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The segregation of native and foreign extra-chromosomal genetic elements in *Saccharomyces cerevisiae*: stable propagation by hitchhiking on chromosomes

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Dedication

To my family and friends

Acknowledgements

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The segregation of native and foreign extra-chromosomal genetic elements in *Saccharomyces cerevisiae*: stable propagation by hitchhiking on chromosomes

Yen-Ting Liu, Ph.D.

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The 2 micron plasmid of the budding yeast *Saccharomyces cerevisiae* resides in the nucleus as an extra-chromosomal element with a steady state copy number around 40-60 per cell. As a benign but selfish DNA element, the plasmid utilizes a self-coded partitioning system and an amplification system to exhibit nearly chromosome-like stability in its host. Plasmid behavior under conditions that missegregate chromosomes suggest that the partitioning system couples plasmid segregation to chromosome segregation. However, the mechanism of this coupling has not been elucidated. A plausible model, consistent with current evidence, is the hitchhiking model, in which plasmid-chromosome tethering provides the basis for faithful plasmid partitioning. Testing this hypothesis unequivocally has been difficult, primarily because of the technical limitations posed by the small size of the budding yeast nucleus and poor

resolution of chromosomes. As a result, cell biological assays based on fluorescence microscopy have had only modest success in addressing this problem.

In the present study, I devised an experimental verification of the hitchhiking model using a single copy derivative of the 2 micron plasmid as a reporter. The rationale was to establish various conditions that force sister chromatids to co-segregate during mitosis in a bias-free manner or with a bias towards the daughter. The segregation patterns of plasmid sisters were followed under these conditions. The sum of the results from this analysis is accommodated by the hitchhiking model, with sister plasmids associating with sister chromatids in a one-to-one fashion.

Episomes of mammalian viruses belonging to the gamma-herpes and papilloma families utilize a hitchhiking mechanism to persist in cells during the latent phase of their infection. Two of the viral partitioning systems have been reconstituted in *S. cerevisiae*. We wished to exploit these systems to characterize the efficiency of non-native chromosome tethering systems in promoting equal segregation of viral plasmids in *S. cerevisiae*. We find that the 2 micron plasmid partitioning system is considerably superior to the viral systems. This could be due to the higher efficiency of plasmid-chromosome association and/or due to the ability of plasmid sisters to tether to sister chromatids.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

Introduction

1.1 Selfish genetic elements as extra-chromosomal circles

Faithful propagation of the genome is critical for the survival of an organism, which devotes considerable metabolic effort towards accomplishing this goal. For a genetic element that does not provide a selective advantage to its host organism, but nevertheless depends on the genetic endowments of the host, long-term survival poses a serious challenge. The evolutionary success of such elements depends on turning selfish, that is, acquiring the capacity to replicate efficiently and often to increase their copy number without imposing a significant fitness cost upon the host (Doolittle and Sapienza, 1980; Orgel and Crick, 1980). A variety of mobile genetic elements, the most abundant among the selfish genes, have evolved sophisticated mechanisms for replication and integration into multiple chromosomal sites in order to spread within a genome and between genomes (Burt and Trivers, 2006). Extra-chromosomal selfish DNA molecules, which by definition cannot become an integral part of the host genome via covalent linkage, stable propagation demands not only efficient replication but also efficient partitioning of replicated molecules to daughter cells in a dividing and growing host cell population. The central theme of the studies presented in this thesis concerns molecular mechanisms that underlie the partitioning systems harbored by eukaryotic extra-chromosomal elements.

1.1.1 Extra-chromosomal circles in prokaryotes

An important class of extra-chromosomal elements in prokaryotes consists of conjugative and non-conjugative plasmids as well as viruses such as P1 that do not integrate into the host chromosome. The F factor of *Escherichia coli*, responsible for sex determination, can have either an autonomous existence as an episome or can be integrated into the chromosome. The majority of prokaryotic plasmids has a circular organization, and can vary in copy number from one or a few to tens of plasmid molecules per cell. Linear plasmids do exist in prokaryotes, spirochetes, gram positive and gram negative bacteria, but are encountered much less frequently than circular plasmids (Barbour and Garon, 1987; Hinnebusch and Tilly, 1993). The ends of the plasmid DNA are stabilized by covalently attached proteins or by the formation of covalently closed hairpin loops (referred to as telomeres). For example, in Borellia (the spirochete responsible for lyme disease), the genome is organized as multiple linear segments with hairpin ends (Chaconas and Kobryn, 2010). Some of these linear fragments do not possess essential genes, and thus fall into the category of extra-chromosomal elements.

Most high copy plasmids segregate by a random mechanism. For a mean copy number of, say, 10 plasmids per cell, the probability $P_{(0)}$ that a cell without plasmid will arise during a cell division is close to zero. The differences in copy number resulting from such a partitioning mechanism is corrected by replication control, that is, higher than normal copy number suppresses replication and lower than normal copy number induces multiple rounds of replication. Thus the mean plasmid copy number in the population is centered at a steady state value.

Low copy plasmids harbor active partitioning mechanisms for promoting their stable segregation during cell division (Gerdes et al., 2000; Jayaram et al., 2004a; Schumacher, 2012). The general organization of bacterial plasmid partitioning systems consists of two partitioning proteins (Par proteins) and a partitioning locus (Par locus), often referred to as the plasmid centromere. The Par locus contains iterated copies of a consensus repeat element. One of the Par proteins is a DNA binding protein that interacts with the Par locus, often oligomerizing along the DNA. The second Par protein, which interacts with its Par partner protein, is an active ATPase belonging to the Walker family or the actin family of ATPases or a tubulin related GTPase. In a few instances, the association of Par ATPase with DNA can occur independently of its interaction with the centromere binding Par protein. Individual plasmids utilize variations of this shared organizational and functional theme to execute distinct partitioning pathways (Fig. 1.1).

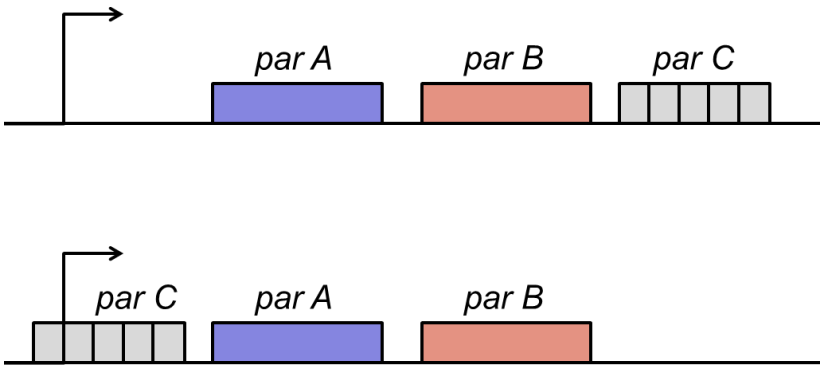


Figure 1.1 Organization of plasmid partitioning system in bacteria. The plasmid partitioning system consists of two *trans*-acting proteins and a *cis*-acting iterated sequence element, often called the plasmid centromere. For simplicity and generality, in this figure, the protein coding loci are referred to as *parA* (blue box), *parB* (red box), and the centromere as *parC* (grey box). The ParA protein is an NTPase, while the ParB protein is a DNA binding protein that associates with the *parC* locus. The *parC* locus can either lie downstream from the coding regions of the Par proteins, or may be located upstream of these loci. In the latter case, *parC* harbors the promoter for the *parA* and *parB* genes. Arrows mark the locations of the *par* promoters and denote the direction of transcription. The figure is modified from Jayaram et al., (2004).

In the well characterized partitioning mechanism of the low copy P1 plasmids (the Type I system), the ParA protein (an ATPase) polymerizes along the nucleoid in an ATP-dependent manner (Ringgaard et al., 2009), or undergoes a multi-step transition that licenses its association with the nucleoid (Vecchiarelli et al., 2010). The interaction of the Par ATPase with its plasmid bound partner ParB protein stimulates ATP hydrolysis causing the retraction of the filament along with plasmid, or uneven localization of the filament along the nucleoid to provide the motive force for plasmid partitioning (Fig. 1.2A). By contrast, in the type II system, the actin-like ParM ATPase forms dynamic filaments that search the cell volume for plasmids, and physically push ParR (ParM partner) associated with ParC (plasmid centromere) (Salje et al., 2010). As a result, the replicated sister plasmids are moved away from each other to be localized in daughter cells (Fig. 1.2B).

The more recently discovered type III system utilizes a tubulin based mechanism for plasmid segregation (Chen and Erickson, 2008; Larsen et al., 2007; Tang et al., 2007). In this system, the active nucleotide is GTP rather than ATP. The nucleotide binding Par protein forms double stranded filaments that associate with the second Par protein bound to the plasmid centromere (Aylett et al., 2010; Ni et al., 2010). This interaction stimulates GTP hydrolysis and treadmilling, with elongation of the filament at the plus end and regression at the minus end. This tram-like mechanism causes the filament and the attached plasmid to be translocated to one pole of the cell (Fig. 1.2C).

Thus, a key step in bacterial plasmid segregation is the polymerization of a nucleotide binding protein into filaments that searches for and associates with the second partitioning protein bound to the plasmid. This encounter is followed by the generation of

the motive force by pushing sister plasmids apart, or pulling plasmids away from each other by filament contraction or biased localization or by treadmilling and filament translocation. The type III tubulin mechanism is perhaps the archetype of the more sophisticated spindle based mechanism for the segregation of eukaryotic chromosomes.

