Mutations in the fission yeast silencing factors *clr4*+ and *rik1*+ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function

Karl Ekwall1,*, Elaine R. Nimmo1, Jean-Paul Javerza1,†, Britta Borgström1,2, Richard Egel2, Gwen Cranston1 and Robin Allshire1

1MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, Scotland, UK
2Department of Genetics, University of Copenhagen, Øster Farimagsgade 2A, DK-1353, Copenhagen K., Denmark

*Author for correspondence (e-mail: karl@hgu.mrc.ac.uk)
1Present address: CNRS Institut de Biochimie et Génétique Cellulaires, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France

**SUMMARY**

Transcriptional silencing is known to occur at centromeres, telomeres and the mating type region in the nucleus of fission yeast, *Schizosaccharomyces pombe*. Mating-type silencing factors have previously been shown also to affect transcriptional repression within centromeres and to some extent at telomeres. Mutations in the *clr4*+, *rik1*+ and *swi6*+ genes dramatically reduce silencing at certain centromeric regions and cause elevated chromosome loss rates. Recently, Swi6p was found to co-localise with the three silent chromosomal regions. Here the involvement of *clr4*+, *rik1*+ and *swi6*+ in centromere function is investigated in further detail. Fluorescence in situ hybridisation (FISH) was used to show that, as in *swi6* mutant cells, centromeres lag on late anaphase spindles in *clr4* and *rik1* mutant cells. This phenotype is consistent with a role for these three gene products in fission yeast centromere function. The Swi6 protein was found to be delocalised from all three silent chromosomal regions, and dispersed within the nucleus, in both *clr4* and *rik1* mutant cells. The phenotypic similarity observed in all three mutants is consistent with the products of both the *clr4*+ and *rik1*+ genes being required to recruit Swi6p to the centromere and other silent regions. Mutations in *clr4*, *rik1* and *swi6* also result in elevated sensitivity to reagents which destabilise microtubules and show a synergistic interaction with a mutation in the β-tubulin gene (*nda3*). These observations suggest that *clr4*+ and *rik1*+ must play a role in the assembly of Swi6p into a transcriptionally silent, inaccessible chromatin structure at fission yeast centromeres which is required to facilitate interactions with spindle microtubules and to ensure normal chromosome segregation.

Key words: *S. pombe*, Kinetochore, Telomere, Spindle, Heterochromatin

**INTRODUCTION**

The proper segregation of eukaryotic chromosomes in mitosis and meiosis is ensured by interactions of microtubules of the spindle and a complex of chromosomal factors forming the kinetochore. The kinetochore is defined as the nucleic acid and protein complex which resides at the centromere. The functions of the centromere and kinetochore are to capture microtubules emanating from opposite spindle poles, to maintain sister chromatid attachment until the metaphase/anaphase transition and during the first meiotic division, and to mediate chromosome movement towards the spindle poles during anaphase.

The specific centromeric DNA sequences which nucleate kinetochore assembly remain enigmatic in higher eukaryotes. However, in many organisms the centromere is located within repetitive sequences which are packaged into transcriptionally inert heterochromatin, for example, alphoid DNA repeats in human and minor satellite in mouse (as reviewed by Sunkel and Coelho, 1995). In *Drosophila melanogaster* a functional centromere has been shown to lie within heterochromatin and to consist of a 200 kb core, containing DNA sequences of a more complex nature, flanked by approximately 200 kb of simple repetitive DNA (Murphy and Karpen, 1995).

The DNA required for complete centromere function has also been defined in both the budding yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*). Although the genome sizes of the two yeasts are comparable *S. cerevisiae* centromeres occupy as little as 125 bp (Cottarel et al., 1989), whereas approximately 25 kb of centromeric DNA is required to provide full mitotic function in *S. pombe* (Baum et al., 1994). *S. pombe* centromeres consist of a central core composed of unique sequences surrounded by inner (*imr/lB*) and outer (*otr/K+L*) repetitive DNA elements (Takahashi et al., 1992; Steiner et al., 1993).

Insertion of the marker genes *ura4*+ and *ade6*+ within the centromeric sequences of fission yeast results in reversible repression of gene expression (Allshire et al., 1994). This phenomenon is similar to transcriptional repression, or silencing, of marker genes observed in the vicinity of telomeres in *S.
*Drosophila* su(var)2-5 gene encodes the chromo domain protein HP1 which localises to pericentric heterochromatin, and mutations in HP1 suppress repression of marker genes lying in close proximity to centromeric heterochromatin and cause defective chromosome segregation (Kellum and Alberts, 1995). The chromo- and newly defined shadow chromo domains seem to be required to target HP1 to heterochromatin and to recruit other heterochromatin factors allowing the formation of a large macro-molecular complex (Aasland and Stewart, 1995; Platero et al., 1995).

