Expression-State Boundaries in the Mating-Type Region of Fission Yeast

Geneviève Thon,*[,1] Pernilla Bjerling,[†] Camilla Marie Bünner* and Janne Verhein-Hansen*

**Department of Genetics, Institute of Molecular Biology, University of Copenhagen, DK-1353 Copenhagen K, Denmark and [†]Karolinska Institute, Södertörn University College, S-14104 Huddinge, Sweden*

Manuscript received November 26, 2001
Accepted for publication March 27, 2002

ABSTRACT

A transcriptionally silent chromosomal domain is found in the mating-type region of fission yeast. Here we show that this domain is delimited by 2-kb inverted repeats, *IR-L* and *IR-R*. *IR-L* and *IR-R* prevent the expansion of transcription-permissive chromatin into the silenced region and that of silenced chromatin into the expressed region. Their insulator activity is partially orientation dependent. The silencing defects that follow deletion or inversion of *IR-R* are suppressed by high dosage of the chromodomain protein Swi6. Combining chromosomal deletions and Swi6 overexpression shows that *IR-L* and *IR-R* provide firm borders in a region where competition between silencing and transcriptional competence occurs. *IR-R* possesses autonomously replicating sequence (ARS) activity, leading to a model where replication factors, or replication itself, participate in boundary formation.

MOST current models of genomic organization in eukaryotes involve specialized DNA elements, such as insulators or boundaries, whose role is to partition chromosomes into domains with different transcriptional states (for review, see Bell *et al.* 2001). How insulators or boundaries function and how many different types are represented in nature are matters of speculation since relatively few have been described to date, especially in their own chromosomal contexts. One means of identifying and characterizing insulator or boundary elements is to examine the edges of chromosomal regions in which transcription is either constitutively or conditionally repressed.

The chromosomal region that contains the silent *mat2* and *mat3* mating-type cassettes of fission yeast is in many respects similar to heterochromatic regions of higher eukaryotes. Reporter genes artificially placed into that region are subject to a position effect by which transcription is stringently repressed (Thon and Klar 1992; Thon *et al.* 1994; Grewal and Klar 1997). This is in contrast to the more common situation in fission yeast where expression of marker genes follows insertion at ectopic sites. The transcriptional repression observed in the mating-type region is mediated by a specialized chromatin structure established in part by the histone deacetylases Clr3 and Clr6 (Ekwall and Ruusala 1994; Thon *et al.* 1994; Grewal *et al.* 1998) and by the histone methyltransferase Clr4 (Ekwall and Ruusala 1994; Thon *et al.* 1994; Ivanova *et al.* 1996; Rea *et al.* 2000; Bannister *et al.* 2001; Nakayama *et al.* 2001). The *Schizosaccharomyces pombe* chromodomain protein Swi6, a member of the HP1 family (Lorentz *et al.* 1994), associates with the modified nucleosomes of the mating-type region (Ekwall *et al.* 1995, 1996; Nakayama *et al.* 2001) and, together with its paralog Chp2, participates in the silencing process (Lorentz *et al.* 1992; Thon and Verhein-Hansen 2000). This association parallels that of mammalian HP1α and HP1β proteins with chromatin modified by SUV39H1, a human Clr4 homolog (Lachner *et al.* 2001). A globally reduced level of histone acetylation such as that due to the action of Clr3 and Clr6 is also a general feature of heterochromatin.

Three *cis*-acting elements are believed to attract silencing complexes to the mating-type region (Figure 1a). Two of the elements are contained within a few hundred base pairs of each silent cassette (Thon *et al.* 1994, 1999; Ayoub *et al.* 1999, 2000). The third element is found in the 11-kb region that separates *mat2* from *mat3*, a region that displays extensive sequence similarity with centromeric repeats over a length of 4.3 kb (Grewal and Klar 1996, 1997; Thon and Friis 1997). The sequence similarity of that region with centromeres underlies other similarities between centromeres and the mating-type region, such as a shared heterochromatic structure (Allshire *et al.* 1995; Nakayama *et al.* 2001).

When modeling silencing in the mating-type region, one envisions that unidentified proteins, possibly products of the *esp* (Thon and Friis 1997) or *clr* genes (Thon and Klar 1992; Ekwall and Ruusala 1994; Thon *et al.* 1994), recognize the aforementioned *cis*-acting elements and attract histone-modifying enzymes and chromodomain proteins. Following that nucleation step, silencing spreads along the chromosome, as observed or proposed for related systems and as supported by the fact that Swi6 can oligomerize and cover large chromosomal regions (Partridge *et al.* 2000; Wang *et al.* 2000). In addition to its SET domain, which has

[1]*Corresponding author:* Department of Genetics, Institute of Molecular Biology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark. E-mail: gen@biobase.dk

histone-methyltransferase activity (Rea *et al.* 2000), the Clr4 protein contains a chromodomain (Ivanova *et al.* 1996), an organization similar to that of the Drosophila protein Suv39h or its human homolog SUV39H1. By analogy with the human system in which SUV39H1 and proteins of the HP1 family biochemically interact (Aagaard *et al.* 1999), Swi6, attracted to the silenced region by its affinity for histone H3 methylated at lysine 9, might in turn recruit the Clr4 methyltransferase and thereby promote propagation of the silenced domain (Bannister *et al.* 2001). Once established, the silenced domain can be inherited in a manner partially independent of the silencing elements (Grewal and Klar 1996; Thon and Friis 1997), in a self-templating process possibly facilitated by the same properties of the silencing proteins that cause spreading of modified chromatin.

Neither the length of the silenced mating-type region nor the mechanisms that determine that length are known. According to all available evidence, silencing affects the 11-kb DNA segment that separates the two silent mating-type cassettes in addition to the mating-type cassettes themselves. At 15 kb on the centromere-proximal side of the *mat2* cassette is the active *mat1* mating-type cassette, a locus whose transcriptional activity has been well characterized (Kelly *et al.* 1988). An essential gene located between *mat1* and *mat2* was identified by Michael *et al.* (1994). Subsequent investigations by Ayoub *et al.* (1999) provide the most precise localization of the edge of the silenced region on its centromere-proximal side, at ~5 kb from the centromere-proximal side of the *mat2* cassette. The position of the centromere-distal edge of the silenced region was not determined, the first active gene identified in that region being *his2*, some 26 kb centromere-distal to *mat3*.

In addition to the elements mentioned above, a 2-kb inverted repeat is found in the mating-type region of *S. pombe* (G. Thon, unpublished observations; Sanger Center *S. pombe* sequencing project; Noma *et al.* 2001). The repeats are placed symmetrically relative to the *mat2* and *mat3* cassettes: One copy, *IR-L*, is centromere-proximal to *mat2* while the other copy, *IR-R*, is centromere-distal to *mat3* (Figure 1a). In wild-type h^{90} cells, *IR-L* and *IR-R* are separated by a chromosomal region of ~16 kb. The *IR-L* and *IR-R* repeats are identical in sequence for a length of 2067 bp. They appear to contain no open reading frame (ORF). Rather, they have features characteristic of intergenic regions of low complexity, such as an AT content of 69%. The homology between *IR-L* and *IR-R* stops abruptly on each side of the identical core, the region of perfect homology on the side distal to the cassettes ending within $G(A)_{3\text{-}6}$ tracts of 21 (*IR-L*) or 32 bp (*IR-R*). The pattern of histone H3 methylation at lysines 4 and 9 changes abruptly at *IR-L* and *IR-R* (Noma *et al.* 2001).

We report here that *IR-L* and *IR-R* colocalize with the edges of the silenced region. We examine their properties in the context of the mating-type region and show that they confer autonomous replication to a plasmid, which suggests that proteins involved in replication, or replication itself, take part in boundary function.

MATERIALS AND METHODS

Yeast media and culture conditions: YES (Thon and Friis 1997) was used as rich medium; MSA (Egel *et al.* 1994) supplemented with 100 mg adenine, 100 mg uracil and 200 mg L-leucine per liter was used as mating and sporulation medium; dropout media (AA; Rose *et al.* 1990) and fluoroorotic acid (FOA) medium (Thon and Friis 1997) were used to test auxotrophies. Yeast nitrogen base and yeast nitrogen base lacking thiamine were purchased from United States Biologicals, agar and yeast extract from Difco (Detroit), amino acids and bases from Sigma (St. Louis) or AppliChem, and salts from Merck (St. Louis). Plates were incubated at 33°.

Construction of *S. pombe* strains: All chromosomal integrations were performed using the lithium acetate method (Moreno *et al.* 1991) and *swi6-115* strains, a background which facilitates recombination and allows the expression of prototrophic markers in the mating-type region (Thon and Klar 1992). Transformants with correct integrations were identified by Southern blots and the *swi6-115* allele was crossed out of such transformants. Strain numbers are provided in the figure legends; alterations introduced in their mating-type region (h^{90} background) are described below and in the figures. All strains are *ade6*$^-$ (*ade6-M210* or *ade6-M216*) and they contain the *ura4-DS/E* allele (Allshire *et al.* 1995) except PG1571 (*ura4*$^+$) and PG2028 (*ura4-D18*). The following strains are *leu1-32*: PG1571, PG1636, PG1898, PG1899, PG1922, PG1928, PG1934, PG1938, PG2029, PG2047, PG2089, PG2096, PG2095, PG2104, PG2173, PG2388, PG2471, PG2510, PG2536, and PG2598.

