The centromere-specific histone variant Cse4p (CENP-A) is essential for functional chromatin architecture at the yeast 2-µm circle partitioning locus and promotes equal plasmid segregation

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The centromere protein A homologue Cse4p is required for kinetochore assembly and faithful chromosome segregation in *Saccharomyces cerevisiae*. It has been regarded as the exquisite hallmark of centromeric chromatin. We demonstrate that Cse4 resides at the partitioning locus *STB* of the 2-µm plasmid. Cse4p-*STB* association is absolutely dependent on the plasmid partitioning proteins Rep1p and Rep2p and the integrity of the mitotic spindle. The kinetochore mutation *ndc10-1* excludes Cse4p from centromeres without dislodging it from *STB*. Cse4p-*STB* association lasts from G1/S through late telophase during the cell cycle. The release of Cse4p from *STB* chromatin is likely mediated through spindle disassembly. A lack of functional Cse4p disrupts the remodeling of *STB* chromatin by the RSC2 complex, negates Rep2p binding and cohesin assembly at *STB*, and causes plasmid missegregation. Poaching of a specific histone variant by the plasmid to mark its partitioning locus with a centromere tag reveals yet another one of the molecular trickeries it performs for achieving chromosome-like fidelity in segregation.

Introduction

The multicopy yeast plasmid 2-µm circle is organized as a cluster of approximately three to five foci in the nucleus and segregates as a cluster during cell division (Scott-Drew and Murray, 1998; Velmurugan et al., 2003; Jayaram et al., 2004). The plasmid-coded Rep1 and Rep2 proteins together with the cis-acting locus *STB* are responsible for the nearly chromosome-like persistence of the 2-µm circle. The plasmid provides no obvious advantage to yeast but poses no significant disadvantage either at its steady state copy number. An amplification machinery based on the Flp site-specific recombination system can correct a potential drop in copy number as a result of rare missegregation events (Futcher, 1986; Murray et al., 1987; Reynolds et al., 1987). The amplification system is under both negative and positive controls. The negative control involves repression of the *FLP* gene by the Rep1 and Rep2 proteins acting, presumably, as a bipartite repressor (Som et al., 1988). The positive control is mediated through the plasmid protein Raf1p, which appears to antagonize the action of the Rep1p–Rep2p repressor. Together, the partitioning and amplification systems can account for faithful plasmid propagation as well as maintenance of plasmid copy number.

The Rep-*STB* partitioning system appears to couple 2-µm circle segregation to chromosome segregation using quite unsuspected mechanisms. The plasmid assembles the yeast cohesin complex at *STB* in a Rep1p–Rep2p-dependent fashion, presumably to pair replicated plasmid clusters during S phase (Mehta et al., 2002). Strong circumstantial evidence suggests that sister clusters segregate when cohesin is disassembled by separase action during anaphase. Therefore, the mechanics of plasmid segregation and chromosome segregation appear to be fundamentally similar. In contrast to chromosomes, cohesion between plasmid clusters is absolutely dependent on the integrity of the nuclear microtubules (Mehta et al., 2005). In the *mtw1-1* mutant, in which multiple chromosomes are detached from the spindle during a cell cycle (Pinsky et al., 2003), the 2-µm plasmid tends to cosegregate almost always with the spindle and, thus, spindle-attached chromosomes (Mehta et al., 2005).

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Abbreviations used in this paper: CDE, centromere DNA element; ChIP, chromatin immunoprecipitation.

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These observations are consistent with a spindle-dependent and perhaps chromosome-assisted segregation mechanism for the plasmid.

The partitioning locus *STB* can be divided into two halves, proximal and distal, with respect to the plasmid replication origin (Murray and Cesareni, 1986). The proximal *STB* consists of a tandem array of approximately six units of a 65-bp consensus sequence. The distal *STB* contains a transcription termination signal that prevents plasmid transcription directed toward the origin from entering the repeated segment of *STB*. The SWI/SNF-related yeast chromatin remodeling complex RSC2 (Mohrmann and Verrijzer, 2005) is required for proper chromatin organization at *STB* (Wong et al., 2002; Huang et al., 2004). A lack of functional RSC2 complex prevents cohesin assembly at *STB* and causes high plasmid loss (Wong et al., 2002; Huang et al., 2004; Yang et al., 2004).

In this study, we demonstrate that the histone H3 variant Cse4p (the centromere protein A homologue), which was thought to be exclusive to chromatin at yeast centromeres, is harbored by *STB* and promotes equal plasmid segregation.

Results

Cse4p associates with the 2-μm plasmid in chromosome spreads

In wild-type [cir$^+$] yeast cells (harboring the native 2-μm circle), a fluorescence-tagged *STB* reporter plasmid resides in close proximity to the spindle pole (Mehta et al., 2005). This characteristic localization is lost in the absence of an intact partitioning system, as in a [cir^0] strain lacking the Rep1 and Rep2 proteins. The plasmid foci were almost always coincident with or partially overlapped the kinetochore marker Ndc10p (unpublished data), suggesting that the 2-μm circle shares more or less the same nuclear locale as centromeres. Consistent with these observations, the Cse4 protein and Rep proteins were found to colocalize in chromosome spreads prepared from [cir$^+$] cells (Fig. 1 A). To distinguish between the true association of Cse4p with the 2-μm plasmid and mere overlap between the plasmid cluster and congressed centromeres, we performed the chromosome spread assays in the *ndc10-1* [cir^0] and [cir$^+$] strains at permissive (26°C) and nonpermissive (37°C) temperatures. Instead of the compact focus of Cse4p seen at 26°C, the protein became disbursed over the DAPI staining region at 37°C, often with a punctuate pattern (Fig. 1, B–D). This delocalization was presumably caused by the centromeres being disorganized in the absence of Ndc10 function (Ortiz et al., 1999). However, in the [cir$^+$] strain, a subset of these Cse4p dots showed a near perfect one to one correspondence with the foci formed by a resident *STB* reporter plasmid (Fig. 1 D). The function of the endogenous 2-μm circles in this assay was to provide the Rep1 and Rep2 proteins. As demonstrated previously, in the absence of either Rep1p or Rep2p, the *STB* plasmid failed to localize to chromosome spreads (Mehta et al., 2002; and unpublished data).

When Ndc10p was inactivated, Cse4p was not associated with centromeres, as assayed by chromatin immunoprecipitation (ChIP; see next section; Fig. 2 E). Furthermore, the authentic kinetochore protein Ctf19p was absent from chromosome spreads under this condition (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200603042/DC1; Ortiz et al., 1999). There is precedent for the mislocalization of Cse4p to inappropriate chromosomal loci. Sharp et al. (2002) observed dispersed Cse4p in chromosome spreads in the *cac1 hir1* background. Consistent with this observation, the inactivation of Spt4p,

