The 2 micron plasmid purloins the yeast cohesin complex: a mechanism for coupling plasmid partitioning and chromosome segregation?

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The yeast 2 micron plasmid achieves high fidelity segregation by coupling its partitioning pathway to that of the chromosomes. Mutations affecting distinct steps of chromosome segregation cause the plasmid to missegregate in tandem with the chromosomes. In the absence of the plasmid stability system, consisting of the Rep1 and Rep2 proteins and the *STB* DNA, plasmid and chromosome segregations are uncoupled. The Rep proteins, acting in concert, recruit the yeast cohesin complex to the *STB* locus. The periodicity of cohesin association and dissociation is nearly identical for the plasmid and the chromosomes. The timely disassembly of cohesin is a prerequisite for plasmid segregation. Cohesin-mediated pairing and unpairing likely provides a counting mechanism for evenly partitioning plasmids either in association with or independently of the chromosomes.

Introduction

The yeast plasmid 2 micron circle is a model selfish DNA element (Broach and Volkert, 1991) with an evolutionarily optimized structural and functional design for high-copy and high-fidelity propagation. The plasmid, with a copy number of 60 per cell, neither confers any obvious selective advantage nor imposes any significant metabolic burden upon its host (Futcher and Cox, 1983). The host replication machinery replicates each plasmid molecule once and only once per cell cycle (Zakian et al., 1979). The rate of plasmid inheritance by daughter cells at cytokinesis approaches that of the yeast chromosomes (Broach and Volkert, 1991). A recombination-based amplification system (Futcher, 1986; Volkert and Broach, 1986; Reynolds et al., 1987) and an efficient segregation system are responsible for the remarkable stability and high steady-state copy number of the plasmid.

The stability system consists of two plasmid-coded proteins, Rep1p and Rep2p, and a cis-acting locus, *STB* (Jayaram et al., 1983; Kikuchi, 1983). The Rep proteins show both self- and cross-interactions in vivo and in vitro, and bind to the *STB* DNA with assistance from host factor(s) (Ahn et al., 1997; Scott-Drew and Murray, 1998; Velmurugan et al., 1998; Sengupta et al., 2001). Recent evidence suggests that the carboxy-terminal domain of Rep2p can associate with DNA in a Southwestern assay (Sengupta et al., 2001).

Within the yeast nucleus, the Rep1 and Rep2 proteins tightly associate with *STB*-containing plasmids into well-organized plasmid foci that form a cohesive unit in partitioning (Scott-Drew and Murray, 1998; Velmurugan et al., 2000). It is generally accepted that the protein–protein and DNA–protein interactions engendered by the Rep–*STB* system are central to plasmid partitioning. Point mutations in Rep1p that knock out interaction with Rep2p or with *STB* simultaneously block the ability of these Rep1p variants to support plasmid stability (unpublished data).

Earlier work revealed that the kinetics of segregation of a GFP-tagged 2 micron plasmid derivative and a GFP-tagged chromosome closely parallel each other (Velmurugan et al., 2000). Consistent with a potential plasmid–chromosome connection in partitioning, the Rep–*STB* system also interacts with host-encoded proteins that are suspected to play a role in chromosome segregation (Velmurugan et al., 1998, 2000; Sengupta et al., 2001; unpublished data; results of this study). These include the products of at least three genes: *SHF1/CST6*, *FUN30*, and *BRN1*. The first two are not essential genes, and their functional roles have not been analyzed (Velmurugan et al., 1998; Ouspenski et al.,

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Table I. **Yeast strains and plasmids used in this study**

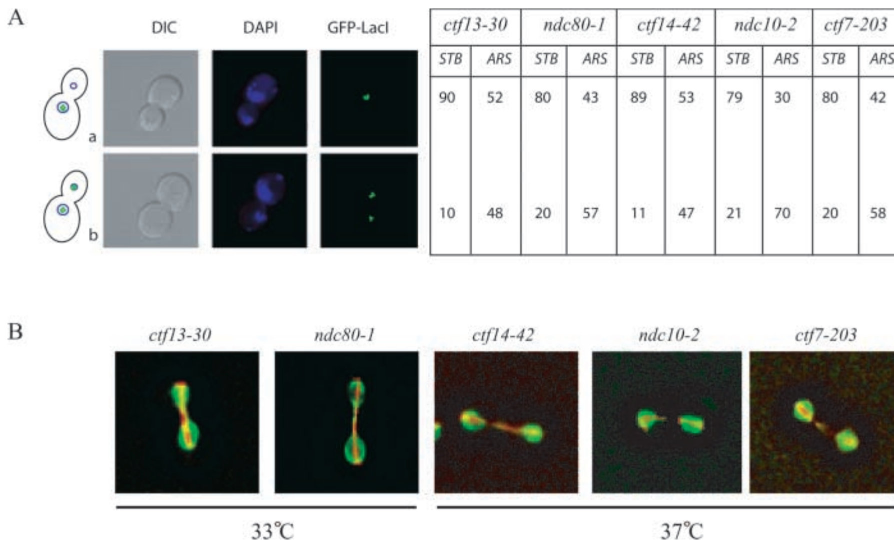
Strain or plasmid	Genotype or salient features
MJY110	*MATa ade2-101 ura3-1 leu2-3, 112 trp1:: MCD1-3HA:: URA3* [cir+]
MJY111	*MATa ade2-101 ura3-1 leu2-3, 112 trp1 :: MCD1-3HA:: URA3* [cir0]
MJY112	*MATa ade2-101 ura3-1::pGAL1-REP1::URA3 leu2-3, 112 trp1 his3-Δ200* [cir0]
MJY113	*MATa ade2-101 ura3-1::pGAL10-REP2::URA3 leu2-3, 112 trp1 his3-Δ200* [cir0]
MJY114	*MATa ade2-101 ura3-1::pGAL1-REP1-pGAL10-REP2::URA3 leu2-3, 112 trp1 his3-Δ200* [cir0]
MJY124	*MATa ade2-101 ura3-1 leu2-3, 112 trp1 his3-11* [cir+]
MJY125	*MATa ade2-101 ura3-1 leu2-3, 112 trp1 his3-11* [cir0]
MJY130	*MATa ade2-101 ura3-1 leu2-3, 112 lys2-801 his3-Δ200 ctf13-30* [cir+]
MJY131	*MATa ade2-101 ura3-1 leu2-3, 112 trp1 his3-Δ200 ctf14-42* [cir+]
MJY132	*MATa ade2-101 ura3-1 leu2-3, 112, trp1 his3-Δ200 ctf7Δ-1::HIS3 ctf7-203::LEU2* [cir+]
MJY133	*MATa ade2-101 ura3-1 leu2-3, 112 trp1 ndc10-2* [cir+]
MJY134	*MATa ade2-101 ura3-1 leu2-3, 112 trp1 his3-Δ200 ndc80-1* [cir+]
MJY135	*MATa ade2-101 ura3-1:: TUB1-YFP:: URA3 leu2-3, 112 lys2-80 his3-Δ200 ctf13-30 :: NUP49-CFP::KanMx* [cir+]
MJY136	*MATa ade2-101 ura3-1:: TUB1-YFP:: URA3 leu2-3, 112 trp1 his3-Δ200 ctf14-42:: NUP49-CFP::KanMx* [cir+]
MJY137	*MATa ade2-101 ura3-1:: TUB1-YFP:: URA3 leu2-3, 112 trp1 his3-Δ-200 ctf7Δ-1:: HIS3 ctf7-203::LEU2:: NUP49-CFP:: KanMx* [cir+]
MJY138	*MATa ade2-101 ura3-1 :: TUB1-YFP:: URA3 leu2-3, 112 trp1 ndc10-2 :: NUP49-CFP::KanMx* [cir+]
MJY139	*MATa ade2-101 ura3-1 :: TUB1-YFP:: URA3 leu2-3, 112 trp1 his3 Δ -200 ndc80-1:: NUP49-CFP::KanMx* [cir+]
MJY140	*MATa ade2-101 ura3-1 leu2-3, 112 :: Lac(O)256 :: LEU2, trp1 :: GAL1p-MCD1-nc :: TRP1 his3 :: GFP-LacI :: HIS3* [cir+]
MJY141	*MATa ade2-101 ura3-1 leu2-3,112 trp1 :: GAL1p-MCD1-nc :: TRP1 his3 :: GFP-LacI :: HIS3* [cir+]
MJY142	*MATa ura3 leu2 trp1 smc1-2MCD1-3HA::KanMx* [cir+]
MJY143	*MATa ade2-1 can1-100 his3-11,15 ura3 leu2-3,112 trp1-1 smc3-42MCD1-3HA::KanMx* [cir+]
MJY144	*MATa ade2-1 his3-11,15 ura3 leu2-3,112 NDC10-3HA::KanMx* [cir+]
MJY145	*MATa ade2-1 his3-11,15 ura3 leu2-3,112 NDC80-3HA::KanMx* [cir+]
pSV1	Lac(O)256 cloned in YEpLac181 (*LEU2*)
pSV4	*GFP–LacI* cloned in YCpLac33 (*URA3*)
pSV5	Lac(O)256 cloned in YEpLac112 (*TRP1*)
pSV6	Lac(O)256 cloned in YRP17 (*TRP1*)
pSV14	mcd1p-nc (noncleavable) expression controlled by the *GAL* promoter in YIpLac204 (*TRP1*)
pSV15	REP1 and REP2 (with native promoter and terminator) cloned into YEpLac181 (LEU2)
pSM41	pESC-Trp plasmid expressing Myc–Mcd1p (Galactose inducible)
pSM42	pESC-Trp plasmid expressing Myc–Smc1p (Galactose inducible)
pSM43	pESC-Trp plasmid expressing Myc–Smc3p (Galactose inducible)
pXY1	Mcd1p cloned into pGAD424
pXY2	*REP1* and *REP2* (with native promoter and terminator) cloned into YEpLac195 (*URA3*)

