A novel type of silencing factor, Clr2, is necessary for transcriptional silencing at various chromosomal locations in the fission yeast *Schizosaccharomyces pombe*

Pernilla Bjerling¹,²,*, Karl Ekwall¹, Richard Egel² and Geneviève Thon²

¹Department of Biosciences, Karolinska Institutet and Department of Natural Sciences, Södertorn University College, 141 89 Huddinge, Sweden and ²Department of Genetics, Institute of Molecular Biology, University of Copenhagen, DK-1353 Copenhagen K, Denmark

Received April 20, 2004; Revised July 23, 2004; Accepted August 2, 2004 DDBJ/EMBL/GenBank accession no. Z98598

ABSTRACT

The mating-type region of the fission yeast *Schizosaccharomyces pombe* comprises three loci: *mat1*, *mat2-P* and *mat3-M*. *mat1* is expressed and determines the mating type of the cell. *mat2-P* and *mat3-M* are two storage cassettes located in a 17 kb heterochromatic region with features identical to those of mammalian heterochromatin. Mutations in the *mat2-P*–*mat3-M* region affects transcriptional repression in the mating-type region, but also centromeric silencing the *mat3-M* gene encodes a 62 kDa protein with no obvious sequence homologs. Deletion of *clr2* not only affects transcriptional repression in the mating-type region, but also centromeric silencing and silencing of a PolII-transcribed gene inserted in the rDNA repeats. Using chromatin immunoprecipitation, we show that Clr2 is necessary for histone hypoacetylation in the mating-type region, suggesting that Clr2 acts upstream of histone deacetylases to promote transcriptional silencing.

INTRODUCTION

Mutant screens aimed at identifying silencing factors acting in the mating-type region of fission yeast have identified several evolutionary conserved proteins required for the formation of heterochromatin. The mating-type region contains three loci: the expressed *mat1* locus that determines the mating type of the cell and the silent storage cassettes *mat2-P* and *mat3-M* (1). Cells switch mating type by transposition of genetic information from *mat2-P* or *mat3-M* to *mat1* (2,3). The mating-type region is localized on chromosome II, the centromere-proximal *mat1* locus being separated from *mat2-P* by a ~15 kb region called L and *mat3-M* being separated from *mat2-P* by a ~11 kb region called ‘K’. The K-region contains sequences with strong similarity to the repeats found in centromeric regions, a 4.3 kb segment of the K-region displaying 96% similarity to the repeats in *cen2* (4). Transcription and meiotic recombination are inhibited in the K-region by a heterochromatic structure spanning ~17 kb of DNA and flanked by two inverted repeats IR-L and IR-R (5–7). The IR-L and IR-R repeats establish the borders between transcriptionally repressed heterochromatin and active euchromatin. The heterochromatic region shares many features with heterochromatin of Drosophila or mammals, including histone hypoacetylation, histone H3 lysine 9 methylation and association with a chromodomain protein, Swi6, bound to the modified histone H3 (6,8–11). The surrounding euchromatin on the other hand shares the features of active chromatin of higher eukaryotes with acetylated histones and histone H3 methylated at lysine 4 (6,10). *Trans*-acting factors required for silencing in the mating-type region were identified in four different mutant screens (12–15). These screens led to propose that Clr1, Clr2, Clr3, Clr4, Clr6 and Swi6 play a role in heterochromatin formation in *Schizosaccharomyces pombe*. Other factors such as Rik1 and Chp2 were identified by other approaches (13,16). Several of these *trans*-acting factors are also central to the formation of heterochromatic structures at the centromeres and telomeres of *S.pombe* (17–19). In addition, Clr3, Clr4, Swi6 and Chp2 are required for transcriptional silencing of PolII-transcribed genes inserted in the rDNA repeats (11,16). Clr3 and Clr6 belong to a family of histone deacetylases (15). Clr3 binds directly to the mating-type region and preferentially deacetylates lysine 14 of histone H3 (11). Clr4 is a methyltransferase that acts specifically on lysine 9 of histone H3 thereby forming a binding site for Swi6 (9,20,21). Moreover, it has recently been shown that a nucleosome remodeling factor, Hrp3 and an NAD⁺-dependent histone deacetylase, Sir2, are needed for transcriptional repression in the mating-type region (22,23).