Various trans-acting factors are required for transcriptional repression at both the silent mating type loci (*HMR* and *HML*) and telomeres in *S. cerevisiae* (Aparicio et al., 1991). It has been demonstrated that the proteins Sir3p and Sir4p interact with each other and with a protein Rap1p, which binds telomeric repeats in addition to other chromosomal sites (Moretti et al., 1994; Cockell et al., 1995). Sir3p, Sir4p and Rap1p act together with amino-termini of histones H3 and H4 to form a heterochromatin-like complex which is normally located at the nuclear periphery and is inaccessible for transcription and to methylation by *dam* methylase (Hecht et al., 1992; Kyrion et al., 1993). Mutations in *SIR3* and *SIR4* affect the peripheral localisation of the Rap1p containing complex (Palladino et al., 1993). It is likely that this compact complex contributes to protection of chromosome ends from degradation since mutations in *SIR3* or *SIR4* cause a reduction in length of the terminal telomere repeat and mutations in *RAP1* destabilise the chromosomes (Palladino et al., 1993; Kyrion et al., 1993). In fission yeast six different genes, *clr1*+, *clr2*+, *clr3*+, *clr4*+, *rik1*+ and *swi6*+ have been identified by mutations which alleviate repression of the silent mating type loci *mat2* and *mat3* (Lorentz et al., 1992; Thon and Klar, 1992; Ekwall and Ruusula, 1994). Mutations at these six loci also affect silencing at centromeres and to some extent at telomeres (Allshire et al., 1995). In particular, *clr4*, *rik1* and *swi6* mutations have a strong effect on silencing within the *imr* and *otr* regions of the centromere and also cause elevated levels of chromosome loss. Intriguingly, the *swi6*+ gene encodes a protein which is 46% identical to *Drosophila* HP1 across the chromo domain (Lorentz et al., 1994) and both *Swi6p* and *HP1* contain a C-terminal shadow chromo domain (Aasland and Stewart, 1995). It has been proposed that this class of chromo domain protein may act as an adaptor molecule which mediates heterochromatin formation leading to repression of genes in that locality (Platero et al., 1995). The *Swi6p* chromo domain protein is located at centromeres, mating type loci and telomeres forming 2-5 discrete spots per haploid nucleus (Ekwall et al., 1995). *Swi6p* clearly plays a key role at fission yeast centromeres since in cells lacking *Swi6p*, transcriptional repression within the centromere is alleviated, chromosomes are lost at a high frequency and centromeres lag on a high proportion of anaphase spindles (Allshire et al., 1995; Ekwall et al., 1995). Thus, the assembly of a fully functional kinetochore in fission yeast is apparently linked to the formation of a heterochromatin-like structure at fission yeast centromeres. Here we further investigate the role of the *clr4*+ and *rik1*+ genes in chromosome segregation.
The probes cos212 (Funabiki et al., 1993) and pRS140 (Takahashi et al., 1992) were used to detect telomeres and centromeres, respectively. Note that the cos212 telomere adjacent cosmid only detects four of the six telomeres, namely those of chromosomes I and II (Funabiki et al., 1993). The probe was prepared by nick translation using Digoxigenin 11 (DIG) dUTP nucleotides (Boehringer). Nick translation was carried out in 40 μl reactions with 1.0 μg of plasmid DNA, 50 mM Tris-HCl, pH 7.5, 10 mM MgSO₄, 0.1 mM DTT, 50 μg/ml BSA buffer and 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.025 mM dTTP, 0.1 mM DIG dUTP, 10 units of DNA polymerase I (Gibco) and a calibrated amount of DNAse I (Gibco) at 15°C for 90 minutes. The probes were separated from unincorporated nucleotides using the Sephadex G-50 spin-column procedure (Sambrook et al., 1989). To determine the optimal probe size the batch of DNAseI (about 1 mg/ml) was diluted to 1/15, 1/30, 1/60, 1/125, 1/250 and 1/500 and 1 μl was used in each test reaction. The filtered products were size separated on a denaturating alkaline gel (Sambrook et al., 1989), blotted to a nitrocellulose filter and detected using anti-DIG alkaline phosphatase (Boehringer) and a detection kit (Vector Labs). The size of the nick translated products was determined by using DIG (VI) labelled HindIII digested size markers (Sigma). The sizes obtained ranged from 1,800 to about 100 nucleotides. The resulting series of probes were tested on the denatured cells and it was found that a size of 100-300 gave the best result. The longer probes did not enter the cells and the shorter ones did not efficiently hybridise to their targets. Anti-DIG-FITC (green) or anti-DIG-Rhodamine (red) secondary antibodies from sheep were used to detect the DIG signal (Boehringer).

### RESULTS

#### Genetic evidence for β-tubulin interactions

We have previously shown that clr4, rik1 and swi6 mutants lose chromosomes at a 50- to 100-fold higher rate than wild-type cells (Allshire et al., 1995). Since microtubules are known to be more stable at higher temperatures (Dustin, 1984) we reasoned that if the clr4+, rik1+ and swi6+ gene products act through the kinetochore then perhaps their phenotype would be more severe at lower temperatures. Therefore control strains and clr4, rik1 and swi6 mutant strains were plated at 18°C and 32°C (Fig. 1A). It is clear from the size of the colonies that...
The viability of *clr4*, *rik1*, and *swi6* cells was reduced about 2- to 3-fold at 18°C as compared to the wild type (data not shown). Thus, the growth and viability of *clr4*, *rik1*, and *swi6* mutants is reduced at low temperature, supporting an interaction with microtubules.