The relevant genotypes of strains and functional attributes of plasmids are briefly outlined. Plasmids YEpLac181, YEpLac195, YEpLac204, and YCpLac33 were obtained from the Gietz laboratory (University of Manitoba, Manitoba, Canada; Gietz and Sugino, 1988). Some of the derivatives constructed from these parent vectors have been described in our previous work (Velmurugan et al., 2000). The pESC-Trp and pGAD424 vectors were purchased from Stratagene and CLONTECH Laboratories, Inc., respectively. The gene for the nondegradable form of Mcd1p was given to us by Mike Christman (Boston University School of Medicine, Boston, MA). The *smc1-2* and *smc3-42* mutant strains were gifts from D. Koshland (Carnegie Institution of Washington, Baltimore, Maryland).

1999, 2000). *BRN1* is essential, and encodes a component of the yeast condensin complex (Lavoie et al., 2000; Ouspenski et al., 2000; Strunnikov et al., 2001). Overexpression of *CST6* and *FUN30* or inactivation of *BRN1* by a conditional mutation results in impaired chromosome segregation.

We discovered that the *ipl1-2* mutation, which leads to chromosome missegregation at the nonpermissive temperature (Chan and Botstein, 1993; Biggins et al., 1999; Kim et al., 1999; Kang et al., 2001), causes a 2 micron circle-derived plasmid to missegregate in tandem with the chromosomes in a Rep1p- and Rep2p-dependent fashion (Velmurugan et al., 2000). The Ipl1 kinase appears to phosphorylate multiple substrates, including histone H3 and the kinetochore component Ndc10p, and may facilitate chromosome biorientation by altering kinetochore–spindle pole connections (Biggins et al., 1999; Kim et al., 1999; Hsu et al., 2000; Tanaka et al., 2002). Perhaps the Ipl1 protein is required for a shared step in the chromosome segregation and plasmid segregation pathways. Or, it may perform independent functions in the two pathways, for example, by phosphorylating separate target protein(s).

In this paper, we demonstrate that mutations in genes required for kinetochore function or the establishment of sister chromatid cohesion (*CTF7*, *CTF13*, *CTF14/NDC10*, and *NDC80*) missegregate the 2 micron plasmid and the chromosomes in a coupled manner. We provide evidence that the cohesin complex, which holds replicated sister chromatids together until they are ready for separation, is important in plasmid partitioning. We propose that the cohesin complex is recruited to the *STB* locus by the cooperative action of the Rep1 and Rep2 proteins. The timings of cohesin association and dissociation during the cell cycle can be essentially superimposed between the plasmid and the chromosomes. As has been observed for chromosomes, when cohesin disassembly is intentionally blocked (Uhlmann et al., 1999), the normal splitting of the 2 micron plasmid cluster into two separate clusters (that would then rapidly move apart) fails to occur.



	ctf13-30		ndc80-1		ctf14-42		ndc10-2		ctf7-203	
	STB	ARS	STB	ARS	STB	ARS	STB	ARS	STB	ARS
a	90	52	80	43	89	53	79	30	80	42
b	10	48	20	57	11	47	21	70	20	58

Figure 1. **Partitioning of *STB* plasmids and ARS plasmids in yeast mutants that are defective in chromosome segregation.** The construction of *STB* and *ARS* plasmids and their functional differences have been detailed earlier (Velmurugan et al., 2000). They are virtually indistinguishable in their segregation properties in a [cir^0] host strain (lacking the Rep proteins). However, in a [cir$^+$] strain (providing Rep proteins in trans), they behave quite distinctly. (A) The temperature-arrested cells with missegregated chromosomes were categorized into two types: a and b. The representative cells shown here are from the *ctf13-30* strain. The chromosome and plasmid profiles were scored by DAPI and green fluorescence, respectively. The values for each cell type were derived from ~450 largely budded cells for each strain. (B) The nuclear membrane and the mitotic spindle were visualized by fluorescence microscopy in live yeast cells expressing Nup49p–CFP and Tub1p–YFP simultaneously. Red and green colors (tubulin and nuclear membrane, respectively) were added artificially using the Adobe Photoshop® software. The patterns shown here are representative of ~80% of the cells shifted to the restrictive temperature.

Results

Design of experiments and organization of data

The yeast strains and plasmids used for the present study and their salient features are compiled in Table I. In some of the experiments, the endogenous 2 micron circles served as the reporter plasmids. Yeast strains harboring them are designated as [cir$^+$]. These plasmids provide a source of the Rep1 and Rep2 proteins for trans complementation of plasmid substrates lacking the *REP1* and *REP2* genes. Strains lacking 2 micron circles are designated as [cir^0]. When complementation was desired in the [cir^0] background, Rep1p, Rep2p, or both were expressed either from their native promoters or from the inducible *GAL1* promoter, *GAL10* promoter, or both. For direct visualization of plasmids by fluorescence microscopy, the association of GFP–Lac repressor (LacI) expressed in yeast to multiple Lac operators ($LacO_{256}$) harbored by a plasmid of interest was exploited (Straight et al., 1997; Velmurugan et al., 2000). It should be pointed out that an *STB* reporter plasmid in the absence of one or both of the Rep proteins is functionally equivalent to an *ARS* reporter plasmid (lacking *STB*).

A 2 micron–derived plasmid stays with chromosomes in mutants defective for chromosome segregation

To further verify the alleged coordination between chromosome segregation and plasmid partitioning, we followed the effects of several mutations that affect fidelity of chromosome transmission on the behavior of 2 micron–derived plasmids. We describe below the results from mutations in the genes *CTF7*, *CTF13*, *CTF14*/*NDC10*, and *NDC80*.

For each mutant strain, logarithmically growing cells were incubated at the nonpermissive temperature for 3 h, and chromosomes and reporter plasmids were monitored in large-budded cells (Fig. 1). Cells showing pronounced missegregation of chromosomes, as judged by a large inequity in DAPI staining, constituted nearly 70–80% of the population. They were divided into two subgroups: those containing the reporter plasmid in one compartment alone (a) or in both compartments (b).

There was a striking correlation between the tandem missegregation of the chromosomes and the 2 micron–derived plasmid at the nonpermissive temperature (Fig. 1 A). The presence of the plasmid in a compartment lacking chromosomes was seen in at most 21% of the cells examined. In contrast, the *ARS* plasmid was found in the chromosome-free compartment in ~50% of the cells for four of the mutants and 70% of the cells for the fifth mutant (*ndc10-1*). Nuclear elongation and spindle assembly were not affected by the nonpermissive temperature (Fig. 1 B).

For the class b cells, containing the majority of chromosomes in one cell compartment but the plasmids in both compartments, the differences in the numbers of fluorescent plasmid dots between the two compartments were counted. When plasmids did segregate, the equal distribution patterns (4:4, 3:3, etc.; Velmurugan et al., 2000) far outnumbered the unequal pattern (4:3/2/1, 3:2/1, etc.; Table II).

In large-budded cells from a wild-type strain grown at 25°C or 37°C or from the mutant strains grown at 25°C, the 2 micron plasmid was almost always present in both cell compartments (unpublished data). The near equivalence in DAPI staining in these compartments indicated normal chromosome segregation. Quantitatively, the results were similar to those published previously (see Fig. 7 and Table II in Velmurugan et al., 2000).

The products of the *CTF13* and *CTF14*/*NDC10* genes are required for kinetochore assembly as well as centromere association with the yeast cohesin complex (Doheny et al., 1993; Goh and Kilmartin, 1993; Jiang and Carbon, 1993; Jiang et al., 1993; Strunnikov et al., 1995; Russell et al., 1999; Tanaka et al., 1999). The Ndc80 protein is part of a kinetochore-associated complex (Wigge et al., 1998; Janke et al., 2001; Wigge and

Table II. **2 micron plasmid distributions when both daughters receive plasmids despite gross chromosome missegregation**

Plasmid foci distribution	Cells containing the indicated plasmid distribution (%)				
	ctf13-30	***ndc80-1***	***ctf14-43***	***ndc10-2***	***ctf7-203***
4:4	86	85	86	88	79
4:3/2/1	14	15	14	12	21
3:3	90	91	83	94	88
3:2/1	10	9	17	6	12
2:2	88	82	81	91	83
2:1	12	18	19	9	17

The data are from large budded cells obtained after shifting the indicated strains to the nonpermissive temperature for 3 h. The cells scored correspond to the small fraction (~20% for each strain) that revealed plasmids in both cell compartments, even though chromosomes were largely confined to one. The values for equal segregation (n:n) and unequal segregation (n:[n − 1], [n − 2],-----, 1) are expressed as %. The n:0 class would represent tandem missegregation of the plasmids with chromosomes.