*To whom correspondence should be addressed at present address: Department of Medical Biochemistry and Microbiology, University of Uppsala, Box 582, SE-751 23 Uppsala, Sweden. Tel: +46 18 471 6652; Fax: +46 18 471 4673; Email: Pernilla.Bjerling@imbim.uu.se*
Here, we describe the cloning and sequencing of the gene encoding the previously uncharacterized silencing factor Clr2. The \(clr2^+\) open reading frame (ORF) encodes a novel type of silencing factor with no obvious sequence homologs. We created a \(clr2\) deletion allele that allowed us to investigate the contribution of Clr2 to transcriptional silencing at several chromosomal locations and we asked whether silencing is dependent on the concentration of Clr2 in the cell. Finally, we examined histone acetylation levels in \(clr2\) cells, thereby gaining insight into the specific molecular defects caused by the lack of Clr2.

**MATERIALS AND METHODS**

**Media**

Media were prepared as in (24).

**Cloning of \(clr2^+\)**

The strain PB1 (Table 1) was transformed with a genomic library in vector pDB248' (25). Transformed cells were selected on PM-leu plates for complementation of the \(S.pombe\) leu1-32 mutation by the \(Sacccharomyces\) cerevisiae \(LEU2\) gene in pDB248'. After 6 days at 30°C Leu+ colonies were replica plated onto sporulation media and incubated at room temperature (20–25°C) for 3 days. The colonies were stained by iodine vapor, and dark-staining colonies were streaked on PM + FOA. A plasmid, pPB9, was recovered from a dark-staining FOA-resistant isolate. A 6 kb HindIII–XbaI fragment was subcloned from pPB9 into pDW232 (26) creating pPB11, which could rescue the Clr2 phenotype upon retransformation. The insert in pPB11 was sequenced.

**Deletion of \(clr2^+\)**

The primers PP45: 5'-CCCCCTGCAGTTTTGCTTTTATACCTCTTTATC3' and PP46: 5'-CCCCCTGCAGTAGCTACTAAAATGACAATAGC3' were used to amplify a 732 bp fragment upstream of the \(clr2\) ORF. The primers PP47: 5'-GGATCCATGCCTGCTATTACTTGTGTTCTGCAGTTTGCTTTTATACCTCTTTATC3' and PP48: 5'-GGCCGCCGGACCTACATTACCTTTGTCTTC3' were used to amplify a 660 bp fragment downstream of the ORF. Restriction sites used for cloning are underlined.

**RESULTS**

**Cloning of the \(clr2^+\) gene**

The \(clr2-E22\) homothallic strain PB1 (Table 1) was transformed with an \(S.pombe\) genomic library. The phenotype of PB1 offers two strategies for cloning the \(clr2^+\) gene. First, PB1 colonies display an aberrant sporulation phenotype due to the simultaneous expression of \(mat2-P\) and \(mat3-M\) in haploid cells, an abnormal event that causes haploid sporulation and reduces the efficiency of mating. As \(S.pombe\) spore asci contain starch molecules that are stained black by iodine vapors, this sporulation phenotype can be assayed at the colony level: \(clr2-E22\) colonies are stained gray by iodine vapors as compared to the black wild-type staining. Second, a \(ura4^+\) gene inserted near the \(mat3-M\) cassette in PB1 is derepressed by the \(clr2-E22\) mutation. This renders PB1 sensitive to the drug 5-fluoroorotic acid [5-FOA; (30)]. Complementation of the \(clr2-E22\) mutation can be expected to partially or totally restore the wild-type iodine staining and FOA resistance.