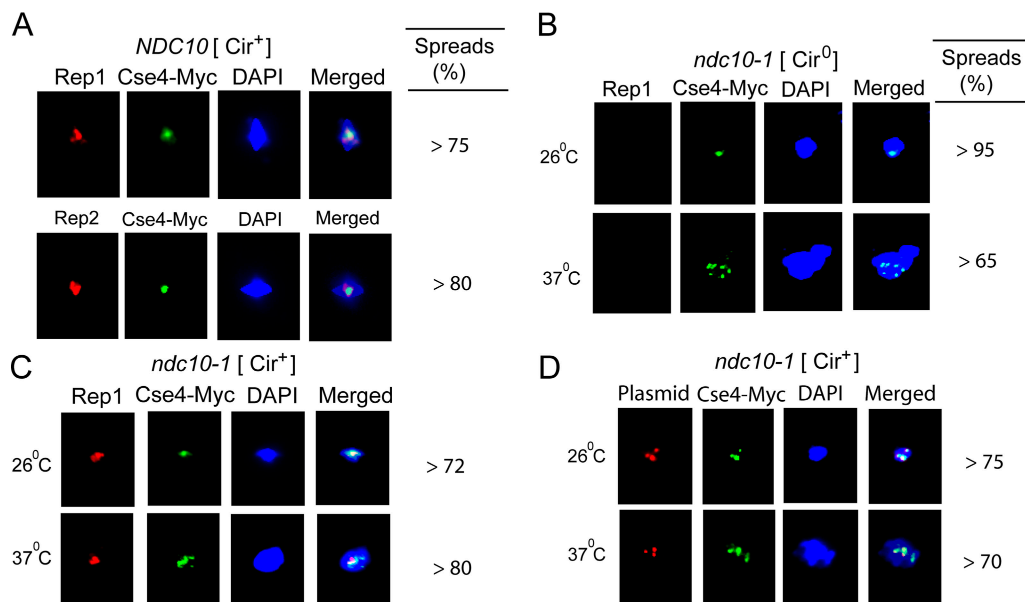


Figure 1. **Localization of Cse4p, Rep proteins, and *STB* reporter plasmid in chromosome spreads.** Chromosome spreads were prepared from wild-type [cir$^+$], *ndc10-1* [cir$^+$], and [cir^0] strains. Rep1p or Rep2p (not depicted) was probed by antibodies to the native protein, and Cse4p was probed by antibodies to the myc epitope harbored by it. The reporter plasmid harboring Lac operator DNA (pSV1; Mehta et al., 2002) was revealed by antibodies to the bound Lac repressor. Secondary antibodies were FITC-conjugated anti–mouse and Texas red–conjugated anti–rabbit antisera. The percentage of spreads (with at least 50 spreads in each assay) that corresponds to a displayed pattern is indicated.

whose localization to kinetochores is dependent on Ndc10p, also causes the mislocalization of Cse4p to noncentromeric locales (Crotti and Basrai, 2004).

One plausible interpretation of the chromosome spread data is that Cse4p, which until now was regarded as a unique centromere marker, is also associated with the 2-μm plasmid. The validity of this interpretation was subjected to further decisive tests as described in the following paragraphs.

Cse4p association with the 2-μm plasmid is specific to *STB* and is dependent on the Rep proteins and spindle integrity

To probe the suspected Cse4p-plasmid association more critically, we performed ChIP in a [cir$^+$] strain expressing myc-tagged Cse4p using myc-directed antibodies. We detected the presence of *STB* DNA in the immunoprecipitate by PCR (Fig. 2 A, first row) but failed to amplify other regions of the 2-μm plasmid (Fig. 2 A, second to fifth rows). At higher inputs of template DNA, a low level of PCR product was observed for the origin region (Fig. 2 A, sixth row) but not for other plasmid loci (not depicted). This background was not unexpected for DNA fragments sheared to a mean length of 500 bp because the origin is located ~340 bp from the proximal end of *STB*. Centromeric DNA was also immunoprecipitated, as exemplified by the amplification of *CEN3* (Fig. 2 A, seventh row). The Cse4p-*STB* association was dependent on both Rep1 and Rep2 proteins (Fig. 2 B). It was positive in a [cir^0] strain harboring an *STB* reporter plasmid and simultaneously expressing the two proteins but was negative in a [cir^0] strain or its derivatives expressing either Rep1p or Rep2p alone (Fig. 2 C).

The Rep1 and Rep2 proteins interact with each other, and each one interacts with *STB* (Ahn et al., 1997; Scott-Drew and Murray, 1998; Velmurugan et al., 1998; Sengupta et al., 2001). At least a subset of these protein–protein and DNA–protein interactions is critical for equal segregation of the 2-μm circle. A Rep1p mutant Rep1(Y43A) that interacts with *STB* but not with Rep2p (Yang et al., 2004) was unable to support Cse4p-*STB* association (Fig. 2 D, second row). In contrast, a second mutant, Rep1(K297Q), that interacts with Rep2p but not *STB* was active in enlisting Cse4p at *STB* (Fig. 2 D, first row).

ChIP analysis has validated the inference from chromosome spread assays (Fig. 1) that an intact kinetochore or Cse4p-*CEN* association is not a prerequisite for Cse4p recruitment at *STB*. Cse4p was detected at *STB* in the *ndc10-1* mutant at both permissive and nonpermissive temperatures (Fig. 2 E, second row), whereas it was absent at *CEN3* under the latter condition (Fig. 2 E, compare the first and second rows).

The nuclear mitotic spindle plays a key role in the clustered organization and precise nuclear localization of the 2-μm circle, cohesin assembly at *STB*, and equal plasmid partitioning (Mehta et al., 2005). We wished to know whether spindle integrity is essential for the acquisition of Cse4p by the plasmid. Depolymerization of the spindle using nocodazole abolished Cse4p-*STB* association, whereas Cse4p-*CEN* association was not affected by this treatment (Fig. 2 F, compare the first and second rows). The incorporation of Cse4p specifically at *STB* within the 2-μm circle genome as well as the requirement of the Rep proteins and an intact spindle for this event implies a potential role for Cse4p in 2-μm circle segregation (see last section of Results).

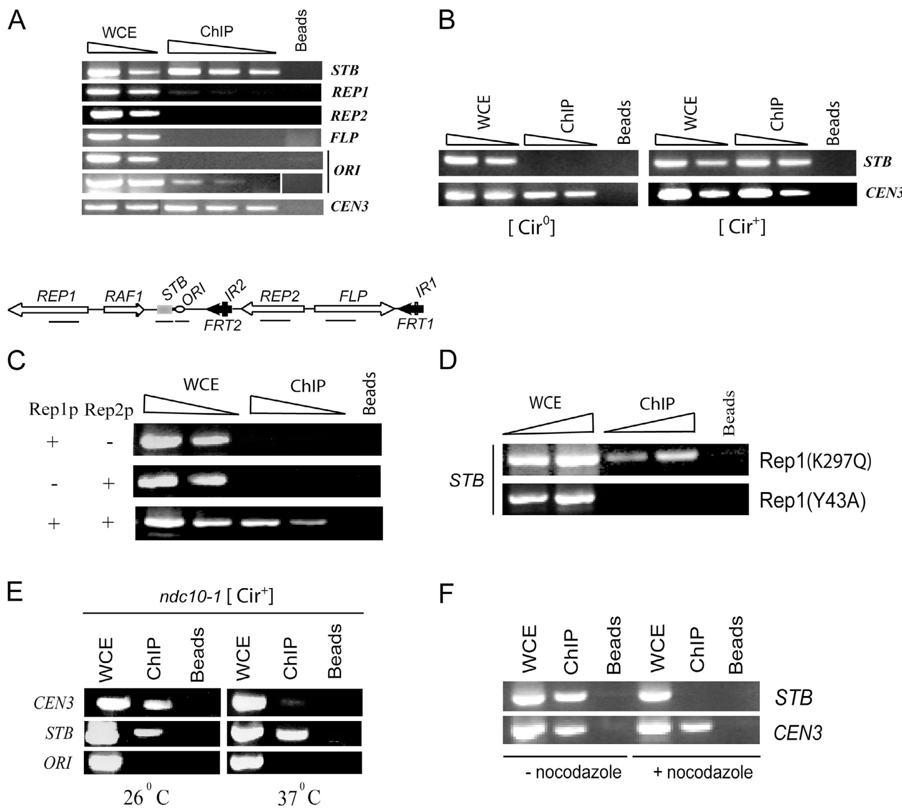


Figure 2. **Localization of Cse4p to *STB* by ChIP.** ChIPs were performed with antibodies to myc-tagged Cse4p, and the precipitated DNA was amplified using primers specific to *CEN3*, *STB*, or *ORI*. In the assays shown in A, in addition to *STB*, other regions of the 2-μm circle genome were probed (horizontal bars in the schematic representation of the plasmid). The PCR products corresponding to *STB* and *ORI* span coordinates 2,969–3,244 and 3,413–3,708, respectively, of the 2-μm circle genome. In the two panels that refer to *ORI*, the bottom one contained twice the amount of input template relative to the top one. The sizes of the PCR products for *REP1*, *REP2*, *FLP*, and *CEN3* are 287, 246, 259, and 222 bp, respectively. The label WCE (whole cell extract) refers to input DNA controls; ChIP indicates experimental samples; and the label beads signifies negative controls.