Kilmartin, 2001), and the Ctf7 protein is important for the establishment of sister chromatid cohesion (Skibbens et al., 1999; Toth et al., 1999). Nonfunctionality in any one of these proteins results in impaired partitioning of chromosomes. Our data argue that the chromosome and plasmid partitioning pathways either overlap with each other in at least some of their steps or the two are coordinately regulated. For the *ctf14* and *ndc80* mutants, missegregation of the 2 micron test plasmid in tandem with chromosomes is dependent on the Rep proteins. In a [cir^0] mutant background, the 2 micron plasmid loses its strong chromosome-directed bias at the nonpermissive temperature (unpublished data). Plasmid segregation in the other mutants has not yet been tested in the [cir^0] background.

Localization of the Rep1 and Rep2 proteins in chromosome spreads

The observed coupling between chromosome and 2 micron plasmid segregation, and the lack of it in the case of an *ARS* plasmid, would imply that the Rep–*STB* system is the likely coupling agent. To probe the potential association (be it direct or indirect) between plasmid and chromosomes, we followed the localization of the Rep proteins and a 2 micron reporter plasmid in yeast chromosome spreads by indirect immunofluorescence (Fig. 2). Only the data for Rep1p are shown; results with Rep2p were essentially identical.

In [cir$^+$] spreads, both Rep1p (green) and the plasmid DNA (red) were localized with the chromosomes in exponentially growing (Fig. 2 A) as well as G1-arrested cells (Fig. 2 B). As was observed previously (Velmurugan et al., 2000),

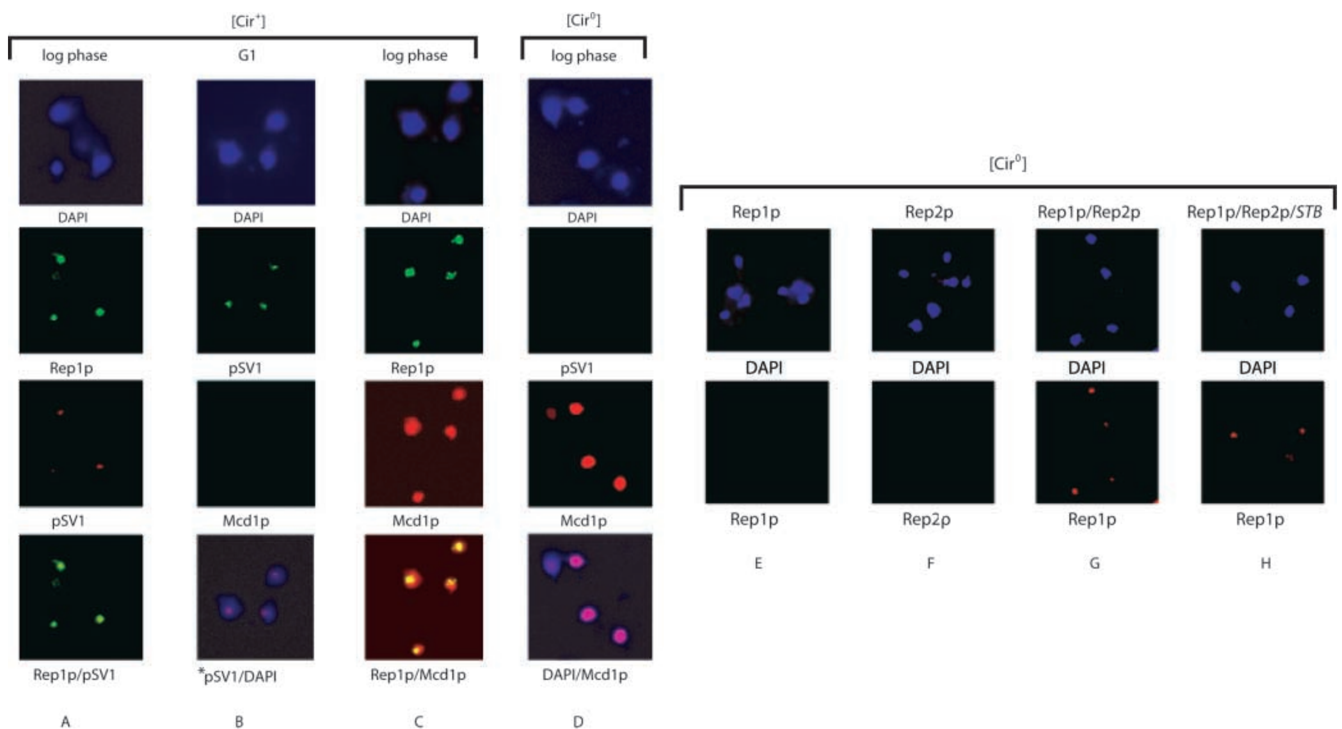


Figure 2. **Localization of the 2 micron plasmid, the Rep proteins, and the Mcd1 protein in yeast chromosome spreads.** Chromosomes were visualized by DAPI, and proteins by immunofluorescence from fluorescein or Texas red–conjugated secondary antibodies. The reporter plasmid (pSV1) harbored Lac operator DNA, and could be revealed by the bound Lac repressor using indirect immunofluorescence. The asterisk over pSV1 (bottom panel of B) denotes that the plasmid fluorescence was artificially changed from green to red using Adobe Photoshop® software for the purpose of overlaying it on the DAPI fluorescence.

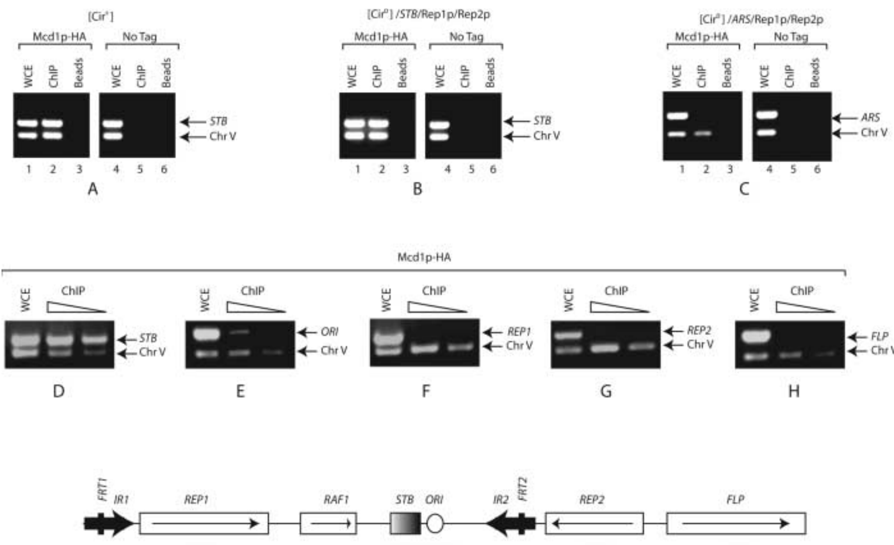


Figure 3. **ChIP assays using antibodies directed to the Mcd1 protein**. The assays were performed with monoclonal antibodies to the HA epitope tag fused to Mcd1p. The relevant genetic features of the yeast strains ([cir^0] or [cir$^+$]; presence of *ARS*- or *STB*-containing reporter plasmids; expression of Rep proteins) are indicated above the experimental panels. The schematic representation of the 2 micron plasmid genome at the bottom shows the location of the origin (*ORI*) and the open reading frames (*REP1*, *REP2*, *FLP*, and *RAF1*) with respect to *STB*. The inverted repeats (*IR1* and *IR2*) contain the Flp recombination target sites (*FRT1* and *FRT2*). The regions probed by PCR are denoted by the horizontal bars. The experimental lanes are captioned ChIP. WCE refers to whole cell extracts from which DNA was PCR amplified without immunoprecipitation. The mock-immunoprecipitated negative controls are indicated by "Beads."

plasmids were confined entirely to the Rep1p zone. The Rep1p foci were smaller and sharper relative to the more spread out pattern obtained with the Mcd1/Scc1 protein (Fig. 2 C), a subunit of the yeast cohesin complex (Michaelis et al., 1997). Mcd1p binds along the entire length of chromosomes, although discontinuously, to establish sister chromatid cohesion (Blat and Kleckner, 1999; Tanaka et al., 1999; Laloraya et al., 2000). The observed profiles suggest either a more restricted set of chromosome association sites for the Rep proteins or, alternatively, the overlap or at least close proximity between nuclear locales occupied by the plasmid and subchromosomal regions. The low resolution of the chromosome spread assay cannot distinguish between these two possibilities. The reporter plasmid present in a [cir^0] strain could not be detected in the chromosome spreads, suggesting that its characteristic localization in the nucleus is mediated by the Rep proteins (Fig. 2 D; see also E–H). Consistent with the stage-specific expression and binding of the Mcd1 protein to the chromosomes during the cell cycle, Mcd1p–chromosome association was absent in the G1-arrested cells (Fig. 2 B) and a subset of the cells from the log phase population (Fig. 2 D). When Rep1p or Rep2p was expressed individually in a [cir^0] strain, no chromosomal association of either protein was observed (Fig. 2, E and F). When the two proteins were simultaneously expressed in the absence of a resident *STB*-containing plasmid (Fig. 2 G) or the presence of one (Fig. 2 H), colocalization of Rep1p with the chromosomes was evident.