Out of 45,000 transformants, one transformant was obtained where the \(clr2-E22\) phenotype described above was complemented. Restoration of a wild-type sporulation phenotype was confirmed by microscopy (Figure 1). The complementing plasmid was rescued from PB1 and its insert was sequenced, identifying an ORF of 1611 bp. This ORF corresponds to a
A. *clr2-E22 + pDW232*

![Image](68x326 to 291x514)

B. *clr2-E22 + pPB11*

![Image](68x526 to 292x714)

Figure 1. Complementation of the sporulation defect in a *clr2-E22* mutant strain with cloned DNA. A black arrow points at a product of haploid meiosis and a white arrow points at a zygotic ascus in (A). Strain KE78(*clr2-E22*) was transformed with vector DNA, pDW232 (A) or the *clr2*-expressing plasmid pPB11 (B).

previously uncharacterized gene in the Sanger Centre *S.pombe* Gene Database (locus C1B3.17, EMBL accession no. Z98598). The 1611 bp ORF is capable of encoding an acidic protein (pI = 5.6) of 537 amino acids (aa) with a predicted MW of 62 kDa (Swiss-Prot accession no. O13881). No obvious homologous protein was found when BLAST searches were performed at the National Center for Biotechnology Information (NCBI). No described motifs were identified using the Swiss Prot or Pfam domain databases. The physical distance between C1B3.17 and the *radl*-1 gene is ~11.2 kb (31). The genetic distance between *radl*-1 (32) and *clr2-E22* was determined by tetrad dissections following a cross between PB1 (*clr2-E22*) and PB65 (*radl*-1). This distance was found to be 1.9 cM. A genetic distance of 1.9 cM in *S.pombe* is in good agreement with a physical distance of 11.2 kb, indicating C1B3.17 is *clr2* and not an extragenic suppressor. We found during the course of the linkage analysis that the *clr2-E22* mutant strain is not sensitive to ultraviolet (UV) irradiation.

Analysis of *clr2* mutant alleles

The *clr2-E22* mutation (13) was sequenced as well as two other mutations in *clr2* described in (14), *clr2-760* and *clr2-785*. The *clr2-E22* mutation is a 16 bp insertion at nucleotide 205 creating a frame shift. The *clr2-760* and *clr2-785* mutations are both non-sense mutations at nucleotide 968 or 903 respectively, introducing a stop instead of a tryptophan at codon 323 or 301. We conclude from our analysis of these truncated alleles that all or parts of the C-terminal 214 amino acids are necessary to make a functional Clr2 protein. The C-terminus of Clr2 also proved sensitive to other modifications since fusions to a green fluorescent protein (GFP) or hemagglutinin (HA) tag resulted in non-functional proteins (data not shown).

Deletion of *clr2*

A construct was made with the *S.pombe his7*+ gene (27) flanked by sequences naturally found on the side of the *clr2*+ ORF. A linear fragment was used to transform a diploid (PB101 X PB102) homozygous for a *his7*-366 mutation in the endogenous *his7* gene. Stable histidine phototrophic transformants were examined by tetrad dissection. A 2:2 segregation pattern was obtained for both the His+/His− and the Clr2+/Clr2− phenotypes, the Clr2− phenotype co-segregating with the His− phenotype in 18 tetrads examined. A Southern blot prepared with one of the diploids and its offspring confirmed integration of the *his7*+ gene into the *clr2*+ locus (data not shown). The *clr2Δ* segregants being viable shows that *clr2* is not an essential gene. The 1611 bp *clr2*+ ORF was cloned into the pREP82X vector to place the ORF under the control of the weakest *mnt1* (no message in thiamine) promoter (28). The resulting plasmid, pPB40, was able to rescue the sporulation defects of a strain where the entire reading frame of *clr2*+ had been deleted, thereby showing that the 1611 bp ORF encodes a functional Clr2 protein.