Certain bacterial plasmids resort to a rather diabolical mechanism to ensure their presence in the host population by killing cells that do not contain the plasmid through the action of addiction modules (Greenfield et al., 2000; Kobayashi, 2004). The molecular basis of addiction is the production by the plasmid of a toxin with a long half-life and an antidote with a short half-life. The toxin is a protein molecule (a restriction enzyme, for example) whereas the antidote may be either a protein (a DNA modification enzyme, for example) or an anti-sense RNA that negatively regulates toxin expression. Presence of the toxin in the absence of the antidote is what causes lethality.

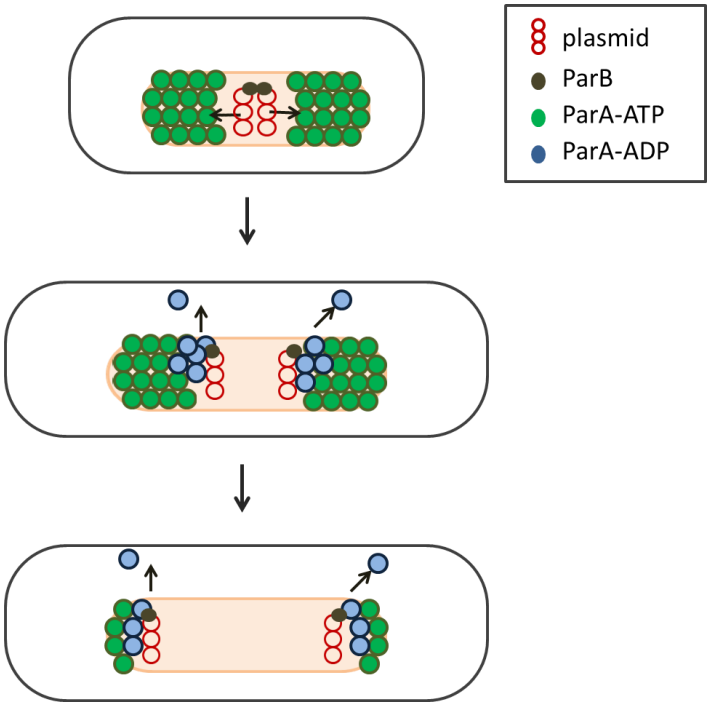


Figure 1.2A Partitioning of the P1 plasmid utilizing the type I system. The ATP bound form of the partitioning protein, [ParA-ATP], binds DNA along the nucleoid. When ParA-encounters its partner protein (ParB) bound to the centromere of the P1 plasmid, its ATPase activity is stimulated. As a result, the ParA-ADP form is displaced from the nucleoid in the vicinity of the plasmid. For a pair of replicated plasmids located near the cell center, the dynamic interaction between ParA and the ParB-centromere complex, causes contraction of the ParA molecules from the vicinity of the plasmid, causing an uneven distribution of ParA along the nucleoid and providing the motive force for plasmid segregation. The ATP bound form of ParA is regenerated from the ADP bound form by nucleotide exchange. The figure is modified from Funnell et al., (2010).

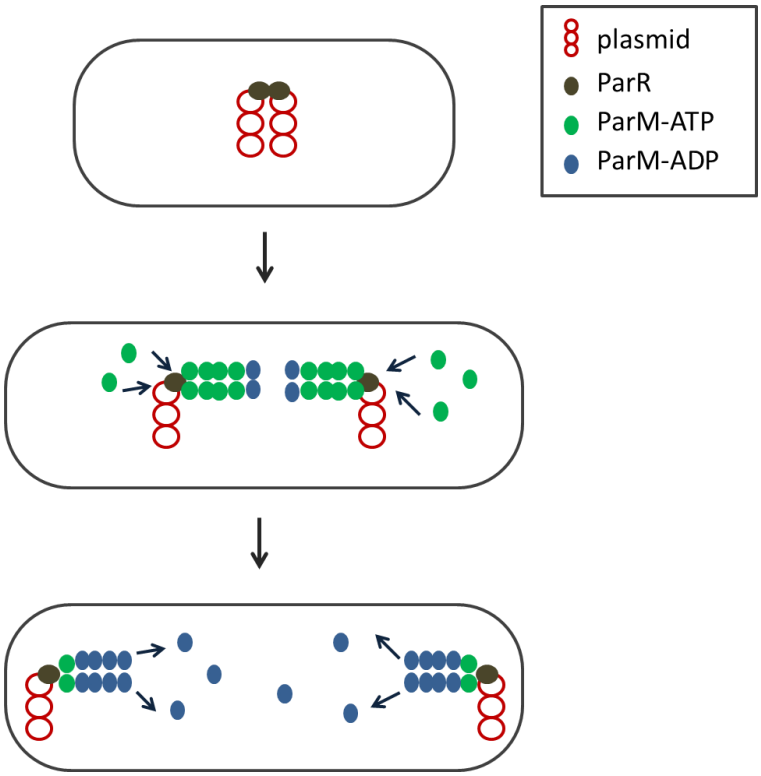


Figure 1.2B Partitioning of the R1 plasmid utilizing the type II system. The replicated copies of the R1 plasmid at the cell center are bridged at their centromeres by the centromere binding Par protein (ParR). This DNA-protein complex nucleates the oligomerization of the second partitioning protein (ParM; ATPase) into a filament which pushes the plasmid sisters away from mid-cell position towards the poles. Filament elongation occurs by the addition of ParM monomers at the end proximal to the centromere. ATP hydrolysis (ATP-- ADP + Pi) results in the disassembly of the filament at the distal end. Nucleotide exchange converts the ParM-ADP into the active ParM-ATP form. The figure is modified from Gerdes et al., (2004).

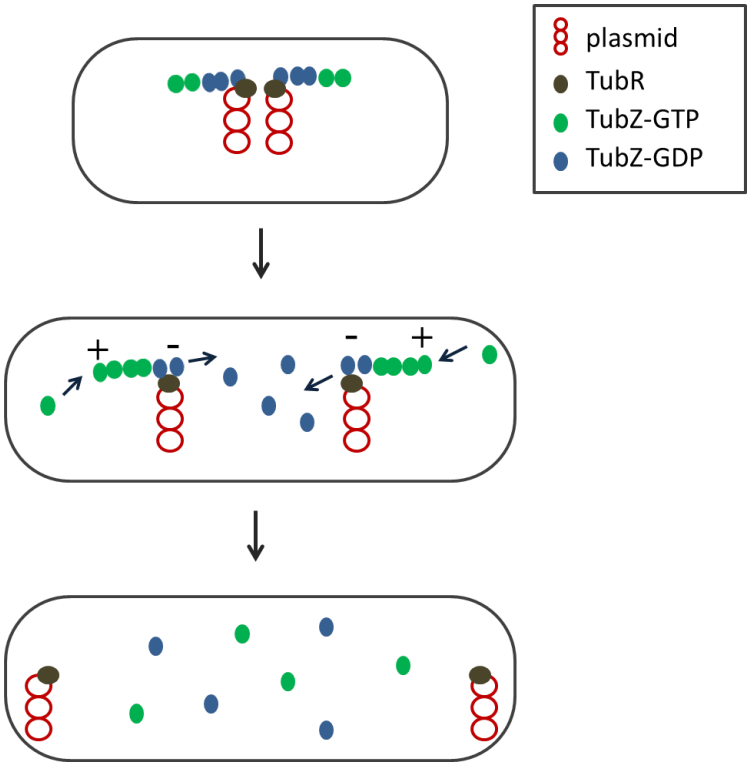


Figure 1.2C Partitioning of the Bacillus thuringiensis plasmid pBtoxis utilizing the type III system. The filament formed by the Par GTPase (TubZ) binds to its partner Par protein (TubR) associated with the centromere locus of each of a pair or replicated plasmid copies. The stimulation of GTPase following this interaction results in the shrinkage of the filament at the centromere proximal end (referred to as the minus end) and growth of the filament at the distal end. This treadmill-like action drags the plasmid towards the cell pole. Note that the nomenclature of the bacterial system refers to the centromere end as 'minus' and the cell pole as 'plus' end, reversing the signs assigned to the spindle ends in the nomenclature of eukaryotic systems. The figure is adapted from Ni et al., (2010).

1.1.2 Extra-chromosomal circles in eukaryotes: yeast plasmids and viral episomes

Plasmids in eukaryotes are restricted to a small group of fungal species belonging to the Saccharomycetaceae lineage (Volkert et al., 1989). These plasmids are similar in size and genetic organization (Blaisonneau et al., 1997; Utatsu et al., 1987). The 2 micron plasmid, which exists as an extra-chromosomal entity in *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, is the most well characterized representative within the group of budding yeast plasmids (Jayaram et al., 2004c). The plasmid utilizes its limited genetic potential to take advantage of the host's metabolic machinery in stably propagating itself with nearly the same stability as chromosomes. Each plasmid molecule replicates once per cell cycle, and replicated copies are partitioned evenly between daughter cells. Any stochastic decrease in plasmid copy number is corrected by a DNA amplification mechanism. The plasmid harbors a regulatory system by which it maintains its steady state copy number (40-60 molecules per haploid cell), protecting against high copy numbers that would pose a metabolic burden to the host. The plasmid, at its normal copy number, is selectively almost neutral, providing no obvious advantage to the host while costing the host little fitness penalty. As such, the 2 micron plasmid offers an excellent model to study the selfishness, as well as moderation of selfishness, in extra-chromosomal DNA elements in eukaryotes (Jayaram et al., 2004a; Jayaram et al., 2004b).

Viruses of the gamma herpes and papilloma families persist for extended periods of time in mammalian cells as extra-chromosomal elements, although they do occasionally integrate into chromosomes. The well characterized among these viruses include Epstein-Barr virus (EBV), Kaposi's sarcoma associated herpes virus (KSHV) and human and bovine papillomavirus (HPV and BPV). The circular, double stranded viral

genomes can be maintained through passive replication for decades during the latent phase of their infection cycles (Botchan, 2004; Frappier, 2004; Ilves et al., 1999; Lehman and Botchan, 1998; You et al., 2004). During the active phase of infection, the viral copy number increases to very high values through active replication.