The tight association of the plasmid with Rep1p and Rep2p and the requirement of both proteins for their colocalization with the chromosomes suggest that this process is functionally relevant to plasmid partitioning. The plasmid and Rep protein patterns are independent of the yeast cohesin complex, as they show no difference between exponentially growing and G1-arrested cells. Because the Rep proteins bind to the *STB* locus, they may act as match makers in the potential association between the 2 micron plasmid and the chromosomes.

The Mcd1/Scc1 protein associates specifically with the *STB* DNA

The yeast cohesin complex plays a central role in chromosome segregation by establishing sister chromatid pairing during the S phase and maintaining it until chromosomes are ready to be separated during anaphase (Uhlmann and Nasmyth, 1998; Skibbens et al., 1999; Toth et al., 1999; Uhlmann et al., 1999, 2000; Wang et al., 2000; Carson and Christman, 2001). Based on the nearly synchronized segregation of the 2 micron plasmid and the chromosomes observed previously (see Fig. 3 in Velmurugan et al., 2000), we entertained the possibility that the plasmid might utilize the cohesin complex to ensure its stable partitioning. A segregation mechanism based on pairing and unpairing of plasmid clusters would be expected to mimic chromosome segregation in its timing if cohesin were responsible for bridging the clusters.

We employed chromatin immunoprecipitation (ChIP)* to test (1) whether a central component of the yeast cohesin complex, Mcd1p, (Guacci et al., 1997; Michaelis et al., 1997) can associate with 2 micron plasmid, and (2) whether such binding (if observed) is discriminatory with respect to specific plasmid sequences. Chromosomal DNA, sheared to an average length of 500 bp, was immunoprecipitated using Mcd1p-directed antibodies and probed for the presence of 2 micron DNA by PCR (Fig. 3, A–C). A 350-bp amplification product signifying *STB* was yielded by a [cir$^+$] strain (Fig. 3 A, lane 2) or a [cir^0] strain expressing Rep1p and Rep2p and harboring an *STB*-containing plasmid (Fig. 3 B, lane 2). When the same [cir^0] host housed an *ARS* plasmid, the predicted 350-bp *ARS*-specific DNA product was not detected (Fig. 3 C, lane 2). Negative results were obtained with the [cir$^+$] immunoprecipitate and primer pairs specific to ~300-bp regions spanning the 2 micron circle replication origin or internal to the *REP1*, *REP2*, and *FLP* genes (Fig. 3, D–H). The weak signal seen for the origin (Fig. 3 E), which

*Abbreviations used in this paper: 3-AT, 3-aminotriazole; ChIP, chromatin immunoprecipitation; Mcd1p-nc, noncleavable Mcd1p.

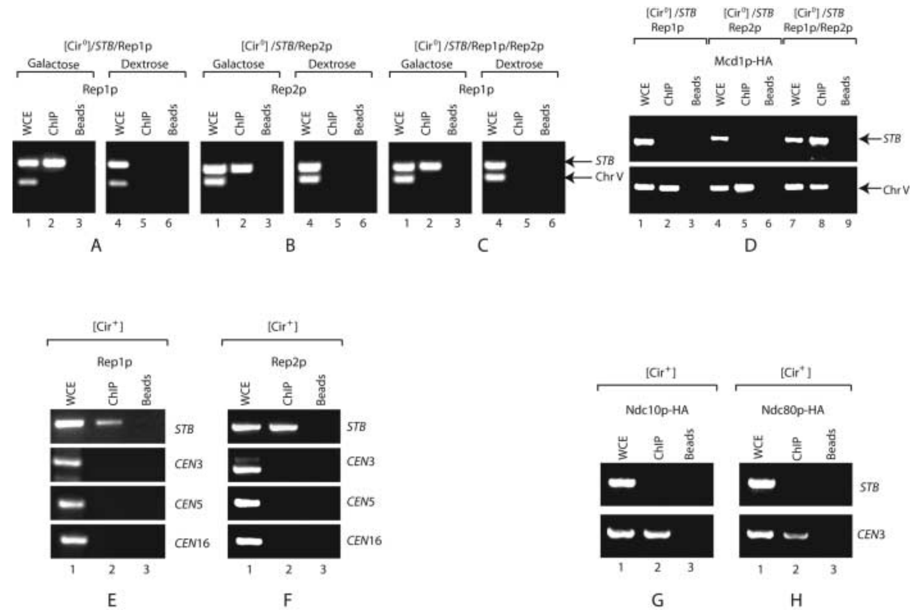


Figure 4. **Probing for *STB* and *CEN* sequences in chromatin immunoprecipitates obtained with antibodies to Rep1p, Rep2p, Mcd1p, or kinetochore proteins.** The proteins targeted for immunoprecipitation are indicated above the respective experimental panels: Rep1p in A, C, and E; Rep2p in B and F; Mcd1p–HA in D; and Ndc10p–HA and Ndc80p–HA in G and H, respectively. As in Fig. 3, the relevant features of the yeast strains are also shown.

is ~600 bp from *STB*, was not surprising. Because of the proximity between the origin and *STB*, some cross-contamination of the two is expected in immunoprecipitates from chromosome fragments of 500 bp average size. The 309-bp PCR product expected for a cohesin binding sequence on chromosome V (Tanaka et al., 1999) was obtained with immunoprecipitates from the [cir^+] strain as well as the [cir^0] strain containing plasmids with or without *STB* (Fig. 3, A–H, lane 2).

The ChIP results demonstrate that the Mcd1 protein, and by extension the yeast cohesin complex (see below), associates specifically with the *STB* region, and this association does not spread significantly beyond *STB*.

The association between Mcd1p and the *STB* locus requires the Rep proteins

To assess the functional significance of Mcd1p–*STB* association, we wished to examine the potential role of Rep1p and Rep2p in this event. As a prelude, we wanted to verify the previously inferred interactions of the Rep proteins with *STB* by ChIP, while simultaneously probing their potential binding to cohesin targets on the chromosomes.

The results displayed in Fig. 4 (A–D) were obtained from a [cir^0] strain harboring an *STB*-containing plasmid. The three variants of this strain contained chromosomal integrations of either *REP1* (Fig. 4 A) or *REP2* (Fig. 4 B) under the *GAL1* or *GAL10* promoter, respectively, or both *REP1* and *REP2* (Fig. 4 C) under the *GAL1-GAL10* bidirectional promoter. ChIP was performed with antibodies to Rep1p (Fig. 4, A and C) or with antibodies to Rep2p (Fig. 4 B). In galactose-induced cells expressing Rep1p alone, Rep2p alone, or both proteins, the immunoprecipitates revealed the presence of *STB* (Fig. 4, A–C, lane 2). When the expression of these proteins was turned off with dextrose, the *STB* DNA was not detected in the mock immunoprecipitates (Fig. 4, A–C, lane 5). The results in Fig. 4 C could be reproduced when Rep2p antibodies were used for immunoprecipitation (unpublished data). Neither Rep1p nor Rep2p antibodies were able to bring down the Mcd1p binding region on chromosome V (Fig. 4, A–C, lanes 2 and 5).

In Fig. 4 D, we arranged the outcomes from three derivatives of a [cir^0] strain expressing Rep1p alone, Rep2p alone, or both Rep1p and Rep2p, respectively, from 2 micron circle-based plasmids containing the *STB* locus. ChIP was performed with Mcd1p-directed antibodies. Presence of the *STB* DNA was not detected in the immunoprecipitate from cells expressing either protein individually (Fig. 4 D, lanes 2 and 5), but was readily seen in the immunoprecipitate from cells coexpressing Rep1p and Rep2p (Fig. 4 D, lane 8). As expected, the expression of the Rep proteins had no effect on the association between Mcd1p and the cohesin binding sequence from chromosome V (Fig. 4 D, lanes 2, 5, and 8).

No association of Rep1p or Rep2p with three centromeric sequences *CEN3*, *CEN5*, and *CEN16* was detected in a [cir^+] strain when ChIP was performed using Rep1p (Fig. 4 E) or Rep2p antibodies (Fig. 4 F). Conversely, antibodies to the kinetochore-associated proteins Ndc10p and Ndc80p failed to bring down *STB*, whereas both antibodies precipitated the *CEN3* sequence (Fig. 4, G and H).

