The Clr4 methyltransferase determines the subnuclear localization of the mating-type region in fission yeast

Jenny Alfredsson-Timmins, Frida Henningson and Pernilla Bjerling*
University of Uppsala, Department of Medical Biochemistry and Microbiology (IMBIM), Box 582, SE-751 23 Uppsala, Sweden
*Author for correspondence (e-mail: Pernilla.Bjerling@imbim.uu.se)
Accepted 11 April 2007
Journal of Cell Science 120, 1935-1943 Published by The Company of Biologists 2007
doi:10.1242/jcs.03457

Summary
The genome has a non-random spatial distribution in the cell nucleus. In Schizosaccharomyces pombe, it has been shown that the centromeres, telomeres and the mating-type region localize to the nuclear membrane (NM), the former by attaching to the spindle pole body (SPB). In addition, reporter genes inserted into these areas are transcriptionally repressed because of the formation of specialized chromatin structures. Performing live cell analysis we found that in a wild-type strain the mating-type region was positioned in the proximity of the SPB, the location where the pericentromeric heterochromatin is also found. In a strain lacking the histone methyltransferase Clr4, crucial for the formation of heterochromatin, the mating-type region had a random localization in the nucleus. Moreover, in a strain in which the two boundary elements IR-L and IR-R had been deleted, the mating-type region was displaced from its position at the proximity of the SPB, but remained in the vicinity of the NM. Moreover, in all investigated strains with silencing deficiencies the distance between the mating-type region and the SPB increased. This result indicates a correlation between transcriptional derepression and displacement of the region. Two different models of how the mating-type chromatin is organized in the nucleus are discussed.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/11/1935/DC1

Key words: Fission yeast, Heterochromatin, Silencing, Subnuclear localization

Introduction
Within the cell nucleus chromatin has a specific spatial organization, and there is growing evidence that this organization affects gene expression by epigenetic mechanisms (Sproul et al., 2005), although very little is known about how this is achieved. In human cells, the chromosomes occupy different territories (Croft et al., 1999). Recent data suggest that these territories are not distinct and that intermingling of the territories can occur (Branco and Pombo, 2006). Moreover, transcribed genes loop out of the chromosomal territories to transcription factories in the interchromatin space (Osborne et al., 2004). During B-lymphocyte development, up- or downregulation of gene expression results in drastic changes in the subnuclear localization of affected genes. CD2 and other genes that are downregulated by Ikaros, a repressor protein, move to a pericentric location, whereas the immunoglobulin (Ig) loci IgH and Igk relocate from the nuclear periphery to a more internal position upon activation (Brown et al., 1997; Kosak et al., 2002).

In the budding yeast, Saccharomyces cerevisiae, specialized chromatin structures are formed by the Sir2, Sir3 and Sir4 proteins at the subtelomeric region and at the mating-type loci, HML, and HMR, located close to the telomeres of chromosome III (Rusche et al., 2003). This organization causes transcriptional repression of reporter genes inserted into these regions. Furthermore, the telomeres are attached to the nuclear membrane (NM) through Ku or Esc1 protein anchors, and tethering a gene to the nuclear periphery facilitates its repression (Andrulis et al., 1998; Hediger et al., 2002).

In interphase cells of the fission yeast, Schizosaccharomyces pombe, the centromeres and telomeres localize to the nuclear periphery, the centromeres by being attached to the spindle pole body (SPB) (Funabiki et al., 1993). The telomeres are found in two to four clusters at the NM at the opposite side of the SPB. How the chromatin of the three S. pombe chromosomes is organized between the centromeres and telomeres is not clear, although it has been suggested that bodies of transcription factor TFIIIC attach certain loci to the NM (Noma et al., 2006). Pericentromeric or subtelomeric areas of the chromosomes are heterochromatic, and genes inserted into these areas are transcriptionally repressed (Allshire et al., 1995; Ekwall et al., 1995; Perrod and Gasser, 2003). Crucial components of this specialized chromatin are conserved between fission yeast and humans (Bjerling and Ekwall, 2002).

In S. pombe it is well established that the formation of heterochromatin is initiated by the formation of small interfering RNA (siRNA) by the RNA interference (RNAi) machinery (Volpe et al., 2002). Recent data suggest that this is also the case in mammals (Fukagawa et al., 2004). Additional similarities between fission yeast and mammalian heterochromatin are low acetylation levels of histones, methylation of histone H3 lysine 9 (H3MeK9), and binding of chromodomain proteins (Jeppesen and Turner, 1993; Bannister et al., 2001; Litt et al., 2001; Noma et al., 2001; Bjerling et al., 2002). The surrounding euchromatin, however, shares features of active chromatin in mammals, including acetylated histones and histone H3 methylation at lysine 4 (H3MeK4) (Litt et al., 2001; Noma et al., 2001). In humans, the H3K9 methylation is performed by the SUV39H1 methyltransferase and in S. pombe by its homologue Clr4. This methylation creates a binding site
for heterochromatin protein 1 (HP1) in humans or its homologue Swi6 in S. pombe (Rea et al., 2000; Bannister et al., 2001).