To test this further, mutants were assayed for their ability to grow in the presence of the anti-microtubule drugs TBZ (thiabendazole) and MBC (methyl-benzimidazol-2-yl carbamate). As shown in Fig. 1B, the *clr1*, *clr2*, *clr3*, and *rad2* mutants and the wild type were able to form colonies in the presence of up to 20 μg/ml of TBZ or 5.0 μg/ml of MBC. In contrast, growth of the *clr4*, *rik1*, and *swi6* mutants was completely inhibited at these concentrations. The *swi6::his1+* strain was highly sensitive to both drugs and could not grow at 10 μg/ml of TBZ and 2.5 μg/ml of MBC. The sensitivity to the anti-tubulin drugs is not just a consequence of the chromosome loss because the control *rad2* mutant strain which also loses chromosomes with a similar frequency but presumably by a different mechanism (see below) as *clr4*, *rik1*, and *swi6* mutants could still form small colonies at 20 μg/ml of TBZ or 5.0 μg/ml of MBC. Thus, the sensitivity of the three mutants to anti-microtubule drugs suggests a specific interaction of the *clr4*+, *rik1*+, and *swi6*+ gene products with spindle microtubules.

To further investigate the interaction with microtubules we tested for a genetic interaction with tubulin by constructing mutants of *clr4*, *rik1*, and *swi6* in combination with a cold sensitive mutation in the β-tubulin gene, *nda3*-KM311 (Toda et al., 1983). Growth of the *clr4 nda3*, *rik1 nda3* and *swi6 nda3* double mutants was significantly slower than the *nda3* single mutant at 28 and 32°C and these only formed microcolonies after 4 days at 25°C while the control *nda3* mutant was still viable (Fig. 2). The normal restrictive temperature for *nda3*-KM311 is 20°C. Thus, the restrictive temperature range of the...
cold-sensitive nda3 mutant was clearly increased by the presence of mutations at clr4, rik1 or swi6. The clr1, clr2, and clr3 mutants, which had only a minimal effect on centromere silencing and were unaffected by anti-tubulin drugs, and the control mutant rad2 (see below) were also combined with nda3-KM311. In these strains the restrictive temperature range was unchanged.

The interaction between the rik1, swi6, clr4 mutations and the nda3 mutation could be rather indirect or unspecific. For example the gene products could act in completely different...
cellular processes and each of them, when mutated, might result in an even more crippled double-mutant. To investigate the specificity of the above interactions the rate of chromosome loss was measured in all single mutants and double mutants. The mitotic loss rate of the 530 kb linear minichromosome Ch16 (Matsumoto et al., 1990) was determined at 32°C (Table 2) by a red-white half-sectoring assay (Allshire et al., 1994). This minichromosome is lost in wild-type cells in less than 0.1% of cell divisions (Table 2 and Niwa et al., 1989). A mutant defective in repair of DNA damage and chromosome segregation, rad2, which encodes DNAse IV was used as a control (Murray et al., 1994; Robins et al., 1994). The rate of loss of Ch16 in rad2::ura4+ is 0.58-1.2% (Table 2 and Murray et al., 1994) and the rate of loss in nda3::KM311 is 0.16-0.33%. Since these two mutations affect different and independent cellular processes there should be no interaction and only an additive effect on chromosome loss should be seen. The loss rate of the rad2::ura4+ nda3::KM311 double-mutant is 1.4-2.6% of cell divisions which is similar to an expected additive loss rate of 0.8-1.7%. The clr1, clr2 and clr3 mutations which do not affect the loss of Ch16 show about the same loss rate in combination with the nda3 mutation as the single mutations (<0.6% of divisions). In contrast, there is clearly a synergistic effect between clr4, rik1 and swi6 mutations and the nda3 mutation with respect to chromosome loss. In the single mutants the loss rate was 3-5% in clr4-S5, 3-7% in swi6::his1+ and 0.5-3% in swi6::his1+ cells whereas the double mutants clr4 nda3, rik1 nda3 and swi6 nda3 showed loss in up to 45% of cell divisions. This clearly indicates that the clr4, rik1 and swi6 mutations act through the same structure or cellular function as the nda3 mutant and it is therefore likely that clr4, rik1 and swi6 interfere with aspects of spindle or kinetochore function.