Cse4p is a component of the nucleosome core at *STB*

Is Cse4p an integral unit of the nucleosome core on which the *STB* chromatin is assembled? We addressed this issue using variations of two assays that were previously used to establish the presence of Cse4p in centromeric nucleosomes. One is based on the ionic strength of buffers required to extract histones out of intact nucleosome oligomers that were isolated from yeast chromatin (Stoler et al., 1995). The other relies on the differential sensitivity of *CEN3* chromatin to the DraI restriction enzyme under conditions in which Cse4p is functional or nonfunctional (Meluh et al., 1998).

In a modified ChIP assay, we pretreated yeast spheroplasts with increasing NaCl concentrations (from 0 to 2.0 M) before performing immunoprecipitations with antibodies directed to myc-tagged Cse4p. As revealed by PCR amplification of the immunoprecipitated DNA, Cse4p association with *STB* could withstand up to 1 M NaCl; at 1.5 and 2.0 M NaCl, *STB* was denuded of Cse4p (Fig. 3 A, first row). The results for *CEN3* were quite similar: Cse4p-*CEN3* association was markedly weakened at 1.5 M NaCl, and it was undetectable at *CEN3* at 2.0 M NaCl (Fig. 3 A, second row). Furthermore, a Western blot analysis of the elution profiles of Cse4p and histone H3 from chromatin were in agreement with the ChIP results (Fig. 3 B). Total release of Cse4p into the supernatant fraction required 1.50 M NaCl; in comparison, H3 release was virtually complete at 1.0 M NaCl.

In the restriction enzyme sensitivity assay, we followed in isolated nuclei the extent of digestion at three DdeI sites located within a PstI–XbaI region of the 2-μm circle genome that covers *STB* (Fig. 3 C). Two of the DdeI sites are harbored within the *ORI*-proximal segment of *STB*, whereas the third one lies just outside of *STB* adjacent to the AvaI site. Nuclei were prepared from two genetically matched yeast strains, one containing *CSE4* and the other containing its temperature-sensitive allele *cse4-107* (Chen et al., 2000), after a 3-h shift to 37°C and were treated with a limiting amount of DdeI for different durations. DNA was isolated, cut completely with PstI plus XbaI, and the composite digestion profiles were displayed by gel electrophoresis and Southern blotting (Fig. 3 D). From the 10–30-min period, the extent of DdeI digestion represented by the abundance of the three lower fragments (632, 508, and 383 bp) relative to the intact PstI–XbaI fragment (1,289 bp) was clearly higher in the *cse4-107* background compared with the wild type (Fig. 3 D). This is graphically represented by ratios of the intensities of

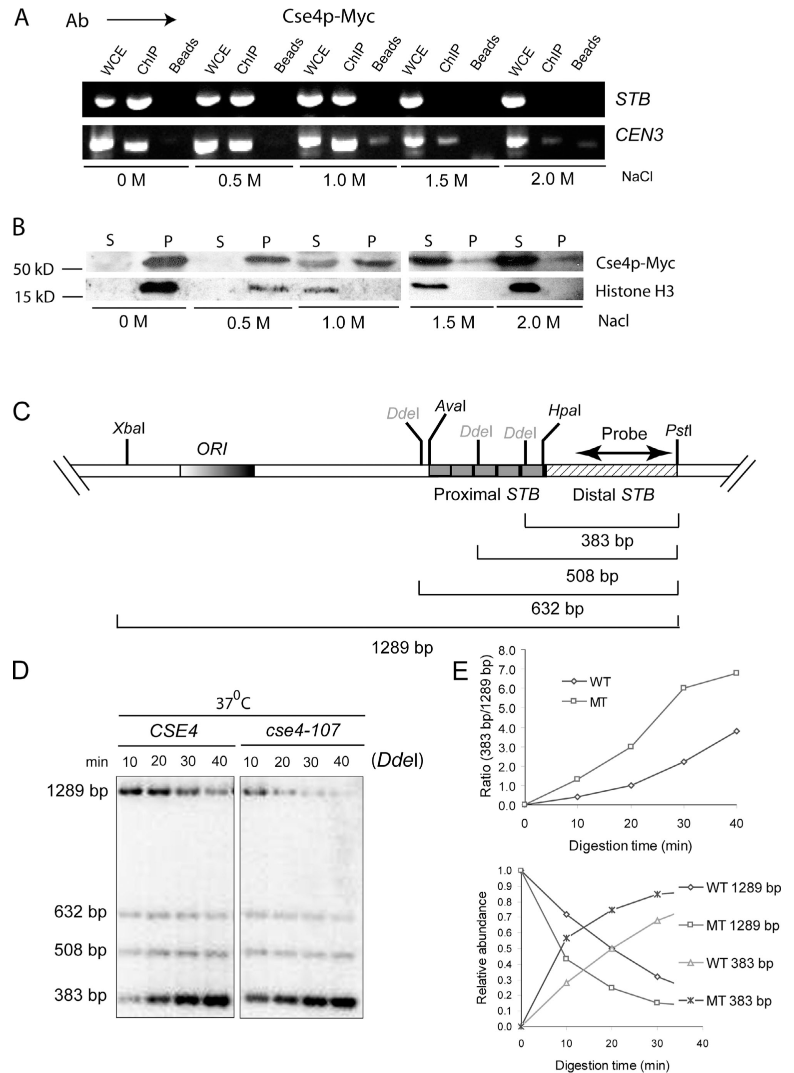


Figure 3. **Cse4p association with *STB* probed by salt extractability of the former and DdeI sensitivity of the latter.** (A) Spheroplasts were treated with indicated salt concentrations before performing ChIP using Cse4p-myc–directed antibodies. The sizes of the PCR products for *STB* and *CEN3* are 275 and 222 bp, respectively. (B) A nuclear pellet preparation was extracted with buffers of increasing ionic strength and separated into pellet (P) and supernatant (S) fractions by centrifugation. Western blots were probed using antibodies to histone H3 and to the myc epitope harbored by Cse4p. Under the electrophoresis conditions used, the myc-tagged Cse4p shows an anomalous mobility above the 50-kD marker (see Fig. 4). (C and D) Nuclei were subjected to partial DdeI digestion followed by DNA isolation and complete PstI plus XbaI digestion. DNA was extracted, fractionated by electrophoresis, and probed with the indicated probes derived from distal *STB*. WT, wild type; MT, mutant.

the 383-bp fragment to those of the 1,289-bp fragment at different time points (Fig. 3 E, top). The same result is also conveyed by plotting the relative abundance of these two fragments with increasing digestion time (Fig. 3 E, bottom). Whereas the half-life of the PstI–XbaI fragment was ∼20 min for the wild-type chromatin under these conditions, it was <10 min for the mutant. This contrast was not detected when a different region of the 2-μm plasmid, namely the *REP1* coding region, was probed by a similar procedure (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200603042/DC1).

As revealed by ChIP, the mutant Cse4 protein was not dislodged, at least not completely, from *STB* or *CEN* after the transfer of cells to the nonpermissive temperature (Glowczewski et al., 2000; and unpublished data). However, centromere function is compromised at the elevated temperature, leading to high rates of chromosome loss (Stoler et al., 1995). The present analysis suggests that the mutant protein, although present at *STB*, cannot support normal nucleosome organization at this locus.

The increased DdeI accessibility of *STB* in the *cse4-107* cells relative to wild type was not caused by differences in their cell cycle stages. The *cse4* mutation triggers the spindle checkpoint, causing the cells to stall in G2/M. However, as revealed by the *cdc20-1* strain, there was little difference in the DdeI digestion patterns at *STB* in cells cycling normally at 26°C or trapped in metaphase at 37°C (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200603042/DC1). The sum of the outcomes from the high-salt ChIP and high-salt protein release assays combined with the DdeI susceptibility analysis strongly argues for Cse4p being an authentic nucleosome component of *STB* chromatin.