Transcriptional silencing through a heterochromatin structure also occurs in the mating-type region, and this region is found at the nuclear periphery, but it is unknown whether or not it associates with other heterochromatic regions (Noma et al., 2006). There is no physical proximity to the centromere or telomere on the chromosome because the mating-type region is found on the right arm of chromosome II, 460 kb from the centromere and 2300 kb from the telomere (http://www.ncbi.nlm.nih.gov). The mating-type region consists of the two silent storage cassettes, mat2-P and mat3-M, and mat1 that is expressed and determines the mating-type, P or M, of the cell (Fig. 1) (Arcangioli and Thon, 2004). The two silent cassettes are utilized when the cells switch mating-type by a gene conversion event, moving genetic information from either mat2-P or mat3-M to mat1 in a homothallic h^{90} strain (Arcangioli and Thon, 2004). Between mat1 and mat2-P there is a 15 kb-long sequence designated L, and between mat2-P and mat3-M there is an 11 kb region called K (Fig. 1) (Grewal and Klar, 1997; Arcangioli and Thon, 2004). Nucleation of heterochromatin occurs in the K-region through two redundant pathways, one consisting of the RNAi machinery acting through cenH, a 4.3 kb sequence sharing 96% identity to the repeats in centromere 2 (cen2), and the other by two DNA-binding proteins of the ATP/CREB family, Pcr1 and Atf1, acting through REIII localized just centromere-proximal of the mat3-M cassette (Grewal and Klar, 1997; Hall et al., 2002; Jia et al., 2004). When the K-region is deleted, a mutation designated h^{K3} for homothallic K, an epigenetic switch occurs between a transcriptionally repressed and derepressed state (Grewal and Klar, 1996; Thon and Friis, 1997; Hall et al., 2002). This inefficiency of heterochromatin formation is probably because of the fact that in the h^{K3} strain only the Pcr1/Atf1 nucleation pathway remains. The borders between transcriptionally repressed heterochromatin and active euchromatin in the mating-type region are established by the IR-L and IR-R inverted repeats and require the binding of TFIIC to B-boxes in these boundary elements (Fig. 1) (Noma et al., 2001; Thon et al., 2002; Noma et al., 2006). Because both TFIIC and the mating-type region are localized to the nuclear periphery, Noma and co-authors suggest that the mating-type region is attached to the NM through the TFIIC bodies.

Even though so much is now known about the molecular mechanisms of setting up the heterochromatin structure, the impact of subnuclear localization on chromatin formation has not been investigated in S. pombe. One group of genes encoding the RNAi machinery, dcr1, ago1 and rdr1, is known to affect subnuclear localization of heterochromatic areas in the S. pombe interphase cells. When these genes are deleted the telomeres remain attached to the NM, but they no longer form clusters (Hall et al., 2003). The subtelomeric heterochromatin structure remains unaffected in these mutant cells because the chromatin in these regions are nucleated through an RNAi-independent mechanism that requires the telomeric Taz1 protein (Kanoh et al., 2005).

In this study we used the mating-type region of S. pombe as a model system to gain further insights into the correlation between transcriptional repression and subnuclear localization. Furthermore, we set out to determine more precisely where the mating-type region is positioned in the cell nucleus and what factors are needed for its correct localization. We found that during interphase in a wild-type strain, the mating-type region resides in the proximity of the SPB where the centromeric heterochromatin is found. This localization was completely dependent on the methyltransferase Cdr4. In addition, changes in the subnuclear localization of the mat2/3 region were also detected in strains with a deletion in the K-region, the two boundary elements IR-L and IR-R flanking the region, or the chromodomain protein Swi6. Furthermore, we demonstrate a correlation between transcriptional derepression and delocalization from the nuclear periphery. Thus, in all strains investigated, mutations that cause the expression of a reporter gene inserted into this normally silent region also caused the mating-type region to be repositioned away from the SPB. We discuss two different models for how the mating-type region is constrained in its subnuclear localization.

Results

Cis-acting mutations alleviate transcriptional repression of an ade6^{+} reporter gene inserted next to mat3-M

A strain background with an ade6^{+} gene at the EcoRV site located 500 bp downstream of mat3-M and a truncated ade6-DN/N allele at the endogenous ade6 locus with lacO repeats inserted at the his2 locus 25 kb centromere-distal from the mat2/3 region, as well as the Lac repressor fused to green fluorescent protein (LacR-GFP) expressed from the his7 locus, was created (Fig. 1) (Shimada et al., 2003). The lacO repeats enable visualization of the nuclear localization of the chromosomal region through binding of the Lac-R-GFP. This strain background was combined with mutations previously shown to affect transcriptional silencing in the mating-type region. The cis-acting mutant strains have either deletions of the two boundary elements IR-L and IR-R, or an 8 kb deletion of the K-region between mat2-P and mat3-M, denoted h^{K3} (Fig. 1) (Thon and Friis, 1997; Thon et al., 2002). As a result of an epigenetic switch, the h^{K3} strain can be isolated in two different states; one with a transcriptionally repressed mat2/3 region similar to a wild-type strain, and one with a derepressed mating-type region (Grewal and Klar, 1996; Thon and Friis, 1997). Switching between these two states occurs at a low frequency (3-8 events per 10^{6} cell divisions), thereby allowing analysis on isolates in both states. In addition, trans-acting mutant strains had either a deletion of the clr4^{+} gene encoding a histone methyltransferase, a deletion of the swi6^{+} gene encoding a chromodomain protein, or a deletion of the dcr1^{+} gene encoding the Dicer protein necessary for RNAi. Moreover, two different wild-type strains were used for the analysis. One wild-type strain has the same truncated ade6-DN/N allele at the endogenous ade6 locus as all the mutant strains used in this study. The ade6-DN/N allele allows for the reverse transcriptase-polymerase chain reaction (RT-PCR) analysis described below. The other wild-type strain instead has the ade6-M210 point mutation as well as the chromophore DsRed fused to the nuclear pore protein Pom152, allowing for the localization studies described below.