The molecular basis for the stable propagation of the viruses through multiple cell divisions is their ability to tether to host chromosomes. By doing so, they avoid the prospect of being excluded into the cytoplasm during the breakdown and reformation of the nuclear envelope, which is a central feature of mammalian mitosis. Some of the papilloma viruses may associate with the mitotic spindle directly, and exploit spindle forces for their segregation (Van Tine et al., 2004).

The viral episomes of mammals and the 2 micron circle and related plasmids of budding yeasts have interesting similarities in their life styles. They normally undergo regulated replication, partition efficiently during cell division, and harbor the capacity to amplify their copy number. There are several lines of circumstantial evidence to suggest that the 2 micron plasmid might also exploit the chromosome segregation machinery for its partitioning (Cui et al., 2009; Ghosh et al., 2007; Hajra et al., 2006; Huang et al., 2011b; Jayaram et al., 2004; Mehta et al., 2002; Velmurugan et al., 2000). Since chromosome segregation has been evolutionarily optimized for maximum fidelity, it is likely that selfish elements as divergent as yeast plasmids and mammalian viruses have independently arrived at logistically related strategies for chromosome coupled propagation. We briefly review below our current understanding of the mechanisms for the stable persistence of these selfish elements, highlighting unifying features among these mechanisms.

1.2 Strategies utilized by viral episomes for stable maintenance

During acute infection by a virus, there is rapid production of progeny virions. However, the abundance of viral antigens also provides the host with the opportunity to launch an aggressive immune response to clear the infection. By contrast, viruses that establish persistent latent infection, maintain their genomes at a low copy number, and manage to escape detection by the host defense system by down-regulating the expression of viral proteins below a threshold level. This mechanism of escaping immune-surveillance is utilized by both viruses that integrate into chromosomes and remain quiescent as well as those that remain as autonomously replicating genomes.

Since our specific interest is in extra-chromosomal selfish genomes, we shall restrict our considerations to gamma-herpesviruses and papillomaviruses, which typify viral genomes with the capacity for autonomous replication and maintenance. We have chosen here as representatives of these two virus classes the Epstein-Barr virus (EBV) and bovine papillomavirus (BPV), based primarily on the information available on their replication and segregation mechanisms. Chapters **4** and **5** will focus on the segregation systems of these viruses reconstituted in *S. cerevisiae*. We shall occasionally refer to the human papilloma virus (HPV), where relevant. EBV and BPV differ markedly in genome size and complexity, the cell types that they infect and in the mechanisms they deploy for cell invasion. Yet, they share several similarities in their strategies for escaping the host defense system and persisting in the nucleus for extended periods.

1.2.1 Life cycles of EBV and PV (papilloma viruses)

1.2.1.1 Life cycle of EBV

As pointed out, Epstein-Barr virus (EBV) belongs to the gamma-herpesvirus family, and is a human herpes virus. It benignly infects more than 90% of the world's population (Evans et al., 1968). The infection of EBV is usually asymptomatic in childhood and causes infectious mononucleosis (IM) in adolescence. Less frequently, EBV infection has been linked to several types of cancers such as Burkitt's lymphoma, nasopharyngeal carcinoma and gastric carcinomas (Tsurumi et al., 2005). The life cycle of EBV is depicted in Fig. 1.3. EBV is capable of infecting epithelial cells and resting B lymphocytes. During primary infection, EBV invades oropharyngeal epithelial cells, where EBV genome replicates efficiently and produces large number of progeny in a localized lytic burst. The virus then passes through the oropharynx and infects circulating B cells, to establish latent infection. During the entry phase, the virus first binds to the CD21 receptor molecule on the cell membrane, and is then internalized into host cells through the mediation of MHC class II molecules (Fingeroth et al., 1984; Li et al., 1997). The establishment of latent infection and the persistence of EBV are coupled to the multistep immortalization of infected resting B cells, which are activated into a state of proliferation, and become permanent lymphoblastoid cell lines (LCLs). The latently infected B cells are grouped into different types based on the expression patterns of EBV latent genes (Table 1.1). With the limited production of viral proteins, EBV-infected B cells can escape being targeted by the immune system. During the final lytic stage of the EBV life cycle, robust replication copiously increases the viral load in the infected B cells in the oropharynx, and infectious virions are released into the saliva.

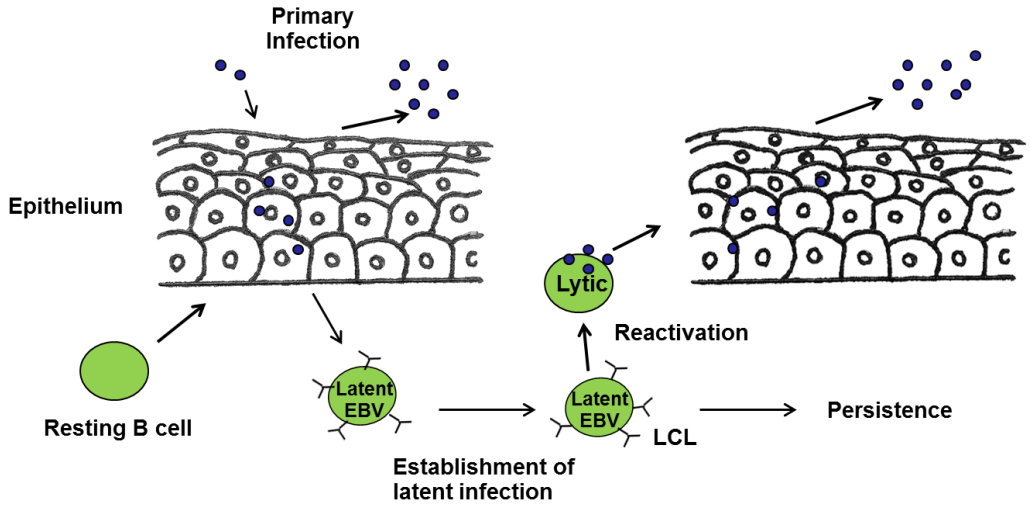


Figure 1.3 The life cycle of Epstein-Barr virus. During primary infection, EBV invades and passes through the oropharyngeal epithelial cells, where localized lytic cycle occurs. The resulting virus particles then infect resting B lymphocytes. The establishment of latent EBV infection leads to activation the B cells and proliferation-mediated establishment of lymphoblastoid cell lines (LCLs). When the latent EBV episomes are reactivated to trigger the lytic stage, the viral genomes amplify more than 100-fold, are packaged into virions, and are finally released into the saliva. The figure is modified from Kieff and Rickenson, Fields Virology, 3rd edition, Chapter 75.

Latency type designation	Proteins expressed
Type 0	
Type I	EBNA-1
Type II	EBNA-1, LMP proteins
Type III	EBNA-1-6, LMP proteins

Table 1.1 Patterns of EBV latent gene expression in B cells. The latently infected B cells can be grouped into four types based on the expression of EBV genes. In some cases, infected B cells re-enter the resting state, and are designated as Type 0. There is little or no EBV gene expression in Type 0 cells and the viral episomes do not replicate in these cells. Infected B cells that go through division belong to two classes, Type I and Type II, depending on which of the viral latency genes are expressed. Type I cells are minimalist, in that only EBNA-1 is expressed in them. Type II cells express latent membrane proteins (LMP proteins) in addition to EBNA-1. Type III cells express all of the latency genes, comprising those coding for EBNA-1 through EBNA-6 and for LMP proteins.

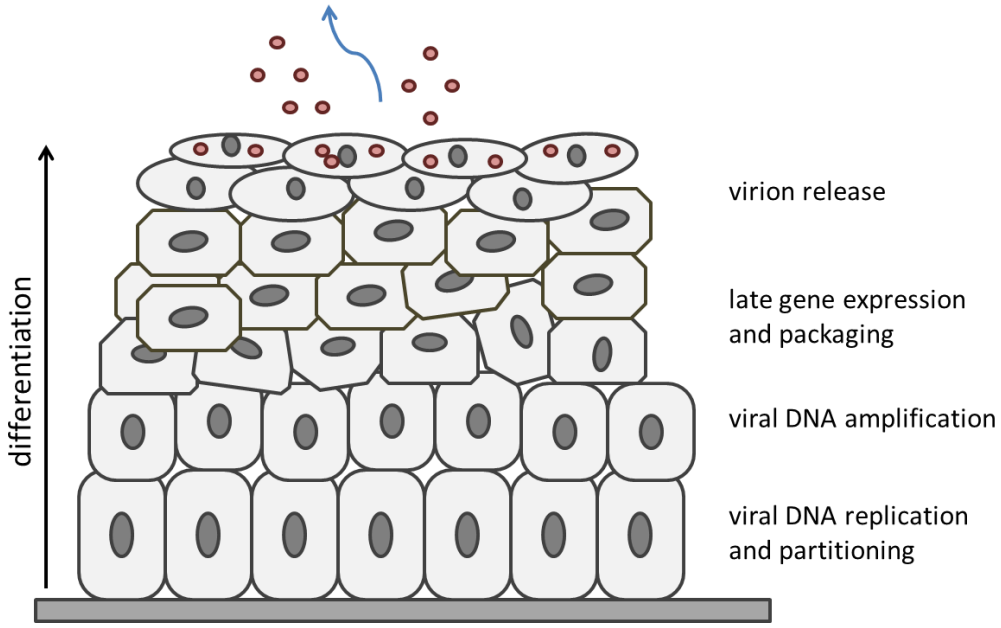


Figure 1.4 The life cycle of papillomavirus. The life cycle of PV is coupled to the differentiation status of host epithelial cells. Infected cells in the basal layer contain relatively low levels of viral episomes, which are maintained stably during cell division. When infected cells are pushed away from the basement membrane, they stop division and enter a phase of programmed terminal differentiation. The differentiation process activates the PV to enter the lytic cycle. Viral genomes are amplified and packaged into virion particles in the cornified layer of the epithelium. Cells from this layer are destined to be sloughed off from the epidermis. This figure is modified from McBride et al., (2012).