### Table 2. The effect of clr1, clr2, clr3, clr4, rik1, swi6, nda3 and rad2 mutations on the segregation of a 530 kb linear minichromosome (Ch16) at 32°C

<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Chromosome loss per division (%)</th>
<th>Mean value chromosome loss (%)</th>
<th>n=1,‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY977</td>
<td>Wild type</td>
<td>0.085-0.19</td>
<td>≤0.17</td>
<td>4†,‡</td>
</tr>
<tr>
<td>FY724</td>
<td>clr1-5</td>
<td>0.19-0.20</td>
<td>≤0.20</td>
<td>2‡</td>
</tr>
<tr>
<td>FY726</td>
<td>clr2-E22</td>
<td>0.10-0.27</td>
<td>0.18</td>
<td>2‡,‡</td>
</tr>
<tr>
<td>FY728</td>
<td>clr3-E36</td>
<td>0.16-0.17</td>
<td>0.32</td>
<td>2‡,‡</td>
</tr>
<tr>
<td>FY730</td>
<td>clr4-S5</td>
<td>3.8-5.0</td>
<td>4.4</td>
<td>2‡,‡</td>
</tr>
<tr>
<td>FY1037</td>
<td>swi6::his1+</td>
<td>0.47-2.9</td>
<td>1.47</td>
<td>4****</td>
</tr>
<tr>
<td>FY1045</td>
<td>rik1::LEU2+</td>
<td>2.5-7.0</td>
<td>4.5</td>
<td>5*****</td>
</tr>
<tr>
<td>FY1207</td>
<td>rad2::ura4+</td>
<td>0.58-1.2</td>
<td>0.93</td>
<td>4***</td>
</tr>
<tr>
<td>FY1096</td>
<td>h+ nda3-KM311</td>
<td>0.16-0.53</td>
<td>0.33</td>
<td>5****</td>
</tr>
<tr>
<td>FY1272</td>
<td>h+ clr1 nda3</td>
<td>&lt;0.17-0.61</td>
<td>≤0.39</td>
<td>2‡,‡</td>
</tr>
<tr>
<td>FY1273</td>
<td>h+ clr1 nda3</td>
<td>0.21-0.49</td>
<td>0.35</td>
<td>2‡,‡</td>
</tr>
<tr>
<td>FY1274</td>
<td>h+ clr2 nda3</td>
<td>&lt;0.29-0.38</td>
<td>≤0.34</td>
<td>2‡,‡</td>
</tr>
<tr>
<td>FY1275</td>
<td>h+ clr2 nda3</td>
<td>0.13-0.15</td>
<td>0.14</td>
<td>2‡,‡</td>
</tr>
<tr>
<td>FY1276</td>
<td>h+ clr3 nda3</td>
<td>0.16-0.46</td>
<td>0.26</td>
<td>3***,†</td>
</tr>
<tr>
<td>FY1277</td>
<td>h+ clr4 nda3</td>
<td>8-18</td>
<td>12</td>
<td>5*****</td>
</tr>
<tr>
<td>FY1278</td>
<td>h+ clr4 nda3</td>
<td>10-20</td>
<td>15</td>
<td>5*****</td>
</tr>
<tr>
<td>FY1194</td>
<td>swi6::his1+ nda3</td>
<td>15-29</td>
<td>23</td>
<td>3***</td>
</tr>
<tr>
<td>FY1195</td>
<td>rik1::LEU2+ nda3</td>
<td>34-45</td>
<td>42</td>
<td>3***</td>
</tr>
<tr>
<td>FY1358</td>
<td>rad2::ura4+ nda3</td>
<td>1.4-2.6</td>
<td>1.8</td>
<td>5***</td>
</tr>
</tbody>
</table>

The rate of minichromosome loss was determined by the half sectoring assay as described in Materials and Methods. n = the number of measurements. A total number of *100-500, † 500-1,000, or ‡ 1,000-1,500 colonies were examined for each strain as indicated.

### Table 3. Frequency of chromosome lagging in clr1, clr2, clr3, clr4, rik1 and swi6 mutants at 18°C

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total number of cells examined</th>
<th>Cells in metaphase or early anaphase</th>
<th>Cells in late anaphase</th>
<th>Late anaphase cells with lagging chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY977</td>
<td>549</td>
<td>30 (5.5%)</td>
<td>19 (3.5%)</td>
<td>0</td>
</tr>
<tr>
<td>FY724</td>
<td>532</td>
<td>37 (7.0%)</td>
<td>20 (3.8%)</td>
<td>1</td>
</tr>
<tr>
<td>FY726</td>
<td>540</td>
<td>28 (5.2%)</td>
<td>21 (3.9%)</td>
<td>0</td>
</tr>
<tr>
<td>FY728</td>
<td>545</td>
<td>28 (5.1%)</td>
<td>26 (4.8%)</td>
<td>0</td>
</tr>
<tr>
<td>FY730</td>
<td>475</td>
<td>15 (3.2%)</td>
<td>24 (5.1%)</td>
<td>11 (46%)</td>
</tr>
<tr>
<td>FY1037</td>
<td>579</td>
<td>25 (4.3%)</td>
<td>29 (5.0%)</td>
<td>10 (34%)</td>
</tr>
<tr>
<td>FY1045</td>
<td>518</td>
<td>18 (3.5%)</td>
<td>26 (5.0%)</td>
<td>9 (35%)</td>
</tr>
</tbody>
</table>

*Late metaphase or early anaphase were defined as cells with a spindle length shorter than 5 μm.
†Late anaphase cells were defined as cells with a spindle length greater than 5 μm.
‡Cells were classified as having lagging chromosomes if DAPI stained material was detected in the midzone of the spindle at least 1.5 μm away from one spindle pole.