To confirm previous reported effects on silencing mediated by these cis- and trans-acting mutations in our strain background, the expression from the ade6^{+} reporter gene inserted next to mat3-M was monitored in two different ways.
First, serial dilutions of cell cultures were spotted onto media with or without adenine (Fig. 2A). As controls we included strains without the mat3-M::ade6+ reporter gene but with the ade6-DN/N allele or the ade6+ allele at the endogenous ade6 locus (Fig. 2A, top lanes). The two wild-type strains with an ade6+ reporter gene inserted next to the mat3-M grew poorly on plates lacking adenine, and there was no difference in growth when comparing the ade6-M210 with the ade6-DN/N strain, which suggests that the endogenous ade6 allele did not influence chromatin structure in the mating-type region. Second, the amount of ade6+ mRNA produced in the cells was measured using RT-PCR (Fig. 2B). All the mutant strains contained the truncated allele ade6-DN/N at the endogenous ade6 locus as an internal control. In the wild-type ade6-DN/N strain the amount of mRNA from the ade6+ reporter was below the detection limit (Fig. 2B). Deletion of the inverted repeats IR-L and IR-R had a strong effect on ade6+ transcription, as detected both by growth on plates lacking adenine and an increase in the amount of ade6+ transcript (Fig. 2). Deletion of the K-region resulted in one strain that maintained a tight repression of the ade6+ reporter gene and one strain with elevated transcription of the reporter (Fig. 2). Transcriptional derepression also occurred in the clr4Δ and swi6Δ strain, but not in the dcr1Δ strain. The results presented here are in agreement with earlier findings (Fig. 2) (Thon and Friis, 1997; Thon et al., 1999; Thon and Verhein-Hansen, 2000; Hall et al., 2002; Thon et al., 2002).

The mating-type region was visualized through the LacR-GFP fusion protein binding to lacO arrays integrated in the proximity of the mating-type region (Shimada et al., 2003). The SPB was detected through the spindle pole component Cut12 fused to the fluorophore cyan fluorescent protein (Cut12-CFP) (Appelgren et al., 2003). Furthermore, the NM was labelled by fusing the fluorophore DsRed to the nuclear pore component Pom152. First, we measured the distance between the SPB and the mating-type region. Although all distances were measured in two dimensions, the optical slice had a depth of 0.8 μm, which is equivalent to one-third of the diameter of the S. pombe nucleus. Only cells where one CFP and one GFP signal could be detected in the same focal plane were used for analysis. In some cells two CFP signals were detected. These signals corresponded to the SPB having split into two and started to migrate towards the poles. Theses cells had left interphase and entered mitosis and were therefore excluded from the analysis. We proceeded to measure the diameter of the cell nucleus and the shortest distance between the mating-type region and the NM, using Pom152-DSRed as a reference. In both wild-type strains, the mating-type region was found next to the SPB close to the nuclear periphery (Fig. 3A). To get an overview of the observed distribution in the wild-type strains, the distance data were plotted as a histogram, first binning the data in groups.
spanning 0.2 μm (Fig. 3A, right panel). The data did not follow a normal distribution. Therefore, a Mann-Whitney rank sum test was used to show that there was no statistically significant difference between the observed median distance of 0.29 μm and 0.27 μm, respectively, between the two different wild-type strains \((P<0.05)\) (Table 1 and Fig. 3A). The distance between the pericentromeric heterochromatin and the SPB is approximately 0.3 μm (Kniola et al., 2001), indicating that the \(\text{mat2/3} \) region may cluster together with this chromatin. For all further studies only the wild-type \(\text{ade6-M210} \) strain was used because it contains the Pom152-DsRed construct, which the wild-type \(\text{ade6-DN/N} \) strain lacks. The mean diameter of the cell nucleus was calculated to be 2.40 μm, using the Pom152-DsRed signal as a reference. Using this value the surface area of the nucleus was calculated and divided into three equally sized concentric zones (Fig. 3B). The distance between the mating-type region and the NM in each cell was then used to classify the mating-type region as being in zone I (0-0.22 μm

Fig. 3. See next page for legend.
Clr4 is essential in chromatin organization

from the NM), zone II (0.23-0.51 μm from the NM) or zone III (0.52-1.20 μm from the NM), where zone I is the nuclear periphery, zone II is next to the nuclear periphery and zone III is the nuclear interior. In the wild-type strain the mating-type region was found in zone II in approximately half of the analysed cells, as visualized by the pie charts in Fig. 3D. The median distance between the mating-type region and the NM was 0.34 μm. In conclusion, the results from Fig. 3 show that the mating-type heterochromatin preferentially localizes next to the SPB, which is where the pericentromeric heterochromatin is found in S. pombe interphase cells (Kniola et al., 2001). This localization correlated with transcriptional repression of the mat2/3 region.

