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Distinct protein interaction domains and protein spreading in a complex centromere

Janet F. Partridge, Britta Borgstrøm,¹ and Robin C. Allshire²

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Fission yeast (Schizosaccharomyces pombe) centromeres are composed of large (40–100 kb) inverted repeats that display heterochromatic features, thus providing a good model for higher eukaryotic centromeres. The association of three proteins that mediate region-specific silencing across centromere 1 has been mapped by quantitative chromatin immunoprecipitation. Swi6 and Chp1 are confined to the flanking outer repeats and Swi6 can spread across at least 3 kb of extraneous chromatin in cen1. In contrast, Mis6 coats the inner repeats and central core. tRNA genes demarcate this transition zone. These analyses clearly define two distinct domains within this complex centromere which interact with different proteins.

[Key Words: Centromere; spreading; chromatin immunoprecipitation; chromodomain; silencing; Schizosaccharomyces pombe]

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Accurate chromosome segregation is dependent on the activity of the centromere, a highly specialized region of chromatin that assembles kinetochore proteins to mediate spindle attachment. Mammalian and Drosophila centromeres are large, cytologically distinct structures, which are composed of repetitive sequences, and show features of heterochromatin [Brown et al. 1994; Karpen 1994, Murphy and Karpen 1995; for review, see Weiler and Wakimoto 1995; Lee et al. 1997; Sun et al. 1997]. Little is known about exact sequence requirements for centromere function in these large regional centromeres. In sharp contrast, the centromeres of the budding yeast are very small and well defined, both in terms of their DNA sequence requirements and the localization of centromeric proteins (Espelin et al. 1997; Meluh and Koshland 1997; Meluh et al. 1998 and references therein). However, fission yeast centromeres are more evocative of higher eukaryotic centromeres than those of budding yeast. They are composed of large inverted repeats with a central core of more unique sequence, surrounded by inner [imr/B] and outer [otr/K+L] repeats (Clarke et al. 1986; Nakaseko et al. 1986, 1987; Fishel et al. 1988). Marker genes inserted within the centromere are transcriptionally repressed (Allshire et al. 1994, 1995). This silent centromeric chromatin is underacetylated, and perturbation of this state by transient treatment with a histone deacetylase inhibitor leads to the epigenetic inheritance of defective centromere function [Ekwall et al. 1997]. Histone deacetylase inhibition also causes delocalization of Swi6, a chromodomain protein that normally localizes to silent chromatin at mating type and telomeres, in addition to centromeres [Ekwall et al. 1995, 1997]. Proper localization of Swi6 is dependent on Clr4 and Rik1, and mutation of any of these genes leads to defective centromeric silencing and impaired centromere function [Allshire et al. 1995; Ekwall et al. 1995, 1996]. Clr4 is the fission yeast homolog of Su(var)3-9, a protein with both a chromo and SET domain, involved in transcriptional regulation in Drosophila [Tschiersch et al. 1994; Ivanova et al. 1998]. The human homolog (SU(V39H1) localizes at centromeres in metaphase-arrested cells, and coimmunoprecipitates with M31, an HP1 family member and homolog of fission yeast Swi6 (Aagaard et al. 1999).

Recently, two essential centromere-specific proteins [Mis6 and Mis12] have been described [Saitoh et al. 1997; Goshima et al. 1999]. These proteins are required for determining correct metaphase spindle length and orienting sister chromatids for efficient segregation. Here, a quantitative chromatin immunoprecipitation [ChIP] assay is used to dissect the protein–DNA architecture of centromere 1 (cen1). The relative distribution of three proteins, Swi6, Mis6, and Chp1 across this centromere is presented. In addition, we demonstrate that both Swi6 and Mis6 are capable of spreading over marker genes inserted within the centromere, and show that Swi6 can coat and mediate silencing of large noncentromeric DNA inserts. Thus, proteins important for full centro-

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mere function can spread, and this lends support to current models for the plasticity of sites of centromere formation.