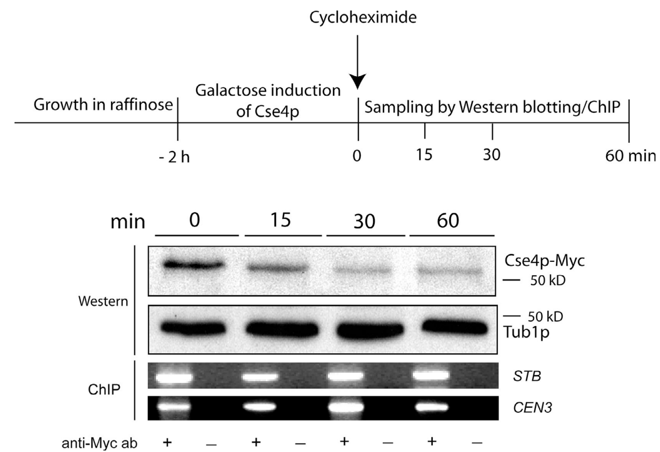


Figure 4. **Depletion of bulk Cse4p after protein synthesis inhibition does not affect Cse4p localized to centromeres or *STB*.** A burst of Cse4p synthesis was induced for a period of 2 h, and translation was arrested by the addition of cycloheximide at time = 0. The cellular pool of Cse4p was assayed by Western blotting, and Cse4p-*STB* and Cse4p-*CEN3* associations were monitored by ChIP. The sizes of the PCR products for *STB* and *CEN3* are 275 and 222 bp, respectively. The loading control for Western blotting was α-tubulin (Tub1p).

Cse4p localized to *STB* is protected from proteolytic degradation

Localization of Cse4p selectively to centromeres is mediated through locale-specific protection of the protein from ubiquitin-mediated proteolysis (Collins et al., 2004). Does a similar protective mechanism apply to Cse4p at the *STB* locus?

We followed Cse4p in wild-type cells, induced for its expression for a 2-h period from the *GAL* promoter, and then treated with cycloheximide by Western blotting and ChIP. The former assay would assess the gross steady state level of Cse4p, whereas the latter would monitor its specific association with *STB* (Fig. 4). The cellular pool of Cse4p was depleted quite rapidly, with a half-life of ∼15 min; however, its association with *STB* was unaffected during the 60-min period of the assay. As expected from a previous study (Collins et al., 2004), Cse4p-centromere association was also independent of the global destruction of the protein. We surmise that the logic of Cse4p localization at the centromeres and *STB* is the same: namely, shielding it against proteolytic degradation.

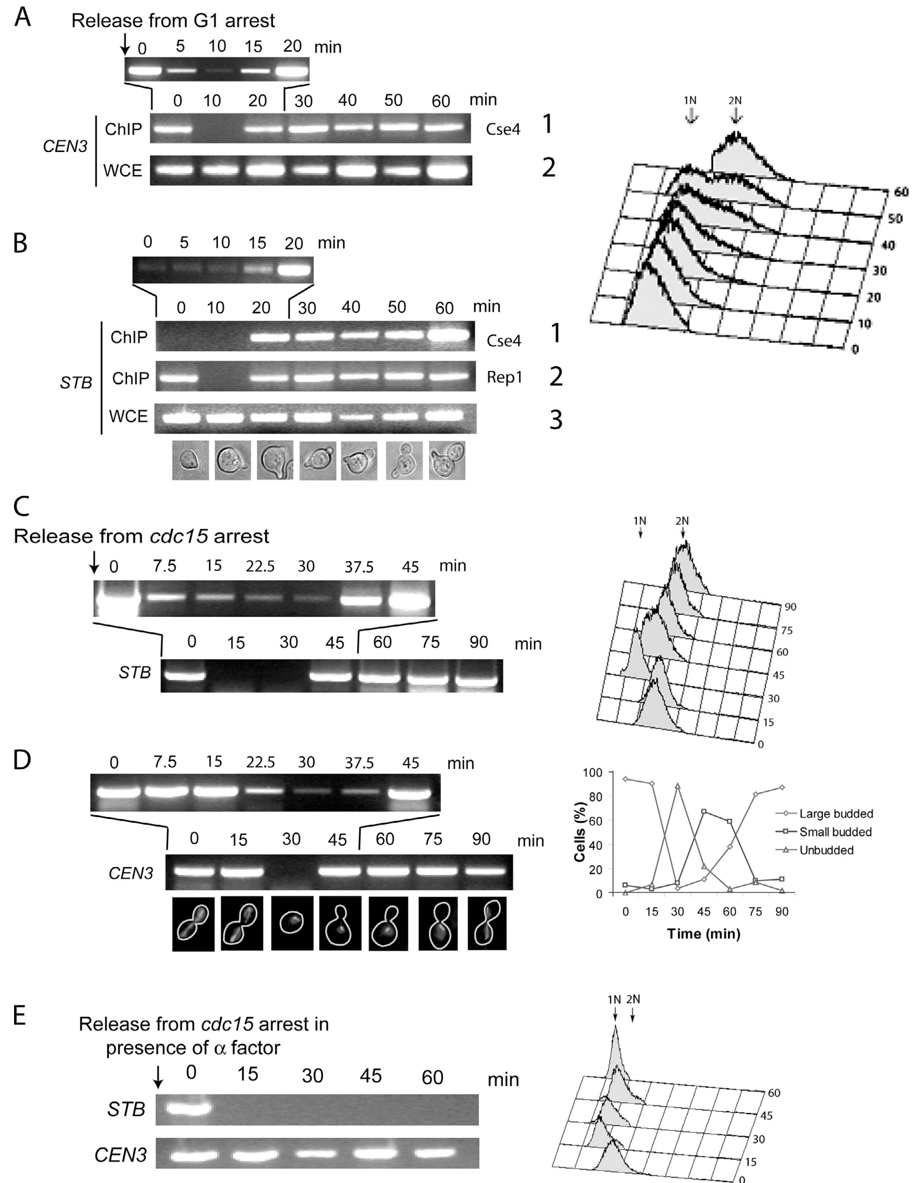
Association of Cse4p with *STB* as a function of the cell cycle

During the yeast cell cycle, newly synthesized Cse4p replaces the old protein at centromeres at the time of DNA replication, as revealed by FRAP analysis (Pearson et al., 2004). Once incorporated, Cse4p remains stable within the centromere chromatin throughout mitosis. The timing of Cse4p exchange presumably coincides with the low frequency orientation switching of sister kinetochores between spindle halves. We have compared the cell cycle dynamics of Cse4p at *STB* and centromeres using a time course ChIP assay.

Consistent with the results of the FRAP analysis (Pearson et al., 2004), we detected the presence of Cse4p at *CEN3* during G1, a brief absence during the G1→S transition, and subsequent stable Cse4p-*CEN3* association (Fig. 5 A, row 1) through the remainder of the cell cycle. The analysis presented in Fig. 5 A followed this association for a period of 60 min after release from G1. In contrast, Cse4p was not present at *STB* in G1-arrested cells (Fig. 5 B, row 1). However, Cse4p-*STB* association was established at the same time during the cell cycle as was Cse4p-*CEN* association (Fig. 5, A and B; row 1; 20 min). Furthermore, this timing matched that of the reassociation of Rep1p (or Rep2p) with *STB* after its ejection from this locus at the time of G1 exit (Fig. 5 B, row 2; and not depicted; Yang et al., 2004). These observations were concordant with very short interval ChIP assays performed from 0 to 20 min with a twofold increase in the template DNA during PCR (Fig. 5, A and B; insets above row 1). After recruitment, Cse4p association with *STB* remained stable until late telophase (see next paragraph).