The sum of the results from Fig. 4 not only establishes the specificity of *STB* in the association of Mcd1p with the 2 micron plasmid but also reveals the absolute requirement of both Rep1p and Rep2p for this association, even though Rep1p can bind to *STB* in the absence of Rep2p and vice versa (Velmurugan et al., 1998; Fig. 4, A and B). Taken together, these data suggest that the Rep proteins act cooperatively to recruit the yeast cohesin complex to the partitioning locus of the 2 micron plasmid. Furthermore, the failure of the Rep proteins to bring down *CEN* sequences and of the centromere binding proteins to bring down *STB* indicate that the association of the plasmid directly to the kinetochore is unlikely.

As a corollary, *STB* and chromosomal cohesin binding sites are not functionally equivalent. Obviously, the Rep proteins have no role in cohesin assembly on the chromosomes. At least the one cohesin target tested here is not

bound by the Rep1 protein, either on its own or in the presence of the Rep2 protein. There is a caveat, though. Approximately 1.3×10^3 cohesin binding sites exist in the yeast genome (roughly one per 9×10^3 bp; Laloraya et al., 2000). If Rep1p binds to these sites randomly and with roughly equal probabilities, the relative abundance of the target site probed in our assays would be 7.7×10^{-4}. This large dilution effect could potentially have interfered with its detection. However, given the sensitivity of the PCR amplification step, we consider this to be an unlikely prospect.

Requirement of the Rep proteins for Mcd1p–*STB* interaction is corroborated by in vivo monohybrid assays

As a further verification of the ChIP results, we scrutinized the role of the Rep proteins in Mcd1p–*STB* interaction by an in vivo monohybrid assay. The assay is based on the enhanced *HIS3* reporter gene expression via the interaction with *STB* of a test protein fused to a transcriptional activation domain. Increased production of the His3 protein allows the tester strain to overcome growth inhibition by the His3p-specific inhibitor 3-aminotriazole (3-AT).

In a [cir^0] host strain, Mcd1p (fused to the activation domain) failed to interact with *STB*, the 3-AT challenge (10–50 mM) yielding no better growth than the control strain containing the empty vector (Fig. 5 A, compare columns 1 and 2). As expected from its ability to bind *STB* in a [cir^0] background, Rep1p conferred 3-AT resistance (Velmurugan et al., 2000; Fig. 5 A, column 3). Coexpression of the Mcd1p fusion protein with Rep1p alone or Rep2p alone also failed to induce 3-AT resistance at 25 and 50 mM concentrations of the drug (Fig. 5 A, columns 4 and 5). The presence of all three proteins simultaneously in the same cell was required for growth at 25 and 50 mM 3-AT (Fig. 5 A, column 6). In a [cir$^+$] strain, the Mcd1p hybrid was active by itself, the Rep1 and Rep2 proteins being supplied by the native 2 micron circles (Fig. 5 A, column 8).

The agreement between the ChIP and monohybrid assays validates the inference that both Rep1p and Rep2p are mandatory for Mcd1p–*STB* association. The involvement of all three components of the Rep–*STB* system in Mcdp1 (and likely cohesin) recruitment suggests that this step is relevant to equipartitioning of the 2 micron plasmid.

Association of Smc1 and Smc3 proteins with *STB*: their absence disrupts Mcd1p–*STB* association

Does the Mcd1p–*STB* interaction truly reflect the recruitment of the cohesin complex by the plasmid partitioning system? We repeated the ChIP assays with antibodies to two other integral cohesin components, Smc1p and Smc3p (Fig. 5 B). As was the case with Mcd1p, both Smc1p and Smc3p associated with *STB* in a [cir$^+$] strain (Fig. 5 B, lanes 2 and 8) and not in a [cir^0] strain (Fig. 5 B, lanes 5 and 11), suggesting that the process requires the Rep proteins supplied by the endogenous 2 micron plasmid. The chromosomal target site was indifferent to the presence or absence of the plasmid (Fig. 5 B, lanes 2, 5, 8, and 11). In host strains carrying temperature-sensitive *smc1-2* and *smc3-42* alleles, Mcd1p–*STB* association was normal at the permissive temperature (Fig. 5 C, lanes 2 and 8) but absent at the nonpermissive temperature (Fig. 5 C, lanes 5 and 11). As expected, the chromosomal association of Mcd1p followed suit in this case (Fig. 5 C, lanes 5 and 11).

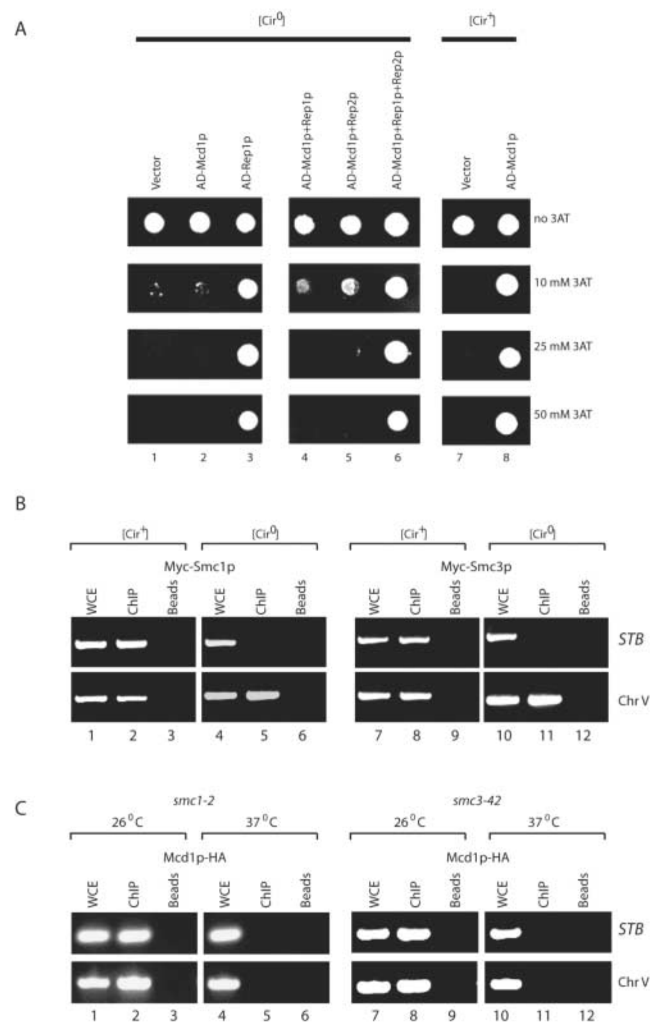


Figure 5. **Monohybrid tests for Mcd1p–*STB* association and ChIP assays for Smc1p–*STB* and Smc3p–*STB* association.** (A) The monohybrid assays were performed in isogenic [cir^0] and [cir$^+$] strains as described previously (Velmurugan et al., 1998). AD, activation domain. (B and C) ChIPs were done with antibodies to the Myc epitope (B) or the HA epitope (C).

The interaction of the *STB* locus with individual components of cohesin and their interdependence in establishing this interaction argue for the recruitment of the whole cohesin complex to the 2 micron plasmid. Yet the plasmid differs from the chromosomes in the mode of cohesin acquisition and in the DNA locale that functions as the cohesin recipient.

Binding of Mcd1p to *STB* as a function of cell cycle progression

The cohesin complex physically bridges sister chromatids as each chromosome is duplicated (Uhlmann and Nasmyth, 1998), and maintains this cohesion from the S phase to the late G2/M phase (Uhlmann et al., 1999). The parallels between the replication and segregation properties of the chromosomes and the 2 micron plasmid have encouraged us to

