

Partitioning of the 2- μ m Circle Plasmid of *Saccharomyces cerevisiae*: Functional Coordination with Chromosome Segregation and Plasmid-encoded Rep Protein Distribution

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Abstract. The efficient partitioning of the 2- μ m plasmid of *Saccharomyces cerevisiae* at cell division is dependent on two plasmid-encoded proteins (Rep1p and Rep2p), together with the cis-acting locus *REP3* (*STB*). In addition, host encoded factors are likely to contribute to plasmid segregation. Direct observation of a 2- μ m-derived plasmid in live yeast cells indicates that the multiple plasmid copies are located in the nucleus, predominantly in clusters with characteristic shapes. Comparison to a single-tagged chromosome or to a yeast centromeric plasmid shows that the segregation kinetics of the 2- μ m plasmid and the chromosome are quite similar during the yeast cell cycle. Immunofluorescence analysis reveals that the plasmid is colocalized with the Rep1 and Rep2 proteins within the yeast nucleus. Furthermore, the Rep proteins (and therefore the

plasmid) tend to concentrate near the poles of the yeast mitotic spindle. Depolymerization of the spindle results in partial dispersion of the Rep proteins in the nucleus concomitant with a loosening in the association between plasmid molecules. In an *ipl1-2* yeast strain, shifted to the nonpermissive temperature, the chromosomes and plasmid almost always missegregate in tandem. Our results suggest that, after DNA replication, plasmid distribution to the daughter cells occurs in the form of specific DNA-protein aggregates. They further indicate that the plasmid partitioning mechanism may exploit at least some of the components of the cellular machinery required for chromosomal segregation.

Key words: *ipl1-2* mutation • tubulin • plasmid cohesion • mitotic spindle • *BRN1*

Introduction

The successful propagation of the 2- μ m circle, a relatively small circular plasmid (6,318 bp) present in most common strains of *Saccharomyces cerevisiae* at a copy number of \sim 60 per cell, is accomplished via a partitioning system and an amplification system (reviewed in Broach and Volkert, 1991). Two plasmid-coded proteins, Rep1p and Rep2p, in conjunction with a cis-acting locus *STB* (also called *REP3*) contribute to the partitioning function (Jayaram et al., 1983, 1985; Kikuchi, 1983; Murray et al., 1987). In addition, host encoded factors may also play a direct or indirect role in plasmid stability (Velmurugan et al., 1998). Recent in vivo and in vitro analyses have demonstrated that Rep1 and Rep2 proteins are nuclear localized, exhibit self- and cross-associations, and bind to the *STB* locus (Ahn et al., 1997; Scott-Drew and Murray, 1998; Velmurugan et al.,

1998). Disruption of their nuclear targeting signals abolishes their function in plasmid partitioning, and fusion to an exogenous nuclear localization signal fully restores their activity (Velmurugan et al., 1998). The functional relevance of these DNA-protein interactions to the plasmid segregation system is supported by the finding that mutations in Rep1p that affect either the interaction with Rep2p or with the *STB* DNA result in reduced plasmid stability (Yang, X.-M., and M. Jayaram, unpublished data). It is not clear whether the association between the 2- μ m plasmid and the Rep proteins is direct, or requires the mediation of other host factors. In vitro studies have demonstrated that urea-solubilized yeast extracts expressing Rep1p and Rep2p, or strains devoid of endogenous 2- μ m circles (*[cir⁰]*)¹ extracts supplemented exogenously with

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¹Abbreviations used in this paper: *[cir⁰]*, strains devoid of endogenous 2- μ m circles; *[cir⁺]*, strains containing 2- μ m circles; DAPI, 4', 6'-diamidino-2-phenylindole; GFP, green fluorescent protein.

Rep1p and Rep2p, can bind *STB* DNA (Hadfield et al., 1995).

Although the apparent copy number of the 2- μ m plasmid is high, the requirement of a partitioning system for stable plasmid inheritance suggests that the effective copy number within the cell may be considerably lower. Impediments to free movement of plasmid molecules are likely imposed by their attachment to subcellular sites. Yeast plasmids containing chromosomal *ARS* elements, but lacking the 2- μ m circle partitioning system, have a propensity to be retained in the mother cell during division (Murray and Szostak, 1983). This mother-daughter bias accounts for their high instability during nonselective propagation of the host cells. The stability of the native 2- μ m plasmid (approximately one plasmid-free cell in 10^4 – 10^5 cells per generation; Futcher and Cox, 1983; Mead et al., 1986) implies that the Rep/*STB* system is able to overcome the segregation bias by one of two plausible mechanisms. Either the plasmids are freed from attachment sites and rendered freely diffusible, or they are actively attached to a cellular entity that divides equally between mother and daughter. Currently available evidence cannot unambiguously distinguish between the two mechanisms.

The need for plasmid amplification arises only if and when there is a decrease in copy number below the steady state value. Normally, each plasmid molecule is replicated once, and only once, per cell cycle (Zakian et al., 1979) and the daughter molecules are partitioned efficiently at nuclear division. When there is a drop in copy number, the amplification system overrides the cell cycle restriction of a single round of plasmid replication during one S phase. Plasmid amplification is absolutely dependent on the 2- μ m circle Flp site-specific recombination system (Volkert and Broach, 1986). A currently favored model for amplification proposes recombinational inversion of a bidirectional replication fork and the resultant double-rolling-circle replication mode as the means for obtaining multiple replicas of the plasmid from a single initiation event (Futcher, 1986, 1988). The cessation of amplification would require a second recombination event that can restore normal direction of fork movement. The time interval between the two successive recombination events would determine the degree of amplification. Based primarily on genetic studies, it has been proposed that a regulatory complex containing Rep1p and Rep2p may provide an indirect readout of the copy number and, at or above a critical concentration, may negatively control amplification by turning down expression of the *FLP* gene (Murray et al., 1987; Reynolds et al., 1987; Som et al., 1988; Veit and Fangman, 1988).

Assuming that the functional copy number of the 2- μ m plasmid is considerably <60 , perhaps as low as unit copy if the individual plasmid copies are coalesced into a single cluster, how does it achieve its remarkably high stability using a seemingly rudimentary partitioning system? The plasmid molecules are resident in the nucleus as minichromosomes with standard nucleosome phasing (Livingston and Hahne, 1979; Nelson and Fangman, 1979; Taketo et al., 1980). Their replication by the cellular replication machine, as already indicated, follows normal cell cycle controls (Zakian et al., 1979; Brewer and Fangman, 1987; Hu-

berman et al., 1987). It seems plausible then that the 2- μ m circle might also depend on the chromosomal segregation apparatus for its stable inheritance. Chromosome segregation in yeast, as in higher eukaryotes, follows a series of precisely coordinated events during the cell cycle. Replication of chromosomes, maintenance of linkage between sisters until their alignment at the mitotic spindle, attachment of kinetochores to opposite spindle poles, loss of sister chromatid cohesion at the onset of anaphase, and the movement of the sisters in opposite directions along the microtubule are spatially temporally orchestrated by the interplay of a number of cellular components (for reviews see Hoyt and Geiser, 1996; Heck, 1997; Nicklas, 1997). In principle, it is possible to ask how loss of function in one or more of these components, leading to chromosome missegregation, affects partitioning of the 2- μ m plasmid.

In this paper, we provide visual evidence that the 2- μ m plasmid and the Rep proteins are associated with each other in the yeast nucleus. The Rep proteins (and by inference, the associated plasmid molecules) are clustered near the poles of the mitotic spindle apparatus. The apparent linkage between the spindle poles and the plasmid-Rep complex suggests a plausible mechanism for the equal partitioning of plasmid molecules after replication. We propose that the plasmid has evolved a strategy to exploit at least certain components of the chromosome partitioning machinery to ensure its own stable propagation. Such a mechanism is consistent with the observed correlation between the segregation kinetics of a 2- μ m-based plasmid and a yeast chromosome (or a centromere containing plasmid) with respect to the cell cycle. Further support for this mechanism is provided by the finding that the yeast chromosomal mutation *ipl1-2* (Chan and Botstein, 1993) results in the missegregation of both chromosome and the 2- μ m-based test plasmid in tandem. This strong correlation in missegregation is lost when the partitioning system is inactivated, or in the case of an *ARS*-plasmid. We consider the potential implications of our findings for the utilization of common cellular components in plasmid and chromosome segregation (see Discussion).

Materials and Methods

Strains and Plasmids

The strains and plasmids used in the present study are listed in Table I. The strain AFS479 was kindly given to us by the laboratory of Andrew Murray (University of California, San Francisco, CA). The [cir⁺] strains were obtained as described previously (Tsalik and Gartenberg, 1998).

The plasmid pAFS59 carrying 256 copies of the lac operator and pAFS144 expressing green fluorescent protein (GFP)-lac repressor from the *HIS3* promoter were provided by the Murray laboratory (Robinett et al., 1996). The iterated lac operator DNA was excised from pAFS59 as a BamHI-SalI fragment and ligated to BamHI plus SalI digested YEplac195 and YCplac111 (Gietz and Sugino, 1988) to obtain the plasmids pSV1 and pSV2, respectively. Similarly, the lac operator repeats from pSV2 were isolated as a SmaI-SalI fragment and cloned into SmaI-SalI digested YRp17 to obtain plasmid pSV3. Plasmid pSV13 was constructed by transferring the KpnI-XbaI fragment that contains the GFP-lac repressor cassette under the *HIS3* promoter into KpnI and XbaI digested YCplac111.

More detailed information on strains and plasmids are available upon request.

