The S. pombe Cdc14-like Phosphatase Clp1p Regulates Chromosome Biorientation and Interacts with Aurora Kinase

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Summary

The S. pombe Cdc14-related phosphatase Clp1p/Flp1p regulates G2/M transition by antagonizing CDK activity and is essential for coordinating the nuclear division cycle with cytokinesis through the cytokinesis checkpoint. At the G2/M transition, Clp1p/Flp1p is released from the nucleolus and SPB and distributes throughout the nucleus to the spindle and the contractile ring. This early relocalization is analogous to vertebrate Cdc14 homologs and stands in contrast to S. cerevisiae Cdc14p, which is not released from the nucleolus until metaphase/anaphase transition. Here, we report that Clp1p/Flp1p localizes to kinetochores in prometaphase and functions in chromosome segregation, since deletion of clp1/flow1 causes cosegregation of sister chromatids, when sister kinetochores are prone to mono-orientation. Genetic, cytological, and biochemical experiments suggest that Clp1p/Flp1p functions together with Aurora kinase at kinetochores. Together, these results suggest that Clp1p/Flp1p has a role in repairing mono-orientation of sister kinetochores.

Introduction

It is crucial that cells faithfully segregate equal amounts of genetic material to daughter cells, since failure to do so leads to aneuploidy, which is often associated with cancer. During mitosis, the sister chromatids attach to spindle microtubules at the centromeres and move along the microtubules to opposite cell ends. For equal segregation, the sister kinetochores need to be attached to microtubules from opposing poles in a bioriented fashion. The kinetochores are not only the site of chromosome attachment to the spindle, but also provide the detection and repair machinery when the attachment is absent or defective. Recent discoveries show that the chromosomal passenger proteins aurora B, survivin, and INCENP, which localize to the kinetochores in early mitosis, play a vital role in the repair of mono-oriented attachment in budding yeast (Biggins and Murray, 2001; Tanaka et al., 2002) as well as in vertebrate cells (Carmena and Earnshaw, 2003; Lampson et al., 2004). Chromosomal passenger proteins concentrate at kinetochores in early mitosis and then to the spindle midzone in anaphase in a variety of eukaryotes (Adams et al., 2001), including the fission yeast Schizosaccharomyces pombe (Morishita et al., 2001; Petersen et al., 2001; Rajagopalan and Balasubramanian, 2002).

The S. pombe Cdc14-like-phosphatase Clp1p/Flp1p (hereafter referred to as Clp1p) was previously found to regulate mitotic entry and coordinate mitosis with cytokinesis (Cueille et al., 2001; Trautmann et al., 2001). As with its Saccharomyces cerevisiae ortholog Cdc14p, Clp1p shuttles between the nucleolus and the cytoplasm (Trautmann and McCollum, 2002). In contrast to Cdc14p, which is released from the nucleolus at metaphase/anaphase transition, Clp1p is released from the nucleolus upon entry into mitosis, when it distributes throughout the nucleus, the cytoplasm, and the contractile ring (Cueille et al., 2001; Trautmann et al., 2001). In late mitosis, S. pombe Clp1p, like its C. elegans ortholog CeCdc14, concentrates at the spindle midzone, reminiscent of the passenger proteins (Grueneberg et al., 2002). The functional significance of the release of Clp1p in early mitosis, as well as whether Clp1p, like the passenger proteins, localizes to the kinetochores in early mitosis, has been unknown. Here, we report that Clp1p localizes to kinetochores in prometaphase and regulates chromosome segregation.

Results

Clp1p Localizes to Kinetochores in Early Mitosis and Deletion of clp1 Causes Chromosome Loss

After analyzing Clp1p localization in early mitosis more closely, we observed Clp1p-GFP not just at the actomyosin ring as expected, but also at foci in the nucleus closely, we observed Clp1p-GFP not just at the actomyosin ring as expected, but also at foci in the nucleus....
Figure 1. Clp1p-GFP Localizes to Kinetochores during Early Mitosis

(A–C, F) Cells in (A) (clp1-GFP nda3-KM311), (B) (clp1-GFP dis1-3HA), and (F) (clp1-C286S-GFP dis1) were grown at 30°C, then shifted to 19°C for 6 hr prior to fixation and visualized for GFP fluorescence (A, B, F) or (C) immunostained for Clp1p-GFP and Cnp1p-HA with GFP and HA antibodies, respectively.

