

Cse4 (CenH3) Association with the *Saccharomyces cerevisiae* Plasmid Partitioning Locus in Its Native and Chromosomally Integrated States: Implications in Centromere Evolution^{∇†}

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The histone H3 variant Cse4 specifies centromere identity in *Saccharomyces cerevisiae* by its incorporation into a special nucleosome positioned at *CEN* DNA and promotes the assembly of the kinetochore complex, which is required for faithful chromosome segregation. Our previous work showed that Cse4 is also associated with the partitioning locus *STB* of the 2 μ m circle—a multicopy plasmid that resides in the yeast nucleus and propagates itself stably. Cse4 is essential for the functional assembly of the plasmid partitioning complex, including the recruitment of the yeast cohesin complex at *STB*. We have located Cse4 association strictly at the origin-proximal subregion of *STB*. Three of the five directly repeated tandem copies of a 62-bp consensus sequence element constituting this region are necessary and sufficient for the recruitment of Cse4. The association of Cse4 with *STB* is dependent on Scm3, the loading factor responsible for the incorporation of Cse4 into the *CEN* nucleosome. A chromosomally integrated copy of *STB* confers on the integration site the capacity for Cse4 association as well as cohesin assembly. The localization of Cse4 in chromatin digested by micrococcal nuclease is consistent with the potential assembly of one Cse4-containing nucleosome, but not more than two, at *STB*. The remarkable ability of *STB* to acquire a very specialized, and strictly regulated, chromosome segregation factor suggests its plausible evolutionary kinship with *CEN*.

The 2 μ m plasmid of *Saccharomyces cerevisiae* is an example of a highly optimized, circular, multicopy extrachromosomal selfish DNA element (25, 27, 49). The plasmid does not seem to contribute to the host's fitness under standard laboratory growth conditions. However, at its steady-state copy number of 40 to 60 molecules per cell, any growth disadvantage imposed by the plasmid is rather small (16, 30). The most remarkable attribute of this selectively almost neutral entity is its ability to propagate with nearly chromosome-like stability at its steady-state copy number. The entire genetic makeup of the 2 μ m circle is devoted to three functions: efficient replication by the host machinery, equal segregation, and maintenance of the copy number. The plasmid accomplishes these goals with minimal metabolic encumbrance to its host.

Direct visualization of fluorescence-tagged reporter plasmids in live cells suggests that 2 μ m circle molecules are organized in the nucleus as 3 to 5 dynamic foci that form a close-knit cluster (47). The plasmid also segregates as a clustered entity; sister clusters part from each other and move away at the anaphase stage of the cell cycle. Population analysis and time lapse assays have revealed close similarities between the 2 μ m circle and the yeast chromosomes or a centromere plasmid (minichromosome) in their dynamics and kinetics of segregation (17, 47). The relevant inference from a variety of

experiments is that plasmid segregation is tightly coupled to chromosome segregation, perhaps by attachment of duplicated plasmid clusters to a pair of sister chromatids (17, 31, 47).

Because of the large reduction in the effective copy number caused by clustering, efficient plasmid segregation is dependent on an active partitioning system comprising two plasmid proteins, Rep1 and Rep2, and the *cis*-acting locus *STB*. The Rep-*STB* system overcomes the mother bias to which *ARS* plasmids, replication competent but lacking partitioning machinery, are subjected (34). Available evidence suggests that negation of mother bias and coupling of plasmid segregation to chromosome segregation are manifestations of the same underlying mechanism. A number of mutations that affect the fidelity of chromosome segregation cause the 2 μ m circle to missegregate in tandem with the chromosomes (31, 47). This chromosome-coupled behavior is abrogated by inactivation of either or both of the Rep proteins or by deletion of the *STB* locus.

The 2 μ m plasmid also harbors an amplification system, consisting of the Flp site-specific recombinase and its target sites (*FRTs*) (31), which can correct a drop in the copy number resulting from rare missegregation events. A Flp-mediated DNA inversion event during bidirectional replication, changing the direction of one fork with respect to the other and thus preventing termination, is believed to be the basis for DNA amplification (15, 39, 48). The amplified DNA may be resolved into individual plasmid copies by Flp-mediated or homologous recombination. A fourth plasmid-encoded protein, Raf1, a positive regulator of *FLP* expression, ensures that the amplification response, when required, is rapidly triggered (36, 42). A combination of negative and positive regulatory circuits involving Rep1, Rep2, and Raf1 serves to minimize deviations of the plasmid copy number from the steady-state value. Recent

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evidence suggests that modification of Flp by the host SUMO attachment system is important in preventing aberrant amplification of the 2 μ m circle (6, 51).

A number of observations suggest that the 2 μ m plasmid partitioning system channels chromosome segregation factors into the plasmid segregation pathway (17, 18, 20, 31, 32, 52). This partitioning system assists the plasmid in utilizing the mitotic spindle and the spindle-associated Kip1 motor to localize to its partitioning center in the nucleus (10). The *STB* chromatin is associated with the centromere-specific histone H3 variant Cse4p (CenH3), and the functional state of *STB* is established by the RSC2 chromatin-remodeling complex (20, 23, 50). The yeast cohesin complex is assembled at *STB* in a Rep1- and Rep2-dependent manner and mediates the bridging of sister plasmid clusters harboring duplicated plasmid copies (17, 18, 31). The disassembly of cohesin during anaphase is essential for the segregation of sister clusters into daughter cells (31). Assays using single-copy derivatives of *STB* reporter plasmids suggest that plasmid pairing by cohesin occurs in a sister-to-sister fashion and that sisters are dispatched in opposite directions upon the dissolution of the cohesin bridge (17, 18). Thus, despite the multicopy nature of the plasmid, there must be a high level of organization within the plasmid cluster to coordinate DNA replication with sister plasmid pairing.

The association of Cse4, presumed to be an exclusive component of centromeric nucleosomes, with the partitioning locus of an extrachromosomal and nonessential entity such as the 2 μ m plasmid is quite intriguing. Our present analyses demonstrate the authenticity, specificity, and *CEN* independence of Cse4-*STB* association and locate it in the origin-proximal region of *STB* (*STB*-proximal) containing five tandem direct copies of an AT-rich 62-bp consensus element. The loading factor for Cse4 at *CEN*, Scm3, interacts with *STB* in a metastable fashion. Depletion of Scm3 causes abrogation of the Cse4-*STB* association. A chromosomally integrated copy of *STB* mimics a plasmid-borne *STB* locus in its association with Cse4. The site of integration, which does not recruit cohesin in its native state, becomes competent for cohesin assembly. Our results, *in toto*, are consistent with a common evolutionary ancestry for the point centromere of budding yeast and the 2 μ m plasmid partitioning locus (29).

MATERIALS AND METHODS

Plasmids. The plasmids employed as reporters or for integration into a chromosomal site are described below.

(i) **Plasmid pSG5.** The construction of the multicopy *STB* reporter plasmid pSG5, used in several of the pulldown assays, has been described previously (18). This plasmid, containing the 2 μ m replication origin and *STB* along with the *TRP1* marker, was composed solely of yeast sequences.

(ii) **Plasmid pCH5.** In the multicopy *STB* reporter plasmid pCH5, the origin-proximal *AvaI*-*HpaI* *STB* segment was flanked by four *BamHI* sites adjacent to the *AvaI* site and four *BglII* sites adjacent to the *HpaI* site. The template for the construction of pCH5 was pSG5-1 (18), which was also the immediate precursor for pSG5 (see the preceding section). First, the entire pSG5-1 plasmid was amplified as a linear fragment using two oppositely oriented primers spanning the *HpaI* site, one of which contained four tandem copies of the *BglII* recognition sequence. The amplified DNA was cut with *HpaI* and was self-ligated to generate plasmid pCH5-1, retaining the *BglII* sites. Next, pCH5-1 was amplified in its full-length linear form by using two inverse primers spanning the *AvaI* site, one of which contained four iterations of the *BamHI* recognition sequence. Both primers contained, in addition, a copy of the *SmaI* recognition sequence, such that *SmaI* digestion and self-ligation generated pCH5-2, harboring four *BamHI* sites next to *AvaI*. Digestion of pCH5-2 by *Sall* and self-ligation generated

pCH5, from which all nonyeast sequences were eliminated. After the transformation of yeast with the ligation mixture, *Trp*⁺ transformants were screened by PCR and Southern blot analysis of total DNA to identify those that had acquired pCH5.