Table I. Strains and Plasmids Used in This Study

Name	Genotype	Source	Reference
Strains			
AFS479	MATa <i>ade2-1 ura3-1 trp1-1 leu2-3,112 lac(O)::LEU2 GFP-lacI::HIS3</i> [cir ⁺]	A. Murray	Robinett et al., 1996
CCY666-7B	MATα <i>ade2 ura3-52 leu2-3,112 his3-Δ200</i> [cir ⁺]	This study	
CCY666-1A	MATa <i>ade2 ura3-52 leu2-3,112 his3-Δ200</i> [cir ⁺]	This study	
MJY30-10a	MATa <i>ade2 ura3-52 trp1-1 leu2-3,112 GFP-LacI::HIS3</i> [cir ⁺]	This study	
MJY31	MATa <i>ade2 ura3-52 trp1-1 leu2-3,112 GFP-LacI::HIS3</i> [cir ⁰¹]	This study	
CCY915-2B	MATα <i>lys2-801 ura3-52 trp1-1 leu2-3,112 his3-Δ200 ipl1-2</i> [cir ⁺]	C. Chan	Kim et al., 1999
MJY43	MATα <i>lys2-801 ura3-52 trp1-1 leu2-3,112 his3-Δ200 ipl1-2</i> [cir ⁰¹]	This study	
Plasmids			
pAFS59	<i>lac(O)</i> repeats in YIplac128	A. Murray	Robinett et al., 1996
pSV1	<i>lac(O)</i> repeats in YEplac195	This study	
pSV2	<i>lac(O)</i> repeats in YCplac111	This study	
pSV3	<i>lac(O)</i> repeats in YRp17	This study	

The yeast strains were obtained from the laboratories of A. Murray or constructed by us. Their relevant genotypes as well as the appropriate references in literature (where available) are listed. Whenever strains devoid of endogenous 2- μ m circles were used in an experiment, the [cir⁺] notation was used to characterize them. Strains without this notation or those with the [cir⁺] notation contained the normal complement of 2- μ m circles.

Plasmid pAFS59 (Robinett et al., 1996) was a derivative of YIplac128 (Gietz and Sugino, 1988) that contained multiple copies of the *lac* operator sequence. The test plasmids used in our studies (pSV1, pSV2, and pSV3) were based, respectively, on the 2- μ m plasmid origin and the *STB* locus, a chromosomal *ARS* sequence and a centromere, and a chromosomal *ARS* sequence without a centromere. They were obtained by transferring the multiple *lac* operator cassette excised from pAFS59 to plasmid YEplac195 (Gietz and Sugino, 1988), YCplac111 (Gietz and Sugino, 1988), and YRp17 (obtained from American Type Culture Collection).

In Vivo Visualization of Plasmids or Chromosome

The yeast strain containing the expression cassette for GFP-lac repressor was transformed with the appropriate plasmid containing the *lac* operator repeats. The expression of the hybrid repressor was induced by the addition of 10 mM 3-AT (3-aminotriazole) for 30 min. The *lac* operator DNA bound by GFP-repressor was visualized by fluorescence microscopy following excitation at the appropriate wavelength. To obtain optimal fluorescence, the pH of the media was maintained at 6.5 by the addition of trisodium citrate (6.5 g/liter). Cells were observed under an Olympus BX-60 microscope with recommended filters for GFP excitation and emission. Images were captured using a Spot Digital Camera from Diagnostic Instruments, and were processed using ImagePro Plus software from Media Cybernetics. Confocal images were taken using the Leica confocal system, TCS4D (Core Facility, Institute for Cell and Molecular Biology, University of Texas, Austin, TX).

Synchronization of Cells in G1 Phase by α -Factor

Cells were grown overnight in selective medium, washed, and resuspended in YEPD at an OD₆₀₀ of ~0.1. The culture was incubated at 30°C for 90 min, and α -factor was added to a final concentration of 7 μ g/ml. Incubation at 30°C was continued for 3 h and the percentage of cells arrested in G1 was monitored by microscopy (Breedon, 1997).

Assay for Plasmid Segregation in Host Strains Harboring the *ipl1-2* Mutation

The yeast strains were grown in appropriate selective media at 26°C, and were arrested in G1 by α -factor treatment. After washing away the pheromone, the cells were allowed to recover from growth arrest at 26°C for 90 min. They were then shifted to 37°C and allowed to grow for 4 h. 4', 6'-diamidino-2-phenylindole (DAPI) was added to the growth medium (final concentration of 2 μ g/ml), and cells were harvested 30 min later. They were washed with sterile water, fixed in 3.5% formaldehyde (0–4°C), and observed under the microscope. Roughly 75–80% of the cells in the population contained large buds. Plasmid and chromosome segregation data shown in Fig. 7 pertain only to the large-budded cells.

Nocodazole Treatment

An exponentially growing yeast culture was treated with either 1% DMSO (control) or 20 μ g/ml nocodazole (Sigma Chemical Co.) in 1% DMSO. The cells were incubated in the presence of the drug at 30°C for 2 h. Examination of the cells under the microscope revealed ~85% of them to be arrested with large buds.

Antibodies

Rep1 and Rep2 polyclonal antisera were generated in rabbits. The antisera were affinity-purified and tested for specificity before use. Antitubulin antibodies were obtained from Serotec. Polyclonal antiserum against the *lac* repressor protein was purchased from Stratagene.

Immunofluorescence Assays

Yeast cells grown to midlog phase (10⁶ cells/ml) were fixed in 5% formaldehyde solution for 60 min at room temperature. The fixed cells were washed once with PBS, once with 1.2 M sorbitol/1mM EDTA, and resuspended in the same medium to a final density of 10⁸ cells/ml. Spheroplasts were obtained by incubating with 1 mg/ml of zymolyase 100T (US Biologicals) in the presence of 10% β -mercaptoethanol for 60 min at 30°C. The spheroplasts were washed with PBS, transferred to poly-L-lysine-coated slides, and flattened using methanol (5 min) and acetone (30 s). Immunofluorescence staining was done according to Adams and Pringle (1984) with some modifications. Blocking was done using 1 mg/ml BSA for 15 min. All the primary and secondary antibodies were diluted in the antibody dilution buffer (1 mg/ml BSA and 0.02% sodium azide in PBS). Incubations with primary and secondary antibodies were done at room temperature for 60 and 30 min, respectively. Observations were made after mounting the samples using mounting solution supplied by KPL Laboratories. Microscopy was carried out using an Olympus BX-60 microscope or the Leica Confocal System, TCS4D. Images were taken at 100 \times and processed in ImagePro Plus (Media Cybernetics) or PhotoShop 5.0 (Adobe Systems Incorporated) software.

Estimating the Copy Number from GFP-LacI-tagged Plasmid Fluorescence

GFP fluorescence intensities from individual plasmid clusters were determined using ImagePro Plus or MetaMorph software (supplied by Media Cybernetics and Universal Imaging Corporation, respectively). Similar estimates were made for the tagged chromosome. The intensities of plasmid fluorescence and chromosome fluorescence were averaged for a large number of cells. The ratio of the averaged values was taken as the mean copy number of the plasmid per cell.

Z-series Sectioning of Yeast Nucleus

The compactness (or the residence zone) of the plasmid clusters was determined by z-series sectioning of the yeast nucleus in the confocal microscope. For each sample, 40 sections at 0.25- μ m thickness were examined,

spanning 5 μm of total thickness. The start point for scanning was set manually ~ 2 – 3 frames beyond the boundary of fluorescence from the GFP-lac repressor-tagged plasmid. Thereafter, the same number of sections (or the same total distance) was scanned for each sample. In every case, the set range completely covered the limits of the plasmid fluorescence zone. An identical procedure was used to obtain the boundary range of the DAPI staining region in each of the cells examined. The ratio of the green fluorescence range to the blue fluorescence range was calculated for each cell. Values from at least 20 individual cells were pooled to express the mean width (\pm SD) of the plasmid residence zone.

Results

Direct Visualization of a 2- μm -derived Plasmid: Comparison to a Yeast Chromosome or a Centromeric Plasmid

To visualize plasmids in live yeast cells, we have used the recognition between multiple copies of the lac operator sequence harbored by the plasmids and a fluorescent version of the lac repressor expressed from an inducible promoter (Robinett et al., 1996). Cells harboring the marked plasmids were examined by conventional (Fig. 1, right) or confocal (Fig. 1, left) microscopy. In a population of strains containing 2- μm circles ($[\text{cir}^+]$) yeast cells growing exponentially in selective media, or in cells synchronized in the G1 phase with α -factor (see Table I for strain and plasmid descriptions), the fluorescently labeled 2- μm -derived test plasmid pSV1 was seen most often as a tetrad cluster within the nucleus ($>50\%$ of the time). The results shown in Fig. 1 were obtained with α -factor-treated cells. The superposition of green plasmid fluorescence (from GFP-lac repressor) and blue nuclear fluorescence (from DAPI) revealed that the plasmid molecules reside within the nucleus (data not shown). At the α -factor concentration used in these experiments (7 $\mu\text{g}/\text{ml}$, in SD medium containing required supplements), the cells did not show the typical shmoo phenotype associated with G1 arrest. However, they did not progress through the cell cycle unless they were washed free of α -factor. The same concentration of α factor in rich medium induced shmooing.