Results

Mis6 and Chp1 silence distinct centromeric domains

The effect on centromeric silencing of a mutation in the gene encoding Mis6 (Takahashi et al. 1994) was examined. Quantitative RT–PCR (Fig. 1) was performed on cDNA generated from strains with ura4+ located at three sites within cen1 (U) and an expressed ura4–DS/E minigene located at the normal ura4 locus (L). A fully expressed random integrant of ura4+, R.Int::ura4+ served as a control. The PCR assay uses one primer pair to amplify different products from full-length ura4+ (U; 694 bp) and the ura4–DS/E minigene (L; 426 bp). At the restrictive temperature, 36°C, mis6-302 significantly derepressed the ura4+ marker gene when positioned within the central core (site 9, Fig.1A) but not when inserted in the flanking outer and inner repeats (sites 6 and 13). Even at 25°C, mis6-302 alleviated central core silencing, which correlates with elevated rates of chromosome loss (Takahashi et al. 1994). mis6-302 is the first mutation identified to strongly alleviate silencing specifically within the central core of the centromere.

Another chromodomain protein, Chp1, has been identified by sequence homology (Doe et al. 1998; B. Borgstrom and R. Allshire, unpubl.). Like clr4Δ, rik1Δ, and swi6Δ, strains lacking chp1+ show little alleviation of central core silencing [site 9, Fig.1B], but strong derepression of ura4+ within the outer and inner repeats of cen1 [sites 6 and 13, Fig. 1B]. This and previous analyses suggest that Mis6 may act and interact exclusively through the central core, whereas Chp1, Clr4, Rik1, and Swi6 might be confined to the flanking repeats.

Mis6 and Swi6 associate with distinct regions of cen1

To test whether the region-specific silencing effects reflect differences in the distribution of proteins, ChIP was used. ChIP has been previously used to examine proteins associated with centromeres and telomeres in budding yeast and fission yeast (Hecht et al. 1996; Ekwall et al. 1997; Meluh and Koshland 1997; Saitoh et al. 1997; Strahl-Bolsinger et al. 1997, Meluh et al. 1998; Goshima et al. 1999). However, determining the relative distribution of Chp1, Mis6, and Swi6 proteins across an entire fission yeast centromere is hampered by the fact that all three centromeres share flanking DNA repeats (Clarke et al. 1986; Nakaseko et al. 1986, 1987; Fishel et al. 1988). Thus, little information on protein localization over the outer repeats can be generated from ChIP experiments, as probes that detect specific centromere outer repeats cannot be designed. To overcome this difficulty, strains with the ura4+ marker located at 13 different positions within, or adjacent to cen1 were utilized (Allshire et al. 1995).

Figure 1. Chp1 and Mis6 silence distinct centromeric domains. (A) mis6-302 specifically alleviates central core silencing, but not silencing of the outer repeats. [B] Chp1, like Clr4, Rik1 and Swi6 is required for silencing of the outer repeats, but not the central core. For both A and B, competitive radioactive PCR was performed on cDNA generated by RT–PCR from strains with ura4+ (U) inserted within the central core [site 9], the inner and outer repeats of centromere 1 [sites 6 and 13] or in euchromatin [R.Int::ura4+] and a fully expressed ura4–DS/E minigene [L] at the ura4 locus. Separated PCR products were quantified. Levels of ura4 (U) were normalized to ura4–DS/E (L) in the mutant strains and expressed relative to values obtained for the wild-type background for each insertion site. In A, mis6-302 and wild-type strains were grown at 25°C, or shifted to the nonpermissive temperature for 4 hr prior to RNA extraction.
Each *ura4* insertion site provides a specific tag for different *cen1* regions and all strains also carry the *ura4*-DS/E minigene at the normal *ura4* locus. Quantitative competitive PCR is performed on immunoprecipitated chromatin to assess enrichment of each centromeric *ura4* relative to the *ura4* locus (Fig. 2A). A strain with the *ura4* gene inserted at a fully expressed site (*RIn-ura4*) serves as a control. To test this assay, we examined the distribution of Mis6 tagged with 3xHA epitopes across *cen1* as Mis6–3xHA is known to immunoprecipitate central core and imr, but not otr, chromatin [Saitoh et al. 1997]. Mis6–3xHA efficiently immunoprecipitated *ura4* at the central core (site 9) and at imr (sites 8 and 10), but little association of Mis6–3xHA was detected at other insertion sites (Fig. 2B,C). Quantification of three independent experiments demonstrates that central core and inner repeat *ura4* insertions are enriched in Mis6–3xHA ChIPs. These data correlate well with the pattern of mis6*+* dependent silencing across *cen1* (Fig. 1A).

