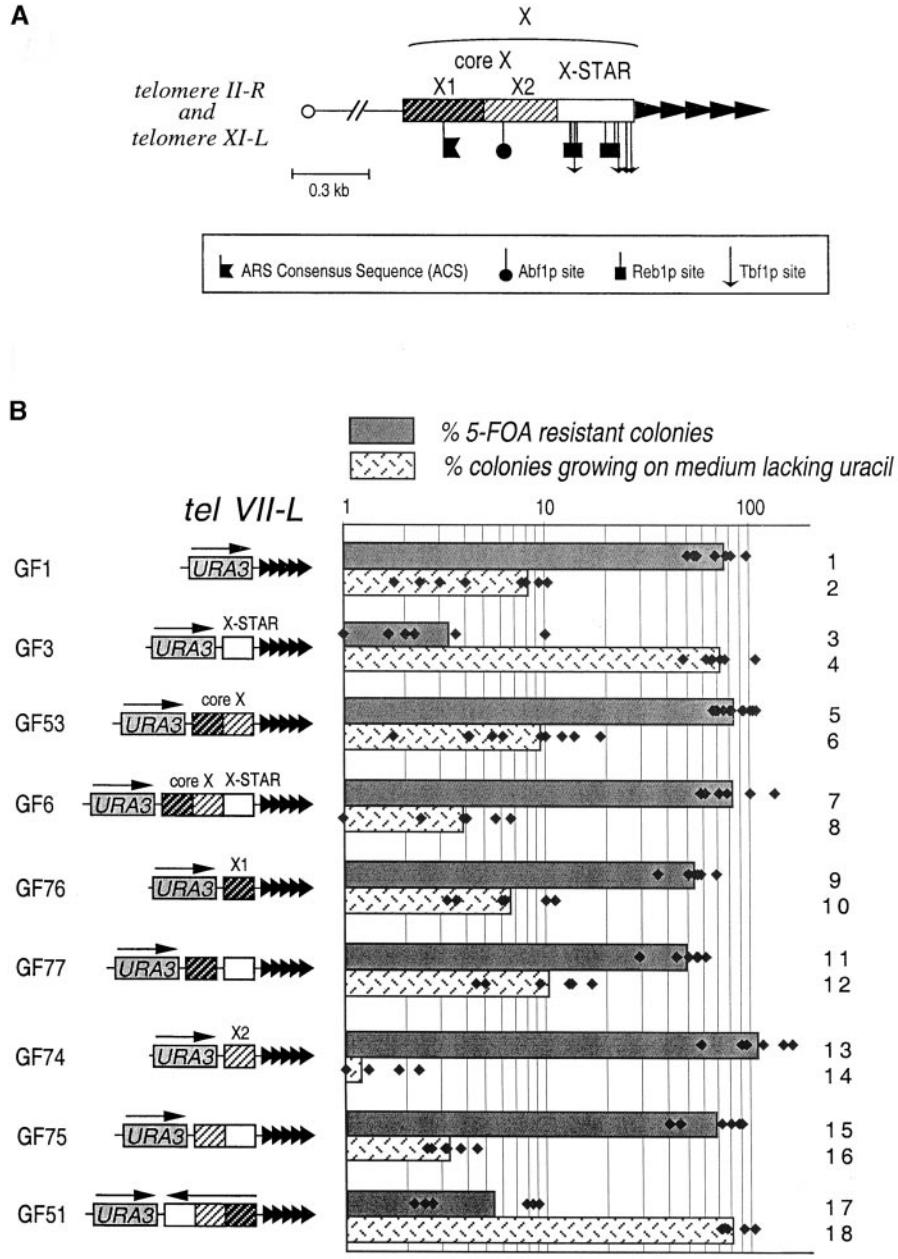

FIGURE 1.—Core X silencing activity involves redundant elements. (A) Schematic representation of *S. cerevisiae* chromosome II right end and chromosome XI left end, with emphasis on the X subtelomeric element. Tandem arrowheads, TG1-3n telomeric repeats. Core X was arbitrarily subdivided into two 300-bp segments, named X1 and X2, and represented as hatched boxes. The STR sequence is shown as a stippled box. The positions of an ACS and of binding sites for Abf1p, Reb1p, and Tbf1p are indicated by symbols. (B) Left: Schematic representation of the left end of chromosome VII after recombination at the *ADH4* locus, telomere seeding, and chromosome fragmentation using pURTEL-based constructs (FOUREL *et al.* 1999), including fragments from the telomere II-R X element (see MATERIALS AND METHODS). Arrows indicate the 5′ to 3′ direction of *URA3* transcription, and the X element reversed orientation in strain GF51. In strain GF77, the STR and the X1 fragments are artificially placed adjacently, which is illustrated by a space separating the corresponding boxes. Shown is a representative experiment. At least three independent transformants were analyzed for each strain, and for each of them cultures started from at least two independent colonies were serially diluted and grown on synthetic complete (SC), on SC + 5-FOA, and on SC − uracil (SC-U) medium. SC + 5-FOA allows only growth of cells that do not express *URA3*. Right: Each diamond indicates the ratio of colonies growing on either SC + 5-FOA or SC-U *vs.* SC for a single culture, considered as measurements of *URA3* repression and expression, respectively. The histogram bar represents the average of the values obtained for a given strain and medium, as indicated above the panel.

previously showed that the effect of deleting the *HDF1* gene on TPE is not as profound for a telomere harboring a core X sequence as for strains carrying only reporter genes next to telomere repeats (FOUREL *et al.* 1999). A similar partial loss of *HDF1* dependency is also observed upon the insertion of either an ACS or an ABF oligonucleotide (Figure 2B, compare GF66, GF67, GF83, and GF84), indicating that individual ACS and ABF sequences can contribute to the overall TPE.