Transcriptional silencing in *clr2Δ* strains

Spontaneous or EMS-induced mutations in the *clr2* gene were shown to affect transcriptional silencing in the mating-type region, in the centromeric *imr* repeats, and near telomeres (13,14,17). In order to determine whether the *clr2* null mutation had a more severe silencing phenotype than these mutations, the *clr2Δ* deletion and the *clr2-E22* mutation were combined with a *ura4*+ reporter gene inserted in the centromere or mating-type region (Figure 2). In addition, we decided to investigate whether Clr2 contributes to the more recently described silencing of PolII-transcribed genes inserted in the meiotic or mating-type region (Figure 2). In addition, we decided to investigate whether Clr2 contributes to the more recently described silencing of PolII-transcribed genes inserted in the *S.pombe* ribosomal DNA (rDNA) (16). To this end, the *clr2Δ* and the *clr2-E22* mutant alleles were each crossed into a strain containing an rDNA::*ura4*+ reporter. For comparison with a more extensively characterized silencing factor a *clr4Δ* (33) allele was also combined with each of the *ura4*+ reporter genes examined here. Expression of *ura4*+ was estimated by plating serial dilutions of cells on selective plates (Figure 2). *clr2Δ* strains with the *ura4*+ gene inserted in the mating-type region [mat3-M(EcoRV)::*ura4*], centromeric repeats [imr1/R(NcoI)::*ura4*], centromeric central core [cen2 (SphI)::*ura4*] or rDNA (rDNA::*ura4*+) all grew poorly on plates lacking uracil and they formed colonies on plates containing FOA consistent with a transcriptional repression of the *ura4*+ gene at these locations.
In contrast, when either clr2 mutant allele or the clr4Δ allele was combined with the mat3-M (EcoRV)::ura4+ reporter, cells grew well on plates lacking uracil but they did not grow on plates containing FOA indicating the mat3-M(EcoRV)::ura4+ reporter is largely derepressed in the mutant backgrounds (Figure 2A, second to fourth rows).

Similarly, all strains with a trans-acting mutation and a ura4+ reporter gene in the imr centromeric repeats [imr1R(Ncol)::ura4+] grew well on plates lacking uracil and poorly on plates containing FOA (Figure 2B, second to fourth rows).

(Figure 2A–D, top rows).
fourth rows). The few large FOA-resistant papilli present in these mutant strains probably contain DNA rearrangements in which the ura4+ gene is lost, as observed in other silencing mutants (16). Transcription is less stringently repressed in the central core of S. pombe centromeres than in the flanking repeats (17). Consistently, we observed a modest repression of cen2 (SphI)::ura4+ in our wild-type background (Figure 2C, first row). This weak repression was partially dependent on Clr2 and Clr4, the clr4Δ allele having a somewhat more pronounced effect than either the clr2Δ or clr2-E22 allele (Figure 2C, second to fourth rows). Finally, cells in which the clr2 or clr4 mutant alleles were combined with the rDNA::ura4+ reporter grew well on plates lacking uracil and poorly on plates containing FOA indicating rDNA::ura4+ is derepressed in the mutant backgrounds. In summary, we found that Clr2 has an effect on transcriptional silencing in the mating-type region, the imr repeats and the central core of the centromere and it also has a clear role in the repression of PolII-transcribed genes inserted in the S. pombe rDNA. The few large FOA-resistant papilli present in our wild-type background (Figure 2C, second to fourth rows). Finally, cells in which the clr2 or clr4 mutant alleles were combined with the rDNA::ura4+ reporter grew well on plates lacking uracil and poorly on plates containing FOA indicating rDNA::ura4+ is derepressed in the mutant backgrounds. In summary, we found that Clr2 has an effect on transcriptional silencing in the mating-type region, the imr repeats and the central core of the centromere and it also has a clear role in the repression of PolII-transcribed genes inserted in the S. pombe rDNA. The extent to which Clr2 represses a reporter gene at these locations is comparable to the repression exerted by Clr4.

Analysis of the effect of Clr2 overexpression

It has been shown that overexpression of Swi6 increases transcriptional repression in the mating-type region (7,34). This finding prompted us to test whether overexpression of Clr2 would affect silencing. In order to monitor protein expression, we used antibodies produced against Clr2. The antibodies failed to recognize Clr2 on a western blot with a protein extract when the protein is overexpressed. Protein extracts from S. pombe transformed into strains carrying a ura4+ reporter gene near the centromere 1 [imr1L(NcoI)] or [otr1L (HindIII)] (17), near a telomere, {Ch16 m23::ura4+-TEL[72]}, (35), or in the mating-type region [mat3-M(EcoRV)::ura4+], (12). Cell suspensions of the transformants were diluted and spotted onto selective plates where the expression of the reporter gene could be estimated. Overexpression of Clr2 had no detectable effect on the expression of the reporter genes at any of the chromosomal locations tested (data not shown). We also investigated whether overexpression of Clr2 could suppress mutations in other trans-acting factors necessary for silencing in the mating-type region. We found that overexpression of Clr2 did not restore the repression of the mat3-M(EcoRV)::ade6+ reporter gene (24) in a clr1-6, clr3-E36, clr4-S5, rik1::ura4, or swi6-S115 mutant background, indicating overexpression of Clr2 does not suppress mutations in these silencing factors. Furthermore, we investigated whether over-expression of Clr2 could restore telomeric silencing in a strain with a swi6-S115 mutation and found that this was not the case. These results all point to silencing not being sensitive to Clr2 dosage.