Next, the distribution of Swi6 across *cen1* was examined (Fig. 2B,C). Swi6 antibodies (Ekwall et al. 1995) were used to immunoprecipitate chromatin extracts prepared from *cen1–ura4* tagged strains. Analysis of the immunoprecipitated chromatin showed strong association of Swi6 with *ura4* tags in the outer repeats (sites 3–6, 12–13). Little or no association of Swi6 was observed with the inner repeat (sites 7, 8, 10, 11) or central core domains (site 9). The symmetry of both *cen1* and the Swi6 distribution profile underscores the reproducibility of this procedure.

Swi6 immunolocalization requires functional Clr4 and Rik1 proteins [Ekwall et al. 1996]. Figure 2D shows that Swi6 immunoprecipitation of *ura4* at site 13 is also abolished in cells lacking functional Clr4 and Rik1. This correlates with the loss of silencing seen at this site in *clr4Δ* and *rik1Δ* mutants (Fig. 1B; Allshire et al. 1995), and demonstrates the specificity of anti-Swi6 ChIP. These data reveal two distinct domains of silencing within *cen1*, with Swi6 and Mis6 distributed in a reciprocal fashion across the centromere. No change in Mis6 association across *cen1* was seen in *clr4Δ, rik1Δ, or swi6Δ* mutants (data not shown), suggesting that Clr4, Rik1, or Swi6 do not act to restrict Mis6 to the central domain.

**Defining the borders of Swi6 and Mis6 cen1 association**

Our analyses indicate that Swi6 does not associate with regions internal to sites 7 [LHS] and 11 [RHS] located between the tRNA^Ala^ and tRNA^Glu^ genes. In contrast, Mis6 appears to be confined to these inner repeats and central core regions. Using the available *cen1–ura4* tags, we cannot clearly define the limits of Mis6 association with *cen1*. To map the boundaries of protein association, primers were designed to specifically amplify sequence adjacent to both the left and right copies of the tRNA^Ala^ and tRNA^Glu^ genes and to quantify enrichment of these sequences in Mis6 and Swi6 chromatin immunoprecipitates (Fig. 3). One example of these immunoprecipitations is shown (Fig. 3A), with the average results for several experiments presented in Figure 3B. Mis6 associates threefold more with sequence just interior to the tRNAAs [Int] than sequence exterior to the tRNAs [Ext]. In contrast, Swi6 immunoprecipitates gave a threefold enrichment of product Ext over product Int. These figures are an underestimate because the chromatin is only sheared to 500–1000 bp and the two probes lie just 700 bp apart. Because both Swi6 and Mis6 are capable of spreading [see below], the observation that these proteins show differential centromere association within a 700-bp region suggests that the region encompassing the tRNA^Ala^ and tRNA^Glu^ genes defines a transition zone between distinct centromere domains.