(D) Individual images from time-lapse analysis of clp1-GFP sid4-GFP expressing cells. Numbers indicate minutes after acquisition of the first image. Kinetochore Clp1p-GFP (arrowhead) and SPB marking Sid4p-GFP (asterisks) are indicated. The movie and further information are in the Supplemental Data.

(E) Images of live cells expressing sid4-GFP clp1-GFP or sid4-GFP in early mitosis. Asterisks indicate SPBs marked with Sid4p-GFP, the arrowhead points to Clp1p-GFP signal between SPBs.

If, as with other Cdc14 proteins, the mutant protein acts in a dominant-negative manner by binding but not re-releasing its substrates (Figure 3F; Xu et al., 2000).

Clp1p Interacts with Aurora Kinase

Like chromosomal passenger proteins, Clp1p localizes to kinetochores in prometaphase and the spindle midzone in anaphase and regulates chromosome segregation defects. Similar to mutants defective in kinetochore proteins, clp1Δ cells showed hypersensitivity to the microtubule depolymerizing drug Carbendazim (MBC, Sigma-Aldrich) (Figure 2A). Comparison of the chromosome loss rate in clp1Δ mutants and wild-type using an assay that monitors loss of a nonessential minichromosome (Niwa et al., 1986) revealed that clp1Δ mutants had a 28-fold increase in chromosome loss compared to wild-type (Figure 2C), consistent with a role for Clp1p in chromosome segregation. To test whether Clp1p phosphatase activity is required for its role in chromosome segregation, we utilized a mutation in the conserved cysteine of the phosphatase domain (C286) to serine, which results in a phosphatase-inactive allele of clp1-C286S-GFP allele was integrated into the genome and expressed from the native clp1 promotor. Despite its correct localization (Figure 1F), clp1-C286S-GFP was hypersensitive to MBC (Figure 2A) and lost the minichromosome at a rate 97.2 times higher than wild-type (Figure 2C). The increased chromosome loss rate of clp1-C286S-GFP compared to the deletion mutation might be explained if, as with other Cdc14 proteins, the mutant protein acts in a dominant-negative manner by binding but not releasing its substrates (Figure 3F; Xu et al., 2000).
Clp1p Regulates Chromosome Segregation

Figure 2. *clp1Δ* Cells Are Sensitive to Perturbations in the Chromosome Segregation Machinery

(A) Wild-type and *clp1Δ* cultures were plated in serial dilutions on YE and YE plates containing 10 μg/ml Carbendazim (MBC) and grown at 36°C. (B) Growth of single and double mutants in drop tests at different temperatures. Serial dilutions were spotted on YE plates and shifted to permissive (30°C) and semipermissive (25°C) temperatures. (C) The rate of minichromosome loss per cell division was determined as described in Experimental Procedures. The numbers in parentheses indicate the total number of colonies screened.

<table>
<thead>
<tr>
<th></th>
<th>chromosome loss rate</th>
<th>fold increase</th>
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<tr>
<td>wild type</td>
<td>4.23 x 10^-4 (4659)</td>
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<tr>
<td><em>clp1Δ</em></td>
<td>1.12 x 10^-2 (1811)</td>
<td>28.2</td>
</tr>
<tr>
<td><em>clp1</em>ΔC286S-GFP</td>
<td>4.11 x 10^-2 (1509)</td>
<td>97.2</td>
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where Clp1p regulates chromosome segregation together with the chromosomal passenger proteins.