Examination of a large sample of cells revealed occasional deviations from the tetrad pattern of plasmid distribution. In $\sim 20\%$ of the plasmid-containing cells, the clusters consisted of triad or diad patterns (Fig. 1, B and C, respectively), whereas, in $\sim 15\%$ of the cells, single fluorescent dots were observed (Fig. 1 D). Occasionally (15% or less), the plasmid foci were constituted by more than four dots (Fig. 1 E). In comparison, a marked yeast chromosome appeared as a single fluorescent dot in $>99\%$ of the cells examined (Fig. 1 F). Similarly, a centromeric plasmid, pSV2, was also detected as a single fluorescent spot in $>95\%$ of the cells (Fig. 1 G), with an occasional cell revealing two fluorescent spots (presumably representing two plasmid copies). By contrast, a population of cells grown selectively for the plasmid pSV3, containing a chromosomally derived replication origin (*ARS*) and none of the components of the 2- μm circle stability system, showed an essentially random distribution of cells containing one to four, and occasionally more than four fluorescent dots. The patterns of plasmid distribution and the frequencies of their occurrence in a G1-arrested cell population are summarized at the bottom of Fig. 1.

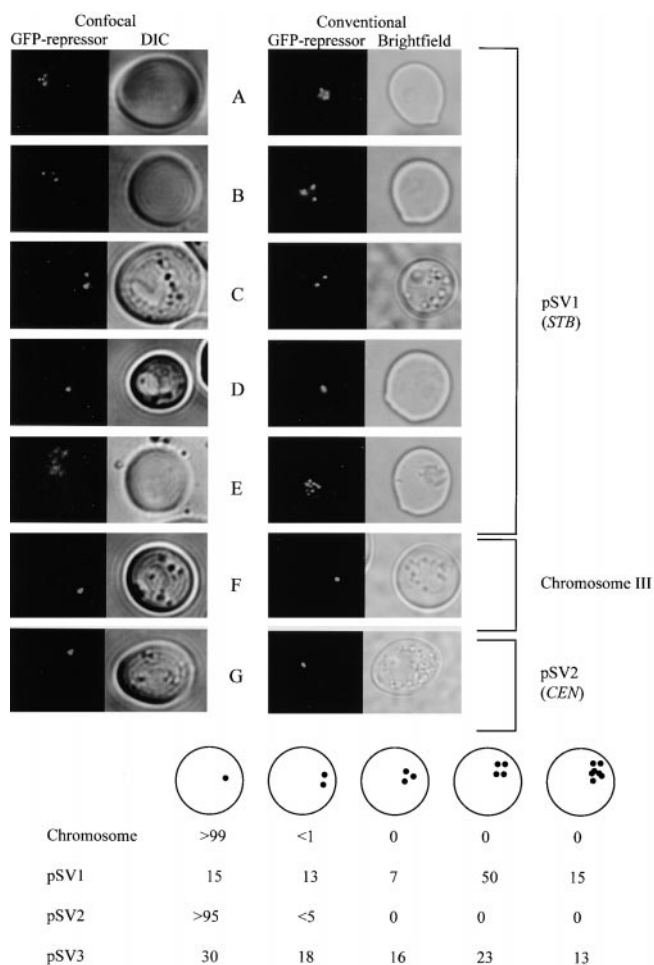


Figure 1. Organization and distribution of a 2- μm circle-based plasmid pSV1 in a $[\text{cir}^+]$ yeast strain. The pSV1 plasmid was visualized by the binding of GFP-lac repressor to plasmid-borne lac operator sequences. The characteristic forms of the pSV1 plasmid in a G1-arrested $[\text{cir}^+]$ cell population, as observed by the green fluorescence, are arranged in rows A–E. The images in the left and right were obtained by confocal and conventional microscopy, respectively. The appearance of a yeast chromosome (chromosome III) and that of a centromeric plasmid pSV2, tagged with the GFP-repressor, as in the case of pSV1, are shown in F and G, respectively. The patterns of distribution of fluorescent dots for the marked chromosome or the different test plasmids (pSV1–3) in a G1 cell population obtained by α -factor arrest are tabulated at the bottom. The *ARS*-based pSV3, unlike pSV1, does not harbor the *STB* locus. The values in the table at the bottom of the figure are averaged from 150–200 cells in each case. Observations on chromosome III and plasmids (pSV1–3) were made in strain AFS479 and MJY30-10a, respectively (see Table I).

Z-series sectioning by confocal microscopy indicated that the 2- μm circle-derived pSV1 plasmid molecules tend to occupy a relatively restricted zone within the yeast nucleus. In a $[\text{cir}^+]$ host, the mean range of the plasmid residence zone expressed as a ratio of the nuclear diameter (derived from DAPI staining) was ~ 0.50 (see Materials and Methods and Fig. 6). The relative intensities of fluorescence yielded an average copy number of ~ 10 – 12 molecules per cell for pSV1 (assuming one copy of the chro-

mosome per cell), and ~ 1 – 2 molecules per cell for pSV2 (the centromere containing plasmid). This estimate of pSV1 copy number refers to cells that contain three or four plasmid foci per cell. Such cells constitute a little over one half the cell population grown selectively (see Fig. 1, table). Estimates from Southern hybridization of total DNA from a selectively grown cell population yielded a pSV1 copy number of 8–10 relative to a single copy gene (data not shown). This value for pSV1 is significantly lower than the steady state copy number of 40–60 molecules per cell for the native 2- μ m circle. However, assuming that the copy number control mechanisms would operate on the plasmid population as a whole, the relevant steady state copy number in a [cir⁺] strain would be the sum of the copy numbers of pSV1 and the endogenous 2- μ m circles.

Association of the 2- μ m Plasmid with the Mother or Bud during the Cell Cycle in a Synchronized Yeast Population

The [cir⁺] host strain containing the 2- μ m test plasmid pSV1, the *CEN*-plasmid pSV2, or the tagged chromosome III was synchronized in G1 phase using α -factor. Following release from α -factor arrest, the plasmid or the chromosome was visualized at various times during cell cycle progression. As indicated by the representative samples displayed in Fig. 2, there was a strong correlation between pSV1 and chromosome III (Fig. 2, compare A to C), or between pSV1 and pSV2 (Fig. 2, compare A to B), with respect to the timing of their appearance in the growing bud. In small-budded cells, pSV1 was almost always associated with the mother cell. As the bud enlarged in size, the plasmid cluster migrated to the bud neck, closely following the dynamics of the chromosome. In large-budded cells, plasmid clusters were almost always detectable in both the mother and the would-be daughter.

To quantitate these observations, we have divided the cell population into four classes (I–IV; see the schematic representation in Fig. 2) with respect to the cell cycle stage as well as the localization of pSV1 within a growing cell (Fig. 3). The relative abundance of each cell type over a period of 180 min after the removal of α -factor indicates that cell synchrony was maintained fairly tightly through the first two generations, but began to break down thereafter. The plot of the distribution of pSV1 between mother and bud as defined by cell types I–IV (Fig. 3, solid line) was nearly superimposable with a similar plot for chromosome III (Fig. 3, dashed line). The synchrony between pSV1 and chromosome III in the [cir⁺] strain was associated with pSV1 being partitioned roughly equally between mother and daughter cells (see Table II). By contrast, in cell types III and IV of the [cir^o] strain, there was a substantial increase in the number buds that contained fewer plasmids than the mother or that were plasmid-free (data not shown; see also Table II).

The above sets of data, obtained by directly following the cellular location and distribution pattern of a multicopy 2- μ m circle derivative, demonstrate that chromosome and plasmid segregation occur as nearly concurrent events during the yeast cell cycle, at least within the limits of the kinetic resolution of our assays. It is possible that

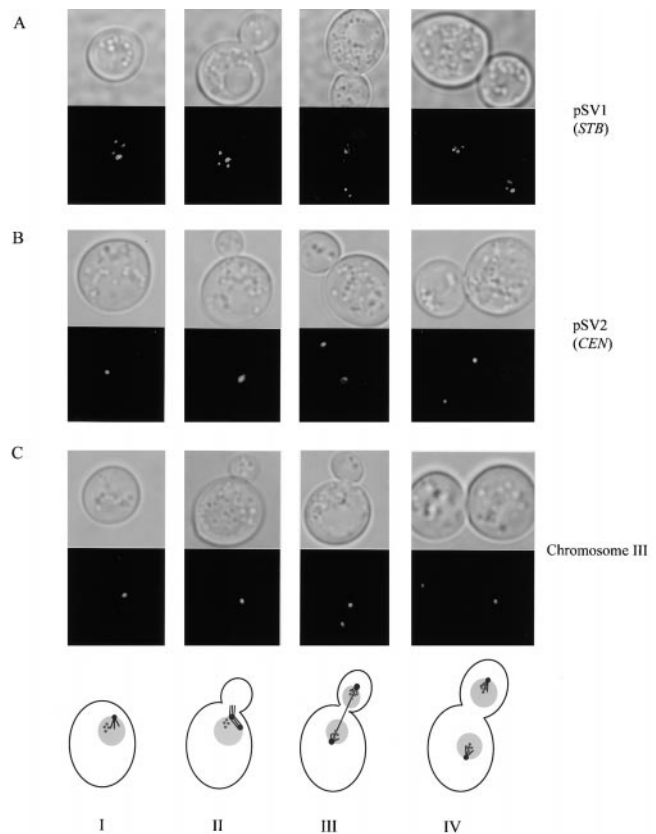


Figure 2. Distribution of the 2- μ m circle-derived pSV1 plasmid in mother and bud during the yeast cell cycle. Cells were synchronized in G1 using α -factor, and then released from G1 arrest. Plasmid or chromosomal DNA, tagged by GFP-lac repressor, was monitored by fluorescence microscopy at various stages of the cell cycle. A, Depicts a representative pattern for the distribution of pSV1 during the progression from G1 through S to G2/M. The corresponding cell types, designated I–IV, are schematically drawn at the bottom of the figure. B and C show the cell cycle distribution of the centromeric plasmid pSV2 and chromosome III, respectively. The yeast stains in this assay were the same as those listed under Fig. 1: AFS479 for following chromosome III and MJY30-10A for following pSV1. At least 200 cells of each type were assayed. Cell types I–IV used for scoring plasmid or chromosome were classified based on bud size as well as DAPI staining. Immunofluorescence assays using antitubulin antibodies, in conjunction with DAPI staining of nuclei, showed that the accuracy of distinguishing type III from IV based on bud size was >90%.

the two processes are mechanistically completely distinct, the observed coordination between them being merely coincidental. Alternatively, the shared timing suggests that the plasmid might utilize at least parts of the chromosomal segregation machinery for its own dispersal.