The plasmids listed below were used to integrate *ura4*$^+$ at sites described by their nucleotide position (nuc.) in GenBank sequences and shown in Figure 1b. *ori1* refers to an integration of *ura4*$^+$ with its promoter centromere-proximal whereas *ori2* refers to *ura4*$^+$ with its promoter centromere-distal. pGT234 (*ori1*) or pGT235 (*ori2*) were used to introduce *ura4*$^+$ at *Xmn*I (nuc. 34563 in AL035065); pAK67, at the *Xba*I site distal to *mat2-P* (nuc. 414 in U57841; Thon *et al.* 1994); pSG4, at *Hin*dIII (nuc. 5345 in U57841; Grewal and Klar 1997); pGT253 (*ori1*) or pGT252 (*ori2*), at *Swa*I (nuc. 9374 in U57841); pGT77, at *Eco*RV (nuc. 2917 in AL353012; Thon and Klar 1992); pPB54 (*ori1*) or pPB55 (*ori2*), at the *mat3-M* centromere-distal *Xba*I site (nuc. 4017 in AL353012); pGT229 (*ori1*) or pGT230 (*ori2*), at *Spe*I (nuc. 6552 in AL353012); and pGT227 (*ori1*) or pGT230 (*ori2*), at *Bam*HI (nuc. 7386 in AL353012).

IR-R was deleted by replacing the *mat3-M (Xba*I*)::ura4*$^+$ allele with a construct containing a 2.1-kb deletion between *Xba*I (nuc. 4017 in AL353012) and *Xmn*I (nuc. 6136 in AL353012), pGT226. *IR-R* was inverted in the same fashion, using a construct containing an inversion of the 2.1-kb *Xba*I-*Xmn*I fragment, pGT243. *IR-L* was deleted by replacing the *(Xmn*I*):: ura4*$^+$ *mat2-P* allele with a construct lacking nuc. 35002 in AL035065 to nuc. 328 in AL353012 (pGT236) or inverted by replacing the deleted nucleotides with the 2.1-kb *Xba*I-*Xmn*I *IR-R* fragment (pGT244).

The *Blp*I site at nuc. 6994 of AL353012 was used to insert the 2.1-kb *Xba*I-*Xmn*I *IR-R* fragment in either orientation or a 1.9-kb *Bst*EII fragment of λDNA. These insertions were combined with *ura4* at the *mat3-M* centromere-distal *Spe*I (nuc. 6552 in AL353012) or *Bam*HI site (nuc. 7386 in AL353012).

pGT289 contains *(Spe*I*)::ura4*$^+$ *ori1 (Blp*I*)::IR-R*; pGT290, *(Spe*I*)::ura4*$^+$ *ori2 (Blp*I*)::IR-R*; pGT287, *(Spe*I*)::ura4*$^+$ *ori1 (Blp*I*)::inv-IR-R*; pGT288, *(Spe*I*)::ura4*$^+$ *ori2 (Blp*I*)::inv-IR-R*; pGT291, *(Spe*I*)::ura4*$^+$ *ori1 (Blp*I*)::*λ*DNA*; pGT292, *(Spe*I*)::ura4*$^+$ *ori2 (Blp*I*)::* λ*DNA*; pGT283, *(Blp*I*)::IR-R (Bam*HI*)::ura4*$^+$ *ori1*; pGT284, *(Blp*I*)::IR-R (Bam*HI*)::ura4*$^+$ *ori2*; pGT282, *(Blp*I*)::inv-IR-R (Bam*HI*):: ura4*$^+$ *ori1*; and pGT281, *(Blp*I*)::inv-IR-R (Bam*HI*):: ura4*$^+$ *ori2*.

Spot tests: Five microliters of 10-fold serial dilutions of cell suspensions were spotted on the indicated selective media and on nonselective media (AA and MSA; not shown). The efficiency of plating on the nonselective media was in each case the same as the highest efficiency of plating on selective media.

Quantitation of *ura4* transcripts: RNA preparation, Northern blotting, and hybridization to a *ura4* riboprobe were performed as described previously (Thon and Verhein-Hansen 2000). Cells were grown in 50 ml YES to 1–2 × 10^7 cells/ml before extraction of RNA. Transcript amounts were quantified using a Storm 840 phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). A truncated *ura4* transcript originating from the *ura4DS/E* allele present in the cells used to extract RNA served as internal standard for quantitation (Allshire *et al.* 1995).

Overexpression of Swi6: The *swi6* ORF was amplified from pAL2 (Lorentz *et al.* 1994) by PCR using as primers 5′-CGGGATCCGCATATGAAGAAAGGAGGTGTTCGATC-3′ and 5′-CGGGATCCCGGGTTATTCATTTTCACGGAACG-3′. The PCR product digested with *Bam*HI was cloned into pREP3X, pREP41X, and pREP81X (Forsburg 1993 and references therein) to create, respectively, pGT245, pGT247, and pGT246. This places *swi6* under the control of three *nmt* promoters of different strength. *S. pombe* strains transformed with pGT245, pGT246, or pGT247 were obtained on selective medium containing thiamine (AA-leu). Expression of *swi6* was subsequently induced by placing the transformants on selective medium lacking thiamine (AA-leu-thiamine) for 24 hr. The strongest expression of *swi6* (pGT245) proved toxic to the cells and was not examined further. Transformants expressing *swi6* from pGT246 or pGT247 were examined further by spot tests on selective media. The expression of *swi6* in these transformants was monitored by Northern blotting (data not shown).

Test for ARS activity of *IR-R*: The 1.8-kb *ura4*$^+$-containing *Hin*dIII fragment (Grimm *et al.* 1988) was cloned into Bluescript SKII(+) (Stratagene, La Jolla, CA) to create pGT188. The 1.2-kb *Eco*RI fragment containing *ars1* (Clyne and Kelly 1995) was cloned into pGT188 to create pGT302. The 2.1-kb *Xba*I-*Xmn*I fragment containing *IR-R* was cloned into pGT188 to create pGT303. One microgram of pGT188, pGT302, or pGT303 was used to transform ~10^8 SP837 cells (*h*^{90} *ura4-D18 leu1-32 ade6-M216*) with the lithium acetate method (Moreno *et al.* 1991). Dilutions of transformed cells were plated on AA-ura. The plates shown in Figure 6 correspond to one-tenth of the transformations.

RESULTS

The two inverted repeats, *IR-L* and *IR-R*, colocalize with the edges of the silenced mating-type domain: We monitored gene expression in the *mat2-mat3* region, with a special focus on the regions surrounding *IR-L* and *IR-R*. As had been done in previous studies (Thon and Klar 1992; Thon *et al.* 1994, 1999; Grewal and Klar 1996, 1997; Thon and Friis 1997), we chose to use the *S. pombe ura4* gene as a marker. The *ura4* gene encodes the *S. pombe* orotidine 5′-monophosphate-decarboxylase. Its expression permits growth on media lacking uracil and inhibits growth on media containing the toxigenic substrate 5-FOA. This reporter gene proved to be silenced when introduced in the chromosomal region that separates the two repeats *IR-L* and *IR-R* (Figure 1b, rows 5–11). Its repression was dependent on the silencing factor Swi6 (data not shown). The same *ura4* marker was expressed at all insertion sites tested on the outer side of the repeats (Figure 1b, rows 3 and 4 and 12–15). Hence, the inverted repeats *IR-L* and *IR-R* mark, or are close to, the edges of the silenced domain of the fission yeast mating-type region.

The inverted repeats *IR-L* and *IR-R* are essential for accurate separation between expressed and silenced chromatin: Whether *IR-L* or *IR-R* have a functional role in transcriptional silencing was investigated by deletion analysis. *IR-L*, *IR-R*, or both elements were deleted from the chromosome and the expression of the *ura4* marker was assayed in strains carrying these deletions. Deletion of *IR-L* or *IR-R* resulted in an increased expression of *ura4* at sites located near the edges of the normally silenced region (Figure 1b, compare row 5 with 20 and 9 with 37; Figure 1c, compare lanes 5 and 6 with 1 and 2). In each case, the effect was local, repression being released preferentially close to the deletion. Simultaneous deletion of *IR-L* and *IR-R* led to release of repression at both edges, the effect at each edge being no stronger than that caused by the individual deletion of its flanking element (Figure 1b, compare row 5 with 44 and row 9 with 48; Figure 1c, compare lanes 5 and 6 with 7 and 8). In addition, a slight derepression was observed at internal sites (Figure 1b, compare rows 6–8 with 45–47).