This led us to compare the localization of GFP-tagged aurora kinase (Ark1p-GFP) in wild-type and *clp1Δ* cells. Cultures were synchronized with hydroxy urea in S phase and Ark1p-GFP localization was followed after release from the block. Live cells were analyzed every 20 min for Ark1p-GFP signal on the kinetochores and seption. In wild-type cells, localization of Ark1p-GFP at the kinetochores peaked prior to septon. The peak was drastically reduced in *clp1Δ* cells (Figure 3C). This behavior was consistent in three independent experiments. Examples of Ark1p-GFP localization are shown in Figure 3D, where, in contrast to the above experiment, cells also express sid4-GFP to confirm the early mitotic stage. Ark1p-GFP at kinetochores of metaphase cells is either much fainter or not detectable in *clp1Δ* cells compared to wild-type. The overall level of Ark1p-GFP was not reduced in *clp1Δ* compared to wild-type cells (Figure 3E, lanes 2 and 3). The effect of *clp1Δ* on Ark1p-GFP localization was specific, as we could not find a difference in localization of the kinetochore proteins Ndc80p-GFP, Mis6p-GFP, Mis12p-GFP, and Bub1-GFP in *clp1*Δ deletion mutants compared to wild-type (data not shown). The percentage of cells localizing Ark1p-GFP to kinetochores was also reduced in the absence of Clp1p in cells arrested in metaphase due to absence of microtubules and an activated spindle checkpoint in the *nda3-km311* mutant (Supplemental Figure S1). The same was true for survivin Bir1p-GFP localization to the kinetochores, while Mad2p-GFP localization was not affected by deletion of *clp1* (Supplemental Figure S1). Interestingly, localization of the INCENP homolog Pic1p, which localizes like Bir1p-GFP and Ark1p-GFP (S.T., unpublished observation), was the same whether Clp1p was present or not (Supplemental Figure S1). Additionally, Clp1p-C286S-13myc and Ark1p-GFP coimmunoprecipitate in extracts from metaphase-blocked cells and to a reduced level in lysates from asynchronous cultures (Figure 3F). Interestingly, wild-type Clp1p and Ark1p only weakly coimmunoprecipitate (Figure 3F). This is consistent with the enhanced phosphatase-substrate interaction of the phosphatase-inactive point mutant Clp1pC286S. These results show that Clp1p exists in a complex with Ark1p and is required for efficient localization of Ark1p and Bir1p to the kinetochores. *S. cerevisiae* Cdc14p is essential for the localization of aurora kinase Ipl1p to the spindle (Pereira and Schiebel, 2003); however, *clp1Δ* mutants were not defective in Ark1p localization to the spindle midzone (Figure 3G).

Deletion of *clp1* Reduces Viability of *dis1* Mutants

Since Clp1p localizes to kinetochores in *dis1* mutants, we tested whether its absence would affect the *dis1* mutant phenotype. Dis1p is an S. pombe XMAP215/TOG/Stu2-family homolog, which binds microtubules and localizes to kinetochores (Nakaseko et al., 2001). Mutants in *dis1* lack the chromosome alignment phase in metaphase and arrest with elongated spindles and segregated but unseparated chromosome pairs (Nabeshima et al., 1999; Ohkura et al., 1988). We constructed double mutants between *dis1* and *clp1Δ* and analyzed their growth rates at the *dis1*-permissive (30°C), -semi-permissive (25°C), and -restrictive (19°C) temperatures. While *dis1* mutants grew fine at 30°C, *dis1 clp1Δ* double mutants showed very poor growth at 30°C (Figure 2B). Double and single mutants containing *dis1* mutations were dead at 19°C (data not shown). In addition, *dis1 clp1Δ* double mutants were dead at 25°C, whereas *dis1* single mutants were able to grow, albeit slowly, at this temperature (Figure 2B). To exclude that deletion of *clp1* enhances all mutants defective in kinetochore proteins and chromosome segregation, we tested whether the *clp1Δ* mutation perturbed growth in kinetochore structure mutants *mis6-302, mis12-537*, and *mal2-1* (Goshima et al., 1999; Jin et al., 2002; Saitoh et al., 1997).
Figure 3. Interactions between Clp1p and Aurora Kinase

(A) Three representative tetrad types from the cross of cut17-275 with clp1Δ and an example of the cut17-275 clp1Δ phenotype are shown.

(B) Growth of single and double mutants in serial dilution drop tests at permissive (25°C) and semipermissive (30°C) temperature.

(C) ark1-GFP and ark1-GFP clp1Δ cells grown at 30°C were blocked in S phase using 11 mM HU for 5 hr, then released and monitored for Ark1p-GFP localization to kinetochores (diamonds) in live cells, as well as septation index (squares).

(D) Live pictures of early mitotic ark1-GFP and ark1-GFP clp1Δ cells carrying sid4-GFP (marked by asterisks) to visualize the SPBs are shown. The arrowhead indicates Ark1p-GFP localization.

(E) Ark1p-GFP levels are compared between wild-type (lane 2) and clp1Δ (lane 3) cells or from an untagged strain (lane 1) after immunoprecipitation and Western blotting with GFP antibodies. The lower lane shows the tubulin loading control from whole extracts prior to immunoprecipitation.