The 2- μ m Circle Partitioning System and the Mother–Daughter Symmetry in Plasmid Distribution

The 2- μ m-derived pSV1 plasmid contains only the cis-acting component, the *STB* locus, of the plasmid partitioning system. It does not encode either of the two Rep proteins. However, the endogenous 2- μ m plasmids in a [cir⁺] strain

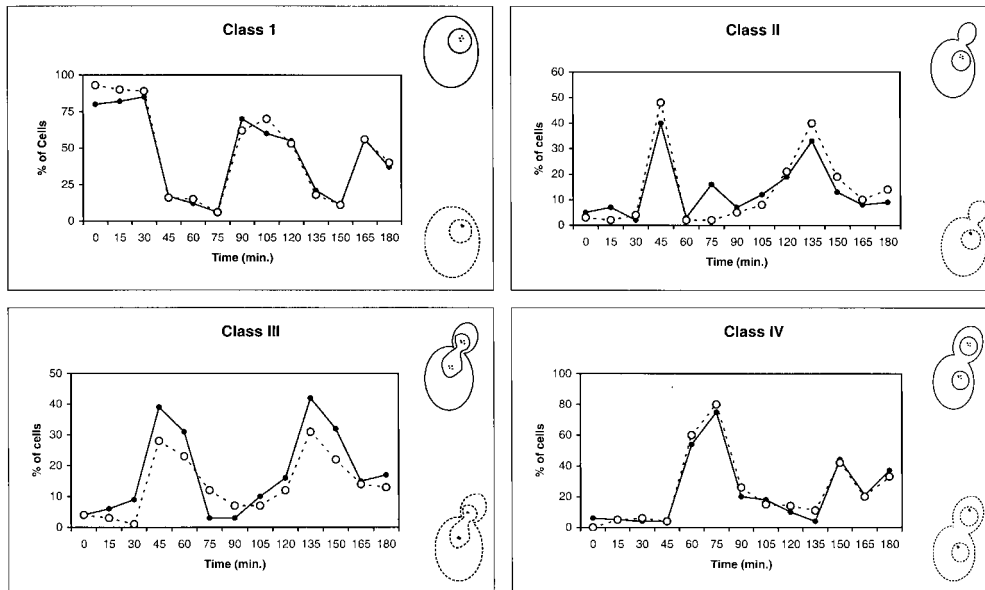


Figure 3. Kinetics of segregation of the 2- μ m circle-based pSV1 plasmid and chromosome III with respect to the cell cycle stages. Cells of strain MJY30-10A harboring pSV1 were released from G1 arrest as described under Fig. 2, and the cell types I-IV (diagrammed at top right corner in each) were scored over a period of 3 h. The results, obtained by examining at least 200 cells for each time point, are graphed as the solid lines connecting the filled circles. An equivalent plot for cells (AFS479) harboring the fluorescent chromosome is shown by the dashed lines connecting the open circles. Cell types I-IV for the chromosome are schematically represented at the bottom

right corner of each. The values in Fig. 3 ignore any quantitative asymmetry during pSV1 segregation, which was noted in a small fraction of the cells (see Table II). They also disregard differences in the pattern of plasmid distribution, that is, whether they were clustered or not, or how many fluorescent dots were associated with a given cluster. The mother or the bud was scored as plasmid positive if either contained at least a single fluorescent plasmid dot. The assay was repeated three times with similar results in each case.

can supply these proteins in trans to reconstitute the partitioning system. The Flp protein, required for copy number amplification, is also not encoded in pSV1. Because of the absence of a functional pair of recombination target sites (*FRT* sites) within it, pSV1 cannot undergo amplification (by the standard Futcher model) even when Flp is provided in trans. The integration of pSV1 (which contains one *FRT* sequence) into a native 2- μ m plasmid via Flp recombination could, in principle, make it a target for amplification. However, the *in vivo* equilibrium of Flp recombination substantially favors plasmid monomers over dimers, trimers, and higher oligomers. Furthermore, the amplifica-

tion system would be nonoperative in a [*cir*⁺] yeast strain containing pSV1 plus 2- μ m circles at steady state levels.

To assess qualitative differences in pSV1 segregation in the presence and absence of a functional partitioning system, the synchronization experiment was repeated in a [*cir*⁰] host strain. The data were recorded at 75 min after release from α -factor-induced cell cycle arrest, and pertain to type IV cells containing four and three fluorescent plasmid foci in the mother cell compartment. They comprised 69 and 40% of the [*cir*⁺] and [*cir*⁰] populations, respectively. Note that at this time point, 75% of the cells in both the [*cir*⁺] and [*cir*⁰] classes were in the large-budded state. The partitioning of pSV1 occurred evenly in the vast majority of the [*cir*⁺] cells (Table II, column 1). By contrast, there was a significantly higher asymmetry of pSV1 segregation in the [*cir*⁰] cells (Table II, column 2). For reference, the 4:n and 3:n segregation patterns of the *ARS* plasmid pSV3 are displayed in Table II, columns 3 and 4. Unlike pSV1, the segregation of pSV3 was unaffected by the [*cir*⁰] or [*cir*⁺] status of the host strain (Table II, compare column 4 to column 3). Note that the data in Table II were derived from cells grown in selective medium. As expected from previous studies (Broach and Volkert, 1991) under nonselective conditions, the pSV1 plasmid was lost from the [*cir*⁰] host at a much higher rate than from the [*cir*⁺] host (results not shown).

An interesting observation was the difference between the [*cir*⁰] and [*cir*⁺] host strains in the relative abundance of cells harboring pSV1 in the high and low copy patterns (four or three fluorescent plasmid dots in the mother cell for the former type; two or one for the latter). The low copy class of cells was nearly doubled for the [*cir*⁰] strain (60%) relative to the [*cir*⁺] strain (31%; data not shown). We also noted that plasmid fluorescence in the [*cir*⁰] back-

Table II. Segregation Patterns of the 2- μ m-based pSV1 and *ARS*-based pSV3 Plasmids in [*cir*⁰] and [*cir*⁺] Cells

Distribution of plasmid foci	pSV1		pSV3	
	<i>Cir</i> ⁺	<i>Cir</i> ⁰	<i>Cir</i> ⁺	<i>Cir</i> ⁰
4:4	70	39	21	19
4:3/2/1	9	33	49	42
3:3	17	13	11	14
3:2/1	3	15	19	25

The host strains (MJY30-10a or MJY31; Table I) harboring pSV1 or pSV3 were released from α -factor arrest, and after a 75-min interval, plasmid distribution was examined by fluorescence microscopy. Only the data from the large-budded stage IV cells containing four and three fluorescent plasmid dots (see Fig. 2) in the mother are tabulated. For pSV1, these cells comprised 69 and 40%, respectively, of the [*cir*⁺] and [*cir*⁰] populations. The corresponding numbers for pSV3 were 56 and 57%. For each plasmid, the percentages of cells of the [*cir*⁰] and [*cir*⁺] hosts harboring the indicated distribution patterns are summarized. The numbers under the distribution pattern are the fluorescent plasmid dots counted by microscopy. Numbers at the left refer to the mother cell compartment and those at the right refer to the daughter cell compartment. It was noticed that during an unequal segregation event, the mother cell almost always acquired the higher number of plasmid foci. The data shown are the composite of two separate experiments. The values in each column were obtained by examining >200 cells.

ground had a tendency to be spread out as individual dots within the nucleus (results not shown; see also Fig. 6). These observations are consistent with a potential role for the Rep1 and Rep2 proteins (supplied by the endogenous 2- μ m circles) in anchoring the plasmid to a nuclear substructure as an aggregate. The apparent drop in plasmid density in the [cir^o] host may be accounted for by missegregation due to lack of a functional partitioning system. Since pSV1 lacks the F1p-mediated amplification system, restoration of copy number would not have been possible. Our failure to observe a significant fraction of cells with greater than their normal share of plasmids (expected to result from missegregation) suggests that there may be some selection against such cells. Overexpression of F1p with consequent artificial amplification of the native 2- μ m plasmid is known to be deleterious to the host (Volkert and Broach, 1986; Reynolds et al., 1987; Scott-Drew and Murray, 1998; Som et al., 1988). Alternatively, the copy number may be balanced by negative control of plasmid replication in cells overpopulated with the plasmid.

Combining the results from Fig. 3 and Table II, we conclude that the Rep/STB system is indispensable for the chromosome-like segregation of the pSV1 plasmid.