A more subtle effect of deleting *IR-L* or *IR-R* could be observed on FOA-containing medium. In the presence of *IR-L* and *IR-R*, *ura4* placed at one of the outer insertion sites is uniformally expressed, in a fashion that allows no detectable growth on FOA-containing medium (Figure 1b, rows 3 and 4). This is, for example, observed at the *mat2* centromere-proximal *Xmn*I site. FOA-resistant cells with *ura4* at this site are very infrequent and they consistently contain mutations in *ura4* (data not shown). Following deletion of *IR-L*, an increased fraction of cells with *ura4* at the *Xmn*I site acquired the ability to form colonies in the presence of FOA (Figure 1b, rows 18, 19, 42, and 43). *ura4* was epigenetically repressed in these cell lineages rather than mutated as found by replating (data not shown). Similarly, following deletion of *IR-R*, *ura4*$^+$ placed in the normally well-expressed region on the *mat3-M* centromere-distal side could be silenced in a fraction of the cell population (Figure 1b, rows 38–41 and 49–52; data not shown). The fraction of cells affected was, however, very small, and Northern blot analysis revealed no changes in the level of *ura4*$^+$ transcript originating from the *mat3-M*-distal *Spe*I or *Bam*HI insertion sites (Figure 2c), indicating that, in most cells, deletion of *IR-R* does not lead to spreading of heterochromatin.

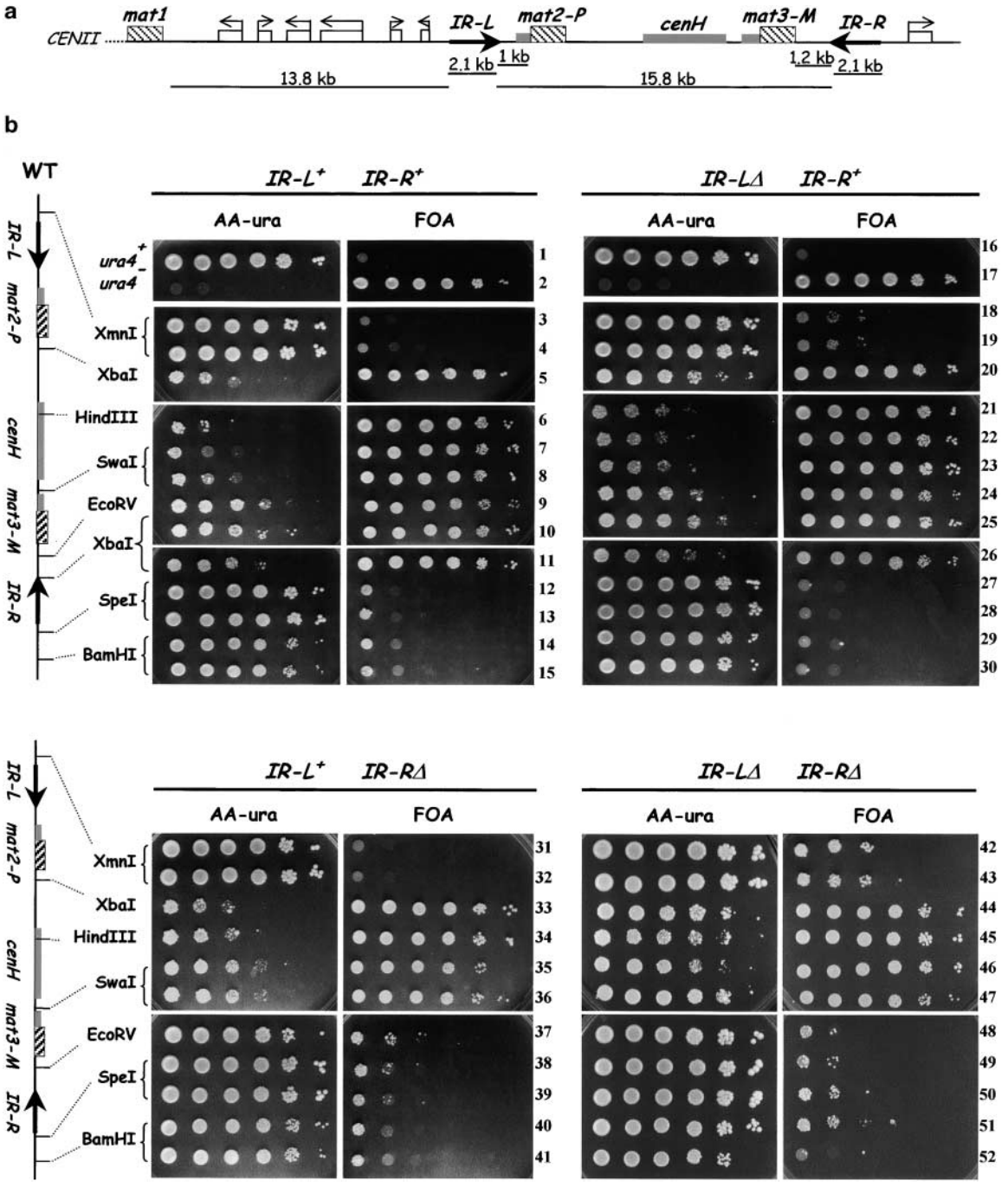


FIGURE 1.—A silenced chromosomal domain in the mating-type region of fission yeast. (a) Mating-type region of *S. pombe*. The three mating-type cassettes (hatched boxes) are linked in the right arm of chromosome 2. Two silencing elements adjacent to, respectively, *ma2-P* and *mat3-M* and a 4.3-kb region with homology to centromeric repeats (*cenH*) are represented by shaded boxes. Open boxes indicate ORFs identified in the *S. pombe* genome sequencing project (Sanger Center). (b) Effects of deleting *IR-L* or *IR-R* on transcriptional competence. Tenfold serial dilutions of strains containing *ura4*$^+$ in the mating-type region were spotted on the indicated media. 1 and 16, PG1571; 2 and 17, PG1636; 3, PG2089; 4, PG2095; 5, PG2104; 6, PG2471; 7, PG2598; 8, PG2388; 9, PG1898; 10, PG2002; 11, PG1997; 12, PG1934; 13, PG1938; 14, PG1922; 15, PG1928; 18, PG2530; 19, PG2537; 20, PG2520; 21, PG2495; 22, PG2505; 23, PG2497; 24, PG2511; 25, PG2541; 26, PG2548; 27, PG2551; 28, PG2555; 29, PG2564; 30, PG2566; 31, PG2077; 32, PG2097; 33, PG2116; 34, PG2400; 35, PG2577; 36, PG2424; 37, PG2045; 38, PG2050; 39, PG2059; 40, PG2028; 41, PG2039; 42, PG2521; 43, PG2524; 44, PG2607; 45, PG2460; 46, PG2484; 47, PG2474; 48, PG2491; 49, PG2588; 50, PG2572; 51, PG2597; 52, PG2526. In cases where two strains are shown for a given insertion site, the strain in the top row contains *ura4*$^+$ inserted with its promoter centromere-proximal and the strain in the bottom row contains *ura4*$^+$ in the opposite orientation. (c) *ura4*$^+$ transcript levels. The amount of *ura4*$^+$ transcript originating from the indicated sites was compared with the amount of *ura4-DS/E* transcript originating from the endogenous *ura4* locus by Northern blot analysis and phosphorimaging. *ura4*$^+$:*ura4-DS/E* transcript ratios are reported below the blots. 1, PG1898; 2, PG1899; 3, PG2510; 4, PG2511; 5, PG2045; 6, PG2047; 7, PG2491; 8, PG2492; 9, PG1934; 10, PG1938; 11, PG1922; 12, PG1928; 13, PG2052; 14, PG2059; 15, PG2029; 16, PG2039.