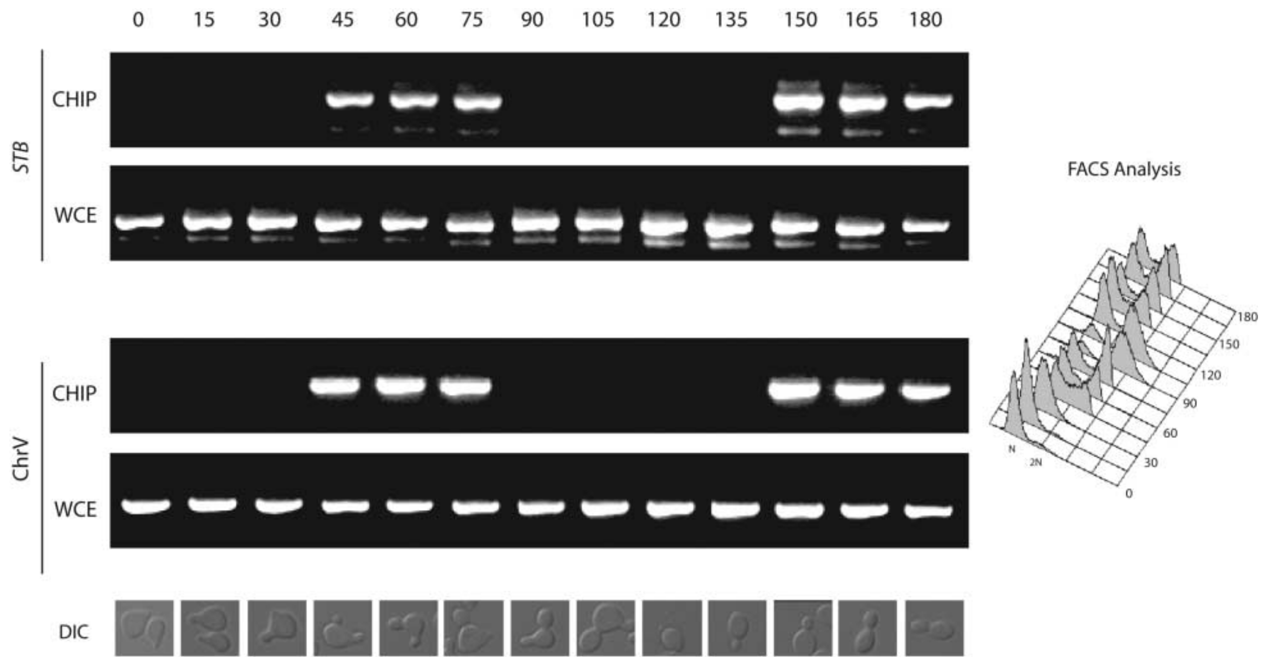


Figure 6. **Cell cycle dependence of Mcd1p binding to *STB*.** Time zero refers to the release of G1-arrested cells from α factor. Representative DIC images of the cells at the different time points after removing the cell cycle arrest are shown at the bottom. The DNA contents of corresponding cell samples derived by FACS® analysis are shown at the right.

test whether the plasmid also behaves similarly in its cell cycle–dependent association with Mcd1p.

G1-blocked [cir$^+$] cells were released from α factor arrest at time zero and allowed to resume the cell cycle in pheromone-free medium. Cell aliquots were sampled at start (time zero) and at 15-min intervals thereafter by ChIP, light microscopy, and FACS® analysis (Fig. 6). The combined results indicated that Mcd1p binding to chromosomal sites and to *STB* DNA is essentially coincident during the cell cycle. The respective associations, established after the onset of the S phase, spanned the S to G2/M window. The timing of Mcd1p binding to and dissociation from *STB* observed in synchronously cycling cells would be consistent with the cohesin complex playing analogous roles in chromosome and plasmid segregation. The plasmid stability system appears to feed into a temporal program preestablished by its host so as to accomplish synchrony of segregation with the chromosomes.

Mechanism of cohesin recruitment by the chromosomes and the 2 micron plasmid can be further distinguished by inappropriate expression of cohesin during the G1 phase

One important issue that is unsettled by the experiments described so far is whether the 2 micron plasmid is actively recruiting cohesin or passively binding cohesin already assembled on chromosomal sites. As a potential means for dissociating the cell cycle synchrony between chromosomes and the plasmid in cohesin association, we induced the expression of Myc-tagged Mcd1p from the *GAL1* promoter in cells arrested in G1 with α factor, and performed chromosome spread assays (Fig. 7 A) and ChIP analyses (Fig. 7, B and C). In [cir$^+$] cells, Mcd1p was detected in the chromosome spreads as a narrowly localized spot that was completely included in the Rep1 protein–localizing region (Fig. 7 A, top). An *STB*-containing reporter plasmid (pSV1), when present in these cells, merged with Mcd1p (Fig. 7 A, middle). In [cir^0] cells containing pSV1, neither Mcd1p nor the plasmid could be detected in the chromosome spreads (Fig. 7 A, bottom). Consistent with these observations, Mcd1p antibodies failed to bring down *STB* or the chromosome V cohesin target in the [cir^0] strain (Fig. 7 B, compare lanes 1 and 2). On the other hand, Mcd1p antibodies precipitated *STB* in the [cir$^+$] background, but not the chromosome V cohesin binding site nor a centromeric sequence (*CEN*3) (Fig. 7 B, compare lanes 3 and 4 and 5 and 6). In addition, binding of the G1-expressed cohesin to *STB* was strictly dependent on Smc1p and Smc3p being functional, when assayed in a temperature-sensitive background for either protein (Fig. 7 C, compare lanes 5 and 2).

Thus, when cohesin is overexpressed at an inappropriate phase of the cell cycle (namely G1), bulk chromosomes are free of it (at the chromosome spread level). Similarly, two chromosomal cohesin binding sites (one of them being a high occupancy centromeric region) remain vacant (by the ChIP test). In sharp contrast, a 2 micron–derived plasmid binds cohesin in G1 and displays the bound form in chromosome spreads.

2 micron plasmid segregation is blocked in cells expressing noncleavable Mcd1p

A critical step in triggering metaphase to anaphase transition in the *Saccharomyces* yeast is the proteolytic cleavage of Mcd1p by the Esp1 endopeptidase by the N-end rule pathway (Rao et al., 2001). When cleavage is prevented, sister chromatids fail to separate from each other (Uhlmann et al., 1999). If the chromosomes and the 2 micron plasmid utilize the cohesin complex for the same end, the noncleavable ver-

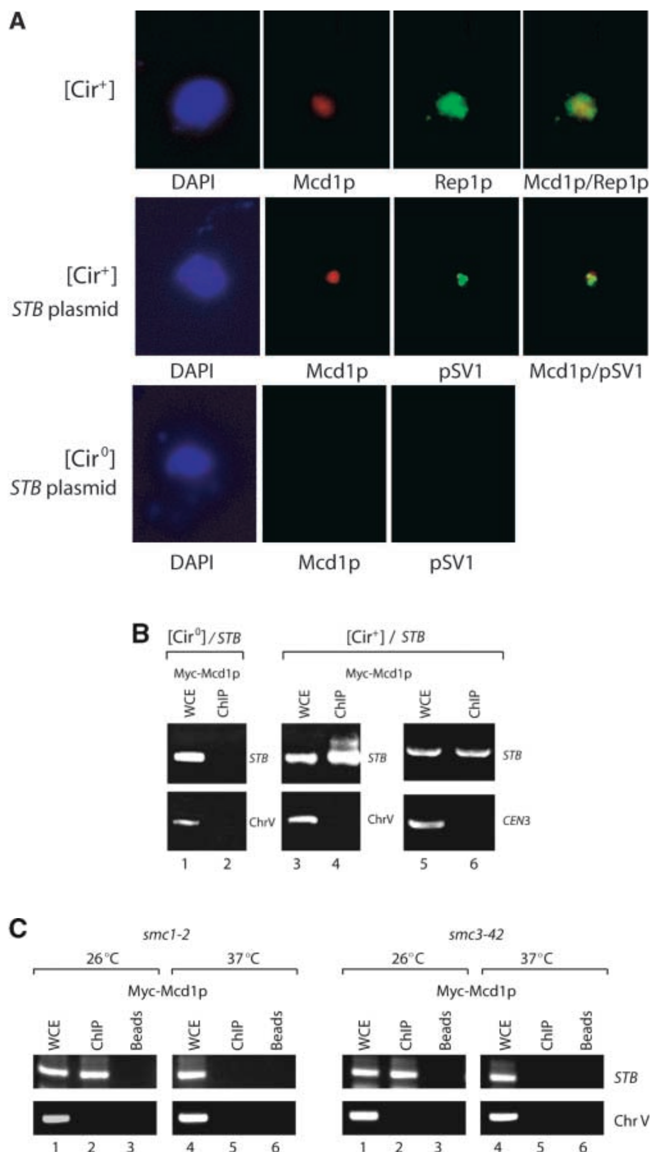


Figure 7. **Distinction between the 2 micron plasmid and the chromosomes in binding G1-expressed Mcd1p.** Myc-tagged Mcd1p was overexpressed from the *GAL10* promoter in G1-arrested cells. (A) Chromosome spreads were probed for Rep1p, Mcd1p, or the *STB*-containing plasmid pSV1 by immunostaining. Antibodies to native Rep1p, the Myc epitope (fused to Mcd1p), or the Lac repressor bound to the operator repeats on pSV1 were used. The signal from Mcd1p expressed inappropriately in G1 was weak. Images obtained with an Optronix Quantix camera were deconvolved and enhanced using the Metamorph software. (B and C) The same antibodies to Mcd1p as in A were used in the chromosome immunoprecipitation assays. Primers specific to *STB*, a cohesin site on chromosome V, or *CEN*3 were used in PCR amplifications.

sion of Mcd1p (Mcd1p-nc) should interfere with plasmid segregation, presumably by blocking the separation of replicated plasmid clusters. We have tested this hypothesis by following the dynamics of fluorescence-tagged reporter plasmids, with or without *STB*, in cells expressing Mcd1p-nc.