The Rep1 and Rep2 Proteins Associate Preferentially with 2- μ m Plasmid DNA in the Yeast Nucleus

The similarity between the timing of pSV1 and chromosomal partitioning during the cell cycle (Fig. 3) observed in the present study raises the intriguing possibility that the Rep/STB system might be involved in coupling plasmid and chromosomal segregation machineries. The nuclear localization of the Rep proteins and their *in vivo* interactions revealed by mono- and dihybrid assays (Ahn et al., 1997; Velmurugan et al., 1998) are consistent with such a

role. For example, it is conceivable that one or both of the Rep proteins can bind to STB on one hand and to a host protein involved in segregation on the other.

To reveal the localization of the 2- μ m plasmid relative to the Rep proteins in yeast cells, immunocytology was employed using mildly fixed cells (Fig. 4, A and B). The Rep proteins were localized by fluorescein-conjugated secondary antibodies and the 2- μ m circle-derived pSV1 plasmid by Texas red-conjugated secondary antibodies (to lac-repressor antibody). The red and green fluorescence could be overlaid on each other in >85% of the cells, and occupied a sublocale within the DAPI-staining region. This strong tendency for colocalization was absent in the case of the ARS-containing pSV3 plasmid and Rep1p (Fig. 4 C) or Rep2p (data not shown). We observed pSV3 dots that were not coincident with the Rep proteins in 50% of the cells. These findings agree with the *in vivo* and *in vitro* evidence for Rep-STB interaction (Hadfield et al., 1995; Velmurugan et al., 1998). It would be consistent with a mechanism by which these proteins might facilitate the docking of plasmid DNA to a cellular entity that is divided evenly between mother and daughter at cytokinesis. Our conclusions regarding the colocalization of the Rep proteins with the 2- μ m plasmid have been further supported by cells in which the plasmid and the chromosomes missegregate in tandem (see Fig. 7, A and B). Immunolocalization of the Rep proteins in such cells reveal their presence only in the cell compartment that displays the bulk of the DAPI fluorescence (data not shown).

Potential Link between the Rep Protein Complex and the Mitotic Spindle of the Host Cell

Intrigued by the similarities between chromosome partitioning and the 2- μ m plasmid segregation (Figs. 2 and 3),

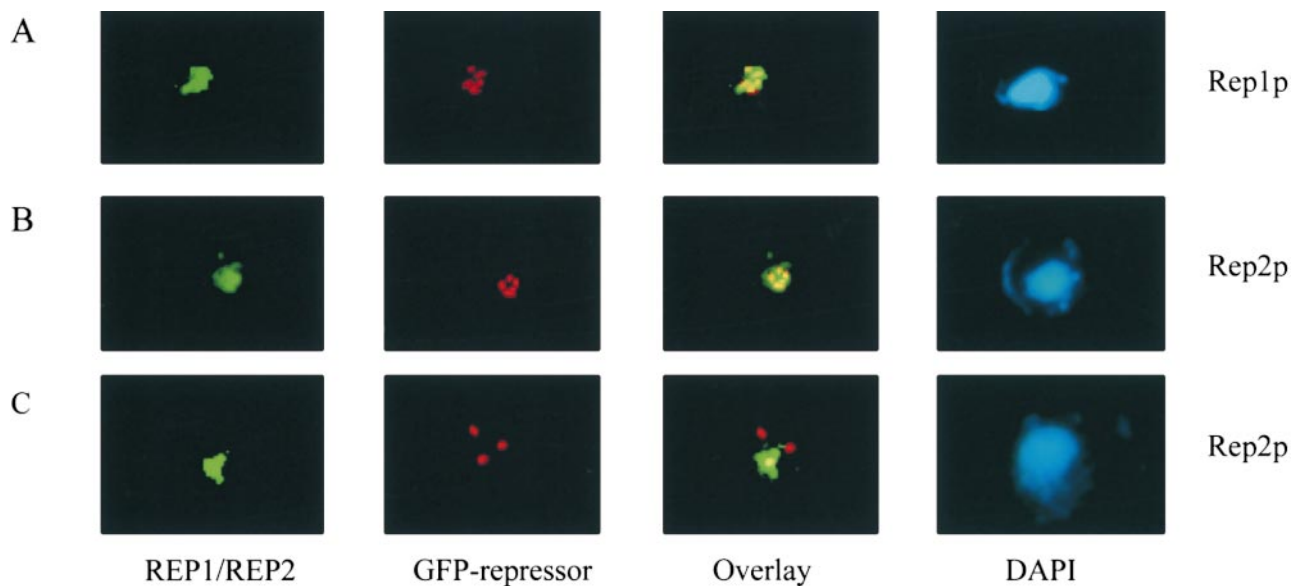


Figure 4. Colocalization of Rep1p and Rep2p with the 2- μ m-derived pSV1 plasmid. Rep1 and Rep2 proteins were visualized by fluorescein-conjugated secondary antibodies. Plasmid pSV1 or pSV3 was localized using Texas red-conjugated secondary antibodies to lac-repressor antibody. Experiments were carried out in strain MJY30-10a (Table I). The Rep proteins were expressed from endogenous 2- μ m circles. The representative data shown in each were derived from examination of \sim 200 cells per assay. The assays were done twice.

we have probed the subnuclear localization of the Rep protein complex with respect to the spindle apparatus during the yeast cell cycle. In these experiments, the chromosomes were visualized by DAPI staining, and the Rep proteins and tubulin were visualized by indirect immunofluorescence using Texas red- and fluorescein-conjugated secondary antibodies, respectively.

The Rep proteins (and therefore the associated 2- μ m circle molecules by inference; see Fig. 4) tended to accumulate at or near the poles of the mitotic spindle (Fig. 5, A and B, rows 2 and 3). In the predominant fraction of stage I and stage IV cells (>90%), there was either perfect or near coincidence between a Rep protein and the spindle pole (Fig. 5, columns I and IV and rows 1–3). In some of the stage II cells (8%), the duplication of the spindle pole and the associated bifurcation of the Rep protein stain were readily discernible (Fig. 5 A, column II and rows 1–3). In the majority of cases (92%), however, a bipartite Rep staining profile was not observed (Fig. 5 B, column II and rows 1–3; also data not shown). In some of these cells, the Rep protein overlapped with both spindle poles as a uni-

formly stained entity with no detectable discontinuity. In others, the Rep protein remained proximal to one of the two poles of a short spindle. In stage III cells, the bulk of the Rep protein was concentrated at or close to each of the poles with a sharp drop in protein levels towards the center of the spindle (>90%). This preferred gradient of Rep localization can be seen distinctly in the confocal images of stage III cells in Fig. 5, A and B, row 5. The correspondence between Rep protein staining and chromosomal staining at all cell stages, I–IV, together with the proximity between the spindle poles and the rep proteins, suggests that plasmid molecules may be directly or indirectly attached to the spindle.

Effect of Microtubule Depolymerization on the Integrity of the 2- μ m Plasmid-Rep Fluorescent Foci

To further examine the potential association between 2- μ m circle and the mitotic spindle, we have assayed the pattern of Rep protein localization as well as the organization of pSV1 plasmid (2- μ m circle based) in nocodazole-