The localization of the mating-type region is dependent on cis-acting sequences

In order to determine what factors are needed for localization of the mating-type region to the nuclear periphery, a strain lacking the boundary elements IR-L and IR-R that surround the mat2/3 region was analysed. Interestingly, there was an increase in the median distance between the mating-type region and the SPB from 0.29 μm in the wild-type strain to 0.40 μm in the mutant strain (Table 1 and Fig. 3A). The range was also much broader in this mutant strain as compared with the wild-type strain (Table 1). The difference between the observed median distance in the wild-type and the IR-LΔIR-RΔ strain was statistically significant (P<0.05). We proceeded to measure the distance between the NM and the mating-type region in order to determine whether the mat2/3 region was found in the nuclear interior or still resided close to the nuclear periphery, but without being next to the SPB. We found that in approximately half of the cells the region was still localized in zone II, similar to the wild-type strain, and the median distance between the mating-type region and the NM increased from 0.34 μm in the wild-type strain to 0.41 μm in the IR-LΔIR-RΔ strain (Fig. 3C,D). Statistically there was no significant difference between the observed distances of these two strains (P>0.05).

| Strain | Mean (μm) | Median (μm) | n | zone I | zone II | zone III | Different from w.t. (P<0.05) |
|--------|-----------|-------------|---|--------|---------|----------|-------------------------------|
| wild type | 0.38      | 0.34        | 61 | 16     | 32      | 13       | n.a.                          |
| IR-LΔIR-RΔ | 0.45      | 0.41        | 69 | 13     | 34      | 22       | no                            |
| hKΔ repressed | 0.50      | 0.52        | 71 | 12     | 23      | 36       | yes                           |
| hKΔ derepressed | 0.42      | 0.38        | 62 | 8      | 41      | 14       | no                            |
| clr4Δ | 0.57      | 0.55        | 62 | 2      | 28      | 32       | yes                           |
| swi6Δ | 0.54      | 0.47        | 64 | 4      | 32      | 28       | yes                           |
| der1Δ | 0.40      | 0.38        | 62 | 17     | 33      | 12       | no                            |
| cut3::lacO | 0.52      | 0.49        | 57 | 7      | 22      | 28       | yes                           |

**Fig. 3.** The mating-type region is localized close to the SPB at the nuclear periphery in the wild-type strains and is delocalized in strains with cis- and trans-acting silencing mutations. (A) Confocal microscopy images of wild-type and mutant cells. Shown in the different panels are: the NM labelled with DsRed (red, first column), the SPB labelled with CFP (blue, second column), the mating-type region or (in the bottom row) the cut3 locus, labelled with lacR-GFP (green, third column), and merged images (fourth column). Bars, 2 μm. Histograms with the distributions of observed distances between the mating-type region (or, in the bottom row, the cut3 locus) and the SPB are shown to the right of the panels. The distribution of the observed distances between the GFP and CFP signals were binned in groups spanning 0.2 μm. The strains analysed were PJ644 (w.t. ade6-M210), PJ654 (w.t. ade6-DN/N), PJ579 (IR-LΔIR-RΔ), PJ577 (hKΔ repressed) and PJ586 (hKΔ derepressed), PJ653 (clr4Δ), PJ668 (swi6Δ), PJ651 (der1Δ) and PJ575 (cut3::lacO). (B) The nucleus divided into three zones of equal surface area. Zone I corresponded to a distance of 0-0.22 μm, zone II to a distance of 0.23-0.51 μm and zone III to a distance of 0.52-1.20 μm from the nuclear periphery. (C) Distribution of mat2/3 localization into zone I, zone II or zone III for each strain. The significance of the differences between each mutant strain and the PJ644 wild type was tested statistically (Mann-Whitney rank sum test, P<0.05). No, no significant difference; yes, a significant difference; n.a., not applicable. (D) The distribution of cells having the mat2/3 region localized into zone I, zone II or zone III plotted as pie charts. Black, zone I; light grey, zone II; dark grey, zone III.
Table 1. Distances between the mating-type region and the SPB

| Strain          | n  | Mean (μm) | Median (μm) | s.d. | Minimum value (μm) | Maximum value (μm) | Different from wild type (P<0.05) |
|-----------------|----|-----------|-------------|------|-------------------|-------------------|---------------------------------|
| w.t. ade6-M210  | 61 | 0.31      | 0.29        | 0.088| 0.15              | 0.82              | n.a.                            |
| w.t. ade6-DN/N  | 64 | 0.29      | 0.27        | 0.106| 0.14              | 0.61              | n.a.                            |
| IR-LΔ/IR-RΔ     | 69 | 0.48      | 0.40        | 0.326| 0.17              | 1.68              | no                              |
| hKΔ repressed   | 71 | 0.38      | 0.30        | 0.257| 0.13              | 1.62              | no                              |
| hKΔ derepressed | 62 | 0.65      | 0.55        | 0.371| 0.16              | 1.68              | yes                             |
| swi6Δ           | 64 | 0.61      | 0.46        | 0.376| 0.22              | 1.48              | yes                             |
| dcr1Δ           | 62 | 0.34      | 0.31        | 0.179| 0.25              | 1.03              | yes                             |
| cut3::lacO      | 57 | 0.47      | 0.35        | 0.327| 0.14              | 1.66              | yes                             |

The mean distance in μm between the mating-type region and the SPB is given together with the number of cells counted for each strain (n), the median, the standard deviation (s.d.) and the minimum and maximum values. The differences in median values between each mutant and the ade6-M210 wild type were tested statistically for significance (Mann-Whitney rank sum test, P<0.05), no significant difference; yes, a significant difference; n.a., not applicable.