1.2.1.2 Life cycle of PV

Papillomavirus are epitheliotrophic viruses that cause warts or papillomas. There are hundreds of papillomavirus types and subtypes, which infect specific species and specific types of cutaneous or mucosal epithelium. The life cycles of papillomaviruses are linked to the differentiation status of the host epithelium (Fig. 1.4). Initial infection leading to the persistence of viral episomes occurs in the basal layer of epithelium, which is still active in mitosis.

After passing through the epidermis via microabrasions or wounds, viral particles bind to membrane-associated heparin sulfate polysaccharides on the host cells (Giroglou et al., 2001; Shafti-Keramat et al., 2003). Internalization of the virus by the host endocytosis pathway is facilitated by the major and minor capsid proteins, L1 and L2 respectively (Day et al., 2003; Schiller et al., 2010; Smith et al., 2007). The uncoated viral genome exits the endosome and migrates into the nucleus through an L2-dependent pathway (Day et al., 2004; Kämper et al., 2006). A brief burst of amplification results in a viral copy number of 10-50 per cell (Schwarz et al., 1985). The viral genomes then enter the latent stage to maintain themselves in dividing basal cells for the duration of the persistent infection. When basal cells leave the basement membrane, they stop division and go through the process of programmed terminal differentiation. The virus is reactivated in these cells to trigger the onset of the lytic stage, during which the viral genome is amplified many fold, late gene expression is fully turned on, and finally the viral genomes are assembled into infectious virions. Thus, the differentiated surface cells of the skin destined to be sloughed off from the epidermis are loaded with the virus progeny in their nuclei (Fig. 1.4).

1.2.2 Genomic organization of viral episomes

1.2.2.1 Genomic organization of EBV

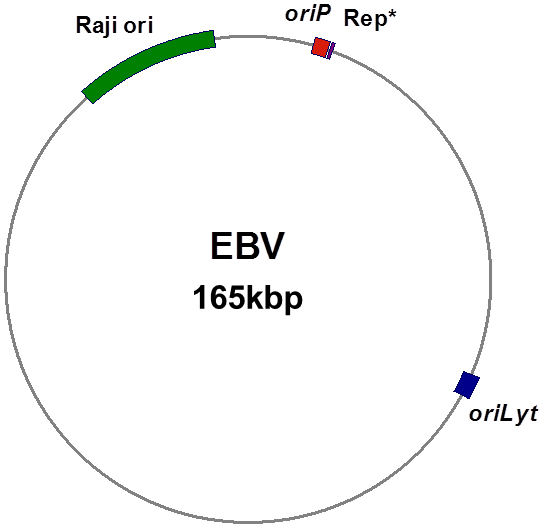
The EBV episome is a large double-stranded circular DNA, 165 kbp in size (Bloss and Sugden, 1994) (Fig. 1.5). Approximately 90 genes are encoded within the genome. And together they account for roughly half the genome size. The primary transcripts go through complicated splicing processes to generate the mature protein coding messenger RNAs (Bodescot et al., 1986; Bodescot and Perricaudet, 1986). Several *cis* elements have been identified for genome replication and maintenance, and are summarized below.

The latent origin of DNA synthesis, *oriP*, is composed of a sequence segment with **d**yad **s**ymmetry (DS) and a **f**amily of **r**epeat (FR) elements (Bear et al., 1984; Reisman et al., 1985). The DS segment contains four binding sites for the viral protein EBNA-1, which are arranged as two pairs, with higher binding affinity vested in the outer binding sites of each pair compared to the two internal binding sites (Reisman et al., 1985; Summers et al., 1996). DS is required for replication initiation during the latent stage. Flanking the pairs of EBNA-1 binding sites of DS are three 9 bp elements, termed nonamers, that resemble the human telomeric repeat sequence (Niller et al., 1995). It has been shown that telomere binding factors (TRF1, TRF2) and the human repressor activator protein 1 (hRap1) are associated with the nonamer sequences. The average copy number of EBV episomes maintained in infected cells decreases when nonamer sequences are deleted from the genome (Deng et al., 2003; Deng et al., 2002). Nonamer sequences also play a role in the replication initiation of the episomes, since TRF2 can

recruit cellular replication factors to the DS element by direct protein-protein interactions (Atanasiu et al., 2006).

The FR element contains 21 copies of imperfect 20bp repeats with 20 high affinity EBNA-1 binding sites, and is required for transcription activation and plasmid retention (Gahn and Sugden, 1995; Längle-Rouault et al., 1998). The Rep* element, which contains two EBNA-1 binding sites, is located 240 bp downstream of DS, and can partially replace the function of DS in DNA synthesis during the latent stage (Kirchmaier and Sugden, 1998; Wang et al., 2006). The lytic origin of DNA synthesis (*oriLyt*) has been mapped approximately 40 kbp downstream of *oriP*, with less defined organizational features compared to *oriP* (Hammerschmidt and Sugden, 1988). When EBV is maintained in Raji cells, an approximately 14 kbp region (Raji ori) instead of *oriP* can support the replication initiation independently of EBNA-1 binding (Little and Schildkraut, 1995; Wang and Sugden, 2008).

A.



B.

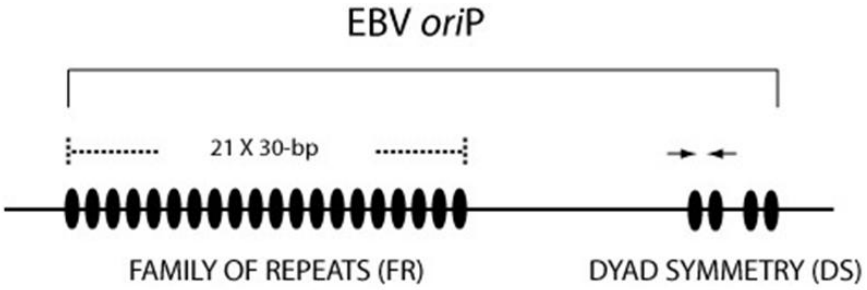


Figure 1.5 The EBV genome and the *cis*-acting elements for replication and maintenance. (A) The *cis*-acting elements for replication and partition of EBV are depicted as filled boxes on the circular EBV genome. The origin of replication (*oriP*) (red box) is responsible for the replication and maintenance of EBV during the latent stage of its infection cycle. *oriLyt* (blue box) lies approximately 40 kbp away from *oriP*, and is responsible for EBV replication during the lytic stage. The Rep* element (purple box), which is located 240 bp downstream of *oriP*, can partially replace the *oriP* function in DNA synthesis during the latent stage. In Raji cell lines, a ~14 kbp region (Raji ori) (green box) can support initiation of DNA synthesis without EBNA-1 binding. (B) An expanded view of the organization of the latency origin of replication (*oriP*) of EBV is shown. The principal features of *oriP* are the family of repeats (FR) and the dyad symmetry (DS) element. EBNA1 binding sites are depicted as solid ellipses. The dyad above DS element indicates the orientation of EBNA1 binding sites. The figure is modified from Lindner and Sugden, (2007).

1.2.2.2 Genomic organization of PV

The genomic organization among various papillomaviruses is well conserved in overall size and functional organization. BPV-1, which is well characterized among papillomaviruses and whose segregation mechanism is investigated in Chapter **5**, will serve as the model papilloma genome for our interests. The BPV-1 genome is a circular, double-stranded DNA with a relatively small size of ~8000bp (Chen et al., 1982) (Fig. 1.6). The protein coding regions are divided into early and late categories according to their locations within the genome and the spatial and temporal differences in their expression controls (McBride, 2008). The early region encodes viral proteins E1 through E8, which are mainly expressed in the less differentiated layers of epithelium. The late region encodes the capsid proteins L1 and L2, which are expressed in superficial, differentiated cells. A region of approximately 1 kb between nucleotides (nt) 7093 and 89, where no coding region has been mapped, is referred to as the **l**ong **c**ontrol **r**egion (LCR) (Haugen et al., 1987; Spalholz et al., 1987). The LCR region contains the sequences required for viral replication and maintenance, and also houses transcriptional enhancer elements and promoters activated by the binding of the viral E2 protein. The BPV-1 replication and maintenance sequences, which are functionally similar to the DS and FR sequences of EBV, are constituted by the **m**inimal **o**rigin of replication (MO) and the **m**inichromosome **m**aintenance element (MME) (Piirsoo et al., 1996; Ustav et al., 1991). The DS segment and the FR element are physically separate and functionally distinct, although they both contain binding sites for viral protein EBNA-1. By contrast, the boundaries within the MME region that demarcate replication and maintenance have not

been well delineated (Piirsoo et al., 1996). The functional contributions of MO and MME are described in more detail in the sections below dealing with viral replication.