(iii) **The pSTB₂₋₅ series of plasmids.** The series of four plasmids comprising pSTB₂ to pSTB₅ (pSTB₂₋₅ plasmids) contained, in addition to the 2 μ m circle origin, two, three, four, and all five copies, respectively, of the 62-bp repeat element that is the building block for *STB*-proximal. The repeat elements were present in these plasmids in their native order, starting with the repeat element closest to the origin. The steps in their construction were as follows. The *STB*-proximal plus *ORI* fragments were amplified using a fixed primer adjacent to the origin and distinct individual primers, within or at the far border of *STB*-proximal, marked by an *HpaI* site. The primer pairs were designed to contain *BamHI* recognition sequences adjacent to their annealing regions. Because of the repeated nature of the *STB* region that served as the template for PCR, the amplified DNA contained, in addition to the authentic product, extra products due to primer annealing at secondary sites. Amplification reaction products were fractionated by agarose gel electrophoresis, and DNA bands of the expected sizes were isolated and purified by chloroform-phenol extraction and ethanol precipitation. The *TRP1* marker without its associated *ARS1* sequence was amplified from a template plasmid using a pair of primers both of which contained a *BamHI* recognition sequence at their nonannealing termini. Each of the *STB* fragments was digested with *BamHI* and was ligated to a similarly digested *TRP1* fragment. The pSTB₂₋₅ plasmids were recovered from the corresponding ligation mixtures by transformation in yeast, followed by Southern blotting and PCR analysis of total DNA prepared from the transformants.

(iv) **pSH-*STB*, pSH-*STB*-Prox, and pSH-*STB*-Dist.** Plasmids pSH-*STB*, pSH-*STB*-Prox, and pSH-*STB*-Dist were used to integrate the full *STB* locus and the origin-proximal or origin-distal region of *STB* into the *HIS3* locus on chromosome XV. The *STB*, *STB*-proximal, and *STB*-distal regions, bordered by *PstI*-*AvaI*, *HpaI*-*AvaI*, and *PstI*-*HpaI* restriction enzyme sites in the 2 μ m circle genome, were amplified by PCR using DNA prepared from a [*cir*⁺] yeast strain. The forward and reverse primer pairs employed in the amplification reaction were designed to contain an *EcoRI* and a *BamHI* recognition sequence, respectively, within their nonannealing terminal regions. After digestion of the amplified DNA with *EcoRI* and *BamHI*, the fragments were cloned into the *EcoRI*-*BamHI* backbone fragment from the yeast integrative plasmid pRS403. The resulting plasmids were linearized by cutting within the *HIS3* marker using *NdeI* and were used to transform a [*cir*⁰] *his3* recipient strain harboring a galactose-inducible *REP1-REP2* cassette. The correct integrants among the His⁺ transformants were identified by PCR screening of total DNA prepared from them.

(v) **p5015.** p5015 is a *CEN* reporter plasmid, provided by D. Ivanov and K. Nasmyth (24), harboring *CEN4* as well as *TRP1*. It was employed as a control in a number of assays.

Plasmid pulldown using an anti-Myc antibody. Pulldown assays were carried out in [*cir*⁺], [*cir*⁰], or [*cir*⁰]-P_{GAL}-*REP1-REP2* host strains expressing Myc12-tagged Cse4. The modified *CSE4* gene was present at the normal chromosomal location and was driven by the native promoter. In a standard assay, spheroplasts obtained from 250 ml of a log-phase culture (optical density at 600 nm [OD₆₀₀], ~0.8) by lyticase treatment were lysed in 2.5 ml of lysis buffer according to published protocols (24). The crude lysate was spun at 12,000 × *g* and 4°C to produce the cleared lysate. Five hundred microliters of the cleared lysate was saved as the input fraction, and the remainder was incubated with an anti-Myc antibody overnight at 4°C. Protein A-Dynabeads (500 μ l) were added, and incubation was continued for 5 h at 4°C. The beads were harvested, and 500 μ l of the supernatant was saved as the “unbound” fraction. The beads were washed three times with lysis buffer containing 200 mM NaCl. Finally, DNA was eluted from the beads using 1% sodium dodecyl sulfate (SDS) elution buffer (24) at 65°C. All the fractions were adjusted to a final concentration of 1% SDS, extracted with phenol-chloroform, and precipitated with ethanol. DNA from the input, unbound, and bound fractions was dissolved in 50 μ l 1× Tris-EDTA (TE). Aliquots from these fractions were analyzed by electrophoresis in agarose gels followed by Southern blot analysis. The input and unbound fractions corresponded to one-eighth of the 1× bound fractions.

Restriction enzyme digestion of plasmids following pulldown. After the pulldown of a reporter plasmid, the protein A-Dynabeads were resuspended in 1 ml of lysis buffer (see above) and were incubated with or without appropriate restriction enzymes for 4 h at 4°C. An excess of enzymes was employed to ensure nearly complete digestion of plasmid DNA. Beads were harvested, and the supernatant was collected. The different fractions were processed further, and DNA was recovered from them by ethanol precipitation as described above for the plasmid pulldown assays. Samples were subjected to agarose gel electrophoresis and Southern hybridization using specific DNA probes.

Micrococcal nuclease digestion of chromatin and analysis of nucleosomes for Cse4 association. Nuclei were isolated from 500-ml (large-scale) or 200-ml (small-scale) log-phase cultures (OD_{600} ~0.8 to 1.0) by following published protocols (13). The nuclear pellet from the small-scale culture was resuspended in 1 ml SPC buffer [1 M sorbitol, 20 mM piperazine- N,N' -bis(2-ethanesulfonic acid) (PIPES; pH 6.3), 5 mM $CaCl_2$] and was then digested with 200 U of micrococcal nuclease (Worthington Biochemicals) at 37°C for 30 min. The digestion buffer included 1 mM phenylmethylsulfonyl fluoride (PMSF) as well as 1× LPC (10 μ g per ml each of leupeptin, pepstatin A, and chymostatin). These conditions were standardized to yield predominantly mono- and dinucleosomes. After the addition of 10 mM EDTA, the reaction mixture was spun down at 13,200 rpm for 10 min in an Eppendorf centrifuge (model 5415R) at 4°C. The supernatant was incubated with an anti-Myc antibody; the mixture was adsorbed on protein A-Dynabeads; and the bound DNA was isolated as described under "Plasmid pulldown using an anti-Myc antibody" above. The input DNA and bound DNA were analyzed by PCR using a defined set of primer pairs. For each primer pair, signal intensities were estimated from reactions employing a set of dilutions of the immunoenriched and input DNAs as templates. Corrections were made for differences in the amplification efficiencies of primer pairs by normalizing the signals from the immunoprecipitated samples to those from the corresponding input DNAs.

Depletion of *Scm3*. The strain expressing *SCM3* from the *GAL* promoter was grown in galactose to an OD_{600} of ~0.3 and was then shifted to glucose medium for 3 h. Under these conditions, the vast majority of cells were arrested in the G_2/M phase.

Cell cycle arrest using α -factor, nocodazole, or the *ndc10-1* mutation. For G_1 arrest, cells grown to mid-log phase were treated with 15 μ g per ml of α -factor. The efficiency of arrest was nearly 90 to 95%. For spindle depolymerization and G_2/M arrest, nocodazole was added as a solution in dimethyl sulfoxide (DMSO) to mid-log-phase cells at 20 μ g/ml. More than 85% of the cells were arrested in the large budded state with a single nucleus at the mother-bud neck. The corresponding control cells were treated with the same amount of DMSO, but without nocodazole. A fresh culture of the *ndc10-1* strain, seeded by an overnight inoculum, was grown to early-log phase at 26°C and was shifted to 37°C for 2.5 h to inactivate kinetochore function.