Inspection of core X sequences did not reveal the presence of any potential Rap1p-binding site complying with the previously defined consensus. However, short tracts of TG1-3 repeats are frequently found on the centromere proximal side of Y′ elements, embedded between X and Y′ sequences. These provide Rap1p-binding sites that might potentially cooperate with core X. Therefore, a Rap1p oligonucleotide (named RAP) was also assayed. It was found to counteract X-STAR-mediated antisilencing when inserted as a single copy, much like ACS and ABF oligonucleotides (Figure 2A, GF82).

Thus, core X subtelomeric silencing activity is partially recapitulated by individual binding sites for the ORC, Abf1p, and Rap1p proteins, and elementary protosilencers seemingly cooperate in an additive manner to yield full core X silencing activity.

Adjoining X-STAR sequences does not improve core X silencing capacity at *HML*: Yeast subtelomeric insulator and silencing elements seemingly operate in an independent manner (FOUREL *et al.* 1999). However, core X

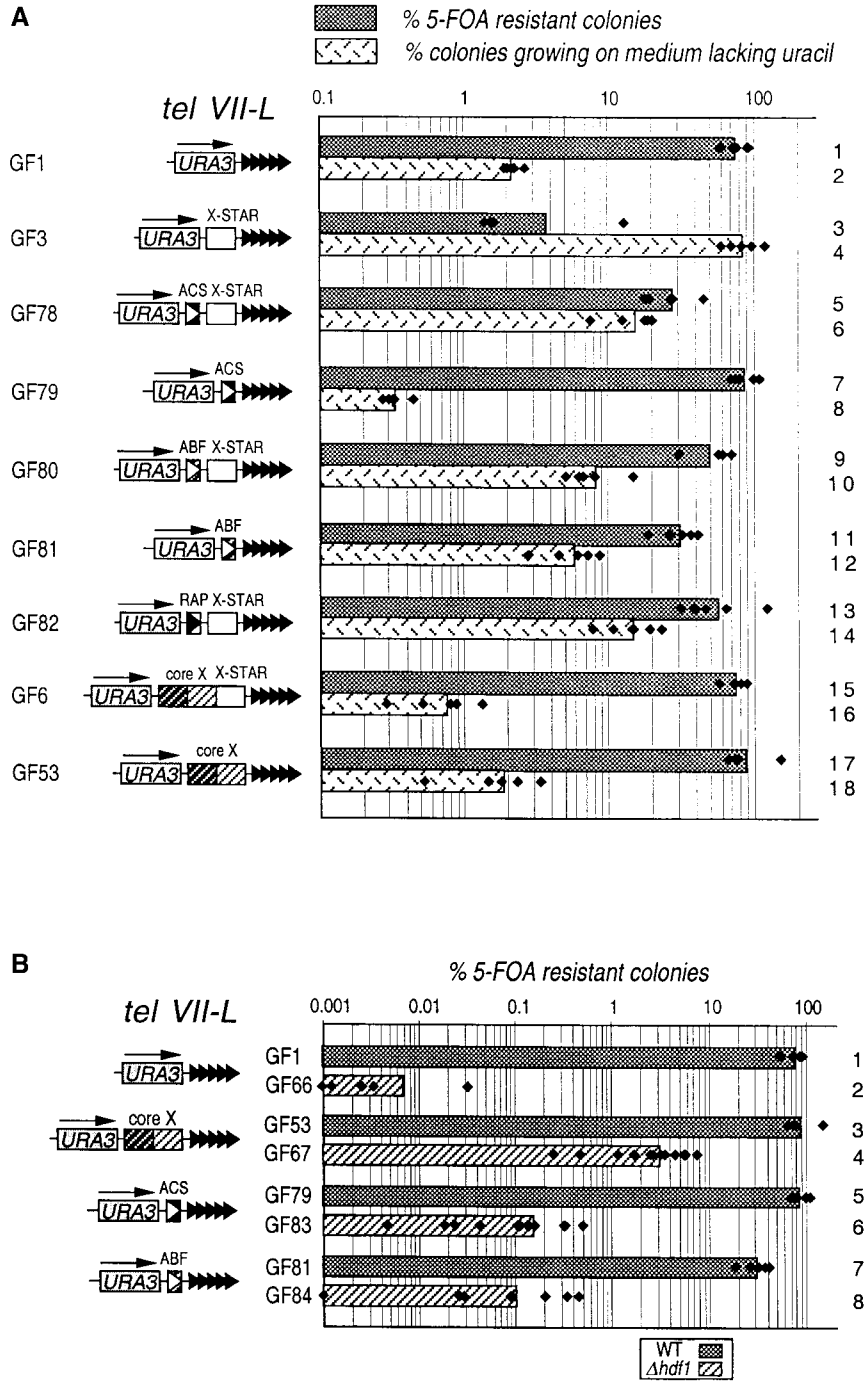

FIGURE 2.—Core X silencing activity is recapitulated by single binding sites for either Rap1p or Abf1p proteins or the ORC complex. The ACS and Abf1p- and Rap1-binding sites inserted in the subtelomeric position at a fragmented VII-L telomere included consensus sequences and were of distinct origins (see MATERIALS AND METHODS). (A) The isolated STR segment in strains GF3 and GF78–82 is derived from chromosome XI left telomere. (B) Deletion of the *HDF1* ORF was carried out in strains GF1, GF53, GF79, and GF81 to obtain strains GF66, GF67, GF83, and GF84, respectively, as described in MATERIALS AND METHODS. Details are otherwise the same as in the legend for Figure 1.