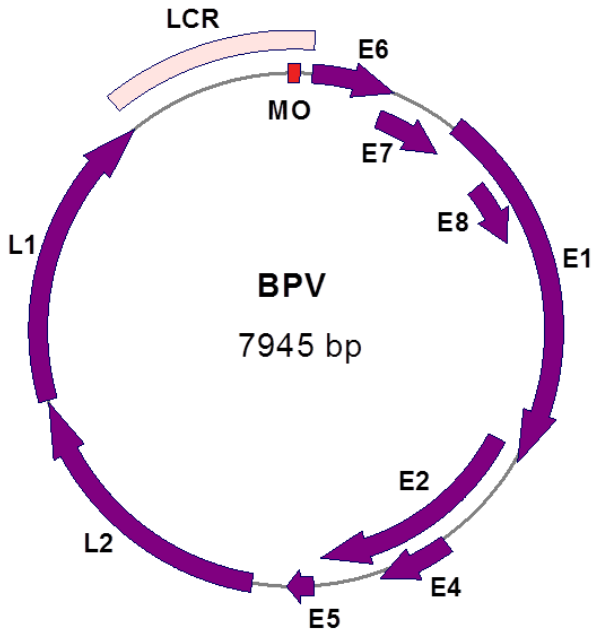


Figure 1.6 The bovine papillomavirus genome. BPV has a circular, double-stranded DNA genome of approximately 8000 bp. It encodes proteins E1 and E2, which function in both viral replication and partitioning. L1 and L2 are capsid proteins, which are expressed in superficial, differentiated cells. Other viral encoded proteins have mainly auxiliary roles in manipulating host cell proliferation and cell cycle checkpoints as well as in escaping host immune defense. The long control region (LCR) contains E1 and E2 protein binding sites, and is required for viral replication and maintenance and for transcriptional regulation of viral genes. The minimal origin of replication (MO) sequence is depicted as a red box. The boundaries of the minichromosome maintenance element (MME), also located within LCR, are not well defined. The figure is modified from McBride et al., (2012).

1.2.3 Replication of viral episomes

1.2.3.1 Replication of EBV

The mechanisms of replication of EBV in latent stage and lytic stage of infection are quite different. The only requirements for latent viral replication are the *cis* element *oriP* and the viral protein EBNA-1 (Yates et al., 1984; Yates et al., 1985). As EBNA-1 does not have DNA polymerase activity, episome replication must rely on the cellular replication machinery (Frappier and O'Donnell, 1991). This inference is consistent with the finding that viral plasmids replicate once per cell cycle during the latent stage (Adams, 1987). Several host replication factors, such as ORC (origin recognition complex) and the MCM (mini chromosome maintenance) helicase complex bind specifically to the DS element of *oriP* (Chaudhuri et al., 2001; Dhar et al., 2001; Ritzi et al., 2003). It is generally agreed that the FR element of *oriP* is dispensable in replication initiation of plasmid since a plasmid containing the DS element alone can still replicate in the host cell (Harrison et al., 1994). At least one of the two pairs of EBNA-1 binding sites within DS is necessary for the replication activity, defining the minimal replicator within *oriP* (Shirakata and Hirai, 1998; Yates et al., 2000). The nonamer sequence, flanking the EBNA-1 binding sites of DS, increases the replication potential significantly, although it is not absolutely required for replication (Yates et al., 2000). The function of the nonamer sequence in replication initiation is believed to enhance the recruitment of ORC complex to DS by the direct interaction of TRF2, which binds to the nonamer sequences, with the BAH domain of ORC1 (Atanasiu et al., 2006; Ritzi et al., 2003). Sequential recruitment of ORC and the MCM complex to DS leads to the assembly of the pre-initiation complex, which licenses DNA synthesis to be initiated at the origin (Lindner and Sugden, 2007).

It is important to note that, while *oriP* is the major replicator in most situations, other secondary replicators do exist, and are required for replication in certain special circumstances. The Rep* sequence referred to earlier, which contains a pair of EBNA-1 binding sites, is less efficient in replication compared to DS in supporting the once-per-cell-cycle replication (Kirchmaier and Sugden, 1998; Wang et al., 2006). The 14 kb Raji ori is responsible for replication when an EBV based plasmid is maintained in Raji cell lines (Little and Schildkraut, 1995). The replication mechanism at the Raji ori is different from that at DS and Rep*, as no EBNA-1 binding site is present in this region. Instead of one replication initiation site, multiple replication initiation sites appear to operate in this broad region (Little and Schildkraut, 1995). Thus, replication at the Raji ori resembles the delocalized patterns of replication initiation observed at several loci in mammalian chromosomes.

In contrast to the latent stage, multiple rounds of replication are triggered from a separate origin, termed *oriLyt*, during the lytic stage of EBV infection (Hammerschmidt and Sugden, 1988). During this stage, a number of virally encoded proteins required for replication are expressed (Fixman et al., 1995). The expression of viral protein BZLF1 coincides with the entry of EBV into the lytic cycle, which is also marked by the arrest of the host cells in the late G1-S phase. This cell cycle arrest is believed to be conducive for the preferential lytic replication of the viral genome (Kudoh et al., 2003). The virally encoded proteins which are necessary for *oriLyt*-dependent DNA replication, and the key steps at which they act, are briefly summarized below.

BZLF1 binds to the BZLF1-responsive elements (ZRE) within *oriLyt*, and this association is required for DNA replication and transcriptional activation of the lytic

genes (Schepers et al., 1996). The viral protein BALF5 is the DNA polymerase catalytic subunit (Tsurumi et al., 1993) whereas BMRF1 is identified as a virus DNA polymerase accessory protein (Tsurumi, 1993). The BALF2 gene encodes the single-stranded DNA-binding protein (SSB) (Tsurumi et al., 1998). The proteins BBLF4, BSLF1 and BBLF2/3 assemble as a complex, and they likely function as the helicase, primase and the helicase-primase associated proteins, respectively (Fixman et al., 1995; Yokoyama et al., 1999). In general, the initial binding of BZLF1 within *oriLyt* provides the scaffold for recruiting the BBLF4/BSLF1/BBLF2/3 complex to *oriLyt*. The opening of the *ori* DNA and the binding of SSB (BALF2) completes the assembly of the pre-priming complex, which further recruits the polymerase holoenzyme BALF5 with the help of the accessory subunit BMRF1 to initiate DNA replication (Tsurumi et al., 2005).

The EBV genome is amplified approximately 100-1000 fold by lytic stage replication over the viral levels present during the latent stage. Since DNA intermediates of lytic replication are found as concatemers of head-to-tail copies of the EBV genome, it is postulated that the amplification of EBV results from rolling-circle DNA replication (Hammerschmidt and Sugden, 1988). These concatemers are resolved into unit length genomes and packed into virions (Challberg, 1996). The mechanisms by which the postulated rolling circle replication is triggered still remain elusive.

1.2.3.2 Replication of PV

The replication of papillomaviruses falls into three temporally distinct stages. When the virus first infects the dividing basal cells, it goes through a limited level of amplification to establish the basal pool of viral episomes. During the maintenance stage (latent cycle), the episome copy number remains more or less constant by the coupling

between viral replication and host replication. During the final stage of infection (lytic cycle), the episomes are vegetatively amplified to produce a large number of progeny genomes, which are packaged into infectious virions. Each stage is briefly described below.

The initial amplification process, during viral establishment, requires the replication origin of the episome, the viral proteins E1 and E2 and cellular replication proteins. The minimal replication origin sequence (MO) identified in BPV contains an E1 binding site, an E2 binding site and an A/T rich region (Ustav et al., 1993; Ustav et al., 1991). The E1 protein is the main viral replication protein, which is an ATP-dependent helicase responsible for melting and unwinding the replication origin and thus facilitating subsequent replication fork progression (Yang et al., 1993) (Fig. 1.7). The role of the E2 protein in the initiation of replication is mainly auxiliary. By interacting with the E1 helicase, which has low binding discrimination for DNA sequences, E2 increases the binding specificity of E1 to the origin (Stenlund, 2003). The initial cooperative binding of a complex between an E1 dimer and an E2 dimer is followed by the dissociation of E2 and the conversion of E1 into its active ring structure (E1 double hexamer), which encircles the ssDNA (Enemark and Joshua-Tor, 2006; Sanders and Stenlund, 1998; Schuck and Stenlund, 2005). Cellular replication factors recruited to the origin with assistance from the E1 protein and/or the E2 protein include the replication protein A, topoisomerase I, DNA polymerase α-primase, polymerase δ, and replication factor C., among others (Clower et al., 2006; Conger et al., 1999; Kuo et al., 1994; Loo and Melendy, 2004). The details of the mechanisms by which replication is regulated to accomplish limited amplification are not understood.

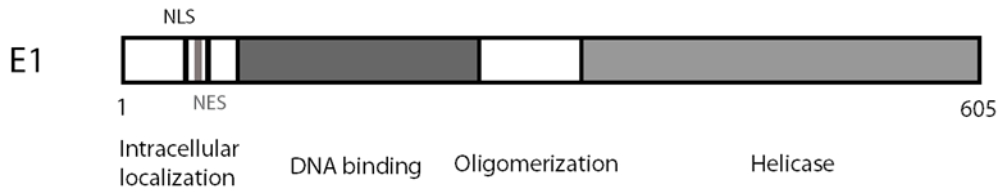


Figure 1.7 Functional domains of E1 replication protein of BPV. The 605 amino acids long E1 protein consists of four domains. the N-terminal domain contains a nuclear localization sequence (NLS) and a nuclear export signal (NES), and is responsible for specific intracellular localization. A DNA binding domain confers sequence specific binding activity with E1-binding sites. An oligomerization domain is required for E1 dimerization. The C-terminal domain, which is responsible for helicase activity of E1, also binds to DNA with low specificity.

The stage of limited amplification is believed to be quite short. Once the virus enters the maintenance stage, the episome copy number is maintained stably during the subsequent divisions of the basal cells. In principle, this could be achieved if the episomes double (or nearly double) their copy number during a cell cycle, and segregate equally (or roughly equally) into daughter cells during cell division. It is known that, during the latent stage of EBV infection, the replication of episomes is coupled to the replication of the host genome, which occurs strictly once per S phase (Adams, 1987). However, whether papillomaviruses follow this regulated replication is still being debated. Early studies seemed to support a model in which each BPV-1 episome undergoes one round of replication per cell cycle during the maintenance stage (Botchan et al., 1986). However, later studies suggested that BPV performs random-choice replication, with a subset of the episomes replicating more than once per cell cycle and

others not replicating at all (Gilbert and Cohen, 1987; Ravnan et al., 1992). However, both scenarios could lead, statistically, to “once per cell cycle” replication per episome (Ravnan et al., 1992).