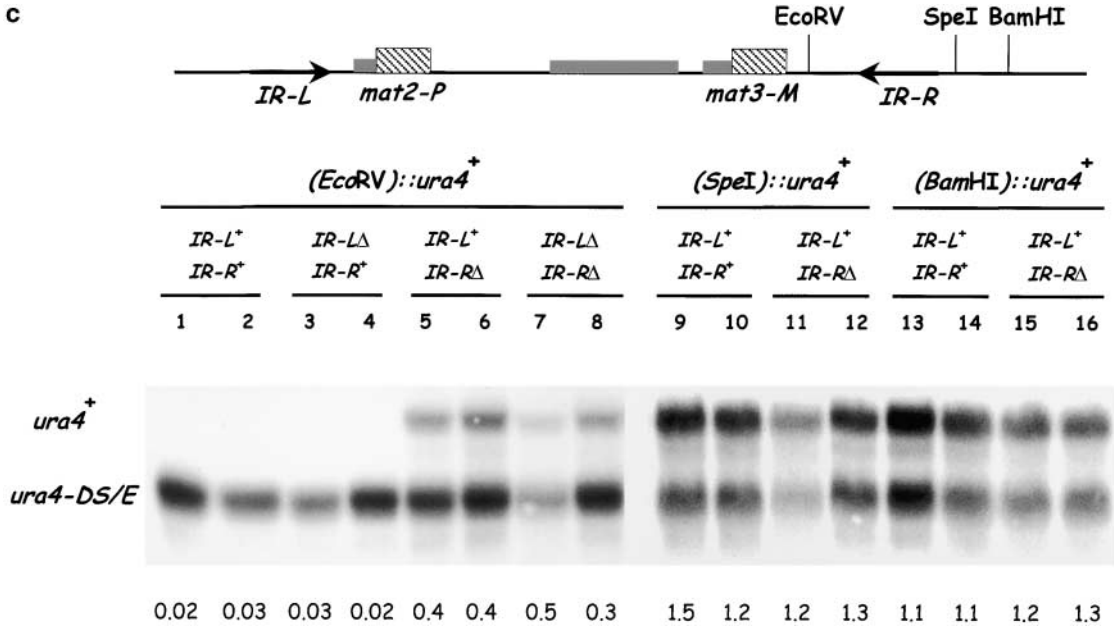


FIGURE 1.—*Continued.*

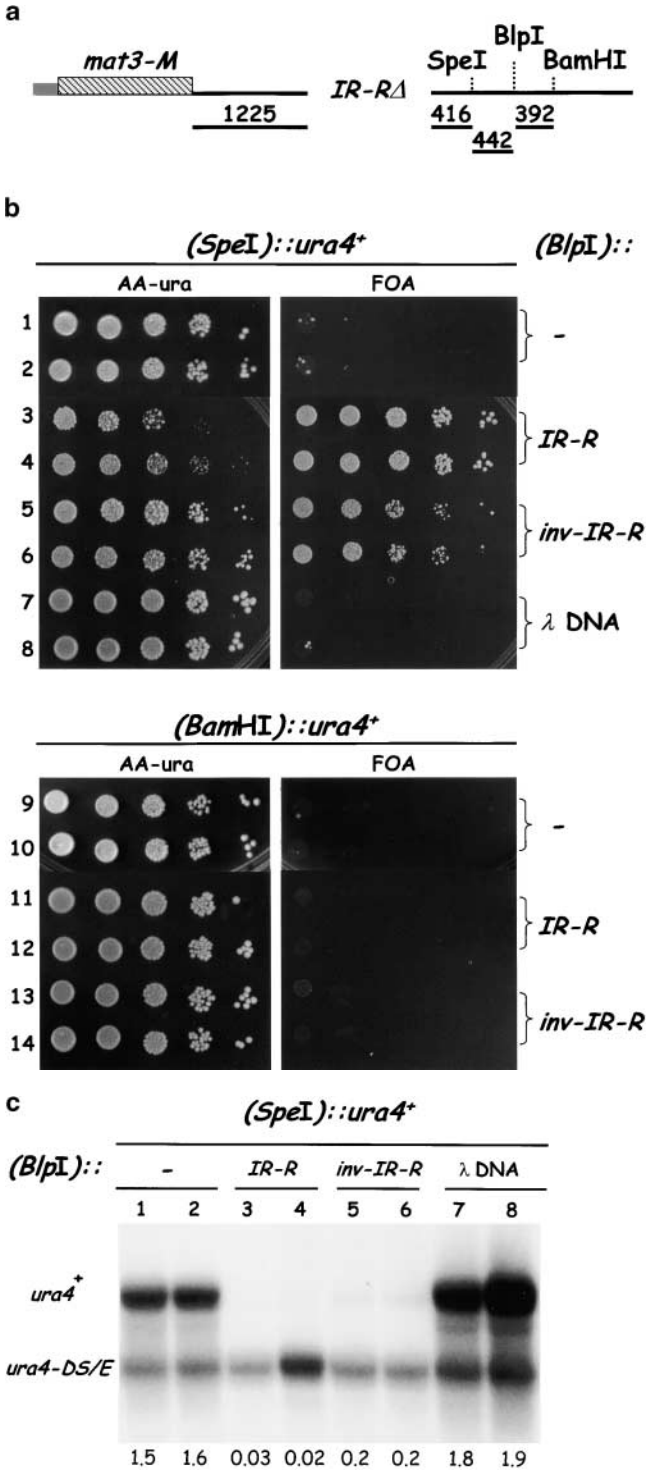
The deletion analysis of *IR-L* and *IR-R* shows that the two elements normally participate in the repression of transcription observed in the chromosomal region surrounding *mat2* and *mat3*. They might act as unidirectional silencers whose action would be directed toward the mating-type cassettes, or they might function as insulators. Our observations are consistent with their major role being preventing the expansion of an expressed state from regions flanking the silenced region into the silenced region. However, they also reduce the spreading of silencing into transcriptionally active regions, an observation supported by further experiments involving the overexpression of the chromodomain protein Swi6 (see below). The two elements *IR-L* and *IR-R* can accomplish these functions independently of each other. Whether they act simply as spacers between regions that have adopted opposite and competing states of expression or whether they have a more active role that is dependent upon their particular sequence is addressed in experiments described below.

Translocation of *IR-R* away from *mat3-M* expands the silenced chromosomal region: *IR-R* was moved from its wild-type chromosomal location to a more centromere-distal location, in the region that is normally expressed. More specifically, the element was moved 850 bp to a *Blp*I restriction site located between an *Spe*I and a *Bam*HI restriction site (Figure 2a), at which the *ura4* reporter gene had previously been integrated and found to be expressed (Figure 1b, rows 12–15; Figure 1c, lanes 9, 10, 13, and 14). Following the placement of *IR-R* between the *Spe*I and *Bam*HI sites, a significant repression of *ura4* was observed at *Spe*I (Figure 2b, rows 3 and 4; Figure 2c, lanes 3 and 4) while expression at *Bam*HI was unaltered (Figure 2b, rows 11 and 12). A fragment of DNA of similar size originating from the bacteriophage λ had no repressive effect (Figure 2b, compare rows 7 and 8 with 1 and 2; Figure 2c, compare lanes 7 and 8 with 1 and 2). Hence, the *IR-R* element positions the edge of the silenced region. It does not simply provide spacing between silenced and expressed regions, but rather some specific aspect of its sequence is required for its effects.

An inverted *IR-R* element also blocks the spreading of active chromatin: Because they are placed as inverted repeats, *IR-L* and *IR-R* face the silenced region in the same fashion. That orientation might be coincidental, or it might reflect a functional requirement, as it would, for example, if *IR-L* and *IR-R* were unidirectional silencing elements. If this were the case, changing their orientation would lead to transcriptional repression on the normally expressed side. The orientation of *IR-L* and *IR-R* relative to each other could also determine their degree of efficiency. In this case, inverting one element at a time might perturb its function whereas inverting them both at once might be less detrimental.

We examined first the effects of inverting *IR-R*. We inverted it at its normal chromosomal location (Figure 3) or at the *Blp*I site mentioned above (Figure 2). Similar observations were made in both cases. First, *ura4* placed at sites centromere-distal to *IR-R* was always well expressed, indicating that *IR-R* does not act as a simple unidirectional silencing element. Second, *ura4* placed at sites centromere-proximal to an inverted *IR-R* element achieved a level of repression intermediate to that conferred by *IR-R* in its wild-type orientation and that observed in the absence of *IR-R*. This was observed for *ura4* at the *Eco*RV site close to *mat3* in the case of the inversion of *IR-R* at its wild-type location (compare Fig-

ure 3a, row 7, with Figure 1b, rows 9 and 37; and in Figure 3b, compare lanes 3 and 4 with 5 and 6) or for *ura4* at the *Spe*I site in the case of the inversion of *IR-R* at the *Blp*I site (Figure 2b, compare rows 5 and 6 with 1 and 4; Figure 2c, compare lanes 5 and 6 with 1 and 4). Hence, *IR-R* has the ability to strengthen silencing on its heterochromatic side when placed in either orientation, the wild-type orientation being slightly more effective than the reverse.



Since the effect of *IR-R* was partially orientation dependent, we tested whether inverting *IR-L* and *IR-R* in the same chromosome would lead to an increased repression at the sites affected by the inversion of *IR-R*. We found that this was not the case (Figure 3a, compare, for example, rows 7 and 18; Figure 3b, compare lanes 7 and 8 with 5 and 6). This supports our previous conclusion derived from the deletion analysis, that *IR-L* and *IR-R* can function independently of each other.

Duplication of *IR-R* leads to variegated boundary formation: We duplicated the *mat3-M* boundary element by inserting a second copy of *IR-R* centromere-distal to the endogenous *IR-R* element (Figure 4). The second copy of *IR-R* was introduced in either orientation at the *Blp*I site. We monitored gene expression between the endogenous and duplicated *IR-R* elements, at the *Spe*I site used previously. We found that insertion of an *IR-R* copy in the wild-type orientation allowed expansion of the silent state past the endogenous *IR-R* element toward the telomere in a fraction of the cell population (Figure 4). No repression was observed in a *swi6*$^-$ background (data not shown), suggesting the repression was due to an extended chromosomal coverage by Swi6. Insertion of a spacer fragment of λDNA or insertion of the *IR-R* element in the opposite orientation did not lead to repression of *ura4* at *Spe*I (Figure 4, rows 3–6). Some FOA-resistant cells were present in strains with the inverted duplication of *IR-R* (Figure 4, rows 3 and 4). However, these FOA-resistant cells had lost the ability to mate or sporulate. Using Southern blot analysis, we determined the loss was due to recombination events between *IR-L* and *inv-IR-R*, which removed *ura4* together with the entire *mat2-P-mat3-M* region (data not shown).