is almost always found adjacent to an X-STAR sequence (LOUIS 1995). Analogous silencer/insulator combinations have been previously described in other systems and organisms (MIHALY *et al.* 1997; AYOUB *et al.* 1999; DONZE *et al.* 1999; SRIVASTAVA *et al.* 2000), which might point to common functional determinants. Therefore, although X-STAR sequences are not required for core X silencing activity under conditions of optimal operation in telomere vicinity, we thought it was possible that they may contribute to core X silencing activity in less favorable settings such as at the *HM* loci. We thus compared the silencing capacity of a full-length X element to that of core X at the *HML* locus.

A series of isogenic yeast strains was generated in which most *HML* was replaced by *URA3*, flanked by various combinations of *HML*-E, *HML*-I, deletion derivatives, and telomere II-R X-derived sequences. The presence of either *HML*-E or *HML*-I in control strains bearing no insert resulted in significant silencing levels of *URA3*, although not as high as with both silencers intact (FOUREL *et al.* 1999; see also Figure 3 and compare GF25, GF19, and GF48). In contrast, not a single colony

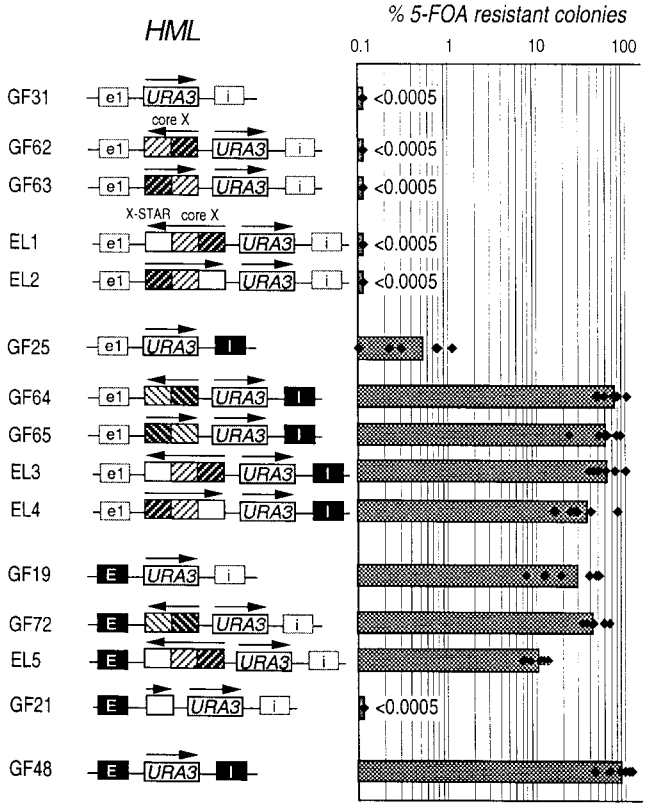

FIGURE 3.—Adjoining X-STAR sequences do not improve core X silencing capacity at *HML*. The *HML*-E and I silencers are drawn as boxes, solid when intact and open when carrying the e1 and i (I^{-242}) deletions (MAHONEY and BROACH 1989; BOSCHERON *et al.* 1996). Most of the HML locus was replaced by *URA3*. Fragments derived from the telomere II-R X element were inserted between *HML*-E and *URA3*. When no colony grew on SC + 5-FOA from 10 μl of undiluted culture, the fraction of 5-FOA-resistant cells was estimated to be <0.0005%, as indicated. Details are otherwise the same as in the legend for Figure 1.

grew out of 300,000 cells on 5-FOA-containing medium in the context of both *HML*-E and -I silencers deleted, even in the presence of core X (FOUREL *et al.* 1999; see also Figure 3, GF62 and GF63) or of a full-length X sequence (Figure 3, EL1, EL2). The full-length X element is therefore as incapable as core X of autonomously eliciting silencing at the *HML* locus.

Adjoining an X-STAR sequence to core X did not enhance its protosilencer activity as revealed in cooperation with a distant *HML*-I silencer (Figure 3, compare EL3 and EL4 with GF64 and GF65). We previously reported that, at telomeres, the synergy between core X and telomeric repeats is not disrupted by interposed STAR sequences (FOUREL *et al.* 1999). This holds true also at *HML* when core X is separated from *URA3* and *HML*-I by an intervening X-STAR sequence (Figure 3, compare GF65 and EL4). Moreover, the antisilencing effect of X-STAR is overcome by core X when X-STAR is bracketed between core X and a silencer (Figure 3, compare GF19, GF21, and EL5), similar to previous observations at telomere (FOUREL *et al.* 1999).