As discussed above, the two hKΔ strains, which contain an 8 kb deletion of the K-region between the mat2-P and the mat3-M cassettes, have two different epigenetic states of the mat2/3 region, one with transcriptional repression of the region and the other with a derepressed region (Fig. 2) (Grewal and Klar, 1996; Thon and Friis, 1997). The observed distance between the mat2/3 region and the SPB in the repressed hKΔ was not different from the wild-type strain (Table I and Fig. 3A). However, in the repressed hKΔ isolate the observed longer distance between the NM and the mat2/3 region, with a median of 0.52 μm, was statistically different from the wild-type strain (P<0.05), as shown in Fig. 3C. In the derepressed hKΔ isolate, however, the mating-type region was delocalized from the SPB and separated from it with a median distance of 0.55 μm (Table I and Fig. 3A), but remained close to the nuclear periphery with a median distance of 0.38 μm (Fig. 3C). We conclude that the cis-acting element in the K-region, and the inverted repeats IR-L and IR-R surrounding this region, influence the subnuclear localization of the mating-type region.

Localization to the nuclear periphery is completely lost in the trans-acting mutant clr4Δ

A strain lacking the methyltransferase Clr4 and a strain lacking the chromodomain protein Swi6 were next investigated. In the clr4Δ mutant strain, the mating-type region was completely delocalized from the SPB (Table I and Fig. 3A). Thus, in this mutant strain the mating-type region did not remain in the proximity of the nuclear periphery, but was found in the nuclear interior, zone III, in half of the measured cells, as displayed in the pie charts in Fig. 3D. The median distance between the NM and the mat2/3 region was increased to 0.55 μm (Fig. 3C). In the swi6Δ mutant strain the mat2/3 region was also delocalized from both the SPB and the NM but not to the same extent as in the clr4Δ mutant strain (Table I and Fig. 3). These results suggest that formation of heterochromatin through the Clr4/Swi6p pathway was important for positioning the mating-type region at the nuclear periphery. To confirm the severe effects on the localization of the mating-type region that we observed in the clr4Δ mutant strain, we analysed the position of an euchromatic locus located in the middle of a chromosome arm, cut3::lacO, both in relation to the SPB and to the NM (Nabeshima et al., 2001). The cut3::lacO displayed characteristics of a random distribution in the cell nucleus, with a broad range in distances to the SPB, and was frequently detected in the nuclear interior (Table I and Fig. 3). This distribution was very similar to the localization characteristics of the mating-type region in the clr4Δ mutant strain, supporting the conclusion that this region has a random localization in a strain lacking the histone methyltransferase Clr4.

The RNAi machinery was dispensable for the localization of the mating-type region in the proximity of the SPB

Because the Dicer protein involved in the formation of siRNA in S. pombe has been shown to be necessary for the clustering of telomeres (Hall et al., 2003), we decided to investigate whether the localization of the mating-type region in the vicinity of the SPB [and thus also in the vicinity of the pericentromeric heterochromatin (Kniola et al., 2001)] was affected in a dcr1Δ mutant strain. However, this was not the case; the mating-type region showed the same localization in relation to both the SPB and the NM in a dcr1Δ strain as compared with the wild type (Table I and Fig. 3). We conclude that the RNAi machinery is not needed for correct localization of the mat2/3 region to the vicinity of the SPB. This is consistent with the finding that telomeres, although no longer clustered, still remain associated with the NM in a dcr1Δ strain (Hall et al., 2003). The fact that the mat2/3 region remains close to the SPB in the dcr1Δ strain, and therefore presumably also close to the pericentromeric heterochromatin, does, however, suggest that clustering of heterochromatin in this case can occur in the absence of the RNAi machinery, unlike the case with telomeres.

Discussion

At interphase, we found that the mating-type region localized in the proximity of the SPB, which is where the pericentromeric heterochromatin is found (this study) (Kniola et al., 2001). This observation is consistent with other reports of sticky heterochromatin localizing together within the nucleus. For example, the telomeres of both budding and fission yeast cluster together in discrete foci (Funabiki et al., 1993; Gotta et al., 1996). Moreover, in the fruit fly Drosophila melanogaster, the bwD allele has a large block of heterochromatin inserted into the bw locus. In heterozygote bw+/bwD flies, the two bw alleles are associated with
Cis-acting elements contribute to the chromatin organization

DNA sequences within the mating-type region including the boundary elements IR-L and IR-R and the K-region are important for subnuclear positioning of the mating-type region. Thus, in a strain in which the boundary elements IR-L and IR-R are deleted, the mat2/3 region was detached from its position next to the SPB, although it remained close to the nuclear periphery (Table 1 and Fig. 3). Interestingly, the derepressed h^KΔ strain showed a similar phenotype as the IR-LΔ/IR-RΔ strain, with the mat2/3 region being detached from the SPB but remaining constrained in its localization, staying at the proximity of the nuclear periphery. Finally, in the repressed h^KΔ strain, the mating-type region also had a slightly different localization as compared with the wild-type situation. In this strain the mat2/3 region remained in the vicinity of the SPB, although at a position further into the nuclear interior away from the NM. Taken together, our results suggest that all three cis-acting elements influence the subnuclear localization of the mating-type region.

The Clr4 histone methyltransferase is essential for organizing the mating-type chromatin

Results presented here indicate that the Clr4 methyltransferase is needed for correct localization of the mat2/3 region. The Clr4 methyltransferase specifically methylates histone H3K9, thereby creating a binding site for the heterochromatin protein Swi6. We found that in strains in which either the clr4+ or the swi6+ gene had been deleted, the mat2/3 region no longer resided in the proximity of the SPB. In the clr4Δ mutant strain the localization of the mat2/3 region to the SPB was completely lost, and its localization resembled that of the euchromatic cut3::lacO locus in a wild-type background (Table 1 and Fig. 3A). In the strain with a deletion of the swi6+ gene we also saw a delocalization of the mating-type region, but it was not as severely mislocalized as in the clr4Δ strain. One possible explanation for this observation is that other chromatin proteins, like the chromodomain protein Chp1, are still bound to the mating-type region in the swi6Δ strain but not in the clr4Δ strain (Noma et al., 2004; Sadaie et al., 2004). Moreover, because the Dicer protein has been shown to be important for the clustering of subtelomeric heterochromatin, we investigated whether the dcr1Δ mutation influenced the positioning of the mating-type region and found that this was not the case. Therefore, we conclude that theClr4 methyltransferase was crucial for a correct subnuclear localization of the mating-type region.