Deletion of clr2+ results in hyperacetylation of histones in the mating-type region

Silencing being affected in the mating-type region of clr2Δ cells suggested that the chromatin structure of that region might be altered in the mutant cells. Histones are hypopainted in the mating-type region of wild-type cells (11). We asked whether Clr2 was necessary for this low acetylation level by performing Chromatin Immunoprecipitations (ChIP) with antibodies directed against histone H3 acetylated at lysine 14 (α-H3AcK14), histone H4 acetylated at lysine 8 (α-H4AcK8), or histone H4 acetylated at lysine 12 (α-H4AcK12). Chromatin extracts were prepared from strains with a ura4+ reporter gene near the mat3-M cassette and a truncated ura4 allele, ura4-DS/E, at the endogenous ura4 locus (Figure 2). Figure 4 shows the result of the ChIP assays. The top panel displays results obtained with the wild-type strain (FY597), and the bottom panel displays results obtained with the clr2Δ strain (Hu582) (Table 1). The acetylation levels of mat3-M(EcoRV)::ura4+ were low in the wild-type strain consistent with previous observations (11). Deletion of

Figure 3. Antibodies against Clr2 recognize Clr2 from a total S. pombe protein extract when the protein is overexpressed. Protein extracts from S. pombe were (A) stained with Coomassie or (B) blotted and incubated with the Clr2p antibody. M, protein size markers; lane 1, PB254 (clr2Δ); lane 2, PB141 (clr2+) transformed with vector pREP82X; lane 3, PB141 (clr2+) transformed with plasmid pPB40 with the clr2+ gene under the control of the weakest nmt1 promoter; and lane 4, PB141 (clr2+) transformed with plasmid pPB65 where the clr2+ gene is under the control of the strongest nmt1 promoter.
H4K12 were used in the immunoprecipitations. Those were conducted in recognizing specifically H3AcK14 (H3K14), H4AcK8 (H4K8) or H4AcK12 antibody was added in the immunoprecipitation step. Antibodies ura4-DS/E chromosomal location (IN) or immunoprecipitated chromatin. strains used were FY597 (wild type) and H u582 (clr2).

clr2+ resulted in increased acetylation of the three lysine residues examined, the histones associated with the mat3-M(EcoRV)::ura4+ reporter gene in clr2Δ cells being as highly acetylated as those associated with the ura4-DS/E control. We conclude that Clr2 is necessary for the low histone acetylation level observed in the mating-type region.

**DISCUSSION**

**Clr2 is a novel type of silencing factor**

We found that the Clr2 silencing factor is encoded by a previously uncharacterized sequence orphan, designated as C1B3.17, in the *S. pombe* GeneBank at the Sanger Genome Sequencing Center. Most of the silencing factors that have been characterized in *S. pombe* are conserved in other organisms. Swi6 has homologs in the HP1 proteins found in *Drosophila melanogaster* and mammals (9,36). Clr4 also has homologs in Su(var)3-9p of *D. melanogaster* and Suv39h1 in mammals (20,21). Clr3 and Clr6 belong to a large conserved family of histone deacetylases (15,37). Other silencing factors in *S. pombe* contain recognizable motifs or domains. For example, Clr1 and Rik1 contain respectively three C2H2 Zinc fingers and a β-propeller domain of the UV-DDB-127 family, indicating these proteins might interact with DNA/RNA (G. Thon, unpublished data) (38,39). Chp2 contains a chromo- and chromoshadow domain (16). These similarities underscore the mechanistic conservation of heterochromatin formation in eukaryotes. Sequence orphans are present in the genomes of all organisms. In the *S. pombe* proteome there are 454 sequence orphans among 4965 proteins in total, or in other words ~9% of the proteins encoded in its genome are unique to *S. pombe* (Sanger Genome Sequencing Center). Orphans might perform functions that are specific to the organism in which they are found, or they might perform conserved functions that do not require sequence conservation. Understanding their origin and evolution is a challenging task. Characterizing orphans, for whom a biological function has been uncovered, such as Clr2, will help understand the place of these proteins in evolution.