(F) Anti-myc and anti-GFP Western blots of anti-GFP-immunoprecipitates from cell lysates of the following cultures are shown: ark1-GFP (1), clp1Δ clp1Δ-13myc (2), nda3-km311 ark1-GFP clp1Δ clp1Δ-13myc synchronized in metaphase (3) and asynchronous (4), clp1-13myc (5), and nda3-km311 ark1-GFP clp1Δ clp1Δ-13myc blocked in metaphase (6) and asynchronous (7).

(G) Live pictures of Ark1p-GFP anaphase localization merged with the dic-image in clp1Δ and clp1Δ strain are shown.

However, double mutants between clp1Δ and the above mutants did not show genetic interactions (data not shown).

clp1Δ Causes Aneuploidy in dis1 Cells

To identify the cause for the reduced viability in dis1 mutants due to deletion of clp1, we examined the phenotypes on a cellular level. Chromosome segregation was compared by DNA staining in asynchronous dis1 and dis1 clp1Δ mutant cultures grown at 30°C or after incubation at 25°C, the dis1 clp1Δ-restrictive temperature for the duration of one cell cycle (4 hr). At 25°C, dis1 clp1Δ cultures showed an increase in cells with uneven DNA segregation (Figure 4A, inset) as compared to dis1 single mutants (Figure 4A). The same phenotype was observed in dis1 clp1Δ C286S-GFP double mutants (Figure 4A).

Next, we examined how the deletion of clp1 caused chromosome segregation defects. dis1 mutants arrest with an activated spindle checkpoint and appear to experience an increase in sister chromatid cosegregation (70%) at full restrictive temperature. Such cosegregation results when mono-oriented attachment of sister kinetochores to microtubules from the same SPB is not corrected. Since dis1 mutants lack the phase of constant spindle length when chromosomes are properly aligned (Nabeshima et al., 1998), it is possible that the chromosomes do not have enough time to become bioriented before spindle elongation and anaphase B chromosome movement. Therefore, the chromosome segregation defect caused by deletion of clp1 could be due either to failure in the spindle checkpoint or to failure to establish biorientation. We first tested whether dis1 clp1Δ double mutants are defective in spindle checkpoint activation.
Since Mad2p localizes to kinetochores when the spindle checkpoint is active, we compared Mad2p-GFP localization in dis1 and dis1 clp1Δ double mutants grown at 30°C or after 4 hr at 25°C. At 25°C, the percentage of cells with Mad2p-GFP at the kinetochores was the same with or without Clp1p (Figure 4B). As shown above, localization of Mad2p-GFP to the kinetochores was also unperturbed in absence of Clp1p in a nda3-km311-induced metaphase block. Although checkpoint response seems to be intact in clp1Δ cells, the possibility remains that Clp1p could be specifically involved in checkpoint response to mono-oriented chromosome pairs as was shown for Ipl1p in S. cerevisiae. However, it has not been demonstrated whether the spindle
checkpoint monitors mono-orientation and lack of tension in *S. pombe*.

To examine whether *dis1 clp1Δ* double mutants would experience a higher frequency of sister chromatid cosegregation compared to *dis1* single mutants, we created *dis1* and *dis1 clp1Δ* strains containing the centromere I-linked LacO array and GFP-LacI-NLS and analyzed the GFP localization at 30°C or after shift to 25°C (Nabeshima et al., 1998). Cells that had completed anaphase, as judged by septum formation, were analyzed for the presence of one GFP dot in each daughter nucleus (equal segregation) or, for GFP, dot(s) in only one daughter nucleus (cosegregation). Examples are shown in Figure 4C. At 30°C, 20% of *dis1 clp1Δ* cells showed cosegregation of chromosome I, compared to only 1% of *dis1* single mutant cells (Figure 4C). Cosegregation of chromatids in the absence of Clp1p was even more severe at 25°C (Figure 4C). Chromosome I segregation was not affected in *clp1Δ* single mutants (Figure 4C). The absence of severe chromosome segregation defects in *clp1Δ* single mutants suggests that Clp1p is required for a mechanism to repair defects such as mono-orientation that occur in *dis1* mutants.