Overexpression of Sir3p and Sir4p enables core X silencing activity at *HML*: We next wondered whether overexpression of Sir3p and Sir4p might confer silencing capacity to core X at *HML* in the absence of any silencer. As shown above (Figure 3) and confirmed here using a more sensitive assay (see MATERIALS AND METHODS), *URA3* did not exhibit any silencing at *HML* in the absence of a silencer and under wild-type expression levels of Sir proteins, regardless of the nearby insertion of X-derived sequences (Figure 4, GF31, GF62, and EL1). An average of 30 colonies grew from 4.5×10^7 cells spread on 5-FOA-containing medium, all of which had acquired a mutation in the *URA3* gene (data not shown). However, overexpression of Sir3p and Sir4p in the absence of any known silencing element yielded a ratio of 4.4×10^{-5}% 5-FOA-resistant colonies that had an intact *URA3* gene, suggesting a weak but statistically significant silencing of *URA3* (Figure 4, EL15 and data not shown). Three hypotheses may be proposed to account for this unanticipated observation. First, this silencing might depend upon unknown silencing elements within the *HML* locus that would remain unaffected by the deletions of the silencers. Alternatively, overexpression of the Sir proteins might allow propagation of silenced chromatin along chromosomal DNA all the way from the III-L telomeric area to the *HML* locus, *i.e.*, >13 kb. In support of this hypothesis, silencing has been reported to extend up to 22 kb away from telomere V-R upon Sir3p overexpression (RENAULD *et al.* 1993). Finally, it is also conceivable that promiscuous nucleation of silent chromatin complexes might occur under high intranuclear Sir concentration, as previously proposed by HOLMES *et al.* (1997).

Silencing levels of *URA3* increased to 0.01% upon Sir3p and Sir4p overexpression when full-length X or core X sequences were inserted (Figure 4, EL16 and EL17). Importantly, Sir3p and Sir4p overexpression did not improve *HML*-E and -I silencing capacity at *HML* but rather had a slight negative effect (Figure 4, EL18 and EL19). This modest *trans*-dominant effect might be accounted for by the perturbation of the optimal stoichiometry between Sir proteins, as previously suggested (BUCK and SHORE 1995; MAILLET *et al.* 1996). Thus, overexpression of Sir3p and Sir4p enables a weak core X-mediated silencing at *HML* and, again in this situation, X-STAR sequences seem not to contribute to X activity.

Plasmid-borne core X behaves as a protosilencer: One may hypothesize that the absence of detectable, autonomous silencing activity of core X inserted at *HML* results from topological constraints due to the position of this locus within a chromosome, whereas X elements naturally stand in an immediate subtelomeric location. In light of the capacity of silencer elements to induce silencing *in cis* when carried on plasmids (ABRAHAM *et al.* 1984; FELDMAN *et al.* 1984), we decided to monitor the silencing capacity of a plasmid-borne core X sequence to address this hypothesis. A series of strains

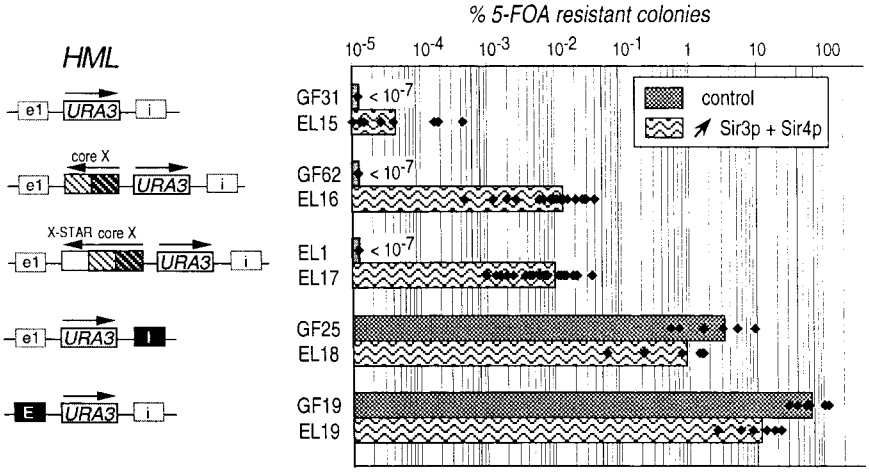

FIGURE 4.—Overexpression of Sir3p and Sir4p enables core X silencing activity at *HML*. Sir3p and Sir4p were overexpressed from high-copy plasmids, and control strains carried the corresponding parental vectors. Silencing was assayed by a particularly sensitive method and integrity of the *URA3* reporter gene was verified prior to taking into account a 5-FOA-resistant colony, as described in MATERIALS AND METHODS. The diamonds indicate the ratio of 5-FOA-resistant colonies for each of the 12–24 individual transformants assayed. The details are otherwise the same as in the legend for Figure 3.

was generated through transformation of W303-1A cells with pRS315-based plasmids carrying copies of *HML* constructs with *URA3* as a reporter gene as described above. These plasmids were selected on leucine dropout medium both following transformation and during the silencing assays. Strain ELC, which harbors the parental pRS315 vector, was found to be resistant to 5-FOA; as expected, as it does not carry a functional *URA3* gene (Figure 5). Some rare 5-FOA-resistant colonies (1 in 7×10^6) grew from cultures containing an *URA3* derivative of pRS315 devoid of any silencer (EL7). Those harbored an intact *URA3* gene as verified by quantitative restreaking on SC-U (data not shown). We believe they arose by spontaneous genetic mutations that confer either silencing-independent 5-FOA-resistant or Sir-independent silencing of *URA3* (LIN *et al.* 1990; CHI and SHORE 1996; DULA and HOLMES 2000). Alternatively, one may envisage the existence of a background level of silencing for plasmid-borne reporter genes, which might be accounted for by the preferential positioning of plasmids at the nuclear periphery, for example. This level of 5-FOA resistance observed for an *HML* locus deprived of silencers sets up the detection limit of our "on-plasmid" silencing assay.