In the absence of such rearrangements, cells with the inverted *IR-R* element were not able to grow on FOA-containing plates. Hence, the repression of *ura4* at the

FIGURE 2.—Effects of moving *IR-R* toward the telomere. (a) Location of restriction sites relative to *mat3-M*. The sizes of DNA fragments are indicated below the map in base pairs. (b) Expression of *ura4*$^+$ assayed by spot tests. Tenfold serial dilutions of strains lacking *IR-R* and containing or not an insertion at the indicated *Blp*I restriction site were spotted on selective media. The insertion at *Blp*I consisted of either a 2.1-kb *Xba*I-*Xmn*I fragment containing *IR-R* in the wild-type (*IR-R*) or reverse (*inv-IR-R*) orientation or the 1.9-kb fragment generated by *Bst*EII in the bacteriophage λ DNA. 1, PG1934; 2, PG1938; 3, PG2684; 4, PG2688; 5, PG2699; 6, PG2701; 7, PG2711; 8, PG2720; 9, PG2028; 10, PG2039; 11, PG2729; 12, PG2733; 13, PG2722; 14, PG2736. The *ura4*$^+$ gene is inserted with its promoter centromere-proximal in the strains in rows 1, 3, 5, 7, 9, 11, and 13 and in the opposite orientation in rows 2, 4, 6, 8, 10, 12, and 14. (c) Expression of *ura4*$^+$ assayed by Northern blot. The ratios of full-length *ura4* transcript originating from *(Spe*I*)::ura4*$^+$ to *ura4-DS/E* transcript originating from the *ura4* locus were determined by phosphorimaging and are indicated below the blot. Lanes 1–8, same as in b.

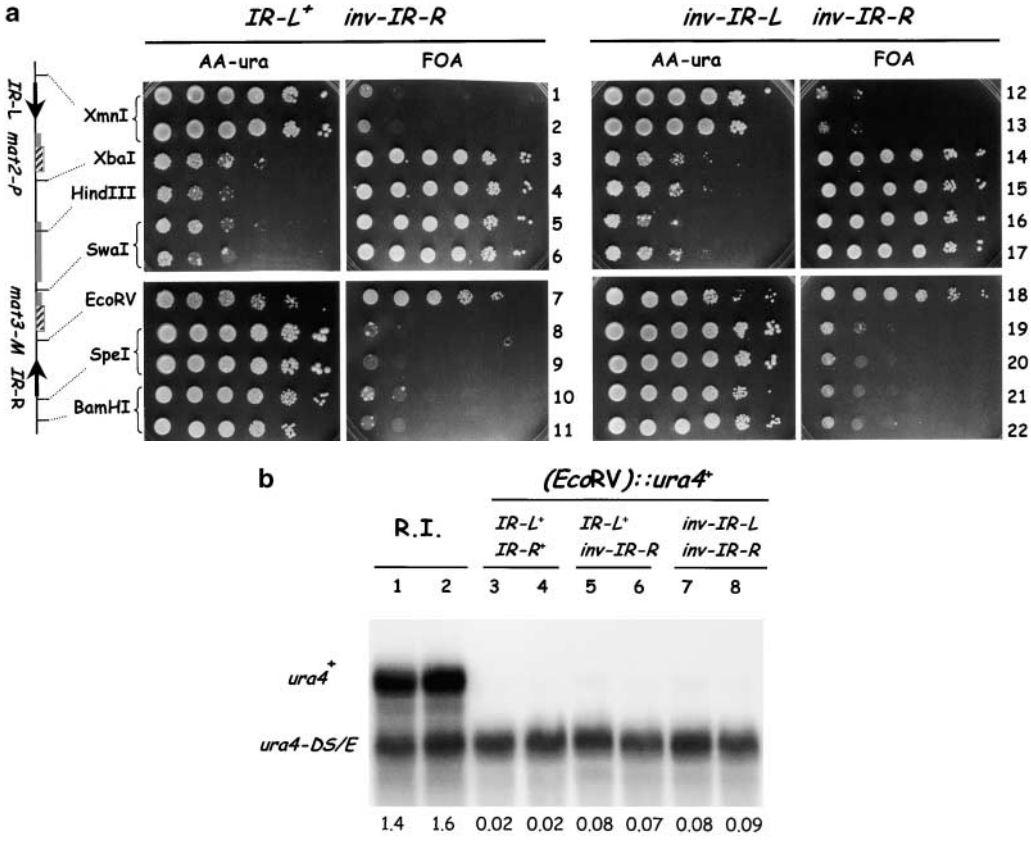


FIGURE 3.—Effects of inverting one or both repeats. (a) Expression of *ura4*$^+$ assayed by spot tests. Tenfold serial dilutions of strains containing either an inversion of *IR-R* (*IR-L*$^+$ *inv-IR-R*) or inversions of both *IR-L* and *IR-R* (*inv-IR-L invIR-R*) were spotted on the indicated media. Two strains are shown in cases where two insertions of *ura4* were examined for a given restriction site, one with a centromere-proximal promoter (top row) and one with a centromere-distal promoter (bottom row). 1, PG2153; 2, PG2161; 3, PG2168; 4, PG2411; 5, PG2390; 6, PG2426; 7, PG2174; 8, PG2124; 9, PG2195; 10, PG2139; 11, PG2198; 12, PG2286; 13, PG2278; 14, PG2585; 15, PG2415; 16, PG2441; 17, PG2433; 18, PG2288; 19, PG2299; 20, PG2303; 21, PG2293; 22, PG2453. (b) Expression of *ura4*$^+$ assayed by Northern blot. Lanes 1 and 2 show the expression of a *ura4*$^+$ gene inserted at a random integration site (R.I.; ALLSHIRE *et al.* 1995). Lanes 3–8 show the expression of *mat3-M (Eco*RV*):: ura4*$^+$ in strains containing the indicated *IR* arrangements. The ratios of *ura4*$^+$ to *ura4-DS/E* transcript are indicated below the blot. 1 and 2, FY340; 3, PG1898; 4, PG1899; 5, PG2173; 6, PG2174; 7, PG2288; 8, PG2290.

*Spe*I site appears to show strong dependence on the orientation of the *IR-R* element introduced at *Blp*I when the endogenous *IR-R* is present (Figure 4, lanes 1–4). The orientation dependence appears much weaker when the endogenous *IR-R* element is absent (Figure 2, lanes 3–6). This difference possibly reflects the ability of the endogenous *IR-R* element to partially block silencing (compare growth in the absence of uracil in Figure 2, lanes 3 and 4, and Figure 4, lanes 1 and 2).

The chromodomain protein Swi6 competes with active chromatin formation in cells lacking boundary elements: The chromodomain protein Swi6 is probably the most extensively characterized of the *S. pombe* silencing factors. It can oligomerize and associates with hetero-

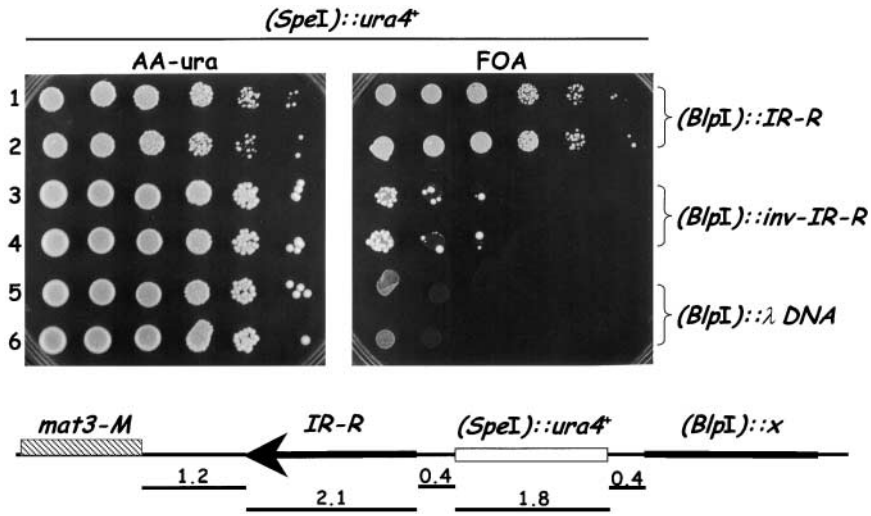


FIGURE 4.—Effects of duplicating *IR-R*. Tenfold serial dilutions of strains carrying *ura4*$^+$ flanked by direct (*Blp*I*::IR-R*) or inverted (*Blp*I*::inv-IR-R*) *IR-R* repeats or by *IR-R* and the 1.9-kb λ *Bst*EII fragment (*Blp*I*::* λ*DNA*) were spotted on selective media. 1, PG2644; 2, PG2653; 3, PG2663; 4, PG2671; 5, PG2674; 6, PG2682. The *ura4* gene is inserted with its promoter centromere-proximal in the strains in rows 1, 3, and 5 and in the opposite orientation in the strains in rows 2, 4, and 6.

chromatic regions over long stretches of DNA, suggesting that it spreads over the silenced regions (Partridge *et al.* 2000; Wang *et al.* 2000; Noma *et al.* 2001). We infer that Swi6 is present in the mating-type region of cells in which *IR-L*, *IR-R*, or both elements have been deleted from the ability of these cells to switch their mating type, an activity that requires Swi6, as well as from their ability to silence sites in the *mat2-mat3* intervening region. However, the reduced silencing observed in regions that flank *mat2* or *mat3* following deletion of *IR-L* or *IR-R* suggests that these regions might be inadequately associated with Swi6. If this inadequate coverage resulted from a competition between, on one hand, the oligomerization of Swi6 and, on the other hand, the propagation of an active state of gene expression, then increasing the intracellular concentration of Swi6 might tilt the balance toward an increasingly silenced state.