**Chp1 associates with flanking centromere repeats**

Because deletion of *chp1* derepresses centromeric silencing [Fig. 1B], attempts were made to use ChIP to examine the distribution of functional-tagged Chp1 (*Chp1–6xMyc, B. Borgström and R. Allshire, unpubl.) across *cen1*. However, no enrichment of any *cen1–ura4* sites was detected in Chp1–6xmyc immunoprecipitates [data not shown]. The primers used for PCR to detect *ura4* in these ChIP experiments were ~570 and 500 bp from the ends of the *ura4* tags and the released chromatin–DNA was sonicated to 500–1000 bp prior to immunoprecipitation. Thus, detection of *cen1–ura4* in immunoprecipitates requires that proteins of interest contact the main body of the *ura4* marker. Swi6 and Mis6 are clearly able to encroach into centromeric *ura4* genes, but our failure to detect association of Chp1–6xMyc might be because Chp1 cannot spread from neighboring centromeric chromatin. We therefore tested for direct interaction of Chp1 with centromeric sequences at two positions within *cen1* that can be specifically amplified by PCR (Fig. 4A). Chp1–6xMyc, like Swi6 immunoprecipitates, are enriched for centromeric imr/otr repeat chromatin, but not the central core region, when compared with immunoprecipitation of the euchromatic control *lpb1* gene locus. This is in contrast to Mis6, which associates only with the central core, and not the centromeric repeat region in this assay.

Chp1 can clearly associate with centromeric chromatin, but cannot encroach on *ura4* genes inserted within the centromere. Therefore, an alternative PCR assay was devised to map interactions of Chp1–6xMyc across *cen1* (Fig. 4B). This assay examines Chp1–6xMyc association with centromeric sequences flanking each *cen1–ura4* tag. A semicompetitive PCR assay was set up with one primer anchored at the end of *ura4* (which also recognizes *ura4*-DS/E) and a primer homologous to centromeric sequences neighboring each centromeric *ura4* insertion site. The centromeric primer is different for each insertion site, and thus generates PCR products of various sizes. The *ura4* primer provides specificity for each *cen1* insertion site. Inclusion of an additional primer from sequence adjacent to the *ura4*-DS/E locus in all reactions yields the larger (550 bp) control product (see Fig. 4B). This assay was tested with anti-Swi6 immuno-
precipitates and the results [Fig. 4C] demonstrate specific association of Swi6 with the outer repeats and fully support those presented in Figure 2B. Our data suggests that there is no significant difference in the ability of Swi6 to associate with centromeric sequences compared with exogenous DNA inserted within the centromere (cf. Fig. 2B,C with Fig. 4C). In contrast, when Mis6–3xHA immunoprecipitates were tested, we saw up to
eightfold more enrichment for binding the flanking centromeric sequences than for binding centromeric ura4+ (data not shown). This indicates that there may be a gradient of Mis6 over these ura4+ inserts. This same PCR assay was then applied to Chp1–6xMyc immunoprecipitates and analyses of all centromeric sites revealed a similar distribution of Chp1–6xMyc and Swi6 across cen1 (Fig. 4C). Chp1 interacts strongly with flanking repeat regions but not with the central domain of cen1. Immunolocalization of Chp1–6xMyc, like Swi6, depends on functional Clr4 and Rik1, but is independent of Swi6 (B. Borgstrøm and R. Allshire, unpubl.). The ability of Chp1–6xMyc to immunoprecipitate cen1-flanking repeat chromatin is also lost in clr4Δ and rik1Δ strains but maintained in swi6Δ, validating this modified ChIP–PCR assay [Fig. 4D].

Thus, two chromodomain proteins, Swi6 and Chp1, associate with the outer repeats of cen1, and require Clr4 and Rik1 for localization. However, they differ in their behavior, because Swi6, but not Chp1, can spread across exogenous ura4+ chromatin assembled within the centromere. The absence of apparent Chp1 spreading could reflect the sensitivity of the method and less total Chp1 in the cell, rather than an absolute difference in the behavior of the proteins.

Swi6 can spread over large insertions of exogenous DNA

To further test this ability of Swi6 to spread, two strains were constructed. In one strain [1.3 kb], 1.3 and 1.7 kb of noncentromeric sequence were used to flank ura4+ at site 13 and in the other [3 kb], this flanking sequence was increased to 3 and 5 kb. The anti-Swi6 ChIP assay showed [Fig. 5] that equivalent levels of ura4+ at site 13 are associated with Swi6 irrespective of the length of the surrounding noncentromeric DNA. Thus, Swi6 can spread over at least 3 kb of additional noncentromeric sequence flanking the ura4+ gene inserted within cen1, and this spreading correlates with efficient silencing of the ura4+ gene [data not shown].