**Clr2 is necessary for silencing at various chromosomal locations**

We determined that *clr2+* is not an essential gene. Mutated *clr2* alleles were previously shown to alleviate silencing in the mating-type region and in the *imr* repeats of the centromere (13,14,17). Deletion of *clr2+* caused silencing defects similar to those caused by deletion of the histone methyltransferase gene *clr4* at all chromosomal locations tested; the mating-type region, the *imr* repeats, the central core of centromere 2, and the rDNA (Figure 2), indicating that Clr2 is a general mediator of transcriptional silencing in *S. pombe*. The effects observed at the central core of centromere 2 ([cen2](SpHl)::ura4+) are noticeably smaller than those observed at the other locations. Previous studies have shown that the central cores of *S. pombe* centromeres are associated with proteins not found in the flanking centromeric repeats such as Mis6, Sim4 and Cnp1 (40,41). Conversely, Swi6 is physically associated with centromeric repeats rather than with the central core (40). In spite of the undetectable association of Swi6 with central core sequences however, Swi6 and other heterochromatic proteins have some influence on the transcriptional repression occurring in central cores since a derepression of *ura4+* placed within the central core of *cen1* [TM1(NcoI):: *ura4+*] can be detected by growth assays in *swi6Δ, rik1-304* or *clr4-S5* mutant backgrounds (17). Our observations are consistent with Clr2 having an effect of the same magnitude as these other proteins involved in heterochromatin formation, whether the effect is directly exerted on the central core, or whether it is an indirect effect reflecting changes occurring in the neighboring heterochromatin.

**Overexpression of Clr2 does not affect silencing**

Transcriptional silencing in the mating-type region varies with the concentration of Swi6 or Clr4 in the cell. High dosage of the Swi6 protein strengthens silencing, whereas a high dosage of Clr4 alleviates silencing. Overexpression of other silencing factors such as Clr1 or Clr3 has no effect on mating-type silencing (7,20,34). Swi6 is a structural component of heterochromatin (34). If Clr2 also enhanced silencing when overexpressed it could be argued that Clr2 is a structural component of chromatin like Swi6. Here we report that overexpression of Clr2 does not affect the degree of silencing in the mating-type region, centromeres or telomeres. This indicates that the amount of Clr2 is not the limiting factor for heterochromatin formation and that Clr2 does not titrate other factors away from their point of action.

**Clr2 promotes histone deacetylation in the mating-type region**

In a strain with a *clr2Δ* deletion, the histones in the mating-type region are hyperacetylated compared to a wild-type strain (Figure 4). This indicates that histone deacetylases such as Clr3, Clr6 and Sir2 are not operating properly in the absence
Table 1. List of S.pombe strains

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<th>Source</th>
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<td>h, imm1(Bcoc1)::ura4* orl1, clr2::his7, ura4-DSE, leu1-32, his7-366, ade6-M210</td>
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h+. Matting type is ambiguous because the silent mating-type loci are deregressed in this background.

of Clr2, possibly because they are not recruited to the normally silent region. Alternatively, these enzymes might be properly recruited to elements nucleating heterochromatin formation, but heterochromatin might be unable to spread in the absence of Clr2. Histone hypoacetylation is not required solely for transcriptional silencing in the mating-type region, but it also mediates silencing at centromeres and in the rDNA repeats, which suggests that Clr2 affects silencing in these regions via increased histone acetylation levels as well. It is difficult to speculate about the precise mechanism of action of Clr2 since the protein sequence does not give any clues. Genetic selections using the S.pombe clr2 mutant strains described here might help identify functional homologs from other species, with unrelated or largely diverged sequences.

ACKNOWLEDGEMENTS

P.B. acknowledges a grant from the Swedish research council (2002-4948); G.T. acknowledges grants from the Novo Nordisk Foundation and from the Danish Research Council.

REFERENCES