In a first set of experiments, we examined the effects of Swi6 overexpression on transcriptional silencing at the *mat3* centromere-distal *Eco*RV site. The *ura4* reporter is repressed at that site in the presence of *IR-R*, partially repressed in the presence of an inverted *IR-R* element, and derepressed in the absence of *IR-R*. We found that overexpression of Swi6 could partially suppress the silencing defects caused by deleting or inverting *IR-R* (Figure 5a).

In a second set of experiments, we examined the ability of Swi6 to expand the silenced domain in the *mat2* centromere-proximal region and whether that ability depended on the presence of *IR-L*. *ura4* is normally well expressed when placed at the *mat2* centromere-proximal *Xmn*I site (Figure 1b, rows 3 and 4). Increased concentrations of Swi6 had no detectable effect at that site in the presence of *IR-L* and a repressive effect when *IR-L* was deleted (Figure 5b). These phenotypes confirm that the *IR-L* and *IR-R* elements can reduce the expansion of heterochromatin in addition to that of euchromatin. They also demonstrate that Swi6 becomes locally limiting in the mating-type region in the absence of *IR-L* or *IR-R*.

***IR-R* confers autonomous replication to a plasmid:** *S. pombe* autonomously replicating sequences (ARS) are several hundred base pairs long and AT-rich, with clusters of A and T residues (Maundrell *et al.* 1988). Since their features are similar to those of the inverted repeats found in the mating-type region, we tested whether *IR-R* possessed ARS activity. A 2.1-kb *IR-R*-containing fragment with 99% identity to *IR-L* was cloned into an *Escherichia coli* plasmid together with the *S. pombe ura4* gene. *IR-R* allowed the plasmid to replicate in *S. pombe* in transformation experiments, with an efficiency similar to that of *ars1* (Clyne and Kelly 1995; Figure 6).

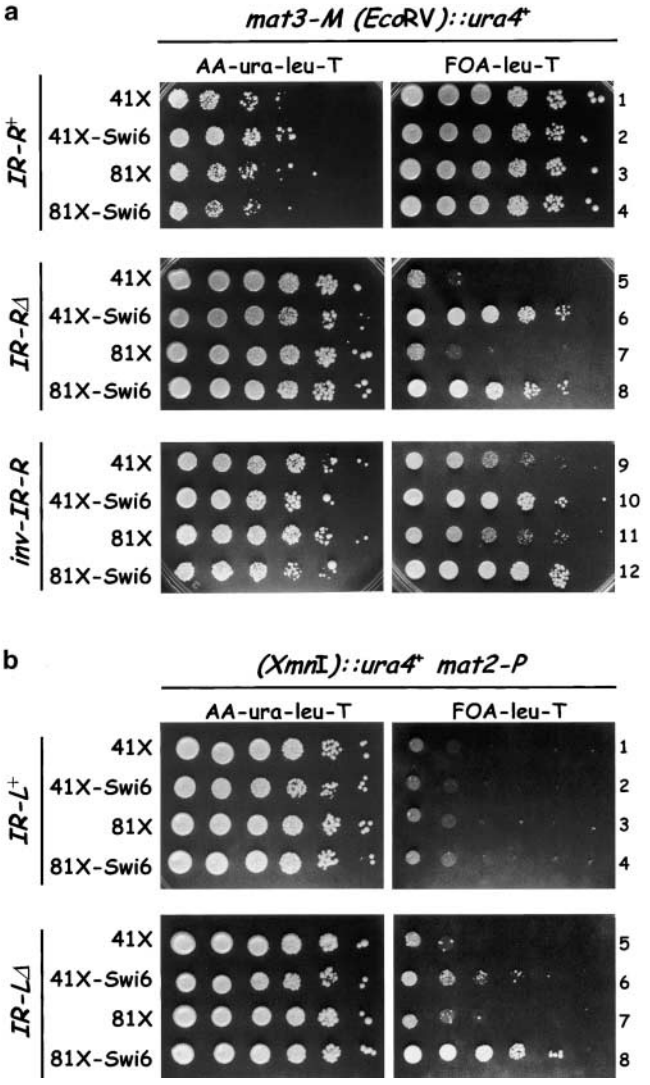


Figure 5.—Transcriptional silencing induced by overexpression of Swi6. Tenfold serial dilutions of cells transformed with the plasmid pREP41X (41X), pREP41X encoding Swi6 (41X-Swi6), pREP81X (81X), or pREP81X encoding Swi6 (81X-Swi6) were propagated in media lacking thiamine for five to six generations to induce transcription from the *nmt1* promoter and spotted on media lacking thiamine. (a) Effect of Swi6 overexpression on *ura4*$^+$ placed near *mat3-M*. 1–4, PG1899; 5–8, PG2047; 9–12, PG2173. (b) Effect of Swi6 overexpression in the *IR-L* centromere-proximal region. 1–4, PG2096; 5–8, PG2536.

DISCUSSION

We have presented evidence that the *IR-L* and *IR-R* repeats mark the edges of the silenced mating-type region of fission yeast (Figure 7a). The limits of transcriptional repression coincide with the limits of a specialized chromatin structure in which histone H3 is hypermethylated at lysine 9 and hypomethylated at lysine 4 and that is associated with the chromodomain protein Swi6 (Noma *et al.* 2001).

We found that *IR-L* and *IR-R* have a dual effect under normal physiological conditions. First, they prevent the spreading of the neighboring transcription competence into the silenced region. Consequently, one phenotype of an *IR-L* or *IR-R* deletion is a reduced repression of

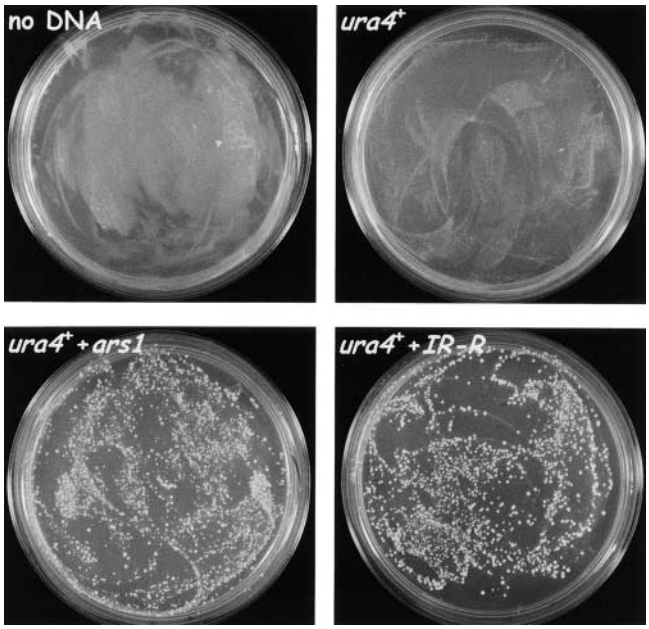


Figure 6.—Test of *IR-R* ARS activity. SP837 cells transformed with pGT188 (*ura4*$^+$), pGT302 (*ura4*$^+$ + *ars1*), pGT303 (*ura4*$^+$ + *IR-R*), or no DNA were plated on AA-ura and incubated at 33° for 3 days.

markers near the deleted repeat. Second, *IR-L* and *IR-R* block the spread of silencing. Following deletion of *IR-L* or *IR-R*, a *ura4*$^+$ marker located outside of the normally silenced region can be repressed, allowing papillation on FOA (Figure 1b, compare, for example, rows 3 and 4 and 18 and 19, 31 and 32 and 42 and 43, 12–15 and 38–41, and 27–30 and 49–52). Although the repression takes place in only a small fraction of the cell population, the silenced state is partially stable once established, allowing improved growth on FOA (data not shown). The ability of *IR-L* or *IR-R* to block the expansion of silencing becomes more obvious in conditions where the Swi6 protein is overexpressed. Under these conditions, marker genes centromere-proximal to *IR-L* are frequently inactivated in cells lacking *IR-L*, but not in cells containing *IR-L* (Figure 5b). Taken together, these phenotypes indicate that *IR-L* and *IR-R* act as insulators between competing states of expression. The possibility that deleting *IR-L* or *IR-R* might decrease silencing was overlooked in the chromatin study of Noma *et al.* (2001) in which the effects of deleting *IR-L* or *IR-R* were examined solely under conditions of Swi6 overexpression. Under such conditions, both studies lead to compatible results, demonstrating the ability of *IR-L* and *IR-R* to block the expansion of silenced heterochromatin. In wild-type cells, however, the major role of *IR-L* and *IR-R* appears to be to maintain the integrity of the silenced region.