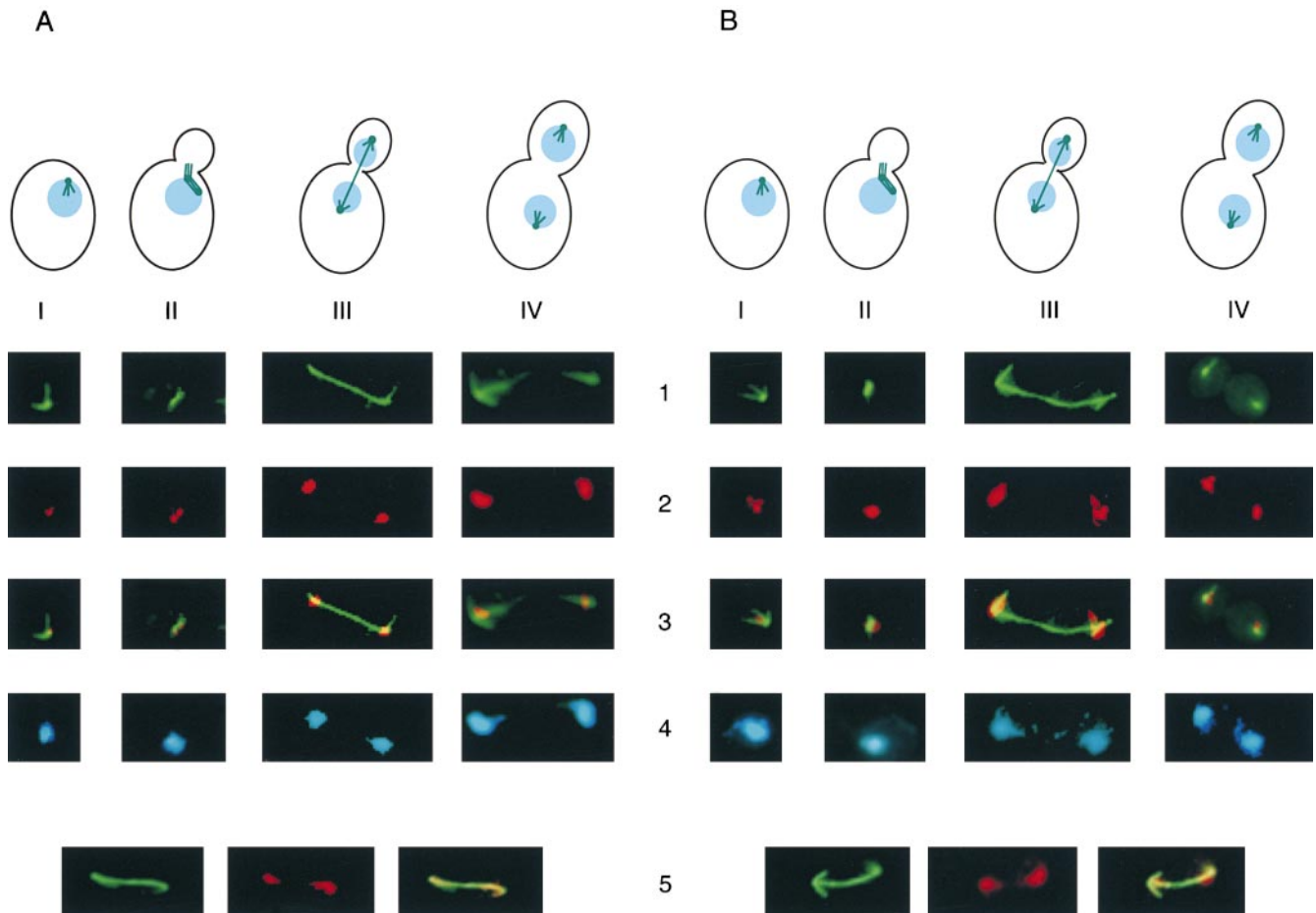


Figure 5. Potential linkage of the 2- μ m plasmid to the host mitotic apparatus. Typical staining of tubulin (fluorescein; row 1) and the Rep protein (Texas red; row 2) are shown in cell types I–IV (drawn at the top). The overlay of the fluorescein and Texas red images is displayed in row 3. The chromosome stained with DAPI is depicted in row 4. Confocal images of tubulin and Rep protein immunofluorescence (left and middle, respectively) and their overlay (right) in cell type III are arranged from left to right in row 5. A and B represent results with Rep1p and Rep2p, respectively. The assays were done four times in strain CCY6661A (Table I), and ~50–100 cells of a given type were scored each time.

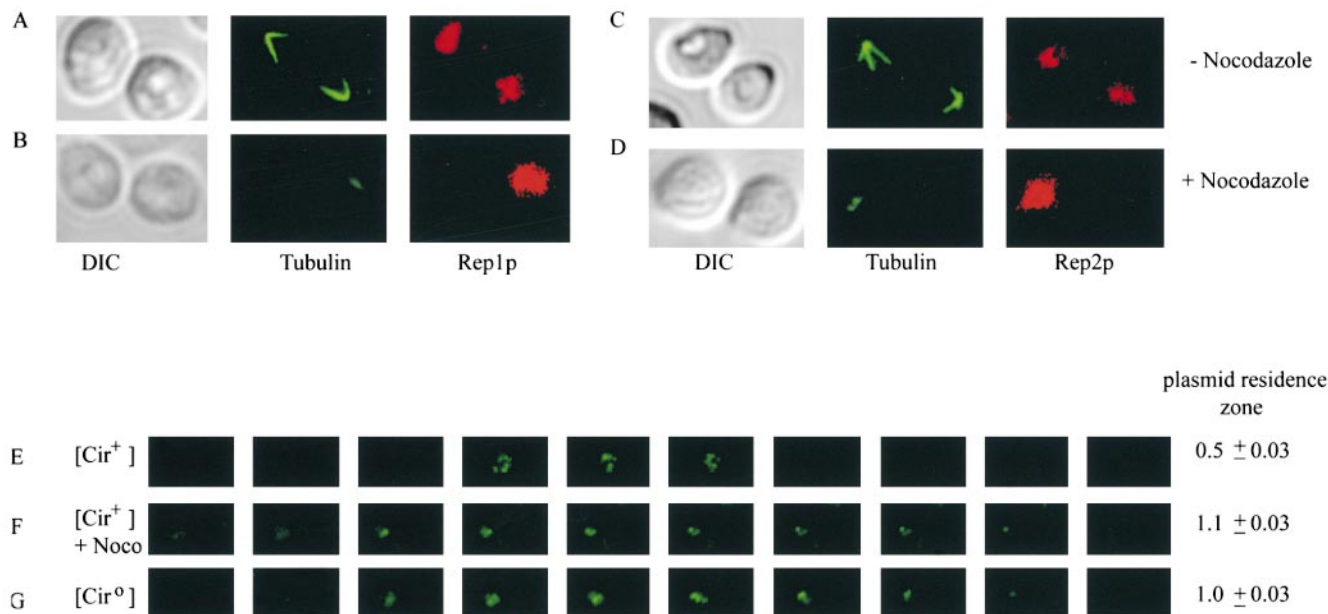


Figure 6. Nocodazole treatment alters the pattern of Rep protein and pSV1 plasmid distribution in the nuclei. After a 2-h treatment of [cir⁺] cells (strain CCY666-1A; Table I) with nocodazole at 30°C, the Rep proteins and tubulin were visualized by immunofluorescence using Texas red- and fluorescein-conjugated secondary antibodies, respectively. The results for Rep1p and tubulin localization in the control and nocodazole-treated cells are shown in A and B, respectively. The corresponding results for Rep2p and tubulin are displayed in C and D, respectively. Typical results of scanning the pSV1 plasmid by z-series sectioning using confocal microscopy are presented in E–G. The leftmost frame in each row represents the arbitrarily fixed boundary beyond the edge of plasmid fluorescence. From this start point, a total of 20 consecutive frames (each 0.25 μm) were scanned for each sample. Only ten of these frames, representing every alternate one, are shown here. For each cell examined, a similar z-series scan was performed for the DAPI fluorescence also. The value for the size of the plasmid zone listed at the right of each row is the mean ratio of the green fluorescence range (emitted by GFP-repressor-bound plasmids) to the blue fluorescence range (emitted by DAPI-bound bulk chromosomes). The results were derived from averaging at least 20 experimental determinations. Note that frames with very faint fluorescence (for example, the two leftmost frames in F) were not included in the estimates. Thus, the zone widths for the nocodazole-treated [cir⁺] cells (F) or the untreated [cir⁰] cells (G) are underestimated. Untreated [cir⁺] cells are shown in E.

treated [cir⁺] cells. Under our assay conditions, 80–85% of the cells were arrested in the G2/M phase as judged by microscopy. Immunofluorescence staining for tubulin in these large-budded cells revealed nearly complete disassembly of the mitotic spindle, although limited residual fluorescence was detectable at some spindle poles (Fig. 6, B and D, middle). Along with the disassembly of the spindle, the Rep proteins showed a less compact, more disperse, pattern of nuclear localization (Fig. 6, B and D, right). Nocodazole did not affect the steady state levels of the Rep1p or Rep2p, as assayed by Western blot analysis of total yeast cell extracts (data not shown). The normal tubulin and Rep protein patterns (in untreated cells at the G2/M phase) are shown in Fig. 6, A and C, for reference.

In addition to the nocodazole-induced scattering of the Rep proteins, the pSV1 plasmid clusters were more loosely organized after disassembly of the spindle (Fig. 6, E and F). The assay was performed by confocal z-series sectioning of the yeast nucleus in a large number of cells (see Materials and Methods), and estimating the mean range of the plasmid occupancy zone. The values were then normalized to the range of DAPI fluorescence in the same cells, also estimated by z-series sectioning. In the control [cir⁺] cells, the range of the plasmid zone was 0.5 ± 0.03 (Fig. 6 E). By contrast, this range was nearly doubled in nocodazole-treated [cir⁺] cells (1.1 ± 0.03 ; Fig. 6 F) or

in untreated, but [cir⁰], cells (1.0 ± 0.03 ; Fig. 6 G). Estimates of cell and nuclear sizes (from scanning DIC and DAPI images, respectively) showed that the cell enlargement or nuclear expansion as a result of nocodazole treatment was no more than 20% (data not shown). Thus, the lack of an intact microtubule array or the absence of a functional Rep system (as in the [cir⁰] host) has the common effect of slackening the cohesive forces between plasmid molecules.

Based on the sum of the results shown in Figs. 5 and 6, we argue that the Rep proteins likely act as bifunctional coupling agents: complexing with the plasmids on one hand, and effectively cross-linking them directly or indirectly to some component(s) of the spindle apparatus.