Discussion

Quantitative mapping by ChIP of three proteins across a large and complex fission yeast centromere has been performed. These analyses reveal the presence of discrete domains of protein interaction within cen1, with Mis6 restricted to the central core and inner repeats, and Swi6 and Chp1 associated with the outer repeats. Swi6 and Mis6 are both capable of spreading over silent ura4+ chromatin placed within cen1, and Swi6 can efficiently coat and silence >3 kb of exogenous DNA inserted within cen1.

Mutational analysis of cen1 on minichromosomes has revealed the importance of both a central core and some flanking repeat sequence for the formation of a mitotically stable centromere. This minimal centromere corresponds to sequences distal to site 5 and encompasses sequence close to site 12 [Baum et al. 1994]. Further truncation of this minimal centromere, causing removal of site 5 and some core proximal sequence, leads to loss of
Figure 4. Chp1 displays a similar cen1 association pattern as Swi6. 

[A] Multiplex PCR was performed to detect association of Chp1–6xMyc with centromeric chromatin. Primers were designed to two sites (imr/otr junction and a region of cnt1), which give amplification specifically from cen1 sequences, and to the euchromatic fbp1+ gene locus to act as a control for nonspecific association. Chp1–6xMyc and Swi6 immunoprecipitates both showed enrichment of the imr/otr product relative to fbp1 and showed no enrichment for the central core sequence (cnt1). In contrast, Mis6–3xHA immunoprecipitates showed enrichment for cnt1 and not for imr/otr. 

[B] Chp1–6xMyc interactions across cen1 were mapped by specific PCR from immunoprecipitates of strains with different cen1–ura4 insertions using various primers from cen1 and one primer anchored in the ura4+ gene. Enrichment of centromeric ura4+ by immunoprecipitation is reflected by increased intensity of the smaller PCR products, which vary in size from different strains, depending on the location of the centromeric primers, relative to the large PCR product of constant size that reflects association with the euchromatic ura4–DS/E locus. 

[C] Using this assay, Chp1–6xMyc and Swi6 associate with the flanking repeats but not the central core of cen1. Relative ip values are an average of 2 (Swi6), and 3 (Chp1–myc) experiments. 

[D] Chp1–6xMyc immunoprecipitation at site 13 is dependent on Clr4 and Rik1 but not Swi6.
mitotic function [Hahnenberger et al. 1991]. Our protein
mapping data suggests, therefore, that both inner repeats and central core sequences (capable of being bound by
Mis6), and sequences capable of high levels of Swi6/Chp1 association, are required for efficient mitotic seg-
regation of minimal centromere constructs.

The transition between the outer repeat and central core/inner repeat protein complexes is demarcated by a
region encompassing the inner repeat tRNA genes. tRNA genes are present within the inner repeat se-
dquences of all three centromeres [Takahashi et al. 1991], and may contribute to the definition of distinct protein
domains in natural centromeres. However, hybrid mini-
mal centromeres can be formed that lack these tRNA genes, but these minichromosomes require additional
spacer DNA to maintain spatial separation of the do-
mains for function [Baum et al. 1994]. Recently, a 1-kb
region including a tRNA gene was identified as having
insulator function at the silent HMR locus in Sacchar-
omyces cerevisiae [Donze et al. 1999]. An interesting
possibility is that the tRNAAla and tRNAGlu genes play a role in demarcating the two distinct protein interaction
domains in fission yeast cen1.