Several models that are not mutally exclusive can account for the ability of a DNA element to prevent the spreading of active or repressive chromatin (for review, see, *e.g.*, Bell *et al.* 2001). In the "looping models," elements with similar properties act in pairs to promote the formation of DNA loops. They either interact directly with each other or mediate attachment to the nuclear matrix, in either case creating a chromosomal domain partially independent from its flanking regions. Looping models are supported by the observation that boundary elements are often found in pairs. This is, for example, the case with the Drosophila scs and scs′ elements (Udvardy *et al.* 1985) and the chicken β-globin 5′ and 3′ boundaries (Saitoh *et al.* 2000 and references therein). Further, boundary elements work in pairs to protect genes from position effets (*e.g.*, Kellum and Schedl 1992). Enhancer-blocking experiments involving duplicated insulators have also been interpreted to support looping models (Cai and Shen 2001; Muravyova *et al.* 2001). In these experiments, a single Su(Hw) insulator placed between an enhancer and promoter blocks enhancer-promoter interactions, whereas two intervening insulators do not. This suggests that interactions between two insulators in tandem preclude the formation of other loops that would include either the enhancer or the promoter and affect their ability to communicate with each other. The perfect sequence identity of *IR-L* and *IR-R* supports models in which the two repeats physically interact. The existence of interactions is further supported by the occurrence of recombination events leading to deletions between *IR-L* and *inv-IR-R* (Figure 4; data not shown). However, we found that *IR-L* and *IR-R* can function independently of each other to establish the boundaries of the silenced mating-type region. Hence, direct interactions between *IR-L* and *IR-R*, although they may take place, are not a requirement for boundary activity.

Alternatives, or additions, to looping models propose that nucleoprotein structures formed on boundary elements create discontinuities in the nucleosome array or in arrays of chromatin-associated proteins, thereby blocking processive activities such as the oligomerization along the DNA fiber of proteins required for either silencing or transcription. Many unrelated proteins could have that effect when complexed with DNA, accounting for the diversity of insulator elements found in *Saccharomyces cerevisiae* and other organisms (Bi and Broach 2001). We note that the sequence characteristics of *IR-L* and *IR-R* are similar to those of cohesin binding sites in *S. cerevisiae* (Blat and Kleckner 1999; Laloraya *et al.* 2000), suggesting that cohesins, which have been implicated in boundary function in *S. cerevisiae* (Donze *et al.* 1999), might participate in boundary activity in *S. pombe*. The ARS activity of *IR-R* suggests a more direct affinity for the origin recognition complex (ORC), a complex of proteins involved in DNA replication as well as other processes, such as transcriptional silencing in *S. cerevisiae* (reviewed by Kelly and Brown 2000). The *S. pombe* ORC comprises six subunits (Moonl *et al.* 1999), including an AT-hook domain protein, Orp4,

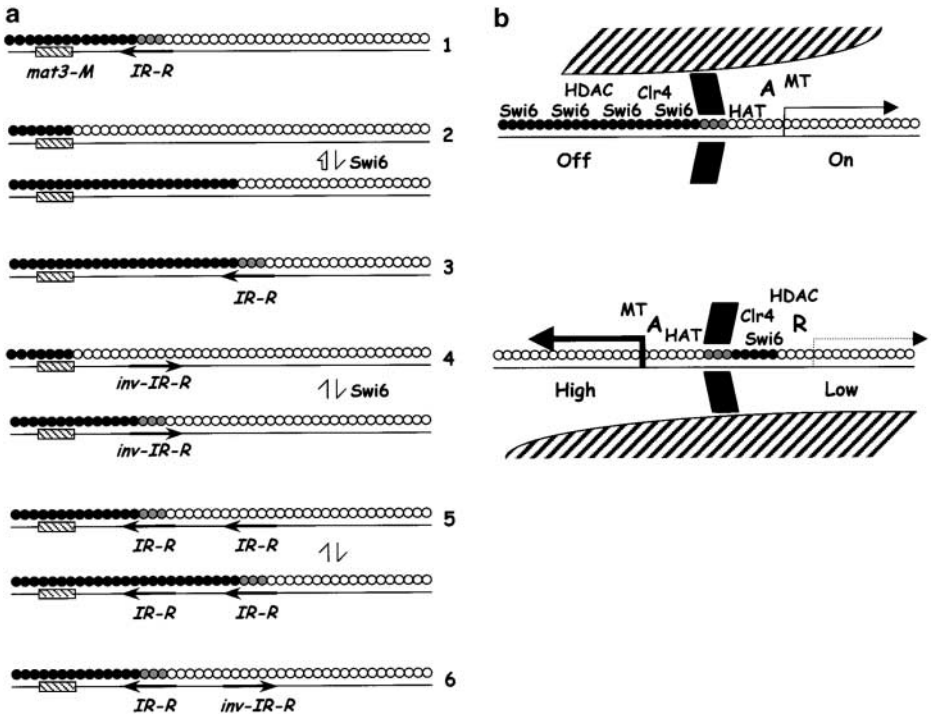


FIGURE 7.—Summary and models. (a) The *IR* elements function as boundaries in the mating-type region. The schematic representations 1–6 summarize the effects of deleting, translocating, or inverting *IR-R* on the expression states adopted at the centromere-distal edge of the mating-type region. Transcription-competent chromatin is represented by open circles, chromatin associated with silencing factors by solid circles, and chromatin associated with an active *IR-R* element by shaded circles. In cases where an equilibrium between two states is observed, high concentrations of Swi6 promote increased repression (2 and 4). (b) ORC as an insulator. ORC (solid parallelogram) separates expressed regions (On) from heterochromatin (Off) and, more generally, active promoter regions with euchromatic acetylation and methylation patterns from repressed promoter regions with heterochromatic features. HDAC, histone deacetylase; HAT, histone acetyltransferase; MT, methyltransferase; A, transcriptional activator; R, transcriptional repressor.

which binds strongly to AT-rich DNA (CHUANG and KELLY 1999). The assembly of ORC with the inverted repeats of the mating-type region might create a chromatin discontinuity displaying boundary function. Further, ORC subunits might contribute to the recruitment of Swi6 on the heterochromatic side of the boundary, by analogy with the Drosophila system in which interactions between ORC and HP1 are observed (PAK *et al.* 1997; HUANG *et al.* 1998).

IR-R promoting replication in plasmids raises the possibility that *IR-L* and *IR-R* also initiate DNA replication in the chromosome. By acting as origins, they might ensure that the region separating them is replicated at a time suitable for silencing. If *IR-R* is an active origin, the phenotype created by its duplication, which reduces the boundary activity of the endogenous *IR-R* element, could be due to origin interference, a phenomenon by which origins of replication suppress initiation of replication at nearby origins (DUBEY *et al.* 1994).

S. pombe origins of replication lie preferentially in intergenic regions. They are associated with promoters rather than with regions downstream of ORFs (GÓMEZ and ANTEQUERA 1999). The functional relevance, if any, of this association is not understood. The placement of ORC-binding sites close to promoters might fulfill a requirement similar to the requirement for *IR-L* and *IR-R* in the mating-type region, namely the separation of regions with various expression states. Proteins involved in the formation of heterochromatin have emerged as participants in the local regulation of gene expression. In mammalian cells, the SUV39H1 methylase and HP1 are recruited by the retinoblastoma (Rb) protein to the cyclin E promoter, which they repress (NIELSEN *et al.* 2001). In *S. pombe*, Clr4 regulates genes outside of the mating-type region (IVANOVA *et al.* 1996). The involvement of histone deacetylases in the regulation of gene expression is widely recognized (reviewed by RICE and ALLIS 2001). Hence, small regions with the characteristics of heterochromatin are very likely formed at the proximity of some promoters in cells where these promoters have to be repressed. Some mechanism must ensure that the heterochromatic state does not spread to affect the transcription of nearby genes and vice versa. ORC proteins placed between transcription units might have that effect (Figure 7b). Their proximity to proteins involved in both activation and repression of transcription would have led to the evolution of interactions, such as those of ORC1 with HP1 (PAK *et al.* 1997; HUANG *et al.* 1998), with the histone acetyltransferase HBO1 (IIZUKA and STILLMAN 1999), or with c-Myc (TAKAYAMA *et al.* 2000). In this model, only a fraction of the ORC associated with DNA would be activated to promote replication, the rest forming local barriers to the spread of active or repressive chromatin.

We thank our colleagues and friends for their critical input and editorial suggestions, especially Stanley Brown, Klavs Riishede Hansen, Inga Sig Nielsen, and Gerry Smith. The reported work was supported by grants from the Novo Nordisk Foundation and from the Carlsberg Foundation.

LITERATURE CITED

Aagaard, L., G. Laible, P. Selenko, M. Schmid, R. Dorn *et al.*, 1999 Functional mammalian homologues of the Drosophila PEV-modifier *Su(var)3-9* encode centromere-associated proteins which complex with the heterochromatin component M31. EMBO J. **18:** 1923–1938.

Allshire, R. C., E. R. Nimmo, K. Ekwall, J. P. Javerzat and G. Cranston, 1995 Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. Genes Dev. **9:** 218–233.

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.

Bannister, A. J., P. Zegerman, J. F. Partridge, E. A. Miska, J. O. Thomas *et al.*, 2001 Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature **410:** 120–124.