Molecular Connection between 2-μm Circle Partitioning and Genes Required for Chromosome Segregation: Missegregation of the 2-μm Plasmid in the *ipl1-2* Yeast Mutant

To further verify the suspected coupling between chromosomal and 2-μm plasmid segregation, we have examined the partitioning of the plasmids pSV1 (containing the 2-μm circle replication origin and *STB*) and pSV3 (ARS-based and lacking *STB*) in a host strain harboring the Ts⁻ *ipl1-2* mutation. The product of the *IPL1* gene is essential

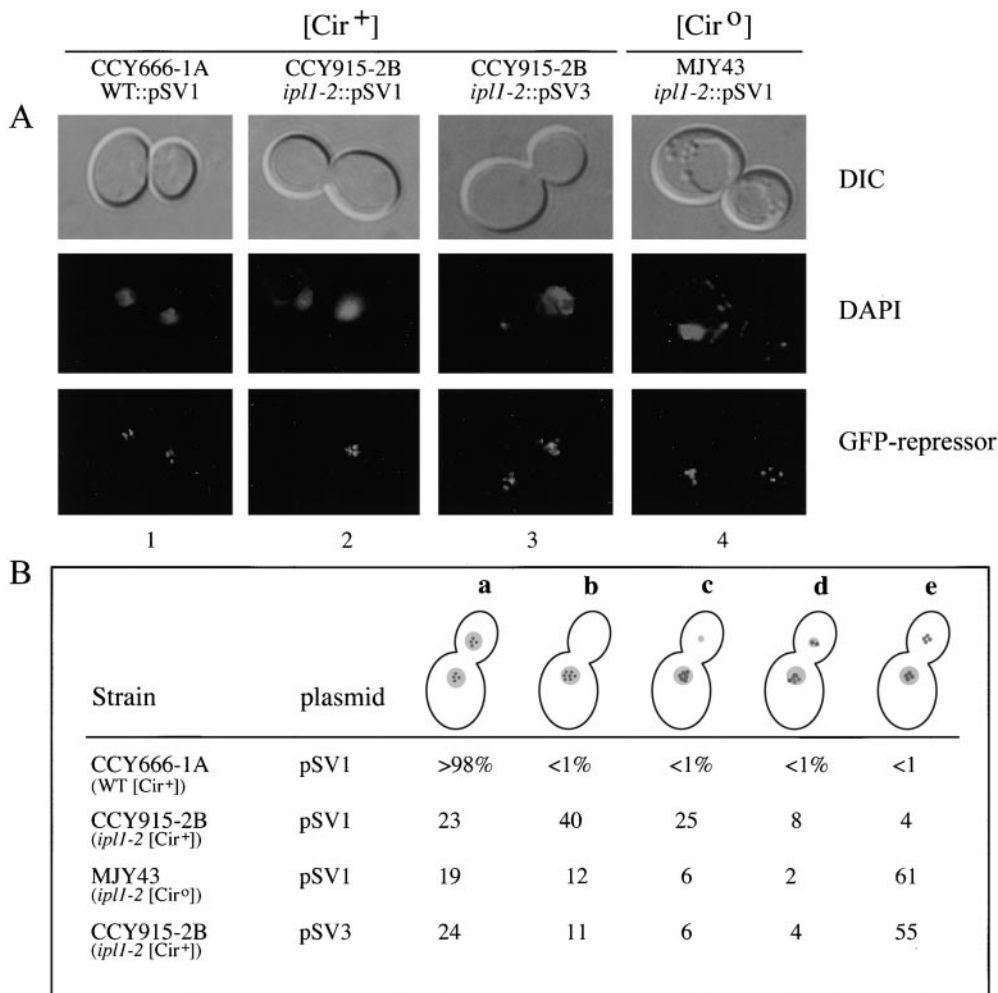


Figure 7. Potential involvement of genes required for chromosome maintenance in 2- μ m plasmid segregation. **A**, The 2- μ m circle-derived pSV1 plasmid or the *ARS*-based pSV3 plasmid was visualized by green fluorescence from the GFP-lac repressor (expressed from pSV13; Table I), and chromosomes by blue fluorescence from DAPI. The outline of the cells are shown by DIC (top). The middle and bottom rows display the DAPI and GFP profiles, respectively, corresponding to the DIC images of the top row. The *ipl1-2* cells were shifted to 37°C for 4 h before they were examined by microscopy. **B**, The plasmid and chromosome segregation data derived by screening 300–400 large-budded cells in each assay are tabulated. In the schematic diagrams of the cells (a–e), the DAPI-staining regions and the fluorescent plasmid dots are indicated. The cells in column a represent normal segregation, and those in b–d denote chromosome and plasmid missegregation in tandem. The cells in column e typify plasmid segregation uncoupled from chromosome segregation. Strains CCY915-2B and MJY43 are isogenic *ipl1-2* strains that differ only in the presence or absence of native 2- μ m circles.

for proper chromosome segregation (Chan and Botstein, 1993; Francisco et al., 1994; Biggins et al., 1999; Kim et al., 1999). When shifted to the nonpermissive temperature, the predominant fraction of *ipl1-2* cells exhibits a severe chromosome missegregation phenotype.

In the experiments depicted in Fig. 7 A, chromosomes were identified by DAPI staining and the pSV1 (2- μ m circle-derived) and pSV3 (*ARS*-derived) plasmids by GFP-repressor fluorescence. Unlike the normal segregation observed in the [cir⁺] wild-type host at 37°C (Fig. 7 A, column 1), the bulk of the chromosomes, along with pSV1, was stuck within the mother or daughter compartment in most large-budded cells from the [cir⁺] *ipl1-2* host (Fig. 7 A, column 2). It is known that the *ipl1-2* mutation does not impart a mother/daughter bias in chromosome missegregation (Biggins et al., 1999; Kim et al., 1999).

In Fig. 7 B, the normal chromosome and plasmid segregation represented by cell type a was contrasted by four types of missegregation represented by cell types b–e. In b and e, the DAPI fluorescence was completely excluded from one of the two cell compartments. In c and d, the flu-

orescence partitioning was strongly (though not absolutely) biased: ~90 to 10 in C and 80 to 20 in D. Whereas chromosome segregation and plasmid partitioning (indicated by the green fluorescent dots) were tightly coupled in cell types b–d, they were strongly uncoupled in e. The correlation between pSV1 and chromosome location during missegregation events was nearly perfect in the *ipl1-2* [cir⁺] host strain (Fig. 7 B, only 4% of type e cells in row 2). In sharp contrast, the segregation of the *ARS*-based pSV3 in the same *ipl1-2* host was not coupled to chromosome segregation (Fig. 7 A, column 3; Fig. 7 B, 55% type e cells in row 4). A similar degree of uncoupling was also observed for pSV1 in an isogenic, but [cir⁰], *ipl1-2* host (Fig. 7 A, column 4; Fig. 7 B, 61% type e cells in row 4).

In an unrelated experiment, we have identified the product of the *BRN1* gene (open reading frame YBL097W on chromosome II in the yeast genome data base) as an interactor with Rep1p as well as Rep2p (data not shown). *BRN1* is an essential gene, and a temperature-sensitive mutation in it leads to increased rate of chromosome loss under the restrictive condition (Lavoie et al., 2000;

Ouspenski et al., 2000). The yeast Brn1 protein has homology to the *barren* gene product of *Drosophila* and to a condensin subunit from *Xenopus*, proteins that are required for proper chromosome segregation (Bhat et al., 1996; Hirano et al., 1997).

Identical partitioning defects in the chromosome and a 2- μ m circle based plasmid due to a mutation in a well characterized yeast gene (*IPL1*) required for chromosomal segregation provides strong circumstantial evidence for a molecular link between the two segregation mechanisms. Notably, the linkage between chromosome and plasmid segregation (or rather missegregation) in the *ipl1-2* background is strictly dependent on the Rep1p/Rep2p/*STB* system. This evidence is further strengthened by the interaction between each of the Rep proteins and a second host gene product, Brn1p, involved in stable chromosome inheritance.

Discussion

The dual strategy employed by the 2- μ m circle plasmid for its stable propagation as a high copy benign parasite genome in yeast utilizes an efficient plasmid partitioning system and a copy number amplification mechanism. Whereas the Flp recombination system is responsible for plasmid amplification, the Rep1p–Rep2p/*STB* system is responsible for faithful plasmid partitioning. The amplification system is not triggered into action unless the copy number drops below steady state levels. The observation that there is little amplification of the plasmid under normal growth conditions (Zakian et al., 1979) implies that deviations from equal partitioning of plasmid molecules at cell division are relatively infrequent. Compared with the elaborate kinetochore/mitotic spindle apparatus involved in the segregation of the chromosomes, the plasmid partitioning system, constituted by two proteins (Rep1p and Rep2p) and five to six tandem iterations of a short DNA sequence (the *STB* locus), would appear to be vastly simpler. How can this apparent simplicity be reconciled with the rather high fidelity of plasmid segregation? The dynamics of a 2- μ m circle–derived plasmid in relation to the chromosome or to a centromere containing plasmid during the yeast cell cycle suggests that the 2- μ m circle may utilize the Rep/*STB* system to attach itself to the mitotic segregation apparatus.

Functional Similarities between 2- μ m Circle and Chromosome Partitioning

The present study has revealed the near equivalence between the chromosome and the 2- μ m plasmid in the timing and pattern of their movement across the cell during the yeast cell cycle and the similarity in their segregation into mother and daughter cells. This chromosome-like behavior is determined by the Rep/*STB* system. In a [cir^o] host, the dynamics of the 2- μ m circle–derived test plasmid pSV1 are significantly altered, resulting in a high frequency of missegregation (see Table II). The Flp recombination system is unlikely to have any direct effect on this process. Note that the pSV1 plasmid is not a substrate for intramolecular recombination, yet is efficiently partitioned in a [cir⁺] host.

Our finding that the Rep1 and Rep2 proteins colocalize with the pSV1 plasmid inside the yeast nucleus is significant, particularly so in conjunction with the preferred accumulation of the Rep proteins near the poles of the mitotic spindle. We interpret this observation to be suggestive of a role for the Rep/*STB* system in coupling the plasmid DNA to the spindle apparatus. This association could be direct or indirect. It might be mediated by binding to the kinetochore complex, to chromosomal DNA sequences, or to chromosome binding proteins. Consistent with the idea of spindle attachment, we also noted that nocodazole treatment results in a more dispersed distribution of the Rep proteins within the yeast nucleus, with a simultaneous decrease in the population of clustered plasmid foci. Based upon the distribution of the Rep1 and Rep2 proteins in cells at different stages of the yeast cell cycle, Scott-Drew and Murray (1998) have suggested that segregation of the 2- μ m plasmid occurs in association with the Rep proteins. Our results are consistent with this model.