There have been several reports of proteins that are
able to spread from specific nucleation points to either activate or suppress gene expression of neighboring chro-
matin. Silencing at S. cerevisiae telomeres is mediated
by a complex including Sir3p, which is recruited to the
telomere by binding of Rap1p to telomeric repeats. The
Sir3p complex normally spreads from the terminal 300-
bp nucleation sites, and associates with and promotes
silencing of 3 kb of telomere adjacent sequence. Upon
overexpression, Sir3p has been detected 16-kb away.
This spreading is thought to be mediated by interaction of Sir3p with the underacylated tails of histones H3 and
H4 [Hecht et al. 1995, 1996; for review, see Grun-
stein 1998]. It has been reported recently that the dosage
compensation complex MSL, which binds the X chro-
mosome in Drosophila, shows high affinity binding to ~35
sites on the chromosome. These sites are thought to be
chromatin entry and nucleation sites, from which the
MSL complex spreads to coat the X chromosome, and
activate gene expression [Kelley et al. 1999]. Interest-
ingly, two of the MSL components [MSL3 and MOF] con-
tain chromodomains [Gorman et al. 1995; Hilfiker et al.
1997]. In addition, Polycomb, a Drosophila chromodo-
main protein can spread from its nucleation sequence,
the Polycomb response element, to alter gene expression
at a distance (for review, see Mochizule and Paro 1994).
Drawing on these models, we propose that at fission
yeast centromeres, Clr4 and Rik1 serve as nucleation
factors for the assembly of a protein complex containing
both Chp1 and Swi6 on the outer repeats of the centro-
mere. Swi6 can then spread from these nucleation
points, possibly via underacylated centromeric chro-
matin [Ekwall et al. 1997], simultaneously creating a
chromatin structure that blocks transcription of embed-
ded genes, and which mediates the assembly of a fully
functional kinetochore.

Current evidence suggests that the formation of cen-
 tromeres is subject to epigenetic regulation with a cer-
tain degree of plasticity in the sites selected for centro-
mere assembly. For example, active new centromeres
(neocentromeres) can be formed in the absence of any
recognizable centromeric DNA sequences in both hu-
mans and Drosophila. These noncentromeric sequences
may be activated by spreading of centromere character in
cis from nearby functional centromeres [Murphy and
Karpen 1998; Williams et al. 1998]. This centromeric
character may take the form of specialized chromatin, or
be induced by the spreading of specific centromere pro-
teins, which promote the formation of an active kineto-
chore. The clear spreading of fission yeast centromere
proteins reported here suggests that the plasticity in cen-
tromere formation may be mediated in part by spreading
of chromatin associated proteins.

Materials and methods

Strain construction

Strains were obtained by crossing centromeric ura4– insertions
1994], chp1Δhis3Δ [B. Borgstrom and R. Allshire, unpubl.],
Mis6–3xHA (Saitoh et al. 1997) and Chp1–6xMyc [B. Borgstrom
and R. Allshire, unpubl.] and verified by the presence of markers
and PCR analysis. To generate strains with additional sequence
flanking ura4– at centromeric insertion site 13, homologous re-
combination was used to replace a centromeric otr1R::ade6–
marker at site 13 [Allshire 1996]. Replacement was performed with a
BamHI–Xhol fragment of ade6 with ura4– inserted
within the HindIII site to generate the strain with 1.3 kb flanking
ura4. To insert 3 kb flanking ura4, the BamHI–Xhol frag-
ment of ade6– was inserted into pJK210 [Keeney and Boeke
1994], and NdeI linearized plasmid was integrated at
otr1R::ade6–.

RNA analysis

Yeast were grown in YES at 32°C to 5 × 106 cells/ml.
mis6–302 and controls were grown at 25°C to 5 × 108 cells/ml or shifted to
36°C for 4 hr prior to RNA extraction. cDNA was prepared by
oligo dT primed RT–PCR and competitive PCR of ura4– and
ura4–Ds/E was performed as described previously [Ekwall et al.
1997]. ura4– levels (U) were normalized to ura4–Ds/E (L) and
quantified relative to wild-type strains.

Formaldehyde cross-linked ChIP

Fission yeast were grown at 32°C in YES to 5 × 106 cells/ml,
shifted to 18°C for 2 hr prior to 30-min fixation with 3% form-
DNAs were assayed by PCR including \( ^{32}P \)dCTP, resolved

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