Bell, A. C., A. C. West and G. Felsenfeld, 2001 Insulators and boundaries: versatile regulatory elements in the eukaryotic genome. Science **291:** 447–450.

Bi, X., and J. R. Broach, 2001 Chromosomal boundaries in *S. cerevisiae*. Curr. Opin. Genet. Dev. **11:** 199–204.

Blat, Y., and N. Kleckner, 1999 Cohesins bind to preferential sites along yeast chromosome III, with differential regulation along arms versus the centric region. Cell **98:** 249–259.

Cai, H. N., and P. Shen, 2001 Effects of *cis* arrangement of chromatin insulators on enhancer-blocking activity. Science **291:** 493–495.

Chuang, R. Y., and T. J. Kelly, 1999 The fission yeast homologue of Orc4p binds to replication origin DNA via multiple AT-hooks. Proc. Natl. Acad. Sci. USA **96:** 2656–2661.

Clyne, R. K., and T. J. Kelly, 1995 Genetic analysis of an ARS element from the fission yeast *Schizosaccharomyces pombe*. EMBO J. **14:** 6348–6357.

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.

Dubey, D. D., J. Zhu, D. L. Carlson, K. Sharma and J. A. Huberman, 1994 Three ARS elements contribute to the ura4 replication origin region in the fission yeast, *Schizosaccharomyces pombe*. EMBO J. **13:** 3638–3647.

Egel, R., M. Willer, S. Kjærulff, J. Davey and O. Nielsen, 1994 Assessment of pheromone production and response in fission yeast by a halo test of induced sporulation. Yeast **10:** 1347–1354.

Ekwall, K., and T. Ruusala, 1994 Mutations in *rik1, clr2, clr3* and *clr4* genes asymmetrically derepress the silent mating-type loci in fission yeast. Genetics **136:** 53–64.

Ekwall, K., J. P. Javerzat, A. Lorentz, H. Schmidt, G. Cranston *et al.*, 1995 The chromodomain protein Swi6: a key component at fission yeast centromeres. Science **269:** 1429–1431.

Ekwall, K., E. R. Nimmo, J. P. Javerzat, B. Borgstrøm, R. Egel *et al.*, 1996 Mutations in the fission yeast silencing factors $clr4^+$ and $rik1^+$ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function. J. Cell Sci. **109:** 2637–2648.

Forsburg, S. L., 1993 Comparison of *Schizosaccharomyces pombe* expression systems. Nucleic Acids Res. **21:** 2955–2956.

Gómez, M., and F. Antequera, 1999 Organization of DNA replication origins in the fission yeast genome. EMBO J. **18:** 5683–5690.

Grewal, S. I., and A. J. Klar, 1996 Chromosomal inheritance of epigenetic states in fission yeast during mitosis and meiosis. Cell **86:** 95–101.

Grewal, S. I., and A. J. Klar, 1997 A recombinationally repressed region between mat2 and mat3 loci shares homology to centromeric repeats and regulates directionality of mating-type switching in fission yeast. Genetics **146:** 1221–1238.

Grewal, S. I., M. J. Bonaduce and A. J. Klar, 1998 Histone deacetylase homologs regulate epigenetic inheritance of transcriptional silencing and chromosome segregation in fission yeast. Genetics **150:** 563–576.

Grimm, C., J. Kohli, J. Murray and K. Maundrell, 1988 Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4* gene as a selectable marker. Mol. Gen. Genet. **215:** 81–86.

Huang, D. W., L. Fanti, D. T. Pak, M. R. Botchan, S. Pimpinelli *et al.*, 1998 Distinct cytoplasmic and nuclear fractions of *Drosophila* heterochromatin protein 1: their phosphorylation levels and associations with origin recognition complex proteins. J. Cell Biol. **142:** 307–318.

Iizuka, M., and B. Stillman, 1999 Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. J. Biol. Chem. **274:** 23027–23034.

Ivanova, A. V., M. J. Bonaduce, S. V. Ivanov and A. J. Klar, 1996 The chromo and SET domains of the Clr4 protein are essential for silencing in fission yeast. Nat. Genet. **19:** 192–195.

Kellum, R., and P. Schedl, 1992 A group of scs elements function as domain boundaries in an enhancer-blocking assay. Mol. Cell. Biol. **12:** 2424–2431.

Kelly, T. J., and G. W. Brown, 2000 Regulation of chromosome replication. Annu. Rev. Biochem. **69:** 829–880.

Kelly, M., J. Burke, M. Smith, A. Klar and D. Beach, 1988 Four mating-type genes control sexual differentiation in fission yeast. EMBO J. **7:** 1537–1547.

Lachner, M., D. O'Carroll, S. Rea, K. Mechtler and T. Jenuwein, 2001 Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature **410:** 116–120.

Laloraya, S., V. Guacci and D. Koshland, 2000 Chromosomal addresses of the cohesin component Mcd1p. J. Cell Biol. **151:** 1047–1056.

Lorentz, A., L. Heim and H. Schmidt, 1992 The switching gene *swi6* affects recombination and gene expression in the mating-type region of *Schizosaccharomyces pombe*. Mol. Gen. Genet. **233:** 436–442.

Lorentz, A., K. Ostermann, O. Fleck and H. Schmidt, 1994 Switching gene *swi6*, involved in repression of silent mating-type loci in fission yeast, encodes a homologue of chromatin-associated proteins from Drosophila and mammals. Gene **143:** 139–143.

Maundrell, K., A. Hutchison and S. Shall, 1988 Sequence analysis of ARS elements in fission yeast. EMBO J. **7:** 2203–2209.

Michael, H., H. Schmidt, O. Fleck, H. Gutz, C. Liedtke *et al.*, 1994 The mating-type region of *Schizosaccharomyces pombe* contains an essential gene encoding a protein homologous to human modulators of HIV transactivation. Gene **145:** 205–210.

Moonl, K. Y., D. Kong, J. K. Lee, S. Raychaudhuri and J. Hurwitz, 1999 Identification and reconstitution of the origin recognition complex from *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. USA **96:** 12367–12372.

Moreno, S., A. Klar and P. Nurse, 1991 Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. **194:** 795–823.

Muravyova, E., A. Golovnin, E. Gracheva, A. Parshikov, T. Belenkaya *et al.*, 2001 Loss of insulator activity by paired Su(Hw) chromatin insulators. Science **291:** 495–498.

Nakayama, J., J. C. Rice, B. D. Strahl, C. D. Allis and S. I. Grewal, 2001 Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science **292:** 110–113.

Nielsen, S. J., R. Schneider, U. M. Bauer, A. J. Bannister, A. Morrison *et al.*, 2001 Rb targets histone H3 methylation and HP1 to promoters. Nature **412:** 561–565.

Noma, K., C. D. Allis and S. I. S. Grewal, 2001 Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science **293:** 1150–1155.

Pak, D. T., M. Pflumm, I. Chesnokov, D. W. Huang, R. Kellum *et al.*, 1997 Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. Cell **91:** 311–323.

Partridge, J. F., B. Borgstrøm and R. C. Allshire, 2000 Distinct protein interaction domains and protein spreading in a complex centromere. Genes Dev. **14:** 783–791.

Rea, S., F. Eisenhaber, D. O'Carroll, B. D. Strahl, Z. W. Sun *et al.*, 2000 Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature **406:** 593–599.

Rice, J. C., and C. D. Allis, 2001 Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr. Opin. Cell Biol. **13:** 263–273.

Rose, M., F. Winston and P. Hieter, 1990 *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Saitoh, N., A. C. Bell, F. Recillas-Targa, A. G. West, M. Simpson *et al.*, 2000 Structural and functional conservation at the boundaries of the chicken beta-globin domain. EMBO J. **19:** 2315–2322.

Takayama, M. A., T. Taira, K. Tamai, S. M. Iguchi-Ariga and H. Ariga, 2000 ORC1 interacts with c-Myc to inhibit E-box-dependent transcription by abrogating c-Myc-SNF5/INI1 interaction. Genes Cells **5:** 481–490.

Thon, G., and T. Friis, 1997 Epigenetic inheritance of transcriptional silencing and switching competence in fission yeast. Genetics **145:** 685–696.

Thon, G., and A. J. Klar, 1992 The clr1 locus regulates the expression of the cryptic mating-type loci of fission yeast. Genetics **131:** 287–296.

Thon, G., and J. Verhein-Hansen, 2000 Four chromo-domain proteins of *Schizosaccharomyces pombe* differentially repress transcription at various chromosomal locations. Genetics **155:** 551–568.

Thon, G., A. Cohen and A. J. Klar, 1994 Three additional linkage groups that repress transcription and meiotic recombination in the mating-type region of *Schizosaccharomyces pombe*. Genetics **138:** 29–38.

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.

Udvardy, A., E. Maine and P. Schedl, 1985 The 87A7 chromomere. Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. J. Mol. Biol. **185:** 341–358.

Wang, G., A. Ma, C. M. Chow, D. Horsley, N. R. Brown *et al.*, 2000 Conservation of heterochromatin protein 1 function. Mol. Cell. Biol. **20:** 6970–6983.

Communicating editor: G. R. Smith