To examine more carefully the difference between *STB* and *CEN* in their cell cycle–dependent Cse4p association, we followed cells arrested in telophase and released into a synchronous cell cycle (Fig. 5, C and D). Cse4p was absent at *STB* at 15 min after release, which is at the time of spindle disassembly but before the completion of cytokinesis (Fig. 5 C). The dissociation of Cse4p from *CEN* occurred later after cell division had been completed and bud emergence was just about to be initiated in the following cell cycle (Fig. 5 D). Results from ChIP assays with better time resolution in the 0–45-min interval and a twofold higher template input during PCR agreed with this inference (Fig. 5, C and D). When telophase-arrested cells were

Figure 5. **Association of Cse4p with centromeres and *STB* as a function of cell cycle.** Immunoprecipitations were performed with antibodies to myc-tagged Cse4p except in row 2 of B, where native antibodies to Rep1p were used. (A–D) Cells arrested in G1 (A and B) or in telophase (C and D) were released at time = 0, and the presence of Cse4p at *STB* and *CEN3* was assayed by ChIP. The progression of the cell cycle was followed by the cell morphology/budding index and by FACS analysis. The mitotic spindle was visualized by indirect immunofluorescence using antibodies to α-tubulin and Texas red–conjugated secondary antibodies. The short interval ChIP data shown in the top rows of A–D contained a twofold higher input of template DNA during PCR. (E) Cells were arrested in telophase as in C and D but were released into medium containing α factor. The sizes of the PCR products for *STB* and *CEN3* are 275 and 222 bp, respectively. WCE, whole cell extract.



released in the presence of α factor to block cells in G1, Cse4p exit from *CEN* was prevented (Fig. 5 E).

Thus, although the de novo association of Cse4p during the cell cycle occurs coincidentally on yeast chromosomes and the 2-μm circle, the lifetime of the associated state is shorter in the case of the plasmid. The release of Cse4p from *STB* does not occur until well past the completion of plasmid segregation. Consistent with a role for Rep1 and Rep2 proteins in Cse4p recruitment by the plasmid, the cell cycle timing of renewal of the association of all three proteins with *STB* is the same.

Cse4p is required for equal segregation of the 2-μm circle: plasmid localization in chromosome spreads, *STB* remodeling by RSC2, Rep2-*STB* association, and cohesin assembly at *STB*

The centromeric nucleosome containing Cse4p is thought to provide a platform for the organization of the kinetochore complex to initiate the spindle attachment of chromosomes during their segregation (Sullivan, 2001; Smith, 2002). Is equal segregation of the 2-μm plasmid dependent on the presence of a Cse4-containing nucleosome at *STB*?

We assayed the segregation of a fluorescence-tagged *STB* reporter plasmid in wild-type and *cse4-107* cells at 26 and 37°C, respectively. Approximately half of the cells in the *cse4* population at 37°C were in G2/M; the others had escaped the delay induced by the spindle checkpoint. Among the latter cells, plasmid foci were counted in the large budded subpopulation showing distinct DAPI staining zones in the two cell compartments (indicating that nuclear elongation had been accomplished and chromosome segregation had been completed, at least at a gross level; Fig. 6 A). However, because of the *cse4* mutation, a majority of this population (∼75%) showed the clear inequality of DAPI between the cell compartments. An equal number of fluorescent foci in the mother and daughter compartments (4:4 and 3:3, etc.) was scored as normal plasmid segregation.

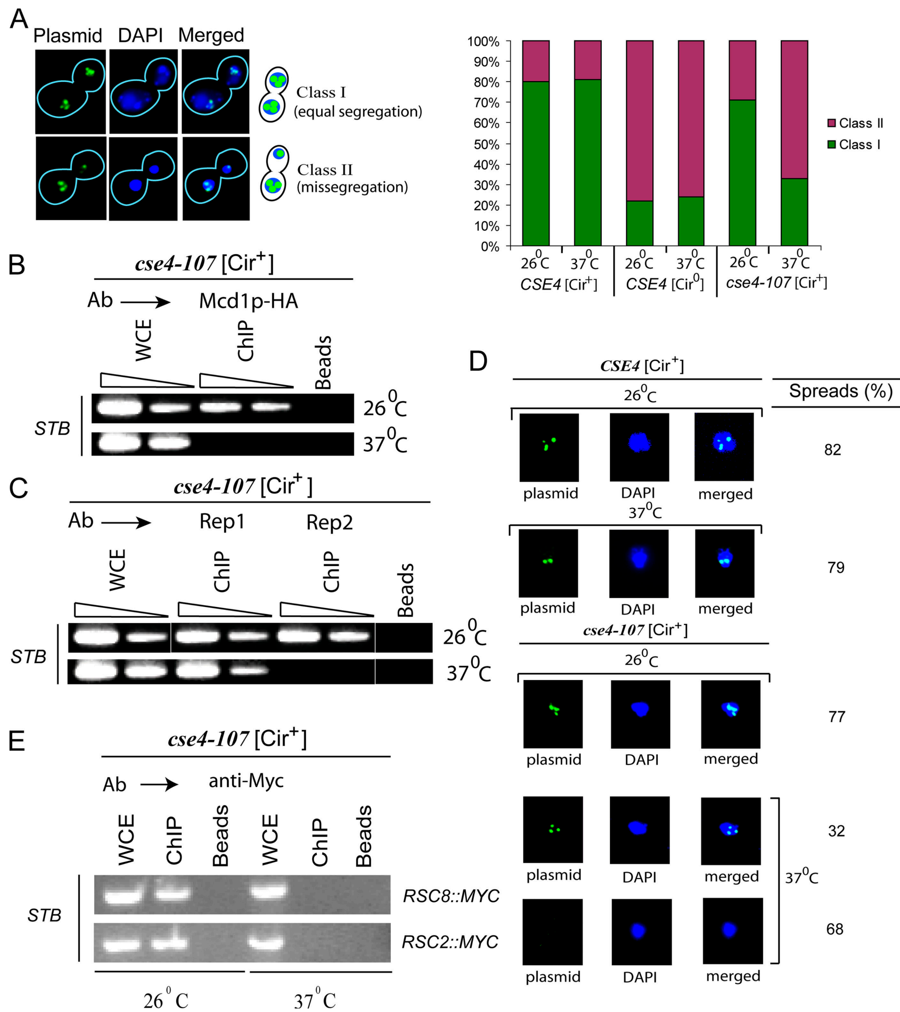


Figure 6. **Effects of cse4-107 mutation on plasmid segregation, cohesin assembly, and binding of Rep proteins at *STB* and plasmid localization in chromosome spreads.** (A) Segregation of the fluorescence-tagged *STB* reporter plasmid was followed in wild-type [cir+] and [cir0] strains and in the *cse4* mutant [cir+] strain. Cultures grown to mid-log phase at 26°C were split into halves and were incubated for a period of 3 h at 26 or 37°C before scoring plasmid segregation. The data were obtained as the means from 150–200 cells for each assay. (B and C) Cohesin recruitment at *STB* was assayed by ChIP using antibodies to the HA epitope carried by the Mcd1 protein. In the ChIP analyses for monitoring Rep1p and Rep2p at *STB*, antibodies to the native proteins were used. The size of the PCR product for *STB* is 275 bp. (D) The *STB* reporter plasmid pSV1 was localized in chromosome spreads (50–100 spreads were scored in each assay) as described in Fig. 1. (E) Rsc2 and Rsc8 proteins were localized at *STB* by ChIP using antibodies to the myc epitope tag harbored by them.

Plasmid missegregation was represented by the collective set of cells showing an unequal number of foci in each compartment. In the mutant strain, the fraction of cells missegregating the plasmid increased from 29% at the permissive temperature to 67% at the nonpermissive temperature, approaching the missegregation rates observed in a [cir0] wild-type strain (lacking the Rep proteins). Complete missegregation (*n*:0) was rarely observed in the *cse4* mutant at the nonpermissive temperature.

As a control, we assayed equal and unequal segregation (1:1 and 2:0, respectively) frequencies of a *CEN* reporter plasmid in the *cse4* background at the permissive and nonpermissive temperatures. Consistent with a role for Cse4p in chromosome segregation, the missegregation rate of the *CEN* plasmid was elevated at 37°C (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200603042). Finally, to circumvent the potential effects caused by cell cycle delay or arrest, we determined plasmid missegregation frequencies in a *cse4-107 mad2Δ* double mutant at 26 and 37°C. The results were not altered significantly by the inclusion of *mad2Δ* (Fig. S4 B).