Correlation between transcriptional derepression and changes in subnuclear localization

In all the strains in which the ade6+ reporter gene was derepressed (the IR-LΔ/IR-RΔ, derepressed h^KΔ, clr4Δ and swi6Δ strains) we found that the mating-type region was either completely or partially delocalized (Figs 2, 3). From our results it is not possible to tell if the changes in subnuclear localization are caused by the increase in transcriptional activity, or if the increase in transcriptional activity occurs because of an initial delocalization away from a heterochromatic environment. By contrast, in the two strains in which the ade6+ reporter gene was still repressed (the repressed h^KΔ isolate and the dcr1Δ strain) the mating-type region remained in the proximity of the SPB. It is clear that there is a strong correlation between transcriptional derepression and subnuclear delocalization.

Two mutually exclusive models of what determines the localization of the mating-type region

One way of interpreting our data is that the amount of heterochromatin in the mating-type region determines its proximity to the SPB. In a clr4Δ strain, other studies show that no chromodomain proteins remain bound to the mat2/3 interval, and we found this region to be completely delocalized in the same mutant background (this study) (Sadaie et al., 2004). In a swi6Δ strain in which the chromodomain protein Chp1 protein still binds the mating-type region, it was a bit more constrained in its localization as compared with the clr4Δ mutant strain (this study) (Sadaie et al., 2004). If this model is correct, the effects on nuclear organization detected in the IR-LΔ/IR-RΔ strain do not depend on the suggested function of these elements as anchors to the NM through TFIIIC (Noma et al., 2006). Instead, in this mutant strain the effects on localization would be a consequence of the reduced amount of heterochromatin formed in the mat2/3 interval. The chromatin immunoprecipitation (ChIP) studies of the mating-type region in a strain without the boundary elements were performed using a strain containing three copies of the swi6+ gene. In this strain background the amount of heterochromatin in the mat2/3 region remains unchanged when the boundary elements are deleted (Noma et al., 2001). The effect of the deletions of the boundary elements in this strain background is that the heterochromatin spreads out to the surrounding euchromatic area. In our strain, with only the endogenous swi6+ gene, it is the euchromatin boundary that spreads into the normally transcriptionally silent region, thereby allowing for expression of the ade6+ reporter gene. However, some heterochromatin must remain in the mat2/3 interval because in a strain with deleted boundary elements there is still transcriptional repression of reporter genes inserted throughout the mat2/3 interval in a strain with one copy of the swi6+ gene (Thon et al., 2002). The remaining heterochromatin would explain why the mat2/3 region is not completely delocalized in this mutant strain.

The effects on subnuclear positioning of the mating-type region in the h^KΔ strains could also be explained by reduced amounts of heterochromatin assembled in the mat2/3 region. Previous studies show that in the derepressed KΔ::ura4+ strain most of the Swi6 protein is dissociated from the ura4+ reporter gene that was used to replace the deleted K-region, although a slight enrichment (1.8-3.2-fold) of Swi6 is still detected in this region in a Swi6 ChIP (Noma et al., 2004). The heterochromatin that remains in the derepressed h^KΔ may explain why it is still localized in the proximity of the nuclear periphery.

An alternative model that could explain our results is that the inverted repeats IR-L and IR-R anchor the mating-type region in the NM in a Clr4-dependent manner. In the IR-LΔ/IR-RΔ strain the mating-type region was no longer attached to the SPB, although remained next to the nuclear periphery. This result indicates that additional factors, besides the inverted repeats, contribute to the restricted localization of the mating-
type region. Clr4 could act by forming the heterochromatin that stabilizes the interaction between the boundary elements and the NM in the vicinity of the SPB. In the clr4Δ strain, in which the chromodomain proteins Swi6 and Chp1 no longer bind to the mating-type region (Noma et al., 2004), the stabilization is lost and the boundary anchors on their own are not strong enough to maintain this region at the NM. This deficiency results in a completely delocalized mat2/3 region, as observed in the clr4Δ strain. Clr4 could have two redundant functions; one to set up the heterochromatin structure in the mat2/3 interval, and the other acting directly through the cis-acting sequences in targeting the inverted repeats to the NM. Finally, we conclude that in both of these models Clr4 has a central role in the organization of the mating-type chromatin in the cell nucleus of S. pombe.

Materials and Methods

Strains and media

All strains used in this study are listed in Table S1 in the supplementary material, and all primer sequences are found in Table S2 in the supplementary material.