**Clp1p Is Required to Prevent Sister Chromatid Cosegregation**

Whether Clp1p is required to prevent sister chromatid cosegregation in more physiological circumstances was not clear. In *S. pombe*, kinetochores are clustered at one spot on the nuclear envelope next to the SPB prior to mitosis (Funabiki et al., 1993). Upon mitotic entry, kinetochores are presumably rapidly captured by microtubules emanating from the duplicated, nearby SPBs. However, in situations when microtubules are depolymerized, such as in the cold-sensitive *nda3-km311* β-tubulin mutant, cells block in prometaphase since they are unable to form a mitotic spindle and the kinetochores often lose their attachment to the SPB (Funabiki et al., 1993; Hiraoka et al., 1984). The displaced kinetochores now need to be captured by microtubules from opposite poles by search and capture mechanisms similar to that used by mammalian cells. This may often lead to a situation where a chromosome pair is closer to one SPB than the result and occur in a mono-oriented attachment (E. Grishchuck and R. McIntosh, personal communication). We tested whether Clp1p was important to promote biorientation in this situation. *nda3-km311* and *nda3-km311 clp1Δ* cells containing a GFP-marked centromere II (Kitajima et al., 2003) were arrested in metaphase by incubation at 19°C and then released from the block at 30°C to allow microtubules to reassemble and attach to kinetochores in a mono- or bioriented manner. The success of the following chromosome segregation can be evaluated by the localization of GFP-marked centromeres after septation. If sister chromatids cosegregate, only one daughter nucleus will receive a GFP focus, as seen in Figure 4F (arrowhead) and Supplemental Movie S4. Centromere II localization was scored 60 min after the release when cells had completed anaphase and were septating (84% wild-type, 87% *clp1Δ*). Due to misplacement of the nucleus relative to the established actomyosin ring, some cells segregate the DNA within one cell compartment, but were scored as equally segregating if the GFP dots appeared in two separate nuclei (Figure 4E, cell #). The culture lacking Clp1p showed a higher frequency of centromere II cosegregation than *clp1Δ* cells (Figure 4D). The cosegregation appearing in *clp1Δ* background after the release is partly due to segregation prior to release from the block without chromosome segregation (16% wild-type, 15% *clp1Δ*). The *clp1Δ* phenotype quantified above was also observed using time-lapse analysis (Figure 4F and Supplemental Movies S3 and S4). GFP-LacI-NLS localizes diffusely throughout the nucleus, which makes the nucleus segregating without chromosome II visible (Figure 4F, arrowhead). To exclude that cosegregation is a randomization of segregation due to premature loss of cohesion when Clp1p is absent, we compared the clustering of sister chromatids of chromosome II prior to release from the restrictive temperature, but found no difference between *clp1Δ* and *clp1Δ* cells (Supplemental Figure S2). This result further supports a role for Clp1p in establishing chromosome biorientation.

**Discussion**

**Chromosomal Passenger Proteins and Clp1p in Biorientation in *S. pombe***

In *S. cerevisiae* as well as mammalian and frog tissue culture cells, aurora B kinase promotes biorientation by disrupting mono-oriented kinetochore-spindle attachments (Carmena and Earnshaw, 2003). Given that Clp1p is found in a complex with Ark1p and deletion of *clp1* reduces Ark1p localization to kinetochores, it seems likely that Clp1p regulates sister chromatid biorientation through aurora kinase Ark1p. However, it is not known whether chromosomal passenger proteins regulate chromosome segregation in *S. pombe*. It is somewhat surprising that Mad2p localization is unaffected in *clp1Δ* mutants, given that Ark1p has been shown to be important for Mad2p localization (Petersen and Hagan, 2003). One explanation could be that there is a sufficient pool of Ark1p at kinetochores to recruit Mad2p in *clp1Δ* cells. How Clp1p regulates aurora kinase remains an interesting question. We expect that the other chromosomal passenger proteins, INCENP and survivin, may also be in the same complex with aurora and Clp1p and could be direct targets of Clp1p. However, we have been unable to resolve this issue because these proteins are difficult to detect by Western blotting (data not shown). Because Clp1p phosphatase activity is required for its role in chromosome segregation, Clp1p presumably dephosphorylates one or more components in the passenger protein complex. One possibility is that a localized reduction of CDK activity due to Clp1p is required for a stable passenger complex in metaphase. This requirement would be dispensable in the absence of high CDK activity as in late anaphase where the passengers localize normally in *clp1Δ* mutants.