According to the currently favored model for plasmid segregation, pairing of duplicated plasmid clusters by the cohesin complex assembled at *STB* and their separation by cohesin disassembly later on are critical steps in equal partitioning of the 2-μm circle (Mehta et al., 2002, 2005). Therefore, we wondered whether plasmid missegregation in the *cse4* mutant could result from the failure of cohesin recruitment or maintenance at *STB*. Results of the ChIP analysis shown in Fig. 6 B demonstrate that in the mutant strain, the cohesin component Mcd1p was associated with *STB* at 26°C but not at 37°C.

As pointed out earlier in the paper, the association between the mutant Cse4 protein and *STB* is retained at the nonpermissive temperature even though cohesin recruitment is blocked, and plasmid partitioning is adversely affected under this condition (Fig. 6, A and B). Also note that the acquisition of cohesin by the 2-μm circle absolutely requires the interaction of Rep1 and Rep2 proteins with each other and at least that of Rep1p with *STB* (Yang et al., 2004). We wished to know whether the association of one or both of these proteins with *STB* might be disrupted by the mutant Cse4p. The results of ChIP assays performed in the *cse4-107* strain using antibodies to Rep1p or Rep2p revealed the presence of Rep1p at *STB* at 26 and 37°C; however, Rep2p was not associated with *STB* at 37°C (Fig. 6 C).

One of the attributes of an *STB*-containing plasmid, which appears to be functionally relevant for its normal segregation, is its ability to localize to yeast chromosome spreads (Mehta et al., 2002, 2005). This may potentially represent direct tethering of the plasmid cluster to a chromosome, which is in agreement with a possible hitchhiking model for plasmid segregation

(Mehta et al., 2002). Alternatively, the plasmid cluster and chromosomal domains may share common anchoring points on the nuclear substratum without direct tethering between the two. Regardless, plasmid foci are not detected in the vast majority of chromosome spreads when either of the two Rep proteins is missing or when the integrity of the mitotic spindle is tampered with (Mehta et al., 2002, 2005). Consistent with the inability of the mutant Cse4p to support Rep2p-*STB* association at the nonpermissive temperature, chromosome spreads from the *cse4-107* strain were predominantly bereft of an *STB* reporter plasmid at 37°C (Fig. 6 D). In contrast, a reporter lacking *STB* was not detected in most of the chromosome spreads either at the permissive or nonpermissive temperature (unpublished data).

The yeast chromatin remodeling complex RSC2 is required for establishing the functional chromatin state of the *STB* locus, stable association of Rep1p with *STB*, and acquisition of cohesin by the 2-μm circle (Wong et al., 2002; Huang et al., 2004; Yang et al., 2004). Consistent with these observations, the plasmid loss rate becomes highly elevated in an *rsc2Δ* background (Wong et al., 2002). It is plausible that remodeling of the *STB* chromatin by the RSC2 complex takes place only in the context of Cse4p-containing nucleosomes. To test this idea, we assayed the association of the RSC2 components Rsc2p and Rsc8p with *STB* after the inactivation of Cse4p. As shown by the ChIP results in Fig. 6 E, both proteins were associated with *STB* in the *cse4-107* background at 26°C, but neither one was associated at 37°C.

The effects of Cse4p inactivation on *STB* association with the cohesin subunit Mcd1p, Rep proteins, and components of the RSC2 remodeling complex (Fig. 6, B, C, and E) are not caused by the relatively large fraction (nearly 50%) of G2/M cells in the *cse4-107* population at 37°C. Our previous study (Mehta et al., 2002) demonstrated that Mcd1p association with *STB* lasts from S phase until anaphase. We also found that the Rep proteins are associated with *STB* throughout the cell cycle except during the window between the late G1 and early S phase (Yang et al., 2004). We have shown that both Rsc2p and Rsc8p are associated with *STB* in *cdc20-1* cells arrested in metaphase at 37°C (Fig. S4 C). The significant fraction of metaphase cells in the *cse4-107* population at 37°C should have displayed Mcd1p, Rep2p, Rsc2p, and Rsc8p at *STB* if Cse4p is not required for their recruitment/maintenance. Finally, even when the metaphase delay was relieved in the *cse4-107 mad2Δ* host, Rsc2p associated with *STB* at 26°C but failed to do so at 37°C (Fig. S4 D).

Thus, when Cse4p is inactivated, the Rep2 protein does not associate with *STB*, the 2-μm circle is not displayed in chromosome spreads, and cohesin assembly at *STB* is blocked. At least part of the effects of inactivating Cse4p is manifested through the lack of functional remodeling of the *STB* chromatin by the RSC2 complex. In a previous study, we noticed that it is the Rep1 (and not Rep2) protein that fails to associate with *STB* in the *rsc2Δ* background (Yang et al., 2004). Overall, these observations suggest that before RSC2-mediated remodeling, the distinct states of *STB* organization conferred by wild-type Cse4p and its mutant counterpart exclude Rep1p and Rep2p, respectively, from occupying this locus. It is only the remodeled *STB* chromatin containing functional Cse4p that can nucleate the stable tripartite Rep1p–*STB*–Rep2p interactions required for faithful plasmid segregation.

Discussion

The Cse4 protein in *Saccharomyces cerevisiae* belongs to the family of histone H3-like proteins (CenH3s) that is conserved throughout the eukaryotic kingdom and is characterized by a common C-terminal histone fold domain and a divergent N-terminal domain (Keith et al., 1999; Chen et al., 2000; Morey et al., 2004). Cse4p substitutes for histone H3 at the point centromeres of yeast chromosomes (Meluh et al., 1998; Keith and Fitzgerald-Hayes, 2000) and is essential for assembly of the kinetochore complex. Our current data argue that a Cse4p-containing nucleosome core also designates an extrachromosomal element for faithful segregation during the yeast cell cycle. The partitioning complexes assembled at loci marked by this specialized chromatin may be quite distinct from each other (for example, the rather extensively characterized kinetochore complex at centromeres as opposed to the as yet poorly understood complex constituted by Rep1p–Rep2p–host factors [the number or types of the host factors involved in this complex is unknown] at *STB*; McAinsh et al., 2003; Jayaram et al., 2004). However, the pathways that emanate from them may point to a common theme for equal segregation; namely, distributing the products of DNA replication through a cohesin-mediated binary counting mechanism.

The *STB* chromatin is assembled on a Cse4p-harboring nucleosome core

By applying the criteria of salt extractability and restriction enzyme sensitivity, we conclude that Cse4p is associated with *STB* as a bona fide nucleosome component. Analogous to Cse4p deposited at centromeres, that resident at *STB* is also selectively protected from ubiquitin-mediated proteolysis. The presence of Cse4p-containing chromatin at *STB* from early S phase to well beyond anaphase during the cell cycle is consistent with a functional role for this protein in 2-μm circle partitioning (Fig. 7). Such a role is compatible with similarities in the dynamics and mechanism between chromosome segregation and 2-μm circle segregation (Velmurugan et al., 2000; Mehta et al., 2002). However, the recruitment of Cse4p at *STB* is not functionally coupled to its recruitment at centromeres. A kinetochore mutation (*ndc10-1*) does not impair the association of Cse4p with *STB*, and, conversely, Rep1 and Rep2 proteins have no role in the incorporation of Cse4p at centromeres. Rather, the 2-μm plasmid has evolved the independent capability to earmark its partitioning locus with a chromatin architecture that on chromosomes is strictly confined to centromeres.