**Missegregation and anaphase defects in clr4 rik1 and swi6 mutants**

The clr4, rik1 and swi6 mutants lose chromosomes at elevated rates and this phenotype is exacerbated by the presence of a mutation in nda3. Chromosome loss during mitosis can be caused by a number of defects resulting in simple loss of a chromatid (1:0) nondisjunction (2:0) or premature disjunction which leads to random segregation and some 2:0 events. To investigate the nature of the chromosome loss events, cultures of clr1, clr2, clr3, clr4, rik1, swi6 mutants and wild-type cells were grown at 18°C and subjected to immunostaining with an anti-α-tubulin monoclonal antibody, TAT1 (Woods et al., 1989) to examine the behaviour of cells during the cell cycle (Hagan and Hyams, 1988). Approximately 500 cells were observed for each culture and based on the absence or presence of the mitotic spindle they were classified to be in interphase, metaphase and early anaphase (spindle <5 μm) or late anaphase (spindle >5 μm). In the clr4, rik1 and swi6 cultures 34-46% of late anaphase cells still retained unsegregated (lagging) chromosomes lying in the spindle midzone (Table 3). Lagging chromosomes in late anaphase were absent or rarely observed in the wild-type, clr1, clr2 or clr3 cultures.

Further cytological examination of the phenotype of clr4, rik1 and swi6 was performed in a different culture of cells grown at 18°C. A portion of the cells were plated for (Ch16) chromosome loss and viability measurements and a portion of the cells were fixed and stained with TAT1 antibodies and subsequently subjected to FISH of centromere DNA and ribosomal DNA. The rDNA gene repeats are located on chromosome III and can therefore be used to visualise the distribution of this chromosome (Takahashi et al., 1994). As in the first experiment after approximately three divisions at 18°C 25-50% of clr4, rik1 and swi6 late anaphase cells showed lagging centromeres as detected by cen-FISH whereas lagging centromeres were not observed in the wild-type culture (Table 4 and Fig. 3). About 3.6-5.1% of the total number of clr4, rik1 and swi6 mutant cells also displayed missegregation of the rDNA cluster on chromosome III. These cells were in telophase or septated and had rDNA FISH signals in only one of the daughter nuclei. The fraction of half-sectoring red/white...
Media of Swi6p localisation in S. pombe 2643

Table 4. Frequency of chromosome loss, nondisjunction of chromosome III and lagging centromeres in wild type and clr4, rik1 and swi6 mutants at 18°C

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Mini-chromosome loss (Ch16)/total divisions</th>
<th>Chromosome III unequal segregation*</th>
<th>Lagging centromeres†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY977</td>
<td>Wild type</td>
<td>0/1510 (&lt;0.07%)</td>
<td>0/227 (&lt;0.4%)</td>
<td>0/98 (&lt;1.0%)‡</td>
</tr>
<tr>
<td>FY729</td>
<td>clr4-S5</td>
<td>17/288 (5.9%)</td>
<td>6/118 (5.1%)</td>
<td>18/48 (38%)</td>
</tr>
<tr>
<td>FY731</td>
<td>rik1-304</td>
<td>28/688 (3.6%)</td>
<td>6/165 (3.6%)</td>
<td>12/45 (27%)</td>
</tr>
<tr>
<td>FY1045</td>
<td>rik1::LEU2</td>
<td>13/516 (2.5%)</td>
<td>6/33 (4.5%)</td>
<td>13/52 (25%)</td>
</tr>
<tr>
<td>FY1037</td>
<td>swi6::his1*</td>
<td>17/370 (4.6%)‡</td>
<td>n.d.</td>
<td>39/80 (50%)‡</td>
</tr>
<tr>
<td>FY733</td>
<td>swi6-115</td>
<td>11/849 (1.3%)</td>
<td>6/138 (4.3%)</td>
<td>15/61 (25%)‡</td>
</tr>
</tbody>
</table>

All measurements were carried out on cells grown at 18°C. The rate of mini-chromosome loss was determined by the half sectoring assay as described in Materials and Methods.

*Unequal segregation of chromosome III was measured by rDNA FISH as described in Materials and Methods. Septated cells with both rDNA signals in one of the daughter cells were scored as unequal segregation events.

†Lagging centromeres in anaphase is defined as cells with one or more centromeric in situ signals in the midzone of the spindle (more than 1.5 µm from one end), and with a spindle greater than 5 µm in length.

‡Data from Ekwall et al. (1995).