Inserting core X by itself did not significantly raise silencing above this limit (Figure 5, compare EL7 and EL8). As expected, significant silencing levels were observed in the presence of *HML*-E and/or *HML*-I silencers (Figure 5, EL9, EL11, and EL13). It is worth noting that, for identical silencing reporter cassettes, silencing is lower in the plasmid context than in the native setting at *HML* (compare Figure 5 with GF25, GF19, and GF48 in Figure 3). These results are consistent with previous reports (ABRAHAM *et al.* 1984; FELDMAN *et al.* 1984; BRAND *et al.* 1985; MAILLET *et al.* 1996).

Finally, core X potently synergized with *HML*-I in establishing a stronger than *HML*-E-driven silencing level (Figure 5, compare EL9, EL10, and EL11). Thus, core X does not appear to act as a silencer when carried on a plasmid, but rather as a protosilencer in that it requires cooperation with a silencer, as previously observed at *HML*.

DISCUSSION

What distinguishes a protosilencer from a silencer? The detailed characterization of core X silencing activity presented here confirms and extends the idea that core X qualitatively behaves like an isolated protosilencer. It is, however, more efficient, owing to the cooperation of several elementary protosilencers, including an ACS and an Abf1p-binding site. It is noteworthy that, although a functional URS1 sequence can strengthen telomeric silencing, we were unable to ascribe any silencing effect to the URS1-like sequence found in core X (G. FOUREL and E. GILSON, unpublished results). Although not characterized in detail, silencing elements previously described in Y′ subtelomeric repeats (FOUREL *et al.* 1999), as well as short internal tracts of TG1-3n telomere-like sequences (LOUIS 1995), may be anticipated to display comparable properties.

Interestingly, core X activity could be demonstrated only upon cooperation with a *bona fide* silencer, with a telomere, or upon Sir protein overexpression. In these environments, core X can amplify preexisting silencing to levels approaching those conferred by classical silencers. In contrast, authentic silencers have been shown to be functional in various chromosomal contexts thought to be exempt of any basal silencing. One may envision that protosilencers and core X qualitatively behave as authentic silencers, *i.e.*, nucleating the *de novo* assembly of a silencing-competent complex, but only when an extremely high concentration of silencing factors is provided (BOSCHERON *et al.* 1996; LUSTIG 1998). In a silencing-free environment, conversely, binding of silent chromatin components by protosilencers would be counter-balanced by their dissociation rate, never reaching the threshold that would allow nucleation of a silencing-competent complex. Thus, in spite of a continuum of organization between core X and authentic silencers,

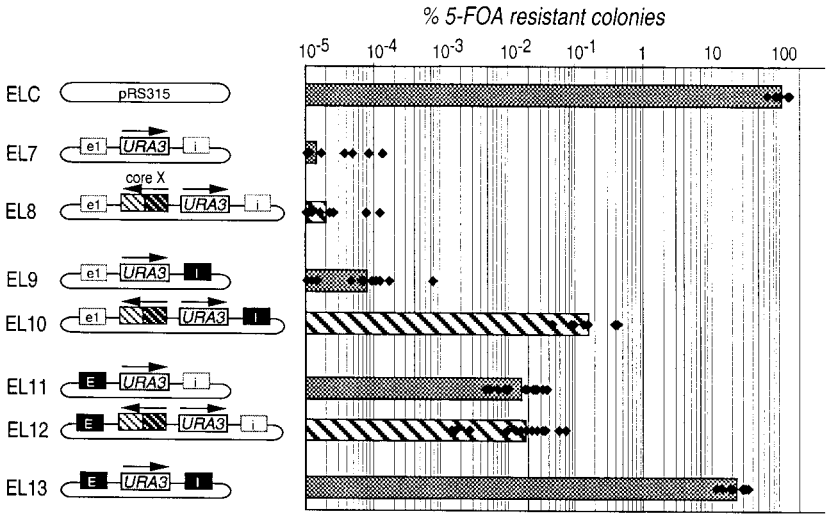

FIGURE 5.—Plasmid-borne core X behaves as a protosilencer. The *HML* locus from appropriate yeast strains described in Figure 3 was amplified and inserted in the pRS315 vector, represented by an oval, giving rise to the plasmids represented on the left. Details are otherwise the same as in the legends for Figures 3 and 4.

both containing protosilencers as active silencing elements but arranged differently, a quantitative difference in their capacity to recruit silent chromatin components would translate into a qualitative one owing to threshold effects. A similar model has been proposed for Drosophila to explain why a single copy of the *white* transgene can recruit the heterochromatin Protein-1 (HP1) but does not display silencing features, whereas multimer arrays of *white* behave similarly to heterochromatin blocks (DORER and HENIKOFF 1994, 1997; FANTI *et al.* 1998). The silencing activity of core X as well as other protosilencers may alternatively depend upon either a particular state of silencing-conducive chromatin or on the presence of classical but unstable silent chromatin, which would besides allow quasi-normal expression of an inserted reporter gene. Both of these hypotheses are in agreement with the recent demonstration that protosilencers stabilize preestablished silent chromatin (CHENG and GARTENBERG 2000).