ChIP. Chromatin immunoprecipitation (ChIP) analyses were performed as described previously (20). In assays using multiple primer pairs, PCR signals yielded by immunoprecipitated DNA were normalized to the corresponding signals given by the input DNA, as described under "Micrococcal nuclease digestion of chromatin and analysis of nucleosomes for Cse4 association" above.

Miscellaneous protocols. Routine experimental protocols, such as bacterial and yeast transformations, total yeast DNA and plasmid DNA preparations, and curing of native 2- μ m circles from [*cir*⁺] yeast strains, are described on the web page of the Jayaram laboratory (<http://www.sbs.utexas.edu/jayaram/jayaramlab.htm>).

Yeast strains and oligonucleotides. The yeast strains used in this study are listed in Table S1 in the supplemental material. The oligonucleotides employed as primers in PCRs pertaining to key experiments are listed in Tables S2 and S3 in the supplemental material. Sequences of additional oligonucleotides, not listed in these tables, are available on request.

RESULTS

An *STB* reporter plasmid can be pulled down by a Cse4-directed antibody in the absence of kinetochore function: concordance between ChIP and pulldown assays. As already pointed out, the presence at *STB* of Cse4, which is the hallmark of the centromeric nucleosome, is quite surprising. A previous demonstration of Cse4-*STB* association was based on ChIP analyses employing formaldehyde-mediated DNA-protein cross-linking (20). Since the 2- μ m plasmid cluster is localized close to the spindle pole body (10, 32), often overlapping clustered centromeres, cross-linking of Cse4 present at *CEN* with *STB* DNA due to their proximity could be a potential pitfall. To rule out such an artifact, we carried out pulldown of *STB* and *CEN* reporter plasmids using a Cse4-directed antibody in the absence of cross-linking and under conditions that keep these loci active or inactive.

In a [*cir*⁺] *ndc10-1* host strain expressing Cse4-Myc12 and

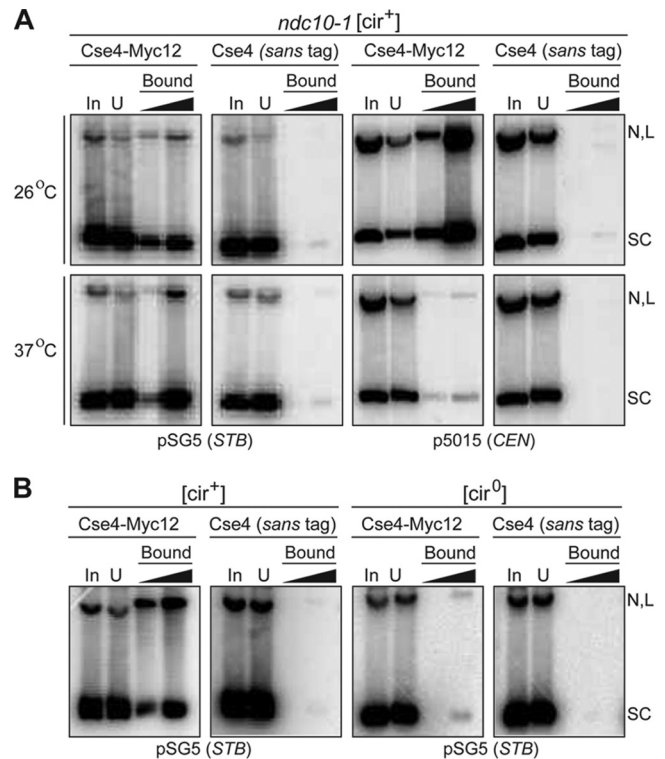


FIG. 1. A Cse4-specific antibody brings down an *STB* reporter plasmid in a Rep1- and Rep2-dependent and Ndc10 independent manner. Plasmid pulldowns in this set of assays and those for which results are shown in subsequent figures were performed as described in Materials and Methods. The antibody employed was directed to the Myc12 epitope fused to Cse4. Plasmid DNA was detected by Southern blot analysis using a radioactively labeled hybridization probe. The DNA load in the second lane of the bound fraction was five times that in the first. In, input; U, unbound; SC, supercoiled plasmid; N, L, nicked and linear plasmids, respectively.

supplying Rep1 and Rep2 from endogenous 2- μ m circle molecules, both the *STB* and *CEN* reporter plasmids were pulled down by the anti-Myc antibody at the permissive temperature, 26°C (Fig. 1A). At the nonpermissive temperature, 37°C, the *STB* plasmid, but not the *CEN* plasmid, was pulled down. Consistent with the requirement of Rep1 and Rep2 proteins for Cse4-*STB* association (20), the *STB* reporter plasmid could not be pulled down in a [*cir*⁰] strain, lacking native 2- μ m circles and thus lacking the Rep proteins (Fig. 1B).

Localization of the 2- μ m plasmid to its specific nuclear address, assisted by the mitotic spindle and the Kip1 motor protein, appears to be an important spatial regulatory step in the plasmid partitioning pathway (10). Consistent with such a mechanism, Cse4 could not be detected at *STB* by ChIP in a *kip1Δ* strain or in nocodazole-treated cells (20). Furthermore, ChIP assays suggested the dissociation of Cse4 from *STB* during late telophase of the cell cycle, in contrast to the persistence of Cse4 at *CEN* through the ensuing G_1 phase (20). Finally, according to ChIP data, the establishment of the functional state of the *STB* chromatin by the RSC2 chromatin-remodeling complex (23, 50) occurs after the recruitment of Cse4 at *STB* (20). In agreement with the ChIP conclusions, the Cse4-directed antibody failed to bring down the *STB* reporter

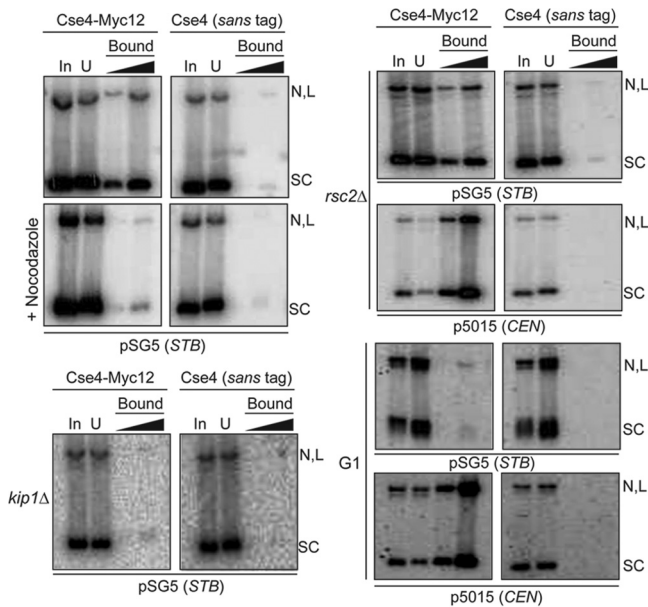


FIG. 2. Spindle depolymerization, G_1 arrest, and the *kip1Δ* mutation, but not the *rsc2Δ* mutation, block Cse4-*STB* association. Pull-down of the *STB* or *CEN* reporter plasmid and DNA analysis were performed as described for Fig. 1.

plasmid in G_1 -arrested or nocodazole-treated cells, as well as in cells lacking Kip1 function (Fig. 2). In contrast, the *rsc2Δ* mutation had no effect on plasmid pulldown. Significantly, G_1 arrest did not impede the pulldown of the *CEN* reporter plasmid; the *rsc2Δ* mutation was in this regard also innocuous.

The results described above attest to the authenticity of Cse4-*STB* association, which is not dependent on chemical cross-linking and is not affected by inactivation of centromere function. They argue in favor of the hypothesis that Cse4 is a genuine component of the 2μ m plasmid partitioning complex.

We have noted that the average pulldown efficiency of the *STB* reporter plasmid was less than 10% that of the *CEN* reporter plasmid. This discrepancy can be readily seen by comparing the lanes for the “input” and “unbound” fractions for the two plasmids in the top panels (26°C) of Fig. 1. Perhaps Cse4-*STB* association is not mediated through a nucleosome, or this association is substoichiometric. These alternative possibilities are being investigated.