For all strains the frequency of lagging centromeres, mini-chromosome loss and unequal segregation of chromosome III were measured in cells from the same culture. n.d., not determined.

colonies reflecting chromosome loss of Ch16 in the clr4, rik1 and swi6 cultures was (by plating cells from the same cultures used for FISH) in the range of 1.3 to 5.9% whereas that of the wild-type culture was less than 0.07%. Thus, the frequency of missegregation of endogenous chromosome III and that of chromosome III derived Ch16 minichromosome is within the same magnitude. It is likely that chromosomes I and II are affected in a similar manner. Clearly cells bearing clr4, rik1 or swi6 mutations missegregate chromosomes and display a specific defect in the movement of centromeres to the spindle poles in mitotic anaphase.

The failure of centromeres to move to the poles concurrently could be due to: (1) a failure of sister kinetochores to capture microtubules from either pole; (2) a failure of sister centromeres to separate following the metaphase to anaphase transition; or (3) the formation of a fragile or incomplete kinetochore which forms weaker interactions with spindle microtubules or is defective in poleward movement. Such defects in mitosis might be expected to elicit some checkpoint mechanism resulting in an accumulation of metaphase or anaphase cells in the culture. However, the fraction of metaphase or early anaphase cells in the clr4, rik1 and swi6 cultures was relatively unchanged as compared to wild type (see also Table 3). Therefore, the slow growth rate of clr4, rik1 and swi6 mutants observed at 18°C must be explained by some other mechanism than a delay at a specific point in the cell cycle. It is possible that the slow growth is due to reduced viability caused by chromosome missegregation events. Hence, it is likely that the segregation defect of clr4, rik1 and swi6 is not controlled by any check-point system monitoring progress through metaphase or anaphase (see Discussion).

The centromeric localisation of the Swi6 protein is disrupted in clr4 and rik1 mutants

It was previously demonstrated that the Swi6 protein is localised to all of the three known silenced regions in the fission yeast nucleus, namely the mating type region, centromeres and telomeres (Ekwall et al., 1995). Since the clr4 and rik1 mutants have very similar phenotypes to those reported for swi6 the question was addressed if the amount of Swi6p is affected in clr4 and rik1 mutant cells. Protein extracts made from clr1, clr2, clr3, clr4, rik1 and swi6 strains as indicated. The blot was probed with pre-absorbed anti-Swi6R4 (top) and counterstained with amido black to compare loading.

Fig. 4. Western detection of Swi6p in wild-type, clr1, clr2, clr3, clr4 rik1 and swi6 cells. Immunoblot of total protein extracts from wild-type, clr1, clr2, clr3, clr4, rik1 and swi6 strains as indicated.

All measurements were carried out on cells grown at 18°C. The rate of mini-chromosome loss was determined by the half sectoring assay as described in Materials and Methods.
Swi6p was no longer localized in discrete spots, but greatly dispersed within the nucleus (Fig. 5A-H). It was evident that the large centromeric spot, most prominent in G2 cells, had completely disappeared. It was also apparent that the Swi6 signal was now often positioned into the hemisphere of the interphase nucleus, which is not stained by DAPI. It is known that this hemisphere contains the nucleolus (Uzawa and Yanagida, 1992). This is in contrast to the wild-type pattern in which the Swi6p spots are exclusively located in the chromosomal (DAPI stained) hemisphere. Even in mitotic clr4 and rik1 cells the Swi6 protein was not concentrated at its normal chromosomal locations, but again it was dispersed in the nucleus. Consequently, the products of the clr4+ and rik1+ genes must be required for localization of the Swi6 protein to transcriptionally silent domains including centromeres.

**FISH analysis of centromeres and telomeres in clr4 rik1 and swi6 cells and telomere length analysis**

In *S. cerevisiae* the absence of silencing factors such as Sir3p results in detachment of Rap1p and presumably also
Mediators of Swi6p localisation in S. pombe

during interphase S. pombe telomeres are also known to cluster at the nuclear periphery and all centromeres are specifically associated with the spindle pole body at the nuclear periphery (Funabiki et al., 1993). Since Swi6p is localised at centromeres and telomeres and this localisation is dependent on fully functional clr4+ and rik1+ gene products, perhaps mutations in these genes would alter the localisation of telomeres and centromeres. To address this a FISH analysis was performed using telomere and centromere probes on clr4, rik1 and swi6 mutant cells. The results are presented in Fig. 6A. No major changes in the clustering of these loci were detected by counting the number of FISH signals in interphase nuclei. As revealed by the centromere probe, both clr4 and rik1 cultures had about 80% interphase cells with one cluster, and about 15% had two spots, and only a few cells had three spots. When the telomere probe was used 40-50% of rik1 and clr4 cells had two signals and about 30% of cells had only one signal. These results are in good agreement with those previously reported for wild-type cells (Funabiki et al., 1993).