We therefore propose that authentic silencers (master silencer in Figure 6) can autonomously elicit silencing (Figure 6a). In contrast, protosilencers, *i.e.*, elementary blocks of silencing elements or more elaborate sequences such as core X, allow silencing to affect neighboring portions of the genome only in a silencing-conducive environment (Figure 6, b–d). These features predict that an array of protosilencers can function as silencing relays and propagate silencing through "spreading" of silent chromatin at an extensive distance away from silencers. In addition, this propagation can be discontinuous, possibly owing to the involvement of insulator elements (FOUREL *et al.* 1999; Figure 6d). Alternatively, the cooperation of multiple protosilencers may alleviate the need for a master silencer and

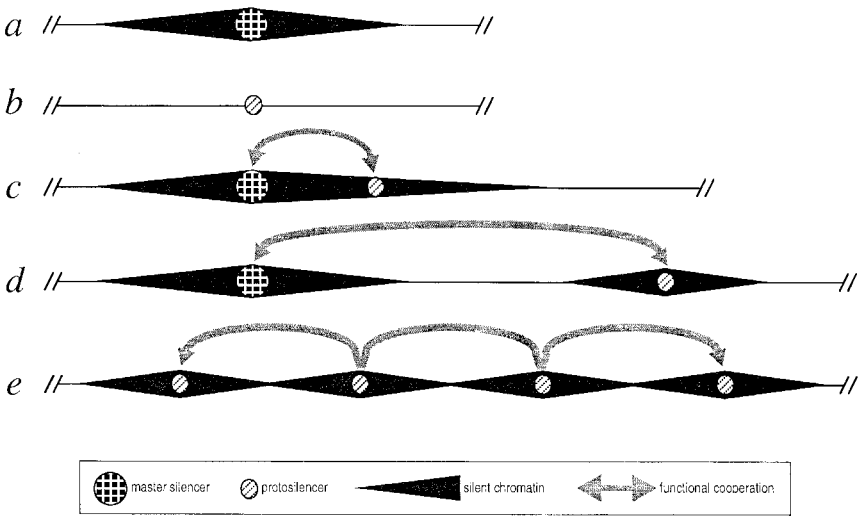

FIGURE 6.—Mechanisms of silencing by silencers and protosilencers. This model emanates from this study as well as other studies quoted in the text. A master silencer can autonomously establish and maintain silencing (a), at least in certain chromosome contexts, whereas a protosilencer cannot do so in the vast majority of settings (b). A protosilencer amplifies, stabilizes, or prolongs silent chromatin propagation only in silencing-conducive environments (c and d). These were found to correspond to chromosomal regions in which silencing preexists, sometimes at very low levels. This is the case, for instance, in the vicinity of telomeres and silencers (c), but also at a larger distance (d) and even in the absence of a residing silencing element at *HML* upon overexpression of Sir3p and Sir4p. Cooperation between the master silencer and the protosilencer must then involve discontinuous silent chromatin propagation. Alternatively, multiple protosilencers may be envisioned to create an autonomous silencing structure (e). Functional cooperation as illustrated by arrows likely involves direct physical interaction and coalescence of the silent domains, as discussed in FOUREL *et al.* (1999).

create a fully functional silencing structure (Figure 6e). A similar model involving cooperating relays was previously proposed to explain the spread of silencing by PcG proteins (Pirrotta 1997; Strutt *et al.* 1997).

General involvement of protosilencers in silencing phenomena: In *S. cerevisiae*, an isolated Rap1p-binding site in the promoter of α1/α2 genes, centrally located in between the *HML*-E and -I silencers, acts as a protosilencer and contributes to the maintenance of silencing at this locus (Boscheron *et al.* 1996; Cheng and Gartenberg 2000). In *Schizosaccharomyces pombe*, elements flanking the mat2-P and mat3-M mating cassettes facilitate the bidirectional spreading of silencing from a central K-region silencer throughout the locus and enhance silencing stability (Thon *et al.* 1999; Ayoub *et al.* 2000). They are seemingly devoid of silencing activity on their own and thus act as protosilencers (Ayoub *et al.* 2000). The REII element may further include a bordering insulator, an arrangement similar to that of X subtelomeric repeats of *S. cerevisiae* (Ayoub *et al.* 1999, 2000; Fourel *et al.* 1999).

Vertebrate *Hox* genes are repressed early on in development through a silencing-type mechanism and are then activated in a spatio-temporal sequence colinear with the position of the genes within their cluster. Dissection of the *HoxD* complex suggested the existence of a master silencer at the 5′ end and of protosilencers, relay elements in between genes (Kondo *et al.* 1998; Kondo and Duboule 1999). Amazingly, the relay element between mouse *Hoxd*-13 and *Hoxd*-12 genes can be functionally replaced by the orthologous zebrafish DNA that displays no apparent nucleotide homology (Kondo *et al.* 1998). A decrease in available components of silent chromatin, following a dilution effect in the course of embryo development, would progressively render relays located the farthest from the silencer inoperative. This would account for the orientated, progressive opening of chromatin toward the 5′ end (van der Hoeven *et al.* 1996; Kondo *et al.* 1998).

Recent studies revealed the possibility of a local and transient break in colinearity upon insertion of a copy of the most anterior *Hoxb* gene at the most posterior position in the *HoxD* complex (Kmita *et al.* 2000). Thus, this master silencer/protosilencer relay system allows silencing discontinuity, as previously shown in *S. cerevisiae* subtelomeric regions (Fourel *et al.* 1999; Pryde and Louis 1999; Figure 6d). In an analogous manner, in female mammals, spreading of heterochromatic inactivation throughout one X chromosome from the inactivation center involves silencing relays that may consist of LINE-1 elements (L1s) and some DNA segments containing significantly fewer L1s can escape inactivation (Bailey *et al.* 2000; Lyon 2000). *Cis* discontinuity in silencing was also recently observed in a classical system of PEV in Drosophila (Talbert and Henikoff 2000).