It is possible that maintenance replication may at least be qualitatively similar to limited amplification in its mechanism, provided some negative regulation of replication limits the copy number increase to just two-fold per cell cycle. Whereas limited amplification is dependent on E1, the role of E1 in maintenance replication is not well understood. Early studies showed that a derivative of BPV-1 encoding a temperature sensitive E1 protein is maintained more or less normally at the non-permissive temperature, suggesting that E1 is not absolutely required for long-term propagation of the virus (Kim and Lambert, 2002). Other lines of evidence suggest that there are differences, at the regulatory levels, between the replication events during early limited amplification and subsequent latent maintenance. The cellular p53 protein appears to inhibit replication of BPV-1 during the amplification period, but does not affect its maintenance replication (Ilves et al., 2003).

For both the limited amplification and the subsequent maintenance stage, the control of virus copy number is important, especially if replication of papillomavirus is not coupled to the replication of the host genome. Copy number maintenance could be mediated through limiting factors that regulate replication during each stage. It has been reported that the expression levels of E1 and E2 are critical in episome replication (Gopalakrishnan and Khan, 1994; Penrose and McBride, 2000). Another layer of copy number control appears to operate at the level of the half-lives of E1 and E2 proteins and thus their steady state concentrations in the cell (Malcles et al., 2002; McBride and

Howley, 1991; Penrose et al., 2004). Furthermore, the E1 and E2 open reading frames may encode more than one polypeptide product as a result of transcription from alternative promoters or by processing of the primary transcript at a commonly utilized 3' splice site located within the coding region (Lambert et al., 1987; Lusky and Botchan, 1986). These additional protein products could negatively control the functions of the full-length proteins. For example, the E2 open reading frame expresses, in addition to full-length E2, a truncated form, E2-TR. Genetic analysis revealed that the episome copy number of a BPV-1 variant that does not express E2-TR is 10- to 20-fold higher than normal (Lambert et al., 1990; Riese et al., 1990). As E2-TR contains only the DNA binding and dimerization domain, but not the transcription activation domain, it can antagonize the activity of full-length E2 (E2-TA) by competing for the E2 binding sites or by forming heterodimers (Barsoum et al., 1992; Lim et al., 1998).

During vegetative replication (lytic infection), the viral episome amplifies to a large number in the differentiated cells. At this stage of differentiation, uninfected cells are mitotically inactive, and cellular replication factors such as DNA polymerase are either absent or expressed at very low levels. However, in infected cells, the expression of viral proteins E6 and E7 causes the differentiated cells to be reactivated for cell proliferation by reprogramming cell-cycle regulation (Cheng et al., 1995; Jones et al., 1997; Stubenrauch and Laimins, 1999). In addition, there is also an increase in E1 and E2 protein levels at this stage, which might also contribute towards vegetative amplification (Burnett et al., 1990; Klumpp and Laimins, 1999).

It is unclear whether, as has been suggested for EBV, there is a switch from the normal θ mode of replication to rolling circle replication when EBV-1 transitions from

latency to the lytic stage. The bubble and Y shaped intermediates predicted for θ replication have not been detected for papillomaviruses during the latent stage of infection. Based on this negative evidence, it has been suggested that papillomavirus replication follows the rolling circle replication even during the latent phase (Dasgupta et al., 1992; Flores and Lambert, 1997). However, in the absence of additional evidence, the issue remains open.

1.2.4 Partitioning of viral episomes

1.2.4.1 Partitioning of EBV

To achieve stable maintenance during the latent stage, viral episomes not only have to duplicate its genome during the cell cycle but also partition roughly equal numbers of replicated copies of the virus into daughter cells at the end of mitosis. During type I latency in B cells, EBV genomes are stably maintained through many generations when only EBNA-1 and small non-coding RNAs (EBERs, stand for Epstein-Barr virus–encoded small RNA) are expressed (Rowe et al., 1987). The function of EBERs is not clear. Although they are constitutively expressed in the latency stage, there is no direct evidence for their role in episome maintenance. Further analysis demonstrated that the FR element of *oriP* and EBNA-1 are essential for the partitioning of EBV plasmids, which is mediated through the tethering of viral DNA to chromosomes (Aiyar et al., 1998; Hung et al., 2001; Kanda et al., 2001).

As the *cis*-acting partitioning locus, FR contains 20 EBNA-1 binding sites, but only six to eight are sufficient for genome maintenance (Wysokenski and Yates, 1989). As the partitioning protein required for viral maintenance, EBNA-1 association with chromosomes depends on its central (amino acids 325-376) and N-terminal (amino acids

33-53) regions that are both Gly-Arg rich (Hung et al., 2001; Nayyar et al., 2009) (Fig. 1.8). Whether EBNA-1 is directly responsible for tethering episomes to chromosomes, or whether it performs this function with the help of cellular proteins, is still under debate. In one set of experiments, the cellular EBNA-1 binding protein 2 (hEBP2) has been shown to be required for EBV partitioning (Kapoor and Frappier, 2003; Wu, 2000). EBP2 is a conserved nucleolus associated protein among eukaryotes, which functions in ribosome biogenesis (Tsujii et al., 2000). After its release from the nucleolus very late in prophase, hEBP2 relocalizes to metaphase chromosomes and interacts with EBNA-1 until telophase (Nayyar et al., 2009; Wu, 2000). Interaction with hEBP2 is mediated by the central region of EBNA-1 spanning amino acids 325-376, which overlaps with the chromosome binding region of EBNA-1 (Ceccarelli and Frappier, 2000) (Fig. 1.8). A deletion derivative of EBNA-1 lacking this region can support replication initiation of viral plasmids, but is defective in its segregation function (Ceccarelli and Frappier, 2000).

Evidence has been provided to suggest that the chromosome tethering activity of EBNA-1 is independent of its association with hEBP2 (Lindner and Sugden, 2007). EBNA-1 may bind to AT-rich sequences within chromatin via its AT-hook motifs (Sears et al., 2004). Consistent with this possibility, the AT-hook motifs of EBNA-1 (amino acids 41-54 and amino acids 329-350) overlap with its chromosome binding Gly-Arg rich regions. Furthermore, EBNA-1 derivatives with AT-hook activity, but lacking hEBP2 interaction potential, can still support the replication and segregation of EBV based plasmids with nearly normal efficiency (Sears et al., 2004). It is possible that direct as well as hEBP2 mediated interactions of EBNA-1 with chromosomes act collaboratively in the maintenance of EBV episomes. In Chapter **4**, we will present our analysis

characterizing the behavior of an EBV reporter plasmid in *S.* cerevisiae under a reconstituted partitioning system. Under these conditions, hEBP2-EBNA-1 interaction is required for the association of EBNA-1 with yeast chromosomes (Kapoor and Frappier, 2003; Kapoor et al., 2001).

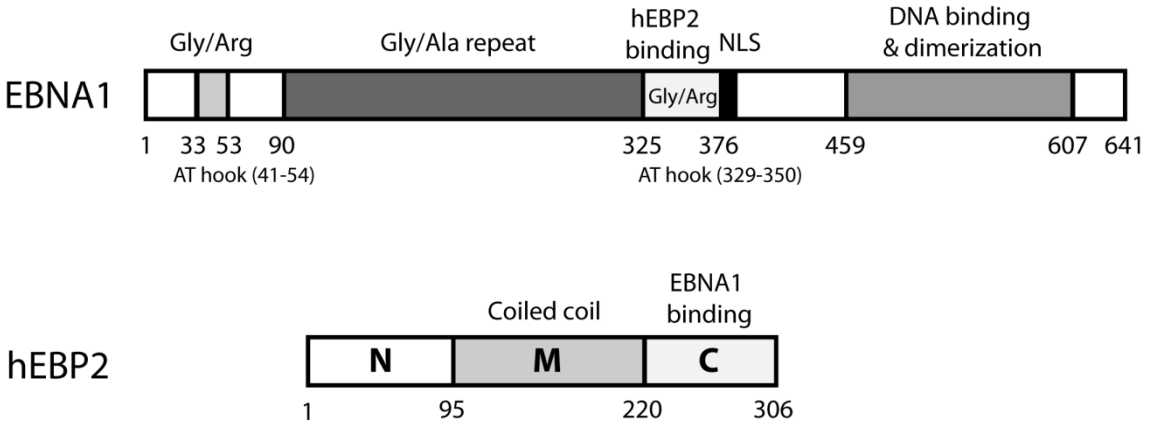


Figure 1.8 Functional domains and structural organizations of Epstein–Barr Nuclear Antigen 1 (EBNA-1) and human EBNA-1 binding protein 2 (hEBP2). The Gly-Arg (glycine–arginine rich) regions present within the N-terminal and central domains of EBNA-1 are likely responsible for mediating chromosome association. The putative AT hook motifs and hEBP2 binding site are within these regions. NLS denotes a nuclear localization signal. In hEBP2, the N-terminal (N) and C-terminal (C) domains are separated by a coiled-coil middle region (M). The C-terminal domain is responsible for interaction with EBNA-1. The numbers below the schematic representations of the polypeptide chains indicate amino acid positions. The figure is modified from Kapoor and Frappier, (2003) and Lin et al., (2008).

1.2.4.2 Partitioning of BPV

As is the case with EBV, most PVs are maintained in proliferating cells during the latent stage by tethering to host chromosomes. The most well characterized BPV-1 partitioning system is composed of the maintenance sequence MME, the viral protein E2 and the host chromosome binding protein Brd4.