In S. cerevisiae mutations which alleviate transcriptional repression of telomere adjacent genes also display alterations in telomere length (Kyrion et al., 1993; Palladino et al., 1993). Since in fission yeast mutations at the clr1, clr2, clr3, clr4, rik1 and swi6 mutants all affect telomeric silencing to some extent, telomere length was measured in all six mutants by Southern blotting. Digestion with NlaIV cleaves a terminally located ura4 gene twice producing a terminal restriction fragment of approximately 1 kb and an internal ura4 fragment of 418 bp. In all six mutants no alteration in the length of the terminal repeat tract could be detected as compared to the isogenic wild-type strain (Fig. 6B). The internal ura4 band provides a convenient internal control for spurious migration in each track. The resolution in this and similar experiments was such that a change in terminal repeat tract length of 50 bp would have been easily detectable. No alteration has been seen in strains carrying null alleles of rik1 or swi6 (data not shown). Although the ura4 gene only analyses the length of one specific telomere no alteration was detected in any of the six mutants of the average length of the bulk telomeric smear detected with a telomeric repeat probe (data not shown).

Fig. 6. Interphase clustering of centromeres and telomeres, and telomere length in wild-type, clr4, rik1 and swi6 cells. (A) Bar diagrams representing the distribution of interphase cells with 1, 2, or 3 centromere FISH signals (left) and 1, 2, 3 or 4 telomere FISH signals (right). Genotypes of the cells are indicated; 150-200 cells were examined in each sample. (B) Telomere length measurements by Southern blotting after NlaIV digestion of genomic DNA as described in Materials and Methods. Strain numbers and relevant genotype are indicated. The 280 bp ura4 probe detects an internal band and a telomeric fragment in each lane.


**DISCUSSION**

In this study it has been demonstrated that the clr4, rik1 and swi6 mutations which have strong effects on chromosome segregation also display cold sensitivity and elevated sensitivity to the drugs MBC and the closely related compound TBZ. Generally mutations in the β-tubulin (nda3) gene are resistant to and mutations in the α-tubulin (nda2) gene are super-sensitive to these drugs (Umesono et al., 1983). MBC has been shown to disassemble yeast microtubules in vitro (Kilmartin, 1981). This indicates that the clr4*, rik1* and swi6* genes are likely to interact, directly or indirectly, with some aspect of tubulin function. By combining mutations in clr4, rik1 and swi6 silencing factors with a mutation in the nda3 gene at a semi-permissive temperature, a synergistic effect on chromosome segregation was revealed. This was not observed with the clr1, clr2 and clr3 mutants or the rad2 control mutant. The rad2* gene is involved in DNA repair and the rad2 mutant has a defect in chromosome segregation of similar magnitude to that of clr4, rik1 and swi6 (Murray et al., 1994). This observation places rad2 in a different pathway from nda3 by epistasis analysis, because an additive effect was observed at the expected range. Our interpretation of the clr4, rik1 and swi6 results is that they affect the same cellular process as nda3, but each mutation is only partially defective (at 32°C) so that the combined effect is synergistic. This synergism is similar to the effect observed in studies in *S. cerevisiae* where compromised centromere DNA sequences were combined with mutations in trans-acting centromere binding proteins (Strunnikov et al., 1995). A ‘synthetically acentric’ screen for mutants successfully identified two components of the *S. cerevisiae* kinetochore NDC10 (or CEP2) and CEP3. In our experiments a partially defective β-tubulin molecule (at 32°C) combined with a change in composition of chromatins at the centromere (caused by mutation in either clr4, rik1 or swi6) appears to cause a similar effect. Previously, we have shown that the segregation function of a compromised minimal centromere is obliterated in a clr4, rik1 or swi6 background (Allshire et al., 1995). Thus, clr4*, rik1* and swi6* gene products act in the chromosome segregation process through β-tubulin, a major component of spindle microtubules.

A high incidence of lagging centromeres on late anaphase spindles was observed in rik1 and clr4 mutants. This is very similar to the swi6 phenotype described previously (Ekwall et al., 1995). The lagging centromeres could be caused by defects in sister chromatid disjunction or defects in attachment of centromeres to the spindle microtubules resulting in inefficient poleward movement. Because more than three centromere spots were frequently observed in these cells we can rule out the possibility of complete nondisjunction. However, it is possible that the lagging centromeres result from premature or delayed disjoining of sister centromeres. Premature disjunction of sister chromatids was tested in rik1, clr4 and swi6 cells arrested at metaphase by imposition of the mts3 (proteosome subunit mutation) temperature-sensitive block (Gordon et al., 1996). In this experiment clr4 mts3 and rik1 mts3 cells were arrested at metaphase with unseparated sister chromatids on a short spindle. If premature disjunction is caused by clr4 or rik1 then chromosomes would move to opposite poles or deattach from the spindle. This would result in a suppression of the metaphase block caused by mts3. No such increase in the frequency of sister chromatid separation was observed (K. Ekwall, unpublished observation). Hence, it is unlikely that the lagging phenotype is caused solely by premature disjunction or complete nondisjunction of sister chromatids.