Tandem repeat-induced silencing in Drosophila (Dorer and Henikoff 1997), the X inactivation center in mammals (Bailey *et al.* 2000), as well as rRNA silencing in *S. cerevisiae* (Bryk *et al.* 1997; Smith and Boeke 1997) may rather be envisioned to operate according to the model in Figure 6e.

Overall, it appears that protosilencers critically assist with silencers and may cooperate to generate autonomously silencing structures in a variety of systems and organisms.

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LITERATURE CITED

Abraham, J., K. A. Nasmyth, J. N. Strathern, A. J. S. Klar and J. B. Hicks, 1984 Regulation of mating-type information in yeast: negative control requiring sequences both 5′ and 3′ to the regulated region. J. Mol. Biol. **176:** 307–331.

Ayoub, N., I. Goldshmidt and A. Cohen, 1999 Position effect variegation at the mating-type locus of fission yeast: a *cis*-acting element inhibits covariegated expression of genes in the silent and expressed domains. Genetics **152:** 495–508.

Ayoub, N., I. Goldshmidt, R. Lyakhovetsky and A. Cohen, 2000 A fission yeast repression element cooperates with centromere-like sequences and defines a *mat* silent domain boundary. Genetics **156:** 983–994.

Bailey, J. A., L. Carrel, A. Chakravarti and E. E. Eichler, 2000 Molecular evidence for a relationship between LINE-1 elements and X chromosome inactivation: the Lyon repeat hypothesis. Proc. Natl. Acad. Sci. USA **97:** 6634–6639.

Boeke, J. D., F. Lacroute and G. R. Fink, 1984 A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:** 345–346.

Boscheron, C., L. Maillet, S. Marcand, M. Tsai-Pfugfelder, S. M. Gasser *et al.*, 1996 Cooperation at a distance between silencers and proto-silencers at the yeast *HML* locus. EMBO J. **15:** 2184–2195.

Brand, A. H., L. Breeden, J. Abraham, R. Sternglanz and K. Nasmyth, 1985 Characterization of a silencer in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell **41:** 41–48.

Bryk, M., M. Banerjee, M. Murphy, K. E. Knudsen, D. J. Garfinkel *et al.*, 1997 Transcriptional silencing of Ty1 elements in the RDN1 locus in yeast. Genes Dev. **11:** 255–269.

Buck, S. W., and D. Shore, 1995 Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between *HMR* and telomeres in yeast. Genes Dev. **9:** 370–384.

Cheng, T.-H., and M. R. Gartenberg, 2000 Yeast heterochromatin is a dynamic structure that requires silencers continuously. Genes Dev. **14:** 452–463.

Chi, M.-H., and D. Shore, 1996 SUM1–1, a dominant suppressor of SIR mutations in Saccharomyces cerevisiae, increases transcriptional silencing at telomeres and HM mating-type loci and decreases chromosome stability. Mol. Cell. Biol. **16:** 4281–4294.

Donze, D., C. R. Adams, J. Rine and R. T. Kamakaka, 1999 The boundaries of the silenced HMR domain in *Saccharomyces cerevisiae*. Genes Dev. **13:** 698–708.

Dorer, D. R., and S. Henikoff, 1994 Expansion of transgene repeats causes heterochromatin formation and gene silencing in *Drosophila*. Cell **77:** 993–1002.

Dorer, D. R., and S. Henikoff, 1997 Transgene repeat arrays interact with distant heterochromatin and cause silencing in *cis* and in *trans*. Genetics **147:** 1181–1190.

Dula, M. L., and S. G. Holmes, 2000 *MGA2* and *SPT23* are modifiers of transcriptional silencing in yeast. Genetics **156:** 933–941.

Fanti, L., D. R. Dorer, M. Berloco, S. Henikoff, and S. Pimpinelli, 1998 Heterochromatin protein 1 binds transgene arrays. Chromosoma **107:** 286–292.

Feldman, J. B., J. B. Hicks and J. R. Broach, 1984 Identification of the sites required for repression of a silent mating-type locus in yeast. J. Mol. Biol. **178:** 815–834.

Fourel, G., E. Revardel, C. E. Koering and E. Gilson, 1999 Cohabitation of insulators and silencing elements in yeast subtelomeric regions. EMBO J. **18:** 2522–2537.

Fourel, G., C. Boscheron, E. Revardel, E. Lebrun, Y.-F. Hu *et al.*, 2001 An activation-independent role of transcription factors in insulator function. EMBO J. **2:** 124–132.

Gartenberg, M. R., 2000 The Sir proteins of *Saccharomyces cerevisiae*: mediators of transcriptional silencing and much more. Curr. Opin. Microbiol. **3:** 132–137.

Giesman, D., L. Best and K. Tatchell, 1991 The role of RAP1 in the regulation of the MAT locus. Mol. Cell. Biol. **11:** 1069–1079.

Henikoff, S., 2000 Heterochromatin function in complex genomes. Biochim. Biophys. Acta **1470:** 1–8.

Holmes, S. G., A. B. Rose, K. Steuerle, E. Saez, S. Sayegh *et al.*, 1997 Hyperactivation of the silencing proteins, Sir2p and Sir3p, causes chromosome loss. Genetics **145:** 605–614.

Kadosh, D., and K. Struhl, 1997 Repression by UME6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. Cell **89:** 365–371.