Recruitment of Cse4p and dynamics of the Rep proteins at *STB*

Although the interaction between Rep1p and Rep2p is critical for the incorporation of Cse4p into *STB*, that between Rep1p and *STB* is dispensable. When the *STB* chromatin harbors a mutant version of Cse4p, Rep2p-*STB* association is disrupted with

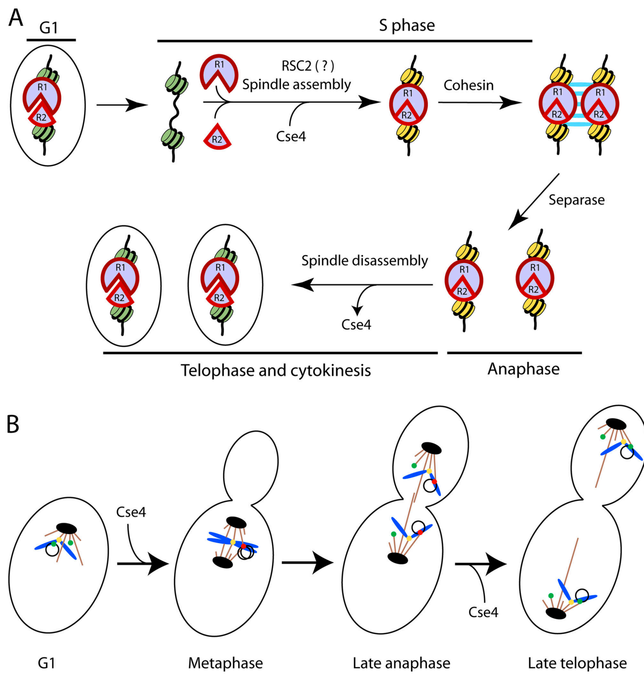


Figure 7. **Cse4p-containing chromatin at *STB* is a prerequisite for equal segregation of the 2-μm plasmid.** (A) Cycling of Rep proteins and Cse4p incorporation at *STB* during G1→S transition, which is likely followed by RSC2-mediated remodeling of *STB* chromatin, are postulated to be key events in establishing the plasmid partitioning complex. After plasmid segregation, Cse4p is released from *STB* during late telophase at spindle disassembly. Cse4p-containing nucleosomes and histone H3-containing nucleosomes are distinguished by coloring them yellow and green, respectively. The associations of Rep proteins with these two chromatin states are thought to be functionally distinct and, thus, are depicted differently. We do not know whether the release of Cse4p from an *STB* nucleosome is accompanied by its replacement with histone H3. (B) Current and previous results can be accommodated by a model in which a Rep1p–Rep2p-containing prepartitioning complex (green nodule) associated with microtubules mediates the capture of the plasmid cluster (drawn as a ring). Establishment of the mature partitioning complex (red nodule) at *STB* occurs concomitant with DNA replication, and further downstream events follow as depicted in A. For comparison, a chromosome (blue) attached to the spindle via the kinetochore (yellow) is also shown.

no obvious adverse effect on Rep1p-*STB* association. Furthermore, this defective chromatin cannot be targeted by the RSC2 complex for remodeling into its functional state. These observations would be consistent with the following sequence of events for the recruitment of Cse4p at *STB* during a normal cell cycle (Fig. 7 A). Recall that Cse4p is absent at *STB* during G1 (this study), whereas both Rep1p and Rep2p are associated with it (Yang et al., 2004). During G1→S transition, the Rep proteins transiently exit *STB* and then reassociate with it. Cse4p may be delivered to *STB* by the incoming Rep1p–Rep2p or a complex containing these proteins. The assimilation of Cse4p may be facilitated by *STB* being at least partially stripped of bound proteins at this stage. Assembly of the functional form of the Cse4p-containing chromatin, perhaps with assistance from the RSC2 complex (Wong et al., 2002; Hsu et al., 2003; Huang et al., 2004), may sanction the more long-lived interactions between Rep1 and Rep2 proteins with *STB* (Mehta et al., 2002; Yang et al., 2004). The downstream steps of the plasmid segregation pathway may then be set in motion. According to this model, it is the flawed chromatin structure of *STB* as a result of the mutant Cse4p that prevents stable Rep2p-*STB* association. A less attractive possibility is that the mutation present in Cse4-107p renders its incorporation into *STB* independent of Rep2p.

A role for the mitotic spindle in assembling the plasmid partitioning complex

We have now discovered that cohesin-*STB* association is provisory to Cse4p-*STB* association, which, in turn, is disrupted by spindle depolymerization. It is likely that the assembly of the plasmid partitioning complex has parallels to kinetochore assembly in being intricately linked to the dynamics of the mitotic spindle (Fig. 7 B). New functional roles for the centromere-binding complex CBF3 in anaphase spindle stability, cytokinesis, and kinetochore attachment have recently come to light (Bouck and Bloom, 2005; Gillis et al., 2005). CBF3 is transported to the spindle midzone during anaphase, localizes to the microtubule plus ends, and retains this position during spindle disassembly. It has been suggested that the CBF3 complex associated with the growing and regressing microtubule plus ends in G1 might provide a prekinetochore structure, which could mature after the "search and capture" of a chromosome by its centromere into a full-fledged kinetochore in the ensuing cell cycle.

The Rep1 and Rep2 proteins, which are primarily localized in proximity to the spindle pole, show a decreasing concentration gradient toward the spindle midzone (Velmurugan et al., 2000). By analogy to the CBF3 complex, it is plausible that the spindle-associated Rep proteins are constituents of a prepartitioning complex that engages the 2-μm plasmid by search and capture (Fig. 7 B). This association may be mediated by a matchmaker mechanism involving interactions between the Rep proteins localized to the spindle on one hand and to *STB* on the other. We showed previously that spindle integrity is not essential for the association of Rep1p or Rep2p with *STB* (Mehta et al., 2005). We imagine that nucleation of the Cse4p-containing chromatin at *STB* and the subsequent events in partitioning, including cohesin recruitment, take place in the spindle-associated state of the plasmid.

Changes at the microtubule plus ends that accompany the dissolution of the spindle midzone are likely responsible for the release of Cse4p from *STB* before cytokinesis. In the budding yeast, by the time the mitotic spindle breaks down, the plus end–directed motor proteins Cin8p and Kip1p are depleted by proteolytic degradation (Gordon and Roof, 2001; Hildebrandt and Hoyt, 2001). Alterations in spindle composition may slacken the high-order protein organization at *STB* and program the dissociation of Cse4p from it. Consistent with this notion, preliminary results demonstrate that the localization of Cse4p at *STB* requires Kip1p (unpublished data).

The 2-μm plasmid partitioning locus: a *CEN* impostor?

The centromere in the budding yeast is assembled from three conserved DNA elements: centromere DNA element (CDE) I, CDEII, and CDEIII. Interactions of the CBF1 and CBF3 complexes with CDEI and CDEIII, respectively, together with

bridging interactions between the two complexes are thought to nucleate the chromatin structure at *CEN* that includes the specialized nucleosome-containing Cse4p (McAinsh et al., 2003). There is some debate over whether the CDEI–CDEII–CDEIII DNA itself is wrapped around the core histone octamer (Meluh et al., 1998; Keith and Fitzgerald-Hayes, 2000) or merely facilitates the assembly of phased Cse4p-containing nucleosomes on either side of it through bound Ndc10p, CBF1, and CBF3 (Espelin et al., 2003). In contrast to *CEN* DNA, the *STB* locus has a bipartite organization: an origin-proximal segment comprising repeats of a consensus element and an origin-distal segment harboring a strong transcription terminator. The distal *STB* element also contains a transcription silencer that can turn down the activity of an upstream promoter (Grunweller and Ehrenhofer-Murray, 2002). Furthermore, *STB* (comprising the proximal and distal segments) can substitute for the native silencing element in conferring transcriptional repression on the silent mating type locus *HML* (Papacs et al., 2004). This silencing activity of *STB* is at least partially dependent on the Rep proteins and cohesin complex and additionally requires the Sir2-4 proteins (Papacs et al., 2004). The only detectable similarity between *CEN* and *STB* is the adenosine-thymidine richness of CDEII and the *STB* repeat element. Even so, although the adenosine-thymidine content of CDEII is 86–98% (Baker and Rogers, 2005), that of the *STB* element is only ∼62–63%.