In mammalian cells a tension sensitive monitor can detect a single kinetochore remaining unattached to the spindle and elicits a delay in metaphase-anaphase transition lasting up to 3 hours (Rieder et al., 1994). A similar checkpoint control has been detected in *S. cerevisiae* where several (but not all) mutations defective in centromere function exhibit a G2/M cell cycle delay (Spencer and Hieter, 1992; Doheny et al., 1993; Goh and Kilmartin, 1993; Strunnikov et al., 1995). In clr4, rik1 and swi6 mutants, although there is a clear defect in centromere function, no accumulation of cells in mitosis could be detected. There are two possible explanations: (1) in wild-type cells the Clr4, Rik1 and Swi6 proteins contribute to the assembly of a structure which is normally scrutinised by a surveillance system and defects in this structure would result in delayed mitosis. However, the absence of any of these gene products disrupts this structure so that no delay is seen in these mutants.

(2) The mutants are only defective in post metaphase centromere function interfering only with movement of centromeres toward the spindle pole during anaphase, and that defects in this anaphase A-like movement are not subject to any form of checkpoint control. In fission yeast ds mutants sister chromatids fail to disjoin but the spindle elongates with a normal cell cycle timing resulting in unequal segregation of chromosomes to the spindle poles (Ohkura et al., 1988). Thus, in the ds mutants a checkpoint control of late anaphase progression appears to be absent.

The Swi6 protein is clearly delocalised from the centromeres in clr4 and rik1 mutant cells. The Clr4 and Rik1 proteins may either directly interact with Swi6p at the centromere or they could be indirectly involved in the recruitment of Swi6p to the centromere. Because Swi6p is synthesised at normal levels and size in clr4 and rik1 we can exclude any indirect model involving the synthesis of Swi6p. We have previously shown that rik1 and clr4 mutants derepress markers placed within the normally very repressed outer repeats of cen1 whereas the swi6 mutant has a more moderate effect at these sites. Mutations in swi6 have been suggested to alter the folding of silent mating-type loci in mating-type switching (Thon and Klar, 1993). This led us to propose a model in which Swi6p facilitates the folding of the centromere into a higher order (Allshire et al., 1995). The clr4* and rik1* factors were hypothesised to also be required for this folded structure. Here we demonstrate that clr4* and rik1* are required to localise Swi6p to the centromeres. This is in good agreement with the proposed model and it follows that a defective higher order structure would persist in rik1 and clr4 mutants due to absence of Swi6p at the centromere. The generation of reagents allowing the localisation of Clr4 and Rik1 gene products will be required to determine if they act directly at *S. pombe* centromeres. The rik1* gene has been cloned and sequenced, but database searches have not been informative about the gene function (Borgström, 1996). Intriguingly, as swi6*, the clr4* gene also encodes a chromo-domain protein (P. Lord, K. Sawin, H. Causton, T. Olsson, K. Ekwall and R. Allshire, unpublished observation) suggesting that it may also be a primary constituent of the centromeric heterochromatin-like structure required for complete centromere function in fission yeast.
The clustering of centromeres adjacent to the spindle pole body during interphase, the aggregation of telomeres at the periphery of interphase nuclei and the length of the terminal repeat tract were all normal in clr4, rik1 and swi6 mutants (Fig. 6). In S. cerevisiae sir3 and sir4 mutants alleviate telomeric silencing, reduce telomere length, and disrupt the clustering of Rap1p at the nuclear periphery (Palladino et al., 1993). It has been proposed that a heterochromatin-like structure is formed at the nuclear periphery by the assembly of a complex which involves Sir3p, Sir4p, Rap1p and telomeres via interactions with the N-terminal regions of histone H3 and H4 (Hecht et al., 1995). The effect of clr4, rik1 and swi6 on silencing at S. pombe telomeres is marginal in comparison to the complete elimination of telomeric silencing seen in mutations in sir3 and sir4 in S. cerevisiae (Aparicio et al., 1991). Although Swi6p is normally localised at S. pombe telomeres, its absence in clr4, rik1 or swi6 mutants does not dramatically alter telomere localisation or length of the terminal repeat. Thus, from our studies in S. pombe disruption of silencing of centromeres and alleviation of silencing at telomeres appears to be independent of the nuclear clustering of these loci and does not affect terminal repeat tract length.

It is likely that the products of the clr4, rik1 and swi6 genes are also required for normal chromosome segregation during meiosis. Consistent with this it has previously been observed that spores germinating from homozygous rik1 zygotes exhibit poor viability and are unequal in size (Egel et al., 1989). In preparation for karyogamy and meiosis in S. pombe telomeres take on a novel role in that they exchange position with the centromeres so that they become associated with the spindle pole body (SPB). The telomeres are then led by the spindle pole body in a series of rapid ‘horse tail’ movements back and forth throughout the cell (Chikashige et al., 1994). It remains to be seen if Clr4, Rik1 and Swi6 play a role in the association of S. pombe telomeres with the SPB during meiosis or in these pre-miotic chromosomal movements.


REFERENCES


encodes a homologue of chromatin-associated proteins from *Drosophila* and mammals. *Gene* 143, 139-143.


(Received 5 June 1996 - Accepted 1 August 1996)