The haploid [cir$^+$] strain harboring the test plasmids contained a copy of the native *MCD1* gene and, in addition, a copy of the variant *MCD1*-nc placed under the control of the *GAL10* promoter. Exponentially growing cells were shifted from dextrose to galactose to induce high level expression of the mutant Mcd1p, and samples were examined by time-lapse fluorescence microscopy. We also analyzed the chromosome behavior in the same host strain lacking a reporter plasmid but containing fluorescence-tagged chromosome III instead. Each set of paired rows in Fig. 8 reports the results from 8 dextrose- and 10 galactose-grown cells.

After shifting to the galactose medium (Mcd1p-nc induced), DNA duplication was normal, but cells were arrested at the large-budded stage with a 2n DNA content, as indicated by FACS® analysis (unpublished data). For chromosome III, 7 out of 10 such cells showed nonseparation of sisters; the corresponding number for nondetached *STB* plasmid clusters was 8 out of 10 (Fig. 8). In sharp contrast, separation of the *ARS* reporter plasmid occurred in all of the cells examined (10 out of 10; Fig. 8). In the case of dextrose-grown cells, chromosome III, the 2 micron plasmid, and the *ARS* plasmid showed separation in every one of the eight cells of each type examined. The results were similar when the assays were done in a synchronized cell population released from α factor arrest (unpublished data). In ~95% of the cells expressing MCd1p-nc, no separation of the 2 micron plasmid clusters was observed.

The above results are consistent with replicated plasmid clusters being held in direct union by cohesin or being bridged indirectly by cohesin because of plasmid tethering to sister chromatids. In both cases, cleavage of Mcd1p would be a prerequisite for the separation of the plasmid clusters. As explained in the Discussion, we favor the former mechanism of cluster to cluster cohesion.

Discussion

The remarkable stability of the 2 micron plasmid is achieved by a surprisingly simple molecular device consisting of two plasmid-coded proteins and a relatively small partitioning locus. The chromosomes, on the other hand, rely on an elaborate segregation apparatus and multiple biochemical steps to ensure that each progeny cell receives a full complement of the genome. We suggest that the effectiveness of the plasmid stability system derives from its ability to appropriate key components of the chromosome segregation pathway. We base this assertion on the accepted functional role of cohesin in chromosome segregation, the chromosome-independent recruitment of cohesin by the plasmid, and the absolute requirement of the Rep–*STB* system for this recruitment. We outline two possible models for plasmid segregation within this conceptual framework for future testing.

Coupling between chromosome and 2 micron plasmid partitioning

The tandem missegregation of chromosomes and the 2 micron plasmid in mutant backgrounds that affect chromosome segregation in different ways provides a strong case for potential molecular links between the chromosome and plasmid segregation pathways. One all-encompassing model that accounts for the above results proposes that plasmids are physically tethered to chromosomes, making the two indistinguishable in segregation. The Rep proteins may bring about this bridging by binding to *STB* on the one hand, and

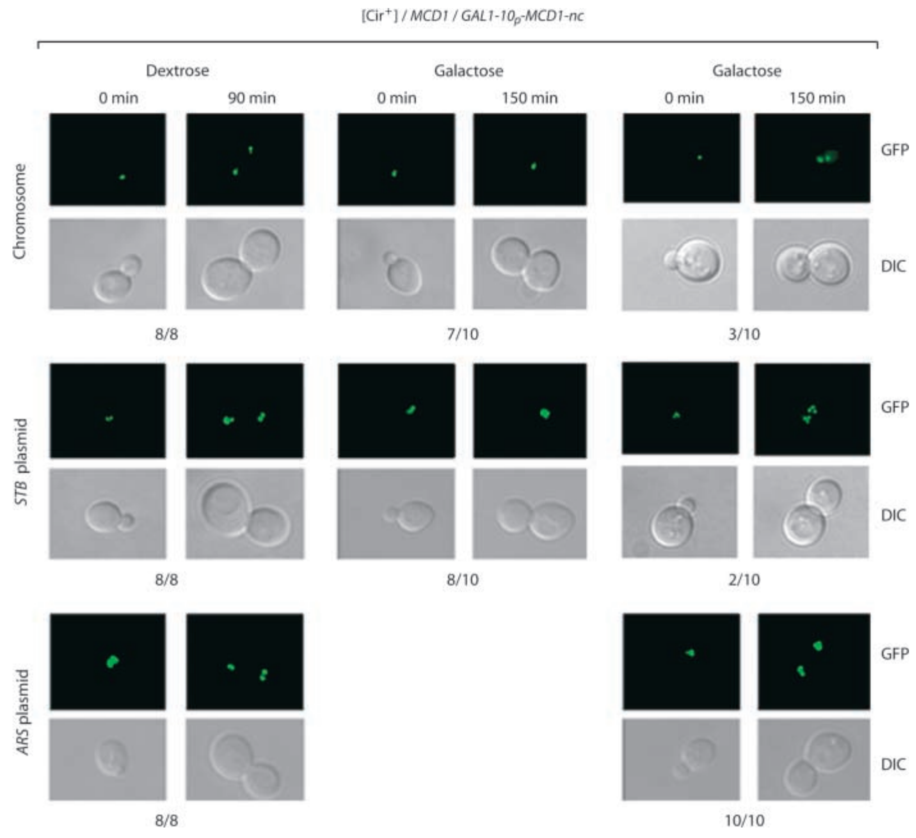


Figure 8. **Effect of noncleavable Mcd1p on the partitioning of an *STB* plasmid and an *ARS* plasmid.** Small budded cells from an exponentially growing culture, induced for GFP–Lac repressor expression were followed by time-lapse fluorescence microscopy for 90 min in dextrose medium or for 150 min after shifting to galactose medium. The representative fluorescence patterns at the initial and final time points are shown. For each experiment, the number of cells that displayed a given pattern is expressed as a fraction of the total number of cells assayed.

interacting with proteins that bind to chromosomes on the other. Data from chromosome spreads are consistent with, but do not provide proof for, the tethering model.

Stable transmission by chromosome attachment is a strategy used by mammalian viruses that replicate as extrachromosomal plasmids in host cell nuclei (Harris et al., 1985; Simpson et al., 1996; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998; Ballestas et al., 1999; Cotter and Robertson, 1999; Ilves et al., 1999; Kanda et al., 2001). An Epstein-Barr virus–based stable partitioning system for plasmids has been reconstituted in yeast (Kapoor et al., 2001). Chromosome tethering provides these viral genomes with a safeguard against their exclusion into the cytoplasm during the breakdown of the nuclear envelope. This argu-

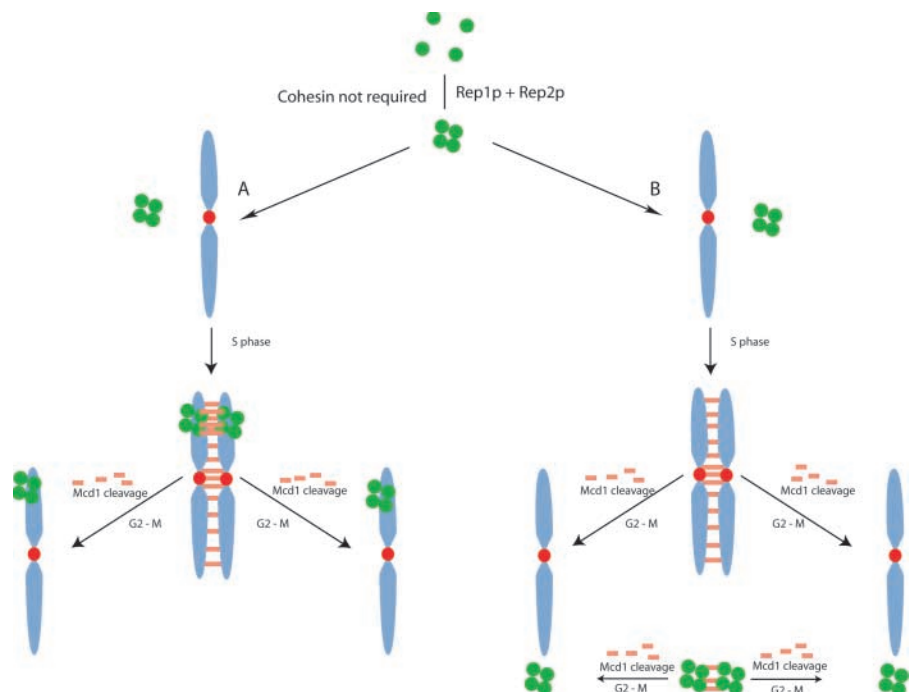


Figure 9. **A model for the role of the cohesin complex in the segregation of the 2 micron plasmid.** The clustering of the 2 micron plasmid and the compactness of the cluster are mediated by the plasmid stability system and are not dependent on cohesin. The duplication and partitioning of the plasmid cluster may occur via mechanisms depicted in A or B or variations of these. (A) Cohesin-mediated pairing of replicated plasmid clusters occurs during the S phase as does the pairing of sister chromatids. The plasmid clusters are tethered to sister chromatids, likely by a cohesin-independent mechanism. (B) Cohesin-mediated pairing and unpairing are common to the chromosomes and the plasmid clusters as in A. The plasmids are partitioned, however, without physical attachment to the chromosomes.

ment does not hold for the 2 micron plasmid, because the nuclear membrane remains intact during yeast mitosis.