The possibility that the partitioning system functions by freeing plasmid molecules from subcellular attachment and facilitating free diffusion is unlikely. Early observations using density shift experiments had suggested that there is little or no plasmid amplification under steady state growth conditions (Zakian et al., 1979). However, this result is not inconsistent with the random segregation model. Because of the high copy number of the 2- μ m plasmid, partitioning by Poisson distribution (with a mean of 60) does not result in a significant fraction of cells that contain fewer than 40 copies of the plasmid (<0.24%). The density shift experiments would not have been sensitive enough to unequivocally rule out or substantiate the low amplification levels predicted by random segregation. Our observations on the location and patterns of plasmid foci during cell cycle progression suggest that the clustering of 2- μ m circles would tend to decrease their effective copy number during segregation (a reduction in the mean of the Poisson distribution). At no stage of the cell cycle have we seen evidence for obvious declustering of the plasmids into multiple foci.

Scott-Drew and Murray (1998) have observed Rep1 and Rep2 proteins to localize in distinct foci during immunofluorescence confocal microscopy. We have also observed a similar pattern when these proteins were expressed from their native promoters, although cells that do not show well-demarcated foci were also frequently encountered during our assays. By counting these foci in cell populations at different stages of division, Scott-Drew and Murray (1998) estimate that their average number increases from 4.5 to 8.1 with the approach of mitosis, with each partner of a cell pair acquiring 4.5 to 5 foci towards the completion of mitosis. These numbers are in remarkable agreement with the most common tetrad pattern of foci for 2- μ m circle–derived plasmids, as well as the coincidence between Rep proteins and plasmids, revealed in our studies. Our localization of the Rep proteins with respect to the mitotic spindle or the spindle pole appears to show some discrepancy with that of Scott-Drew and Murray (1998) at certain intermediate stages of the cell cycle. It is not clear whether this is due to differences between strains or growth conditions, or results from differences in the

technical procedures. We are attempting to clarify this point by simultaneous observation of plasmids and the mitotic spindle in live yeast cells.

Molecular Components of Chromosome Segregation Function in Plasmid Partitioning

Two observations made in this study provide the first strong indication for a molecular link between plasmid and chromosomal partitioning. First, in an *ipl1* yeast strain (Chan and Botstein, 1993) that missegregates chromosomes at the nonpermissive temperature, there is a near perfect correlation in the cosegregation of chromosomes and a 2- μ m circle-derived test plasmid. In particular, in those large-budded cells that contain the bulk of the chromosomes in the mother or bud (as revealed by DAPI), the plasmid is also present almost exclusively in the same half of the cell (as revealed by green fluorescence tagging). Furthermore, this association is lost when the host strain is [cir^o], and thus lacks a functional Rep/*STB* system. Second, in a dihybrid screen, the Rep proteins have been found to interact with the product of *BRN1* gene that, like *IPL1*, is also required for proper chromosome segregation (Lavoie et al., 2000; Ouspenski et al., 2000).

The Ipl1 protein is a kinase that appears to act in association with the Sli15 protein after the sister chromatids have separated from each other (Kim et al., 1999). Failure of chromosome segregation in *ipl1-2* mutant cells is often associated with the abnormal distribution of the spindle pole-associated Nuf2 protein (Kim et al., 1999). The Ipl1 kinase has also been implicated to function in the binding of kinetochores to the spindle (Biggins et al., 1999). Our finding that 2- μ m plasmids, along with the Rep proteins, are either colocalized with the spindle poles or present in close proximity to them is consistent with the suggested role of the Ipl1 protein in normal spindle pole function. Thus, it seems quite plausible that one or more of the steps at which the Ipl1 protein acts in the segregation pathway would be shared by chromosomes and the 2- μ m plasmid.

The Brn1 protein is homologous to the *Drosophila barren* gene product (Bhat et al., 1996) and the XCAP-H protein of *Xenopus* (Hirano et al., 1997). The *barren* product is required for sister chromatid segregation, and in its ab-

sence the sisters remain bridged, even though centromeres move apart at the metaphase to anaphase transition. The XCAP-H protein is a subunit of the 13S condensin complex required for condensing mitotic chromosomes as a prerequisite for their normal segregation. The possible requirement of the Brn1 protein in 2- μ m plasmid partitioning is supported by the finding that a point mutation in Rep1p that nearly abolishes its binding to Brn1p without affecting its interaction with Rep2p or the *STB* DNA also results in high instability of a 2- μ m circle-derived plasmid (Yang, X.-M., and M. Jayaram, unpublished results).

Chromosome segregation requires a number of coordinated steps that include chromosome condensation, sister chromatid cohesion, bipolar mitotic spindle assembly and elongation, kinetochore attachment to microtubules, sister chromatid separation and their movement to opposite poles (Hoyt and Geiser, 1996; Heck, 1997; Nicklas, 1997). The interaction of the Rep1 and Rep2 proteins with a condensin-like protein, as well as the requirement of a protein kinase involved in normal spindle and/or kinetochore function for 2- μ m plasmid segregation, would argue for multiple molecular links in the chromosomal and plasmid partitioning mechanisms.

Plausible Models for Chromosome/Plasmid Cosegregation

Within the general scheme of coordinated segregation of chromosomes and the 2- μ m plasmid using a common machinery, two plausible models can be envisaged. In one, the plasmid adheres to one or more of the chromosomes and hitchhikes with them during segregation; in the other, plasmid attachment to the spindle is mediated independent of the chromosomes. Precedence for plasmid transmission by tethering to chromosomes has been established in the case of bovine papilloma virus (Lehman and Botchan, 1998; Ilves et al., 1999). Such a mechanism would be advantageous to the virus in preventing its exclusion from the nucleus into the cytoplasm during breakdown and reconstitution of the nuclear membrane associated with mitosis. This argument does not apply to the 2- μ m plasmid, since yeast mitosis does not involve breakdown of the nuclear envelope. If chromosomal tethering is not the

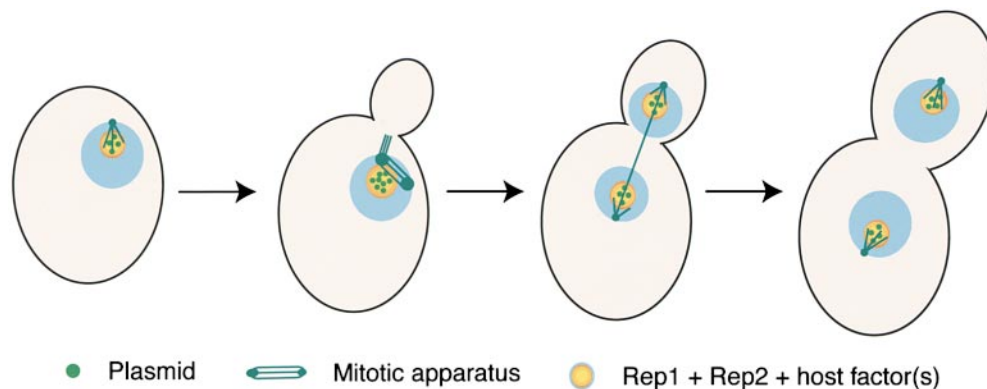


Figure 8. A model for the stable partitioning of the 2- μ m plasmid. The sum of the data presented in this study is consistent with a model in which the Rep proteins provide the 2- μ m plasmid access to the chromosomal segregation apparatus. This is achieved by their bifunctional interactions with the plasmid on one hand (likely via the *STB* locus), and potential host factor(s) on the other. The model accommodates the timing of

plasmid replication by the host replication machinery determined previously (Brewer and Fangman, 1987) and the kinetics of plasmid segregation observed in this study.

means for plasmid partitioning, it should be possible, at least in theory, to identify mutations that missegregate chromosomes without affecting the plasmid.

The 2- μ m Plasmid: A Paradigm for Near-Perfect Optimization of a Selfish Genome

The circular geometry, structural compactness, and functional parsimony of the 2- μ m plasmid appear to represent an optimized evolutionary solution for the high copy maintenance of an extrachromosomal selfish DNA element in yeast. By virtue of harboring a replication origin that is functionally analogous to chromosomal replication origins, the plasmid molecules are able to utilize the host DNA replication machinery for their own replication during the S phase of the cell cycle. However, the normal control of cellular replication prohibits more than one round of replication per plasmid template. To counter the eventuality of a potential fall in copy number due to unequal segregation, the plasmid has evolved the Flp site-specific recombination system as a means for amplification. The Futcher model (Futcher, 1986, 1988), a key prediction of which has been experimentally supported (Volkert and Broach, 1986; Reynolds et al., 1987), provides a mechanism by which more than one round of plasmid replication can be effected without breaking the cell cycle ban on reinitiation at an already fired replication origin. The need for amplification is minimized by the Rep/*STB* partitioning system that ensures equal segregation of plasmids during cell division.

The present study has provided a direct, semiquantitative measure of the segregation fidelity of the 2- μ m plasmid, and suggests a mechanism that accommodates the high functional competence of the Rep/*STB* system within its organizational simplicity. As diagrammed in the model in Fig. 8, the association of the Rep proteins with the plasmid via the *STB* sequence, and with the spindle apparatus or the chromosomes via one or more host factors can coordinate and synchronize plasmid and chromosome segregation spatially and temporally. In conclusion, the success of the 2- μ m plasmid as a stably propagating extrachromosomal element appears to be founded on the plasmid's ability not only to directly utilize its host's replication apparatus, but also to indirectly exploit the chromosomal segregation machine.

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