Only the *STB*-containing DNA fragment of a reporter plasmid stays associated with the Cse4-directed antibody after restriction enzyme digestion. Previous results on the salt extractability of Cse4 from *STB*, as well as differential restriction enzyme sensitivities of *STB* in the presence and absence of functional Cse4, were interpreted as being consistent with the presence of Cse4 as a nucleosome component at *STB* (20). However, since *STB* is the site of multiple protein associations, DNA accessibility could be restricted as a result of cooperative DNA-protein and/or protein-protein interactions. As a result, a nonnucleosomal mode of Cse4 residence at *STB* cannot be ruled out. Regardless of the precise nature of Cse4-*STB* association, the pulldown assays for which results are shown in Fig. 1 and 2 demonstrate that a reporter plasmid can be baited by the Cse4 antibody only when the plasmid-borne *STB* locus is

functional. A reasonable expectation, then, is that fragmentation of the reporter plasmid should free any DNA piece lacking *STB* from its association with the Cse4-directed antibody (Fig. 3A).

We therefore digested an *STB* or a *CEN* reporter plasmid following pulldown so as to split the *STB*- or *CEN*-containing fragment from the plasmid backbone fragment. We then probed the retention of the resulting DNA fragments by the Cse4-directed antibody, or their release from that antibody (Fig. 3B and C). In the case of the *STB* reporter plasmid, the *STB*-containing HindIII-EcoRI fragment (L1; 1,341 bp) remained preferentially associated with the Cse4-directed antibody on protein A beads, whereas the fragment lacking *STB* (L2; 1,051 bp) was released into the supernatant (Fig. 3B). In the control assay with a *CEN* reporter plasmid, the *CEN*-containing EcoRI-BglII fragment (L1; 1,072 bp) stayed bound to the beads, and the fragment lacking *CEN* (L2; 1,288 bp) dissociated from them (Fig. 3C).

Thus, Cse4 association is restricted to DNA regions harboring *STB* or *CEN* in the respective reporter plasmids and is not affected by digestion of the DNA with restriction enzymes. Such a localized and stable DNA-protein interaction would be consistent with the occupancy of *STB* by a Cse4-containing nucleosome(s).

Scm3 associates with *STB* and is required for Cse4 recruitment at *STB*. Scm3 is an essential protein in *S. cerevisiae* that is associated with *CEN*, interacts with Cse4, and is required for the localization of Cse4 at *CEN* (4, 33, 43). There is controversy as to whether Scm3 is a kinetochore protein that functions as a loading factor for Cse4 or an authentic constituent of the centromeric nucleosome (5, 33). The presence of Cse4 at *STB* raises the question of whether Cse4 recruitment by the 2μ m plasmid is also dependent on Scm3.

Scm3 was detected at *STB*, as at *CEN3*, by ChIP (Fig. 4A). Scm3-*STB* association was specific, as suggested by the absence of Scm3 at the *FLP* locus of the 2μ m plasmid. There was a marked diminution of Cse4 association at both *STB* and *CEN3* when cells were depleted of Scm3 (Fig. 4B). However, neither a *CEN* nor an *STB* reporter plasmid could be pulled down using an antibody against Scm3 (Fig. 4C).

Considered together, the ChIP and pulldown results suggest that while Scm3 promotes Cse4-*STB* association, the interaction between Scm3 and *STB* is weaker than that between Cse4 and *STB*. Furthermore, the sharply contrasting behaviors of a *CEN* reporter plasmid during pulldown by antibodies targeting Cse4 or Scm3 argue against Scm3 being an integral component of the nucleosome core at *CEN*.

A chromosomally integrated copy of *STB* is competent for Cse4 recruitment in its ectopic context. As noted before, the precise nuclear address of the 2μ m plasmid appears to be a critical determinant in its equal segregation. We wondered whether the partitioning function of *STB* is context specified, that is, whether it is active only in its native extrachromosomal state. We integrated a copy of *STB* at the *HIS3* locus on the right arm of chromosome XV and assayed its ability to recruit Cse4. The host strain was [*cir*⁰] and harbored a galactose-inducible *REP1-REP2* expression cassette.

The association of Cse4 with the integrated *STB* copy was detected by ChIP, but only under conditions of *REP1* and

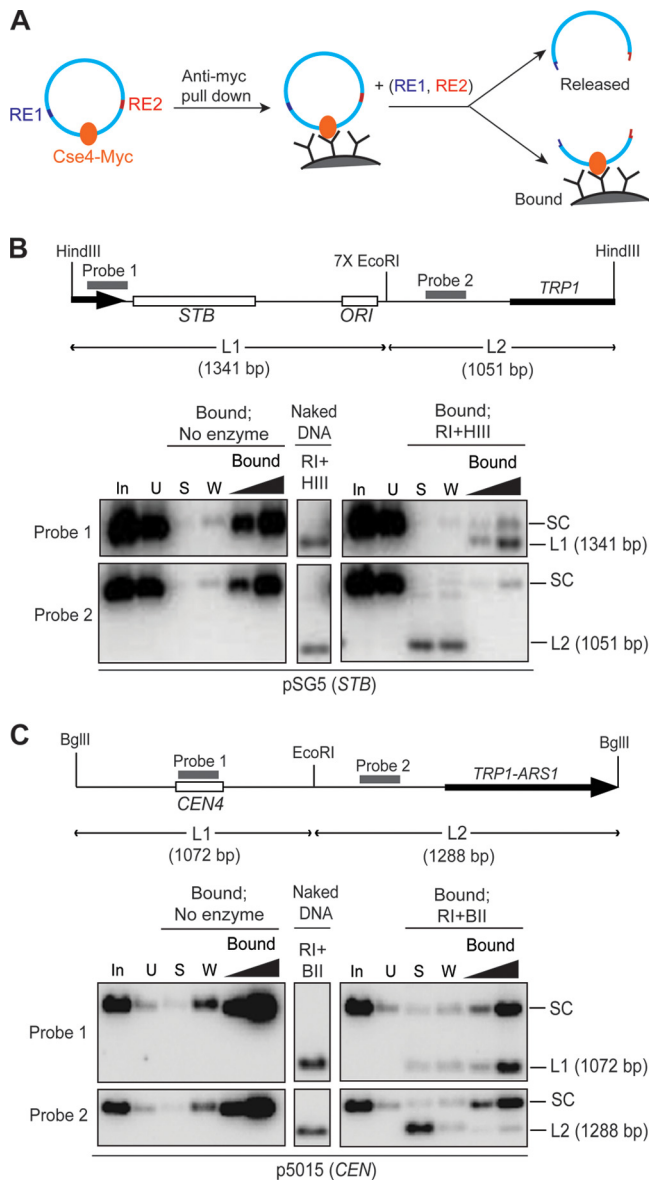


FIG. 3. Cse4 association is specific to *STB*-containing DNA. (A) Schematic diagram of the rationale of the analysis. If Cse4 association is specific to a plasmid locale, DNA digestion with the restriction enzymes RE1 and RE2 should release the fragment lacking Cse4 from its association with the Cse4-directed antibody. (B and C) The *STB* (B) or *CEN* (C) reporter plasmid employed in the assays is schematically diagrammed at the top. Restriction enzyme digestions were performed on protein A-Dynabeads, and DNAs in the released or retained fractions were analyzed by gel electrophoresis and Southern hybridization using the indicated probes. For reference, enzyme digestions performed on total DNA ("naked" DNA) prepared from the experimental strains were analyzed similarly. Hybridization profiles from the relevant portions of the gels are displayed. RI, HIII, and BII stand for EcoRI, HindIII, and BglII, respectively. In, input; U, unbound; S, supernatant; W, wash; SC, supercoiled plasmid.

REP2 induction (Fig. 5A). When the expression of *REP1* and *REP2* was repressed by glucose, Cse4 was absent at *STB*.