The mat3-M(EcoRV):ade6+ segment from plasmid pPB17 was cut out using the restriction enzymes PstI and SalI (Thon et al., 1999) and used to transform strains PJ247 (RP415/MATa/R produce Pst) and PJ249 (mΔ) by electroporation (Prentice, 1992), thereby replacing the mat3-M(EcoRV)::ura4+. The replacement was verified both by growth on media lacking adenine and failure to grow on media lacking uracil, and by PCR using the forward primer A45 and reverse primer A43. In this and all other PCR reactions described below the Expand High Fidelity PCR system (Roche) was used. The resulting strains, PJ286 and PJ289, were then used for further crosses creating strains PJ415 and PJ408. Two hem positive isolates, one red and one white colony, representing a repressed and a derepressed mat2/3 region, respectively, were obtained by streaking out strain PJ408 for single colonies on rich media with low amounts of adenine (YE). These two isolates were then used for further crosses. PompS (RP415) was excised-c terminally with the flavochromes DsRed (Bahrler et al., 1998). The DsRed::kanMX cassette was amplified from the plasmid PTY24 (Yeast Resource Centre, University of Washington, USA) by PCR using the forward primer A25 and the reverse primer A26, and the PCR product was integrated at the endogenous pompS locus in strain PJ236. Integration was verified by growth on YE plates containing 200 μg/ml G418, and by PCR using the forward primer A32 and the reverse primer A10. The kanamycin resistance marker was then replaced by a hygromycin B resistance marker amplified from the plasmid P30438 using primers M4X6/4casAUP and M4X6/4casADOWN (Hengte et al., 2005). The replacement was verified by growth on YE containing 200 μg/ml hygromycin B and by failure to grow on YE containing G418, followed by PCR using the forward primer A32 and the reverse primer B14. Standard genetic techniques were used for the crossing of strains. Media were prepared as described previously (Thon et al., 1999).

Microscopy

Cells were grown at 30°C until mid-log-phase in synthetic media (PMG) containing filter-sterilized glucose, then concentrated by centrifugation and mounted on coverslips. The cells were immobilized by mixing equal amounts of cell suspension and 1 mg/ml lectin (Sigma L1395). All images were captured using a Zeiss LSM510 META confocal microscope with a Plan-Apochromat 63× oil objective lens (NA=1.4) in the multi-track mode using the 16-line average plane scan setting at 1999).

RNA preparation and RT-PCR

Cells were grown to mid-log-phase in synthetic medium (PMG) at 30°C. RNA was extracted using the RNaseasy® Mini kit (Qiagen) according to the Yeast Enzymatic Lysis protocol with the following modifications. Spheroplasts were generated by incubating the yeast cells at 37°C in Buffer Y1 containing 0.1 mg/ml of Zymolyase 100T (Nordic BioSite) with gentle shaking for 60 minutes. Before RT-PCR, all RNA samples were DNaseI treated (RiboPure® Yeast Kit, Ambion). The mat3-M(EcoRV):ade6+ reporter gene and the truncated endogenous ade6-DN/N allele were detected with primers B11 and B12 using the Qiagen OneStep RT-PCR® kit (Djupedal et al., 2005). Control reactions with no RT were run for all RNA samples at the same time. Using the BioRad Gel Doc Volume measure tool of the Quantity One software (Version 4.5.0), the expression from the mat3-M::ade6+ reporter in each strain was measured, after the deduction of the background noise, and calculated as the fraction of the expression from the endogenous ade6-DN/N locus in the same strain.

We thank Geneviève Thon and Ayumu Yamamoto for sending us strains, Iain Hagan for advice on GFP microscopy and Jakub Orzechowski Westholm for advising us concerning statistical analysis. Göran Magnusson and Hans Ronne are thanked for comments on the manuscript. This work was supported by grants 2002-4948 and 2004-3286 from the Swedish Research Council. F.H. is a postdoctoral fellow supported by grant 2006-5437 from the Swedish Research Council.

References

Ahlbäck, R. C., Nimmo, E. R., Ekwall, K., Javerzat, J. P. and Cranston, G. (1995). Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. Genes Dev. 9, 218-233.

Andrusi, E. D., Neiman, A. M., Zappulla, D. C. and Sternberg, R. (1998). Perinuclear localization of chromatin facilitates transcriptional silencing. Nature 394, 592-595.

Appelgren, H., Kniola, B. and Ekwall, K. (2003). Distinct centromere domain structures with separate functions demonstrated in live fission yeast cells. J. Cell Sci. 116, 4035-4042.

Archangiolli, B. and Thon, G. (2004). Mating-type cassettes: structure, switching and silencing. In The Molecular Biology of Schizosaccharomyces pombe (gene: Genetics, Genomics and Beyond (ed. E. Eggle), pp. 129-148. Berlin: Springer.

Bahrler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., 3rd, Steever, A. B., Wach, A., Philippsen, F. and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast 14, 943-951.

Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C. and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410, 120-124.

Bjerling, P. and Ekwall, K. (2002). Centromere domain organization and histone modifications. Braz, J. Med. Biol. Res. 35, 499-507.

Bjerling, P., Silverstein, R. A., Thon, G., Caudy, A., Grewal, S. and Ekwall, K. (2002). Functional divergence between histone deacetylases in fission yeast by distinct cellular localization and in vivo specificity. Mol. Cell. Biol. 22, 2170-2181.

Branco, M. R. and Pombo, A. (2006). Intermingling of chromosome territories in interphase suggests role in translocations and transcription-dependent associations. PLoS Biol. 4, e138.

Brown, K. E., Guest, S. S., Smale, S. T., Hahn, K., Merkenschlager, M. and Fisher, A. G. (1997). Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. J. Cell Biol. 139, 1119-1131.

Crotti, J. A., Bridge, J. M., Boyle, S., Perry, P., Teague, P. and Bickmore, W. A. (1999). Differences in the localization and morphology of chromosomes in the human nucleus. J. Cell Biol. 145, 1119-1131.

Crnogorac, K. and Henikoff, S. (1996). Genetic modification of heterochromatic association and nuclear organization in Drosophila. Nature 381, 529-531.