**Cdc14-like Phosphatases at the Kinetochore**

Our results also suggest that Clp1p functions quite differently from its *S. cerevisiae* ortholog Cdc14p. Similar to Clp1p, Cdc14p localizes to kinetochores and binds to the chromosomal passenger complex; however, it is required to dephosphorylate INCENP, which promotes localization of the chromosomal passenger complex to...
the spindle midzone (Pereira and Schiebel, 2003). We did not observe a defect in aurora localization to the spindle midzone in cdc11 mutant cells. Similarly, knock-down of CeCdc14 expression using RNAi in C. elegans did not cause a total loss of Air1p (aurora B) at the spindle midzone, either (Grueneberg et al., 2002). It is not known whether S. cerevisiae cdc14 mutants are defective in aurora kinase localization to the kinetochores as in S. pombe. One reason for the difference between S. pombe and S. cerevisiae might be the different timing of Cdc1p/Cdc14p release from the nucleolus. Cdc14p is not released from the nucleolus in early mitosis and therefore might not function in the exact same manner as Clp1p. To what extent Cdc14-related phosphatases regulate chromosome segregation in higher eukaryotes is not known. Although CDC14 has not been implicated in chromosome orientation is established in culture cells (Tanaka, 2002), we thank Dr. Mitsuhiro Yanagida, Dr. Takashi Toda, Dr. Ursula Fleig, Dr. Dr. Mohan Balasubramanian, Dr. Kathy Gould, Dr. Chris Norbury, Revised: October 15, 2004

Experimental Procedures

Strains and Culture

Strains used in this study are listed in Supplemental Table S1. Standard fission yeast techniques and media were employed (Moreno et al., 1991). For growth tests, 5 μl of 0.1 OD600 cultures and 1:10 dilutions were spotted onto plates. The entire coding region of the phosphatase inactive allele clp1-C286S was cloned into the pJK210 and Xenopus XCD14α/β do localize to nucleoli during interphase (Kaiser et al., 2002, 2004) and are released upon nucleolar disassembly at entry into mitosis as in S. pombe. This is also the time when biorientation is established in culture cells (Tanaka, 2002), raising the possibility that the role of Clp1p in chromosome segregation may be a conserved function for CDC14-related phosphatases.

Microscopy

If otherwise noted, cells were fixed in MeOH as previously described (Balasubramanian et al., 1997). Indirect immunofluorescence was performed as previously described (Balasubramanian et al., 1997; Hagan and Hyams, 1988) with the following modifications. Cells were fixed for 30 min in 4% paraformaldehyde (Electron Microscopy Science) and treated according to Balasubramanian et al. (1997) with PEM as buffer instead of PBS (Hagan and Hyams, 1988). Monoclonal mouse αHA (HA 11 MMS-101P, COVANCE, dil. 1:1000) and rabbit αGFP serum (A-6455, Molecular Probes, dil. 1:1000) were used as primary antibodies and detected by Alexa Fluor 594 goat-anti-rabbit αGFP serum (A-11011, Molecular Probes, dil. 1:300). Confocal images were captured using a Zeiss LSM700 confocal microscope. Immunoprecipitation was done as described (Sparks et al., 1999). Mouse monoclonal αGFP (2 μl per 1 ml lysate, A-1120, Molecular Probes) antibody was used for immunoprecipitation, and rabbit αGFP serum (dil. 1:1000, A-6455, Molecular Probes) and αmyc mouse monoclonal antibody (dil. 1:1000, clone 9E10) were used for Western blotting. For the immunoprecipitations of Ark1p-GFP, crude cell lysates were cleaved by centrifugation at 3000 × g in the presence of phosphatase inhibitors (1 mM NaVO₃ and 5 mM NaF).

Minichromosome Loss Assay

Minichromosome loss assays were done as described (Allshire et al., 1995; Fleig et al., 1996). Briefly, loss of the nonessential minichromosome causes cells to accumulate a red pigment. The rate of chromosome loss can be determined by counting the frequency of half-sectored colonies, which result from minichromosome loss in the first division. Here, the rate of chromosome loss equals the number of half red sectored colonies divided by the total number of colonies minus red colonies, which had lost the minichromosome prior to the assay.

Acknowledgments

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