Thus, the competence of *STB* to acquire Cse4 is not lost by displacing it from its normal location within the 2μ m plasmid. The chromosomally located *STB*, like its plasmid counterpart,

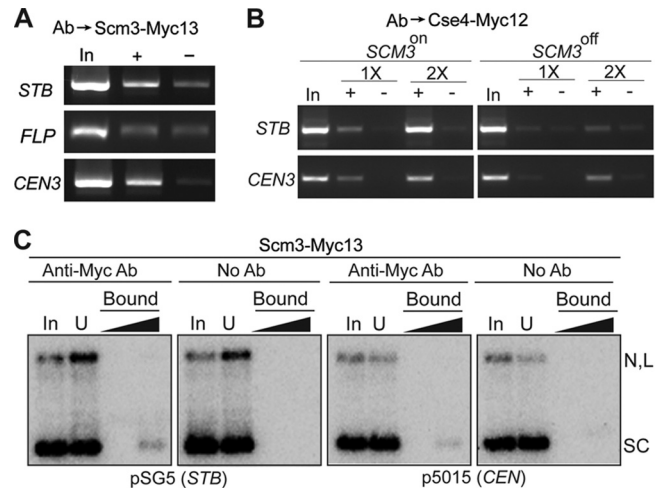


FIG. 4. Scm3 interacts with *STB* and promotes Cse4-*STB* association. (A and B) ChIP assays were performed in [*circ*⁺] host strains using antibodies (Ab) against Scm3-Myc13 (A) and Cse4-Myc12 (B). The immunoprecipitated DNA was probed using primers specific to the *STB* and *FLP* loci of the 2μ m plasmid and *CEN3*, as indicated. "In," "+," and "-" refer to input, immunoprecipitated, and mock-immunoprecipitated (without antibody) DNA samples, respectively. For panel B, the expression of *SCM3*, under the control of the *GAL* promoter, was turned on or off in the presence of galactose or glucose, respectively, as the carbon source. 1× and 2× refer to the relative amounts of template DNA used in the PCRs. (C) The pull-down analyses of the *STB* and *CEN* reporter plasmids were similar to those described in the legend to Fig. 1.

is dependent on Rep1 and Rep2 for Cse4 acquisition. The integrated *STB* fragment does not include the 2μ m circle replication origin, which is approximately 400 bp away from the *STB* border proximal to it. The location of *STB* with respect to the origin or the intervening 2μ m circle DNA between them does not appear to have a strong influence on Cse4-*STB* association.

Cse4-*STB* association is specific to the *ORI*-proximal region of *STB*. The *STB* locus has a bipartite organization, a proximal and a distal region with respect to the origin of replication of the 2μ m plasmid. As noted previously, *STB*-proximal is made up of a tandem array of five directly repeated units of a 62-bp AT-rich consensus sequence element (35). *STB*-distal contains a transcription termination signal that blocks two 2μ m circle transcripts (1,650 nucleotides [nt] and 600 nt) spanning the *RAF1* locus from entering *STB*-proximal (35, 45). Furthermore, *STB* has been shown to mediate transcriptional silencing with the assistance of the Sir2 to Sir4 proteins (37). Consistent with this ability, *STB*-distal contains an element that can down-regulate transcription from upstream promoters (35). We wanted to know whether *STB*-proximal and *STB*-distal are functionally distinct with regard to Cse4 association.

ChIP analyses performed on *STB*-proximal and *STB*-distal integrated at the chromosomal *HIS3* locus revealed that the occupancy of Cse4 was limited to *STB*-proximal (Fig. 5B). The signal from *STB*-distal was nearly the same as the background. The ChIP results were concordant with the results of pull-down assays performed on reporter plasmids containing *STB*-proximal or *STB*-distal (Fig. 5C).

The specific association of Cse4 with *STB*-proximal is con-

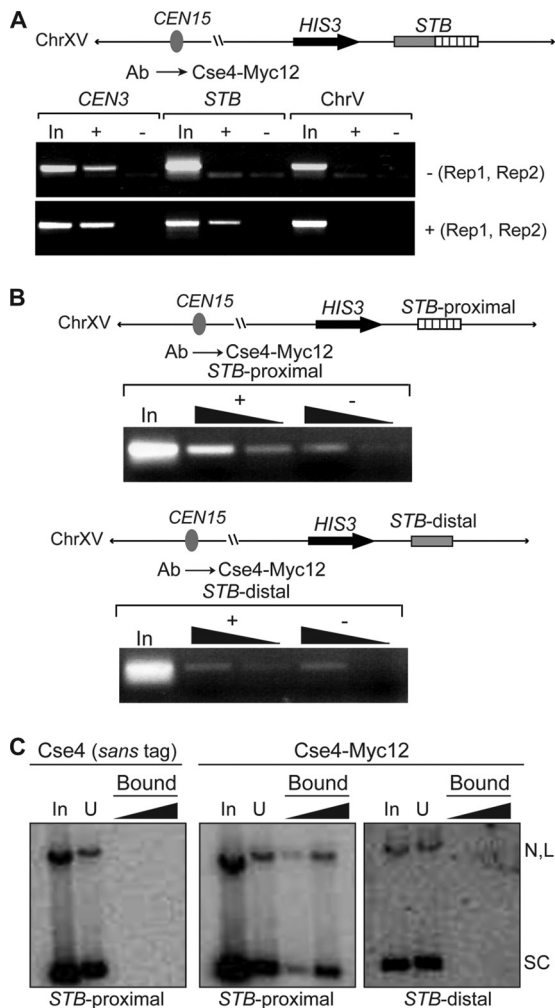


FIG. 5. Cse4 associates with *STB* or *STB*-proximal, but not with *STB*-distal, integrated at the *HIS3* locus on chromosome XV. (A and B) The sequence contexts of chromosomally integrated *STB*, *STB*-proximal, and *STB*-distal are schematically diagrammed. ChIP assays were performed in a [*cir*⁰] host strain harboring an integrated *REP1-REP2* cassette controlled by the *GAL* promoter. “–(Rep1, Rep2)” and “+(Rep1, Rep2)” denote the glucose-repressed and galactose-induced states of the *REP1-REP2* loci, respectively. “ChrV” refers to a location on the chromosome V arm where cohesin is assembled but Cse4 is not present. (C) The *STB*-proximal and *STB*-distal sequences harbored by the pulled down reporter plasmids were the same as those present in the corresponding integrated versions of these loci.

sistent with the observation that this region is sufficient to confer stability on a reporter plasmid in a Rep1-Rep2-dependent manner (26, 35). However, the efficacy of this minimal *STB* region can differ depending on the sequence context in which it is placed. The transcriptional insulation provided by *STB*-distal may be important for realizing the full potential of *STB* in plasmid stability.

Localization of Cse4 in nucleosome fractions produced by micrococcal nuclease digestion. The experimental evidence from Fig. 5 localizes Cse4 to *STB*-proximal but does not rule out the possibility that *STB*-distal may acquire competence for Cse4 association when it is adjacent to *STB*-proximal. We also do not know whether such competence, if valid, might spread

from *STB*-proximal to any DNA sequence placed next to it. Since Cse4 is incorporated into a nucleosome in its only known, and well-characterized, association with *CEN* DNA, we wanted to delimit the region spanning *STB*-proximal and adjacent DNA occupied by Cse4 with respect to nucleosome markers.

Previous analyses employing DNase I and micrococcal nuclease digestion suggested that the organization of *STB* chromatin, which appears to have a relatively low occupancy of nucleosomes, is modulated by the Rep1 protein and the RSC2 chromatin-remodeling complex (12, 46, 50). The size of *STB*-proximal or *STB*-distal, ~300 bp, can accommodate one or two standard H3-containing or Cse4-containing nucleosomes, which wrap ~150 bp and ~125 bp of DNA, respectively, around them. In the experiments described below, we enriched Cse4-associated nucleosome fractions from micrococcal-nuclease-digested chromatin and investigated the presence of *STB*-proximal and adjacent DNA sequences by a PCR-based assay. The rationale was adapted from analogous assays employed to demonstrate the exclusive localization of Cse4 to a single nucleosome present at a centromere (13).