Djupedal, I., Portooso, M., Spahr, H., Bonilla, C., Gustafsson, C. M., Allshire, R. C. and Ekwall, K. (2005). RNA Pol II subunit Rbp7 promotes centromeric transcription and RNAs-directed chromatin silencing. Genes Dev. 19, 2301-2306.

Ekwall, K., Javerzat, J. P., Lorentz, A., Schmidt, H., Cranston, G. and Allshire, R. (1995). The chromodomain protein Swi6: a key component at fission yeast centromeres. Science 260, 1429-1431.

Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Aguirre, H., Kniola, and Ekwall, K. (2002). Centromere domain organization and histone modifications. Braz, J. Med. Biol. Res. 35, 499-507.

Göran Magnusson and Hans Ronne are thanked for comments on the manuscript. This work was supported by grants 2002-4948 and 2004-3286 from the Swedish Research Council. F.H. is a postdoctoral fellow supported by grant 2006-5437 from the Swedish Research Council.
Nakayama, T. and Oshimura, M. (2004). Dicer is essential for formation of the heterochromatin structure in vertebrate cells. Nat. Cell Biol. 6, 784-791.

Funabiki, H., Hagan, I., Uzawa, S. and Yanagida, M. (1993). Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. J. Cell Biol. 121, 961-970.

Gotta, M., Laroche, T., Formenton, A., Maillet, L., Scherthan, H. and Gasser, S. M. (1996). The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. J. Cell Biol. 134, 1349-1363.

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

Grewal, S. I. and Klar, A. J. (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.

Hall, I. M., Shankaranarayana, G. D., Noma, K., Ayoub, N., Cohen, A. and Grewal, S. I. (2002). Establishment and maintenance of a heterochromatin domain. Science 297, 2232-2237.

Hall, I. M., Noma, K. and Grewal, S. I. (2003). RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. Proc. Natl. Acad. Sci. USA 100, 193-198.

Hediger, F., Neumann, F. R., Van Houwe, G., Dubrana, K. and Gasser, S. M. (2003). Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. Curr. Biol. 15, 1808-1819.

Kniola, R., O’Toole, E., McIntosh, J. R., Mellone, B., Allshire, R., Mengarelli, S., Hulteny, K. and Ekwall, K. (2001). The domain structure of centromeres is conserved from fission yeast to humans. Mol. Biol. Cell 12, 2767-2775.

Kosak, S. T., Skok, J. A., Medina, K. L., Riblet, R., Le Beau, M. M., Fisher, A. G. and Singh, H. (2002). Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. Science 296, 158-162.

Litt, M. D., Simpson, M., Gaszner, M., Allis, C. D. and Felsenfeld, G. (2001). Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. Science 293, 2453-2455.

Nabeshima, K., Kakihara, Y., Hiraoka, Y. and Nojima, H. (2001). A novel meiosis-specific protein of fission yeast. Meu13p, promotes homologous pairing independently of homologous recombination. EMBO J. 20, 3871-3881.

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

Noma, K., Sugiyama, T., Ham, H., Verdel, A., Zofall, M., Jia, S., Moazed, D. and Grewal, S. I. (2004). RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nat. Genet. 36, 1174-1180.

Noma, K., Cam, H. P., Maria, R. J. and Grewal, S. I. (2006). A role for TFIIC transcription factor complex in genome organization. Cell 125, 859-872.

Osborne, C. S., Chakalova, L., Brown, K. E., Carter, D., Horton, A., Debrand, E., Goyenechea, B., Mitchell, J. A., Lopes, S., Reik, W. et al. (2004). Active genes dynamically colocailize to shared sites of ongoing transcription. Nat. Genet. 36, 1065-1071.

Perrod, S. and Gasser, S. M. (2003). Long-range silencing and position effects at telomeres and centromeres: parallels and differences. Cell. Mol. Life Sci. 60, 2303-2318.

Prentice, H. L. (1992). High efficiency transformation of Schizosaccharomyces pombe by electroporation. Nucleic Acids Res. 20, 621.

Rea, S., Eisenhaber, F., O’Carroll, D., Struhl, R. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtl, K., Ponting, C. P., Allis, C. D. et al. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406, 593-599.

Rusche, L. N., Kirschmaier, A. L. and Rine, J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. 72, 481-516.

Sadaie, M., Iida, T., Urano, T. and Nakayama, J. (2004). A chromodomain protein, Chp1, is required for the establishment of heterochromatin in fission yeast. EMBO J. 23, 3825-3835.

Shimada, T., Yamashita, A. and Yamamoto, M. (2003). The fission yeast meiotic regulator Mei2p forms a dot structure in the horse-tail nucleus in association with the smc2 locus on chromosome II. Mol. Biol. Cell 14, 2461-2469.

Sproul, D., Gilbert, N. and Bickmore, W. A. (2005). The role of chromatin structure in regulating the expression of clustered genes. Nat. Rev. Genet. 6, 775-781.

Thon, G. and Fris, T. (1997). Epigenetic inheritance of transcriptional silencing and switching competence in fission yeast. Genetics 145, 685-696.

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

Thon, G., Bjerling, P., Bunner, C. M. and Verhein-Hansen, J. (2002). Expression-state boundaries in the mating-type region of fission yeast. Genetics 161, 611-622.

Vopel, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I. and Martienssen, R. A. (2002). Regulation of heterochromatin silencing and histone H3 lysine-9 methylation by RNAs. Science 297, 1833-1837.