Kmita, M., F. van der Hoeven, J. Zakany, R. Krumlauf and D. Duboule, 2000 Mechanisms of *Hox* gene colinearity: transposition of the anterior *Hoxb1* gene into the posterior *HoxD* complex. Genes Dev. **14:** 198–211.

Koering, C. E., G. Fourel, F. Klein, E. Binet-Brasselet, T. Laroche *et al.*, 2000 Identification of high affinity Tbf1p binding sites within the budding yeast genome. Nucleic Acids Res. **28:** 2519–2526.

Kondo, T., and D. Duboule, 1999 Breaking colinearity in the mouse HoxD complex. Cell **97:** 407–417.

Kondo, T., J. Zàkàny and D. Duboule, 1998 Control of colinearity in AbdB genes of the mouse HoxD complex. Mol. Cell **1:** 289–300.

Lin, C. I., G. P. Livi, J. M. Ivy and A. J. Klar, 1990 Extragenic suppressors of mar2(sir3) mutations in *Saccharomyces cerevisiae*. Genetics **125:** 321–331.

Louis, E. J., 1995 The chromosome ends of *Saccharomyces cerevisiae*. Yeast **11:** 1553–1573.

Louis, E. J., and R. H. Borts, 1995 A complete set of marked telomeres in *Saccharomyces cerevisiae* for physical mapping and cloning. Genetics **139:** 125–136.

Louis, E. J., E. S. Naumova, A. Lee, G. Naumov and J. E. Haber, 1994 The chromosome end in yeast: its mosaic nature and influence on recombinational dynamics. Genetics **136:** 789–802.

Lustig, A. J., 1998 Mechanisms of silencing in *Saccharomyces cerevisiae*. Curr. Opin. Gen. Dev. **8:** 233–239.

Lyon, M. F., 2000 LINE-1 elements and X chromosome inactivation: a function for "junk" DNA? Proc. Natl. Acad. Sci. USA **97:** 6248–6249.

Mahoney, D. J., and J. Broach, 1989 The HML mating-type cassette of *S. cerevisiae* is regulated by two separate but functionally equivalent silencers. Mol. Cell. Biol. **9:** 4621–4630.

Maillet, L., C. Boscheron, M. Gotta, S. Marcand, E. Gilson *et al.*, 1996 Evidence for silencing compartments in the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression. Genes Dev. **10:** 1796–1811.

Marcand, S., S. W. Buck, P. Moretti, E. Gilson and D. Shore, 1996 Silencing of genes at nontelomeric sites in yeast is controlled by sequestration of silencing factors at telomeres by Rap1 protein. Genes Dev. **10:** 1297–1309.

Martin, S. G., T. Laroche, N. Suka, M. Grunstein and S. M. Gasser, 1999 Relocalization of telomeric Ku and SIR proteins in response to DNA breaks in yeast. Cell **97:** 621–633.

McNally, F. J., and J. Rine, 1991 A synthetic silencer mediates SIR-dependent functions in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 5648–5659.

Mihaly, J., I. Hogga, J. Gausz, H. Gyurkovics and F. Karch, 1997 In situ detection of the *Fab-7* region of the bithorax complex into a chromatin domain boundary and a *Polycomb*-response element. Development **124:** 1809–1820.

Pirrotta, V., 1997 Pc-G complexes and chromatin silencing. Curr. Opin. Genet. Dev. **7:** 249–258.

Pryde, F. E., and E. J. Louis, 1999 Limitations on natural TPE in yeast. EMBO J. **18:** 2538–2550.

Pryde, F. E., T. C. Huckle and E. J. Louis, 1995 Sequence analysis of the right end of chromosome XV in *Saccharomyces cerevisiae*: an insight into the structural and functional significance of subtelomeric repeat sequences. Yeast **11:** 371–382.

Renauld, H., O. M. Aparicio, P. D. Zierath, B. L. Billington, S. K. Chhablani *et al.*, 1993 Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. Genes Dev. **7:** 1133–1145.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Smith, J. S., and J. D. Boeke, 1997 An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev. **11:** 241–254.

Srivastava, M., S. Hsieh, A. Grinberg, L. Williams-Simons, S.-P. Huang *et al.*, 2000 *H19* and *Igf2* monoallelic expression is regulated in two distinct ways by a shared *cis* acting regulatory element upstream of *H19*. Genes Dev. **14:** 1186–1195.

Stevenson, J. B., and D. E. Gottschling, 1999 Telomeric chromatin modulates replication timing near chromosome ends. Genes Dev. **13:** 146–151.

Strutt, H., G. Cavalli and R. Paro, 1997 Co-localization of *Polycomb* protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. EMBO J. **16:** 3621–3632.

Talbert, P. B., and S. Henikoff, 2000 A reexamination of spreading of position-effect variegation in the *white-roughest* region of *Drosophila melanogaster*. Genetics **154:** 259–272.

Thon, G., K. P. Bjerling and I. S. Nielsen, 1999 Localization and properties of a silencing element near the mat3-M mating-type cassette of *Schizosaccharomyces pombe*. Genetics **151:** 945–963.

Triolo, T., and R. Sternglanz, 1996 Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. Nature **381:** 251–253.

van der Hoeven, F., J. Zâkàny and D. Duboule, 1996 Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. Cell **85:** 1025–1035.

Vega-Palas, M. A., S. Venditti and E. Di Mauro, 1997 Telomeric transcriptional silencing in a natural context. Nat. Genet. **15:** 232–233.

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