Nuclei were digested with micrococcal nuclease by using the [*cir*⁰] strain containing the integrated copy of *STB* (see Fig. 5), and inducible *REP1-REP2*, to yield a nucleosome population consisting predominantly of mono- and dinucleosomes ($\geq 85\%$), with a significantly smaller fraction of trinucleosomes ($\leq 15\%$). DNA isolated from the immunoenriched subpopulation of Cse4-associated nucleosomes was analyzed by PCR using a series of primer pairs directed to *CEN4* (as a control), *STB*, and DNA regions immediately flanking these loci on either side. Since the position of the *CEN* nucleosome is clearly defined, resolution at the single-nucleosome level was possible for *CEN* analysis (Fig. 6A). In contrast, *STB* analysis could not be as precise. Information on exact nucleosome placements at and around *STB* is lacking. Furthermore, because *STB*-proximal is composed of repeated DNA elements, primer pairs that would specifically amplify selected subregions within this region were difficult to design. Given these caveats, better resolution than that at the dinucleosome level was not possible. As indicated in the schematic in Fig. 6B, the primer pairs for *STB* analysis were spaced two nucleosome lengths of DNA (~300 bp) apart. Although the depiction of nucleosomes within and outside *STB* is rather arbitrary, it is consistent with published micrococcal nuclease digestion results (50). Two nuclease-sensitive sites have been mapped to the borders of *STB*-proximal, with a weaker one at the center, suggesting the presence of internucleosome DNA linkers at these locales. Therefore, two nucleosomes are likely positioned at *STB*-proximal. Assuming no discontinuity in nucleosome spacing or phasing, *STB*-distal, which is nearly identical in size to *STB*-proximal, would also contain two nucleosomes.

The signal intensities of the PCR products were plotted after they were normalized to the corresponding values from reactions with the input DNA as the template (Fig. 6C and D). As expected, a sharp peak signifying Cse4 occupancy marked the *CEN4* DNA (Fig. 6C). A similar peak was positioned at *STB*-proximal in the presence of Rep1 and Rep2, with a drop-off in the signal at *STB*-distal as well as flanking DNAs to the left and right of *STB* (Fig. 6D). The authenticity of this peak was

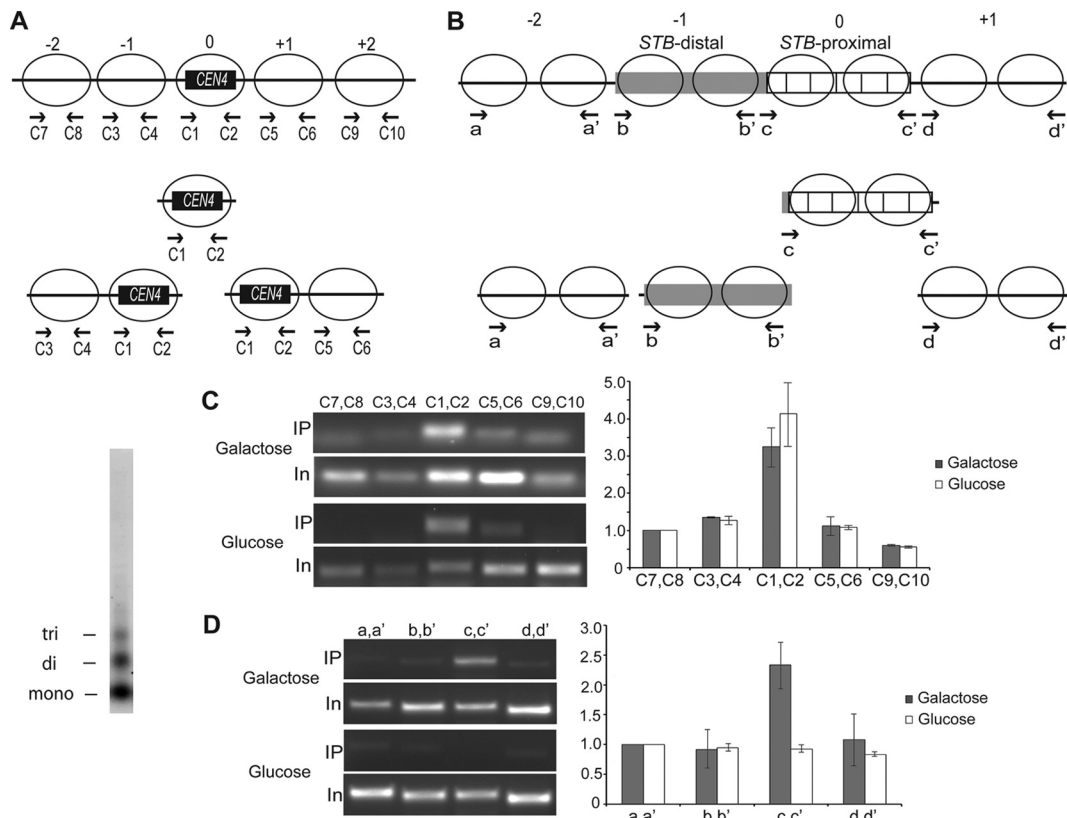


FIG. 6. Association of Cse4 with *STB* probed in chromatin digested with micrococcal nuclease to yield predominantly mono- and dinucleosomes. The assays were conducted in the *STB*-integrated strain, with *REP1* and *REP2* expression either repressed by glucose or induced by galactose. As a control, the *CEN* region of chromosome IV was analyzed in parallel. (A) Expected pattern of mono- and dinucleosomes associated with Cse4 in chromatin spanning *CEN4*. Since Cse4 is present exclusively in the nucleosome positioned at *CEN*(0), only one mononucleosome (0) and two types of dinucleosomes (0, +1 and 0, -1) will be pulled down by the Cse4-directed antibody. (B) Based on their sizes, *STB*-proximal and *STB* distal can each potentially accommodate two adjacent nucleosomes. The placement of two nucleosomes at *STB*-proximal was prompted by the presence of a clear micrococcal-nuclease-sensitive site at each of its borders, plus a weaker one at its center (50). If Cse4 residence is confined to *STB*-proximal, only the c-c' primer pair is expected to yield a positive PCR signal. (C and D) (Left) Amplification outputs from one assay for a given amount of the immunoprecipitated DNA (IP) and a fixed dilution of the input DNA (In) using the indicated primer pairs that probe *CEN4* (C) or *STB* (D) as well as the DNA regions on either side of them. (Right) The cumulative data are displayed as bar graphs. In this representation, the signal given by each primer pair from the immunoprecipitated DNA was normalized to that from the input DNA, and a value of 1 was assigned for the leftmost primer pairs: C7, C8 in *CEN4* analysis and a, a' in *STB* analysis. (Far left) Nucleosome profile of the chromatin digest used for the analyses.

certified by its absence when the expression of *REP1* and *REP2* by the host strain was repressed by glucose.

As a final verification of the Cse4 residence zone, we performed pulldown assays, followed by restriction enzyme digestion, of a reporter plasmid in which *STB*-proximal was bounded by four tandem copies each of BglII and BamHI recognition sequences at its left and right ends, respectively. After digestion with BamHI plus BglII, the *STB*-proximal DNA fragment (L1; 335 bp) remained quantitatively associated with the Cse4-directed antibody in the "bound" fraction (Fig. 7, top). More than 70% of the rest of the plasmid DNA, including *STB*-distal (L2; 2,159 bp), was disjoined from the antibody and appeared in the "supernatant"/"wash" fractions (Fig. 7, bottom).

In summary, the association of Cse4 with *STB* is mediated almost exclusively through the *STB*-proximal region. By analogy to centromeres, this association could involve a Cse4-containing nucleosome, or perhaps two such nucleosomes. However, the possibility that the Cse4-*STB* interaction does

not conform to the conventional Cse4-*CEN* interaction cannot be ruled out entirely.

A subset of the *STB*-proximal consensus elements is necessary and sufficient for Cse4 recruitment. In order to determine whether Cse4-*STB* association is dependent on the full complement of *STB*-proximal, or whether a subset of the 62-bp repeat units would suffice, we tested reporter plasmids containing 2 to 5 tandem copies of the repeat unit in pulldown as well as ChIP assays. The deletions were introduced at the far end of *STB*-proximal from the 2 μ m origin of replication. All of the constructs retained the same native plasmid sequence from their intact *STB* borders to the origin.