An important clue in the characterization of MME came from the finding that MO sequence which includes only one E2 binding site is not sufficient for stable replication/maintenance of viral plasmids (Piirsoo et al., 1996). Based on serial deletion assays, it has been concluded that the activity of MME does not depend on a unique sequence within the LCR region; rather it depends on the number of E2 binding sites harbored by MME. Of the 11 E2 binding sites in the LCR region of the BPV-1 genome, at least 8 are required for proper episome maintenance function (Piirsoo et al., 1996). Further analysis showed that MO is not required for MME activity (Ilves et al., 1999). Similar to EBNA-1 of EBV, E2 is a multi-functional protein, which is involved in replication, transcriptional activation, and viral partitioning (Fig. 1.9). It has been shown that E2 can tightly associate to chromosomes utilizing its N-terminal trans-activation domain (Bastien and McBride, 2000; Skiadopoulos and McBride, 1998). However, in order to mediate the association of viral plasmids to chromosomes, the C-terminal DNA binding domain of E2 is essential. This region is responsible for interacting with the E2 binding sites within MME (Ilves et al., 1999).

Evidence has been presented to show that the cellular BET family protein Brd4 facilitates the association of BPV episomes with host chromosomes (You et al., 2004). Brd4 harbors two bromodomains as well as an extra-terminal (ET) protein interaction

domain (Florence and Faller, 2001) (Fig. 1.10). The bromodomains can interact with acetylated histone proteins H3 and H4, promoting Brd4 association with chromatin throughout mitosis (Dey et al., 2003). In addition, Brd4 is involved in transcriptional regulation by association with mediator complexes (Jang et al., 2005; Wu and Chiang, 2007). A possible role of Brd4 in promoting BPV tethering to chromosomes first came to light by the discovery that Brd4 co-purifies with E2 during affinity purification (You et al., 2004). In cell biological assays utilizing immunofluorescence, the BPV-1 E2 protein and Brd4 form colocalized punctuate spots along mitotic chromosomes. Further analysis identified the C-terminal domain of Brd4 to be responsible for its interaction with the N-terminal trans-activation domain of E2. A truncated version of Brd4 containing only the C-terminal domain competes effectively with full-length Brd4 for interaction with E2. As a consequence of this dominant negative effect, expression of this C-terminal domain of Brd4 also interferes with the colocalization of E2 and native Brd4 on mitotic chromosomes. Under these conditions, BPV-1 episomes fail to associate with chromosomes, suggesting the functional relevance of Brd4-E2 interaction in tethering BPV-1 genomes to chromosomes (You et al., 2004). The interaction between E2 and Brd4 is also required for the transcriptional activation function of E2 (Baxter et al., 2005; McPhillips et al., 2006).

Figure 1.9 Functional domains of E2 protein of BPV. The E2 protein consists of three regions; an N-terminal domain, a C-terminal domain and a hinge region in the middle that connects the two domains. The N-terminal domain is responsible for the interactions of E2 with E1 and Brd4, and for E2 function in transcriptional regulation. The C-terminal region has sequence-specific DNA binding and dimerization properties. Both domains are conserved among PVs. On the other hand, the hinge region varies in sequence and length in different E2 proteins.

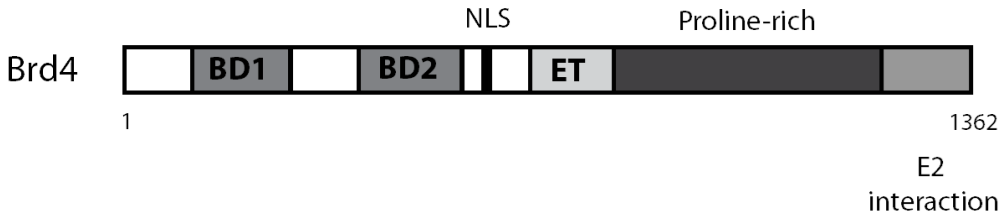


Figure 1.10 Functional domains of bromodomain protein Brd4. As the member of BET family proteins, Brd4 contains two bromodomains (BD1 and BD2) and an extra-terminal (ET) protein interaction domain. The region of interaction with the viral E2 protein lies within the C-terminal tail of Brd4. An NLS and a proline-rich region harbored by E2 are also indicated. This figure is modified from McBride et al., (2004).

Several lines of evidence suggest that the interaction between E2 and Brd4 is not passive, but is rather actively orchestrated and/or modulated within the cell. Brd4 forms a diffused coat around mitotic chromosome in the absence of E2 (Dey et al., 2003). When E2 is present, Brd4-chromosome interaction is refashioned and stabilized. The E2 induced localization of Brd4 on mitotic chromosomes has a characteristic punctuate pattern (McPhillips et al., 2005).

Although Brd4 promotes the attachment of viral genomes to chromosomes, other mechanisms may also be involved in BPV partitioning. It has been reported that a small sub-population of E2 molecules localizes on the central microtubules during late mitosis and interacts with the kinesin-like protein MKI (Yu et al., 2007). It is not clear whether this microtubule association of E2 is related to episome partitioning by a direct spindle mediated mechanism. It is interesting to note that the interaction of the kinesin related (and spindle associated) nuclear motor Kip1 in *S. cerevisiae* with the partitioning locus of the 2 micron plasmid promotes its equal segregation (Cui et al., 2009). It is quite unlikely, though, that the spindle is directly involved in plasmid segregation.

It is important to note that the mechanisms of episome partitioning among different PVs might not be fully conserved. Although the E2 proteins from most PVs are associated to mitotic chromosome, this is not so for all PVs. The E2 proteins of HPV11, 16, and 18 have been shown to associate with the mitotic spindle but not chromosomes (Van Tine et al., 2004). Even among E2 proteins that associate with chromosomes, the patterns of localization can be distinct (Oliveira et al., 2006) (Fig. 1.11). E2 of BPV-1 binds along the entire chromosome length in the form of tiny speckles. However, E2 of HPV-8 binds mainly to the peri-centromeric region of a subset of chromosomes. In the

case of certain members of the alpha-papillomavirus group, the E2 proteins do not stably interact with mitotic chromosome unless specific steps of fixation are employed during immunofluorescence assays (Oliveira et al., 2006). It is likely that Brd4 is not the only cellular mediator protein responsible for promoting the localization of viral episomes on chromosomes. Although E2-Brd4 interaction is a common feature of the trans-activation function of all E2 proteins analyzed (Ilves et al., 2006; McPhillips et al., 2006), this interaction is not required for genome partitioning of all PVs (McPhillips et al., 2006). Whereas the E2 proteins from some PVs show complete colocalization with Brd4 on mitotic chromosomes, the colocalization is only partial for the E2 proteins from other PVs (McPhillips et al., 2006).

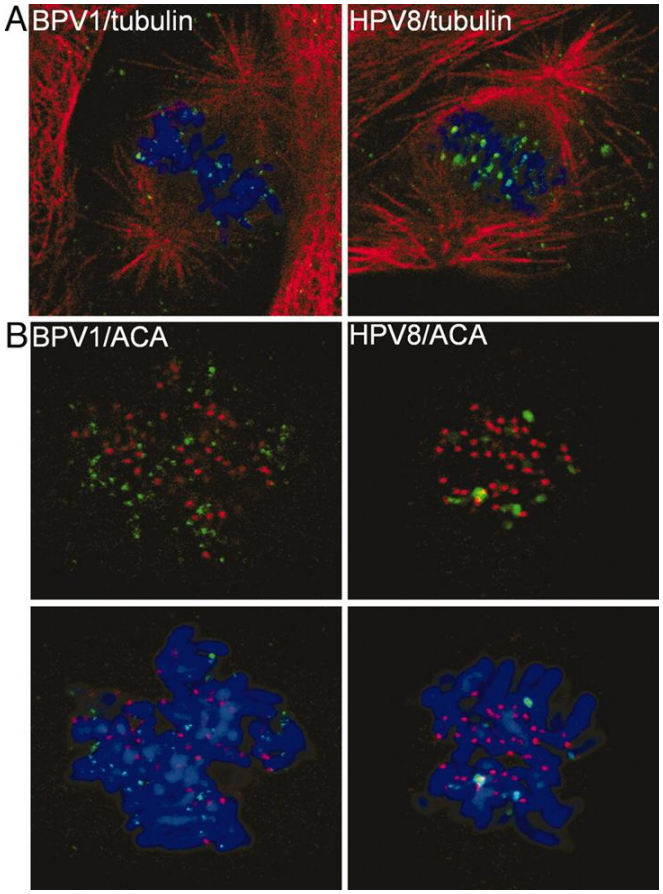


Figure 1.11 E2 proteins from different papilloma viruses associate with mitotic chromosomes in distinct patterns. E2 proteins from BPV-1 and HPV-8 are compared for their localization patterns on mitotic chromosomes in fixed cells (A) and in chromosome spreads (B). Chromosomes (blue) are visualized by staining with Hoechst, and the E2 protein is detected by immunofluorescence (green) in (A) and (B). In (A), tubulin is revealed by immunofluorescence (red). In (B), centromeres are localized by immunofluorescence of a kinetochore protein (red). The figure is reproduced with permission from Proceedings of the National Academy of Sciences of the United States of America (Oliveira et al., 2006).

1.2.5 Non-random segregation of viral episomes

The segregation of EBV episomes during the latent stage appears to be non-random, which suggests that the viral association with chromosomes must take place in an orderly fashion (Nanbo et al., 2007). By measuring the distribution of EBV-derived plasmids in single live cells throughout the cell cycle, Nanbo et al. (2007) showed that replicated viral plasmid sisters are colocalized in the G2 stage, and 88% of them are partitioned equally to daughter cells in the following mitosis (Nanbo et al., 2007). These results suggest that the pair of EBV sisters formed by replication associate with identical or closely spaced sites on sister chromatids. This type of chromosome tethering may be facilitated by the proximity of the sister chromatids, which are held together by the cohesin complex (Nasmyth, 2005). As sister chromatids separate from each other and move into daughter cells during anaphase, the associated plasmids achieve non-random (symmetric) segregation (Nanbo et al., 2007). Consistent with this model, immunofluorescence assays have shown that a significant number of EBNA-1 dots are symmetrically localized on sister chromatids in mitotic chromosome spreads. Furthermore, EBV genomes are seen to be localized in between "EBNA-1 pairs" (Kanda et al., 2007) (Fig. 1.12).