Despite their distinct organizational features, *CEN* and *STB* arrange similar chromatin environments that use Cse4p as a nucleosome component. The functional states of both of these elements require that they be maintained transcription free (Broach et al., 1979; Panzeri et al., 1984; Sutton and Broach, 1985). They define the sites for the organization of specialized, though distinct, partitioning complexes and serve as assembly zones for the yeast cohesin complex. The RSC2 complex promotes chromatin organization not only at *STB* but also at centromere-proximal regions; RSC2 inactivation gives rise to chromosome missegregation and high plasmid loss rates (Wong et al., 2002; Hsu et al., 2003; Huang et al., 2004).

The building/maintenance of centromeric chromatin in budding yeast is facilitated by nucleosome assembly factors such as CAF1 and the Hir proteins (Sharp et al., 2002). A lack of these functions disturbs stable kinetochore–spindle interactions, impairs the spindle checkpoint, and causes increased rates of mitotic chromosome missegregation (Sharp et al., 2002; Sharp and Kaufman, 2003). It would be interesting to see whether these factors also contribute to the integrity of the chromatin structure at *STB* and thereby promote stable plasmid maintenance. The aforementioned studies demonstrate that the ability to program centromere-like chromatin architecture into its partitioning locus is at the heart of the high-fidelity segregation of the yeast plasmid.

Materials and methods

Strains and plasmids

The yeast strains and plasmids used in this study are listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200603042/DC1). The reporter plasmids containing Lac operator arrays has been described previously (Velmurugan et al., 2000; Mehta et al., 2002; Yang et al., 2004). Synchronized cell populations were obtained by α-factor arrest in G1 phase followed by release as described previously (Velmurugan et al., 2000). Cell cycle arrest at telophase was affected by shifting log phase cultures of the *cdc15-2* mutant strain from 26 to 37°C for 2.5 h.

ChIP

The majority of ChIP analyses were performed essentially as described previously (Mehta et al., 2002; Yang et al., 2004). After immunoprecipitation, the beads were washed five times at room temperature as follows: twice with 1 ml of immunoprecipitation wash buffer I (50 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, and 1% Triton X-100) for 5 min each, once with 1 ml of immunoprecipitation wash buffer II (50 mM Hepes-KOH, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, and 1% Triton X-100) for 5 min, and once with 1 ml of immunoprecipitation wash buffer III (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% sodium deoxycholate, and 0.5% NP-40) for 5 min. Finally, the beads were washed with 1 ml Tris-EDTA for 10 min at room temperature. The additional washing steps resulted in a particularly clean PCR background in the negative samples even though there was some loss of signal in the positive ones. Serial dilutions of the template DNA were used in PCR reactions to verify that the yield of the amplified DNA was in the linear range with respect to the input template.

For ChIP assays at the nonpermissive temperature, log phase cultures of *cse4-107*, *ndc10-1*, and *cdc15-2* strains grown at 26°C were preincubated at 37°C before performing formaldehyde cross-linking at this temperature. The durations of thermal shift were 3 h for *cse4-107* and *ndc10-1* and 2.5 h for *cdc15-2*.

In assays designed to test the effect of high salt on Cse4p association with chromatin, the ChIP protocol was modified as follows. Exponentially growing yeast cells were harvested and washed once with spheroplasting buffer (1 M sorbitol and 0.1 M KPO_4, pH 7.4). They were resuspended in the same buffer containing 70 mM β-mercaptoethanol and 50 μg/ml 100T zymolyase (U.S. Biological) and incubated at 30°C for 60 min. The spheroplasts were harvested and treated with various salt concentrations (0–2.0 M NaCl) in spheroplasting buffer for 1 h at 4°C. They were collected by centrifugation, washed, resuspended in spheroplasting buffer containing 1% formaldehyde, and carried through further steps of ChIP (Mehta et al., 2002; Yang et al., 2004).

For probing Cse4p-chromatin association, ChIP assays were performed with antibodies to the myc epitope using a yeast strain that harbors an engineered *CSE4* gene expressing myc-tagged Cse4p (Buvelot et al., 2003). In the ChIP assays shown in Fig. 6 B, antibodies to the HA epitope tag present on the Mcd1 protein were used. Antibodies against native Rep1 and Rep2 proteins were used for monitoring their association with the *STB* DNA. For detecting Rsc2p and Rsc8p at *STB*, antibodies to the myc epitope harbored by them were used.

High-salt extraction of proteins from yeast nuclei

Nuclei were isolated according to Nelson and Fangman (1979) and were extracted with high salt as described by Mirzayan et al. (1992). After resuspending nuclei in 10% Ficoll, 20 mM phosphate buffer (potassium as counter ion), pH 6.5, 0.5 mM $MgCl_2$, and protease inhibitors (Complete Mini; Roche), they were treated for 30 min on ice with 3 vol of extraction buffer (0.5 M sucrose, 20 mM potassium phosphate, pH 6.5, 0.5 mM $MgCl_2$, 1% Triton X-100, and protease inhibitors) that yielded a final concentration of 0–2.0 M NaCl. The samples were spun at 16,000 *g* for 30 min at 4°C. The pellet proteins and trichloroacetic acid–precipitated supernatant proteins were fractioned by 10–15% SDS-PAGE in preparation for Western blotting. Cse4p fused to the myc epitope was detected by the same antibodies as those used in the ChIP experiments. Histone H3 was detected using a polyclonal antiserum.

Chromatin accessibility assay

Restriction endonuclease susceptibility of intact chromatin was assayed using modifications of the procedure outlined by Nelson and Fangman (1979). The wild-type and *cse4-107* mutant cells were grown to early to mid-log phase at 26°C and were shifted to 37°C for 3 h. Cells were harvested, and nuclei were isolated as described previously (Nelson and Fangman, 1979). These nuclei were resuspended in NEbuffer 3 (New England Biolabs, Inc.) and treated with DdeI at 50 U/ml for various times. The nuclease-digested samples were subsequently deproteinized, and DNA extracted from them was digested to completion using XbaI plus PstI or XbaI and PvuI. After electrophoresis in agarose gels, DNA was transferred to Hybond-XL membrane (GE Healthcare) and hybridized to specific radiolabeled probes. Bands were visualized using a phosphorimager

(Molecular Imager FX; Bio-Rad Laboratories), and individual band intensities were quantitated using Quantity One software (Bio-Rad Laboratories). Similar assays were performed in the *cdc20-1* strain using cells from a mid-log phase culture at 26°C and those shifted to 37°C for 3 h.

Antibodies

Antibodies against the myc epitope (9E10) and HA epitope (HA.11) were obtained from Covance. Polyclonal antibodies against histone H3 and the HA epitope were purchased from Abcam and Sigma-Aldrich, respectively. All other antibodies used were described previously (Velmurugan et al., 2000).

Other protocols

The preparation of chromosome spreads, fluorescence microscopy of reporter plasmids, and indirect immunofluorescence assay of microtubules have been described previously (Velmurugan et al., 2000; Mehta et al. 2002). For obtaining chromosome spreads at the nonpermissive temperature, *cse4-107* and *ndc10-1* cultures grown at 26°C were preincubated at 37°C for 3 h. Observations of live or fixed cells and chromosome spreads was performed using a microscope (BX-60; Olympus). The images were captured with a camera (Photometrics Quantix; Roper Scientific) and were processed with MetaMorph software (Universal Imaging Corp.).

Online supplemental material

Table S1 provides a list of yeast strains and plasmids used in this study. Fig. S1 shows the localization of Cse4p and Ctf19p in chromosome spreads in the *ndc10-1* strain. Fig. S2 shows that chromatin integrity within the *REP1* locus is not affected by the inactivation of Cse4p. Fig. S3 shows the accessibility of *STB* chromatin in cycling and metaphase-arrested cells probed by sensitivity to DdeI. Fig. S4 shows the segregation of centromere and 2-μm reporter plasmids in the *cse4-107* background as well as the association of Rsc2 and Rsc8 with *STB* in *cdc20-1* and *cse4-107 mad2Δ* strains. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200603042/DC1.

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