An array of at least three repeat units was necessary and sufficient to elicit optimal Cse4-*STB* association (Fig. 8). There was a sharp drop in the pulldown efficiency (Fig. 8A), as well as in the ChIP signal (Fig. 8B), for a reporter plasmid containing only a two-repeat-unit array.

The requirement of three repeat elements (~180 bp) for Cse4-*STB* association suggests that the minimal DNA region

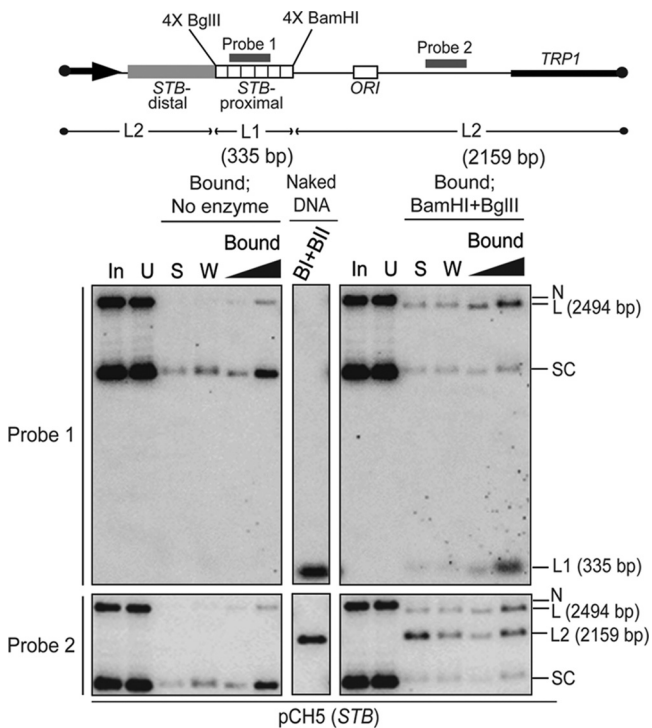


FIG. 7. Only the *STB*-proximal segment can retain association with the Cse4 directed antibody following pulldown and restriction enzyme digestion of a reporter plasmid. The four tandem copies of BamHI and BglIII recognition sites that cordon off *STB*-proximal in the reporter plasmid are diagrammed schematically. Plasmid pulldown and Southern blot analysis of the BamHI- plus BglIII-digested DNA were conducted as described in the legend to Fig. 3. BI + BII (above the lane showing results for the digestion of naked DNA) refer to BamHI and BglIII, respectively.

that interacts with Cse4 is sufficiently long to be wrapped around at least one Cse4-containing nucleosome (~125 bp).

The chromosomally integrated copy of *STB* promotes cohesin assembly. A critical, and perhaps the final, step in the pathway for the assembly of the plasmid partitioning complex is the recruitment of the cohesin complex at *STB* (17, 18, 31). It is not clear how cohesion between sister molecules, observed with the help of a fluorescence-tagged single-copy reporter plasmid (17, 18), translates into cohesion between two sister clusters containing multiple molecules of the 2 μ m plasmid. In one model, every pair of sister plasmids coheres, and each sister within such a pair is distributed one-to-one between sister clusters. It is as if the original plasmid cluster acts as a template for the formation of its sister. A plausible alternative model, in which sister clusters are held together by cohesin bridges formed by a subset of plasmid sisters, cannot be ruled out. Nevertheless, every condition so far tested that blocks cohesin-*STB* association leads to plasmid missegregation, suggesting that cohesin is an authentic component of the plasmid partitioning complex (10, 17, 20, 31, 32). Although Cse4 association satisfies one important criterion for the functionality of the chromosomally integrated *STB*, we wanted to know whether this *STB* is also competent for cohesin assembly. A positive result would imply that the entire pathway for orga-

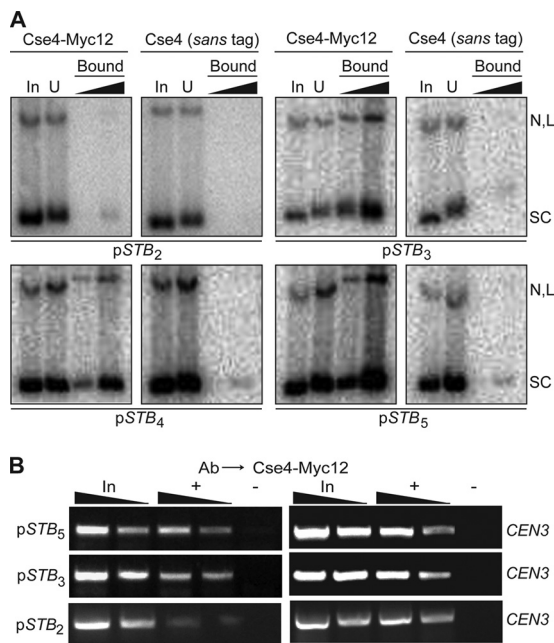


FIG. 8. A subset of three of the 62-bp repeat elements within *STB*-proximal is sufficient for Cse4-*STB* association. Cse4-*STB* association was assayed by pulldown (A) and ChIP (B) assays. The subscript assigned to *STB* denotes the number of intact repeat elements, starting with the origin-proximal repeat element, present in a given reporter plasmid.

nizing the plasmid partitioning complex is unaffected by the transplantation of *STB* from plasmid to chromosome.

As revealed by ChIP, the integrated *STB* copy was capable of cohesin assembly, whereas the proximal *HIS3* locus was not (Fig. 9A). Consistent with this result, a ChIP walk performed along a portion of the integrated region revealed a Cse4 peak positioned over *STB* (Fig. 9B).

The ability of *STB* to license a chromosomal site that normally does not associate with either Cse4 or the cohesin complex to acquire these factors required for 2 μ m circle segregation suggests that the functionality of the Rep-*STB* system in assembling the partitioning complex is preserved in this ectopic environment.

DISCUSSION

In this study, we have characterized the functional association between Cse4, a histone H3 variant regarded as unique to specialized nucleosomes assembled at centromeres (7, 29), and the partitioning locus of the 2 μ m plasmid. Cse4 is recruited to *STB* not only in its native plasmid context but also in an ectopic chromosomal context. The localization of Cse4 strictly to *STB*-proximal, containing five iterations of a 62-bp repeat element, and the DNA length requirement for this localization are consistent with the potential occupancy of *STB* chromatin by a Cse4-containing nucleosome, or perhaps two such nucleosomes. Furthermore, the retention of function by *STB* placed within a chromosome makes it possible to analyze the plasmid partitioning system without the complexities of multiple copies and the clustered organization of *STB* in its extrachromosomal state. We consider below the broader implications of the un-

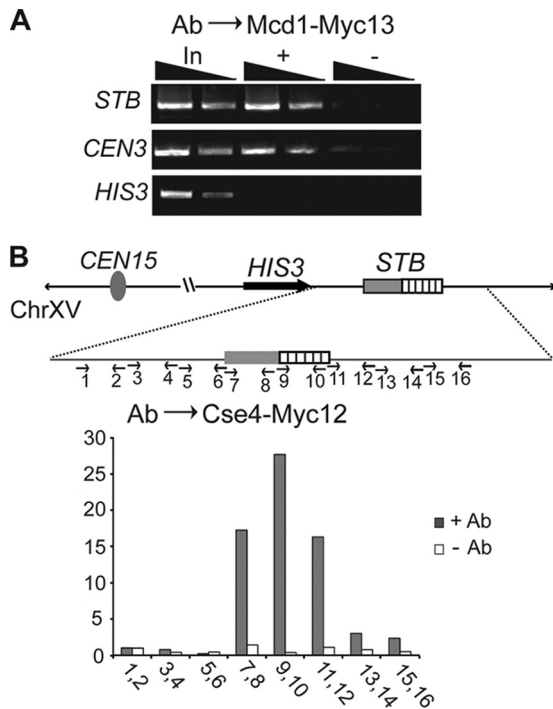


FIG. 9. *STB* integrated at the *HIS3* locus promotes cohesin recruitment at this chromosome locale. ChIP assays were carried out in two derivatives of the *STB*-integrated strain, one expressing *MCD1-MYC13* and the other expressing *CSE4-MYC12* from their normal chromosomal locations. Rep1 and Rep2 were supplied by turning on, in the presence of galactose, the *GAL* promoter-controlled *REP1-REP2* cassette harbored by these strains. (A) The immunoprecipitated DNA was probed using primer pairs specific to *STB* and to the chromosomal *CEN3* and *HIS3* loci, respectively. (B) The indicated primer pairs (each pair encompassing ~300 bp) were used to march along the indicated DNA region. The PCR signals were normalized to those from the input DNA and were plotted on an arbitrary scale, with the signal from the "1, 2" primer pair set as equal to 1.

anticipated presence of the same histone variant at the partitioning loci of chromosomes and a nonessential extrachromosomal DNA element.