The Rep system recruits the yeast cohesin complex to the *STB* locus: cohesin disassembly late in the cell cycle is a prerequisite for plasmid partitioning

The most significant finding from the present study is that the 2 micron plasmid stability system channels the yeast cohesin complex toward its partitioning. Our results best fit the interpretation that Rep1 and Rep2 proteins act in concert to actively recruit cohesin to the *STB* locus. The plasmid is not just passively associating with cohesin already loaded at chromosomal locales. When Mcd1p is inappropriately expressed in G1, it can be detected on the 2 micron plasmid but not on the chromosomes by ChIP or in chromosome spreads. However, at the onset of the ensuing S phase, association of cohesin with *STB* is reinitiated de novo, presumably because of the recycling of the Rep proteins on the *STB* DNA (unpublished data).

Multiple factors contribute to the cell cycle dependence of cohesin to *STB* association. Because Mcd1p is absent in G1, the plasmid would be cohesin free, even though *STB* is binding competent at this stage. *STB* loses its competence during a narrow window preceding the onset of the S phase, and regains it during early S phase. As a result, during the normal cell cycle, the timing of cohesin binding to the 2 micron circle would be indistinguishable from that of cohesin binding to the chromosomes. Cohesin recruitment to the chromosome and the plasmid, although retaining a conserved theme, differs in the details, including the site as well as the mediators of recruitment. The timing and life span of cohesin–*STB* association and the requirement for cohesin disassembly late in the cell cycle suggest that the yeast cohesin complex plays fundamentally similar roles in the partitioning of yeast chromosomes and the 2 micron plasmid.

The tandem missegregation of the 2 micron plasmid with the chromosomes caused by defects in the establishment of sister chromatid cohesion (*ctf7-203*) or the association of centromeres to the cohesin complex (*ctf13-30* and *ctf14-42/ndc10-2*) do not follow directly from our results. Preliminary results suggest that Ctf7p is not essential for plasmid–cohesin association (unpublished data). Perhaps the plasmid may utilize a checkpoint mechanism to abort its partitioning and stay with the bulk of the chromosomes when chromosome missegregation is sensed. Avoidance of a cell bereft of chromosomes would be a wise strategy for a selfish DNA element.

Plausible models for plasmid segregation

We suggest two general models for cohesin-mediated plasmid segregation that need not be mutually exclusive. Because of the timing and half-life of plasmid–cohesin association, both models assume that replicated plasmid clusters are bridged by cohesin.

In model I (Fig. 9 A), cohesin facilitates pairing between the two duplicated plasmid clusters that, in turn, are tethered to a pair of sister chromatids. Because a single plasmid cluster forms the segregation unit, attachment of the duplicated clusters to chromosomes in a random fashion cannot mediate stable partitioning. The coincident dissolution of the cohesin bridge between the sister chromatids and the plasmid clusters would dispatch each cluster in opposite directions in association with the chromosomes. The plasmid–chromosome attachment could be mediated by cohesin itself, although this is unlikely. Its disassembly at the time of segregation would negate the hitchhiking scheme, unless the cohesin bridge between plasmid and chromosome is selectively resistant to disassembly, or its dissolution is delayed until after segregation. If the chromosome spreads are boldly interpreted as evidence for plasmid–chromosome tethering, the observed G1 pattern would exclude cohesin from being the tether. By the same argument, cohesin would be exempt from any role in the primary clustered organization of the plasmid.

In model II (Fig. 9 B), the two postreplication plasmid clusters are bridged by the cohesin complex but are not tethered to chromosomes. Upon disassembly of cohesin, each unpaired plasmid cluster moves to opposite cell poles without assistance from the chromosomes. This movement may be mediated by spindle attachment, by an active transport system unrelated to the spindle, or by association with a subcellular entity that is evenly partitioned at cell division.

In the two models, cohesin provides a gross counting device to partition approximately half the total number of plasmid molecules into each of the two daughter cells. However, a more sophisticated counting mechanism in which the two “sister plasmids” resulting from one duplication event are directed toward opposite cell poles cannot be ruled out.

Persistence of the 2 micron plasmid in yeast

The present study sheds further light on the molecular basis of the evolutionary success of the 2 micron plasmid as a stably propagating extrachromosomal DNA element in yeast. By harboring a replication origin that is functionally equivalent to the chromosomal origins, the plasmid enjoys duplication by the host replication machinery. By pilfering host factors using components of its stability system, the plasmid apparently gains access to the services of a sophisticated partitioning mechanism. And by preserving a recombination-mediated amplification system in readiness, the plasmid ensures that its copy number is maintained at the steady-state value.

Why an active partitioning mechanism for a high copy plasmid?

How does one reconcile the amplification mechanism and the high copy number of the 2 micron plasmid on the one hand with the active partitioning system and the apparently low effective copy number on the other? Perhaps in its early evolutionary history, the plasmid segregated by a random mechanism. The Rep–*STB* system may have originated more recently in response to a reduction in the effective copy number as a result of plasmid clustering. Why does yeast still maintain a high copy extrachromosomal element that apparently makes no contribution to its fitness? The built-in sophistication of the strategies for plasmid maintenance suggests that the plasmid might have, at one time, conferred a significant selective advantage on its host. And paradoxically, this very sophistication may make it hard and slow for yeast to get rid of the plasmid now.

Materials and methods

Strains, plasmids, and growth conditions

Standard tools of yeast genetics were employed for construction of strains with desired genotypes (Adams et al., 1997). Cells were grown in YPD or in SD dropout media at 30°C, unless otherwise mentioned.

Strains expressing the hybrid proteins Nup49–CFP and Tub1–YFP were constructed as follows. A PCR-amplified CFP DNA cassette was inserted at the *NUP49* locus as described by Wach et al. (1997). A *TUB1*–YFP fusion cassette was integrated at the *URA3* locus such that the hybrid gene was expressed from the *TUB1* promoter. CFP and YFP formed the carboxy-terminal portions of the fusion proteins. The plasmids pDH3 and pDH5 obtained from the Yeast Resource Center (http://depts.washington.edu/yeastrc) provided the templates for the construction of the engineered strains. Yeast *Escherichia coli* shuttle plasmids with desired properties were constructed by routine procedures (Sambrook and Russell, 2001).

Chromosome spreads

Chromosome spreads from mitotic cells were prepared by following the procedure of Nairz and Klein (1997) with minor modifications. 20 μl of the yeast spheroplasts were mixed gently with 40 μl of 4% paraformaldehyde/3.4% sucrose and 80 μl of 1% lipsol, and spread on glass slides. After overnight incubation at room temperature, the slides were washed twice with 0.4% photoflo-200 (Kodak) and once with 1× PBS before immunofluorescence assay.

Immunofluorescence

Chromosome spreads were first blocked with 1 mg/ml BSA for 15 min at room temperature. Primary antibody was added and incubated in a humid chamber at room temperature for 3 h. The slides were washed with 1× PBS, and incubated with the secondary antibody conjugated to a fluorescent dye for 1 h at 26°C. 1 μg/ml DAPI in 1× PBS was used as the DNA stain. Slides were mounted with mounting media and cover glass, and examined by fluorescence microscopy.

ChIP

ChIP assays were performed as described by Saitoh et al. (1997) with some modifications as described by Kang et al. (2001).

PCR amplification

An aliquot of the immunoprecipitated DNA (usually 1/25 of each sample obtained from 20 OD_{260} units of cells) was used as a template for PCR detection of potential Mcd1p binding elements. DNA amplification was performed using Taq DNA polymerase, and the PCR products were resolved by electrophoresis in 2% agarose gels containing ethidium bromide for visualization of DNA.

Fluorescence microscopy

Plasmids were visualized by fluorescence microscopy by tagging them with GFP–Lac repressor expressed in yeast (Straight et al., 1997; Velmurugan et al., 2000). CFP and YFP images of the nuclear membrane and the spindle, respectively, in the appropriately engineered strains were acquired using a Nikon inverted microscope and excitation and emission filters recommended by the manufacturer. Images were captured using a Photometrics Quantix camera from Roper Scientific, and the MetaMorph software from Universal Imaging Corp.

Other procedures

Monohybrid genetic assays and cell cycle arrest using α-factor followed by restart were performed as described previously (Velmurugan et al., 1998, 2000).

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Note added in proof. While this paper was under consideration, a report by Wong et al. (Wong, M.C., S.R. Scott-Drew, M.J. Hayes, P.J. Howard, and J.A. Murray. 2002. *Mol. Cell Biol.* 22:4218–4229) demonstrated a central role for the Rsc2 protein, a component of the RSC nucleosome remodeling complex in the maintenance of the 2 micron plasmid. In the absence of Rsc2p, the chromatin structure of the *STB* locus was significantly altered. The growing list of chromosomally encoded factors that functionally interact with *STB* or modify its organization exemplifies the degree of sophistication in the molecular selfishness of the yeast plasmid.

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