Appropriation of a critical chromosome segregation factor by the multicopy $2\mu\text{m}$ plasmid: a functional paradox? Replacement of the canonical histone H3 by members of the CenH3 family, including Cse4, exclusively at centromeres permits unequivocal distinction between centromeric and noncentromeric DNA and provides the spatial signal for the assembly of the kinetochore complex (21, 29, 44). Whether that distinction is imparted by altered histone stoichiometry of the *CEN* nucleosome core or through distinct structural motifs conferred by CenH3 on a histone octamer, or is due to the contrary positive writhing of *CEN* DNA around the CenH3-containing nucleosome, is controversial (5, 14, 33, 40).

Consistent with its highly specialized role in chromosome segregation, Cse4 is present only within the single nucleosome that resides at the short ~125-bp centromere of each *S. cerevisiae* chromosome (13). The cell guards against the adverse consequences of Cse4 association with noncentromeric DNA (2, 9, 41) and potential assembly of ectopic kinetochores by

actively turning over such mislocalized Cse4 via the ubiquitin-proteasome pathway (8, 22, 38). Excess Cse4 not bound to chromatin may also be subject to degradation. It is not clear whether the spread of Cse4 nucleosomes beyond *CEN* DNA is prevented by the establishment of chromatin barriers. Nevertheless, the strict regulation of Cse4 would limit its overall availability. Under this restriction, and since three to four times as many $2\mu\text{m}$ plasmids as chromosomes are present in the nucleus, the association of Cse4 with *STB* was unexpected. Yet the specificity of this interaction and its abrogation under conditions that inactivate *STB*, as revealed in this study, support a functional role for Cse4 in plasmid partitioning. The mechanism by which a high-copy-number selfish DNA element copes with the tight cellular economy of a host factor on which it is dependent is currently being investigated.

Cse4 signals distinct partitioning mechanisms at *CEN* and *STB*. Despite the association of Cse4 with *STB*, we have not detected the presence of any of the kinetochore components at *STB* (31; also unpublished data). Conversely, neither Rep1 nor Rep2 interacts with *CEN* (31). There is also no evidence for direct spindle-mediated segregation of the $2\mu\text{m}$ plasmid. The presence of two copies of *STB* on a reporter plasmid does not induce instabilities analogous to those observed in dicentric minichromosomes (unpublished observations). Currently available evidence would be consistent with a model in which the plasmid cluster segregates in a chromosome-tethered fashion. The pairing of duplicated clusters by the cohesin complex would enhance the probability of their tethering to sister chromatids. Upon the disassembly of cohesin, sister clusters would hitchhike on sister chromatids to daughter cells. Within such a scheme, Cse4-*STB* association would fit into the general strategy by which the $2\mu\text{m}$ circle partitioning system appropriates chromosome segregation factors in order to achieve chromosome-coupled plasmid segregation.

Cse4 associations with *CEN* and *STB* are functionally analogous in that they promote assemblies of partitioning complexes at both these loci. At the same time, they are functionally distinct in that one of these (at *CEN*) is devoted to chromosome segregation and the other (at *STB*) to plasmid segregation. The two separate programs of high-order protein assembly, each temporally ordered and functionally hierarchical on its own, may be set into motion by specific, yet distinct, DNA-protein interactions initiated at these loci. The binding of the CBF3 complex to the CDEIII sequence within *CEN* is thought to provide the trigger for kinetochore assembly (11). Similarly, interactions of the Rep proteins, and perhaps host factors, with *STB* likely nucleate the assembly of the plasmid partitioning complex (19, 31). Critical dependence on specific Cse4-DNA interactions is a shared attribute of both pathways. At *CEN*, this interaction is mediated through a specialized nucleosome. The situation is not as clear-cut at *STB*, although current evidence is consistent with *STB* harboring one or two Cse4-containing nucleosomes. As far as we know, *STB* is the only noncentromeric site, chromosomal or episomal, in a eukaryotic cell where CenH3 recruitment is sequence specific, regulated, and functionally relevant.

The presence of two functional centromeres in a minichromosome in yeast gives rise to frequent nondisjunction or DNA breakage events, presumably due to opposing forces generated by their attachment to spindles from opposite poles. These

instabilities can be effectively prevented by decreasing the distance between the competing centromeres (28). Since *STB*-mediated plasmid segregation does not appear to follow the *CEN*-kinetochore mechanism of spindle attachment (see above), the potential assembly of two Cse4 nucleosomes at *STB* does not pose the threat of instabilities. Furthermore, by analogy to *CEN*, the immediate proximity of the nucleosomes would have prevented such instabilities, even if the plasmid had relied on direct spindle-mediated segregation at some point in its evolutionary history. The mechanistically distinct contributions of the mitotic spindle to the segregation modes of *CEN* and *STB* plasmids may also account for the large differences in their respective copy numbers.

Is the point centromere of budding yeast derived from the partitioning locus of an ancestral 2 μ m plasmid? The simple, genetically defined point centromere of *S. cerevisiae* and a related group of fungi belonging to the Saccharomycetaceae lineage represents a major evolutionary transition from the larger, more complex and epigenetically specified “regional” centromeres found in most other eukaryotes, including the vast majority of fungi (29). Yet both classes of centromeres carry the common epigenetic mark of CenH3-containing nucleosomes. The switch to point centromeres appears to have occurred contemporaneously with the loss of most or all of the machinery required for heterochromatin assembly and RNA interference (1). Quite remarkably, the presence of stably propagating 2 μ m circle-related nuclear plasmids appears to be unique to the Saccharomycetaceae lineage as well (3). It is reasonable, therefore, that the partitioning locus of an ancestral plasmid, following chromosomal integration, could have served as the progenitor of the point centromere (29). It is also noteworthy that the repeat elements in *STB*-proximal can be aligned as two tandem units of 124 bp with 97% homology between them (35) and that each unit is almost identical in size to the 125-bp *S. cerevisiae* centromere.

By the reasoning presented above, the ancestral Rep1 and Rep2 plasmid partitioning proteins are the plausible precursors for the scaffold proteins that support the assembly of the kinetochore complex (29). It is significant in this regard that components of the CBF3 complex, such as Ndc10 and Ctf13, have no identifiable homologues outside the Saccharomycetaceae among fungi and other eukaryotes. Similarly, Rep1 and Rep2 homologues are limited to the family of 2 μ m circle-related plasmids. The Rep2 proteins are barely recognizable as homologues by primary sequence alignment, perhaps suggesting their coevolution with their respective hosts.

Despite a common origin, the rapid divergence between the chromosomal and plasmid partitioning systems would make it virtually impossible at present to recognize their evolutionary kinship at the DNA and protein levels. The finite, though small, fitness penalty to be paid for bearing the plasmid burden would have provided the drive for the chromosome segregation machinery to evolve away from the plasmid partitioning system. Yet the conservation of Cse4 association and cohesin assembly at *CEN* and *STB* may not only point to the common ancestry of these loci but may also signify the plasmid’s counterstrategy to ensure its efficient propagation by exploiting the chromosome segregation pathway.

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