In contrast to EBV, BPV-1 does not appear to follow a symmetric pattern of segregation. The E2 protein is not symmetrically distributed on sister chromatids in mitotic chromosome spreads (Oliveira et al., 2006) (Fig. 1.11). Thus, even though the two viruses resort to chromosome tethering as the basic mechanism for efficient segregation, the individual systems differ in the extent to which they have fine-tuned this mechanism. It should be noted that even if two sister episomes tether to two different

chromosomes, there is still a 50% chance that they will segregate equally. As the number of episomes increase, this efficiency will also increase even though the episomes may be unequally distributed between daughter cells.

(A)

(B)

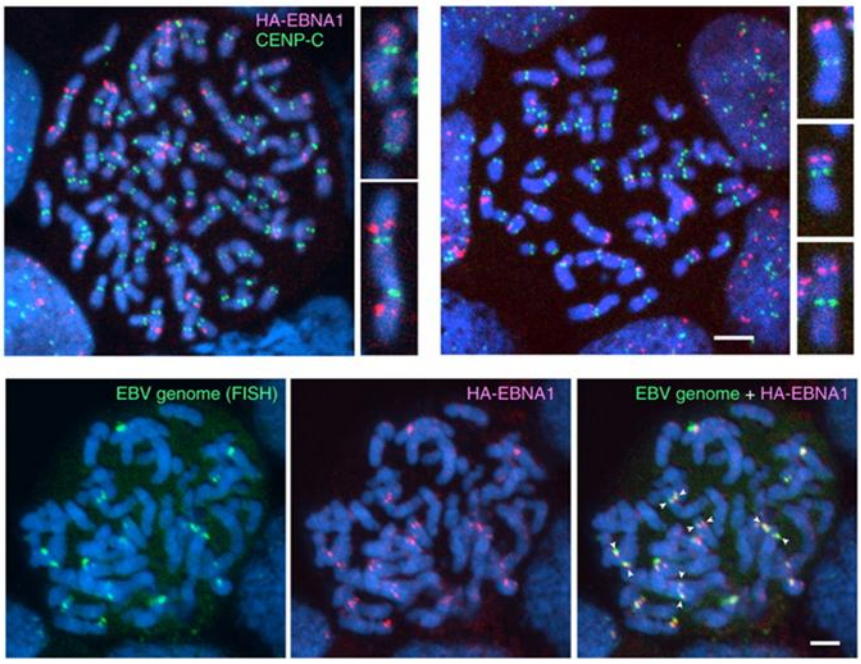


Figure 1.12 Symmetrical distribution of EBNA-1 molecules on sister chromatids of mitotic chromosomes. (A) The localization of EBNA-1 (red) and centromere (green) on mitotic chromosome spreads is performed by immunofluorescence assay. (B) The localization of EBV genome (green) and EBNA-1 (red) in mitotic chromosome spreads is scored by FISH (**f**luorescence **i**n **s**itu **h**ybridization) and immunofluorescence, respectively. The figure is reproduced with permission from Journal of Cell Science (Kanda et al., 2007).

1.3 The 2 micron plasmid: a highly optimized selfish DNA element

The 2 micron plasmid is maintained stably in *Saccharomyces cerevisiae* at an average copy number of 40-60 per cell. The stability of the plasmid is comparable to that of the yeast chromosomes (a loss rate of 10^{-5} to 10^{-4} per cell division). Such a high stability is surprising, since the plasmid provides no advantage to cells harboring it, at least under growth conditions employed in the laboratory. Furthermore, the steady state copy number of the plasmid has been evolutionarily optimized at nearly the maximum value that poses almost no impediment to the normal physiology of the host. In other words, the 2 micron plasmid is a selfish genome that is almost perfectly designed for efficient propagation with little fitness cost to its host. The rather limited genetic potential of this relatively small plasmid is devoted to accomplish three goals: efficient replication with the help of the host replication machinery, equal segregation of replicated plasmids, and maintenance of plasmid copy number without large fluctuations from the steady state norm.

1.3.1 The organization of the 2 micron plasmid genome

The genetic organization of the 2 micron plasmid, in its two isomeric forms (A and B), is depicted in Fig. 1.13. The 6.3 kbp plasmid genome harbors four *cis*-acting DNA elements and four protein coding regions (Broach et al., 1979; Hartley and Donelson, 1980). All of the *cis*-acting loci and *trans*-acting proteins are essential for the stable, high copy maintenance of the plasmid.

The plasmid genome is divided into two unique regions by a pair of 599 bp inverted repeats, each of which includes the target (*FRT* = Flp recombination target) site for the plasmid coded site-specific recombinase Flp. The plasmid replication origin (*ORI*),

which partially overlaps with one of the inverted repeats, is functionally analogous to origins present on chromosomes. The firing of *ORI* follows the once-per-cell cycle rule (Zakian et al., 1979), that is, each plasmid molecule replicates once, and only once, during S phase. The *cis*-acting *STB* (**st**a**b**ility-conferring) locus, essential for plasmid stability, is located a few hundred bp away from the *ORI*. *STB* can be divided into two sub-regions, proximal-*STB* and distal-*STB*, based on their relative locations with respect to *ORI* (Murray and Cesareni, 1986) (Fig. 1.14). The proximal-*STB* contains five tandem direct repeats of a 62 bp AT-rich consensus element (Jayaram et al., 1983). Approximately three copies of the repeat element are sufficient to elicit nearly the full plasmid partitioning function of *STB* (Jayaram et al., 1983; Murray and Cesareni, 1986). The distal-*STB* contains a transcription terminator that keeps the proximal-*STB* and *ORI* as transcription-free zones (Murray and Cesareni, 1986).

Two partitioning proteins Rep1 and Rep2, together with the *STB* locus, are responsible for the faithful segregation of 2 micron circle during cell division (Jayaram et al., 1983; Kikuchi, 1983). The presence of the A and B plasmid forms in host cells is the result of recombination between the two *FRT* sites mediated by the Flp protein (Broach, 1982). The recombination reaction is critical in the copy number maintenance of the plasmid (Futcher, 1986; Volkert and Broach, 1986). The Raf1 protein is also involved in copy number control, and is believed to act by positively regulating *FLP* gene expression (Murray et al., 1987; Reynolds et al., 1987; Som et al., 1988).

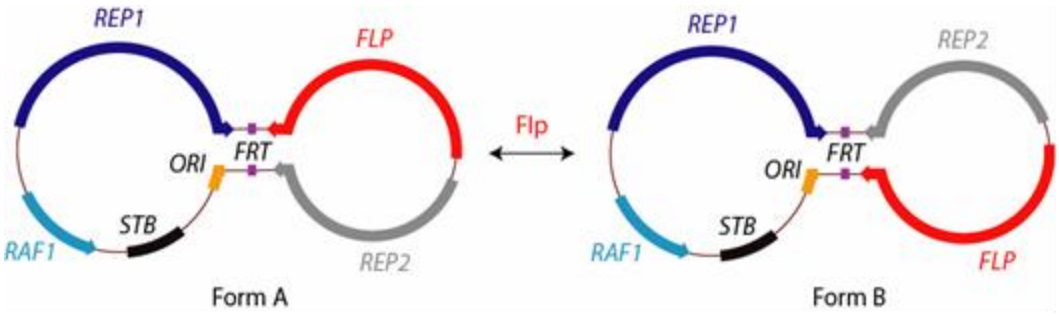


Figure 1.13 Organization of the 2 micron plasmid. The plasmid is shown here in its standard dumbbell representation. The parallel lines signify the inverted repeat regions, each 599 bp long. Four open reading frames (*REP1*, *REP2*, *FLP* and *RAF1*) are highlighted, and the directions in which they are transcribed are indicated by the arrowheads. The two Flp recombinase binding sites (*FRT*) are shown as small purple boxes within the inverted repeats. Interconversion of the plasmid between forms A and B is achieved by Flp-mediated recombination at the *FRT* sites. The replication origin (*ORI*) of the plasmid (orange box) is located asymmetrically relative to the *FRT* sites and is important for plasmid amplification. This figure is adapted from Velmurugan et al., (2003).

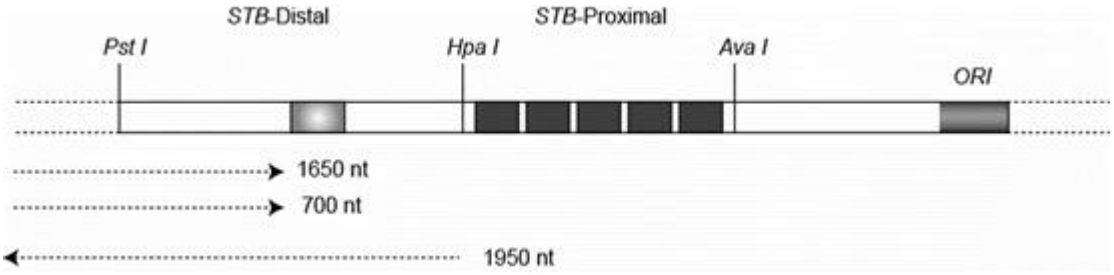


Figure 1.14 Organization of the 2 micron circle *STB* element. The *STB* locus is located between the *PstI* and *AvaI* sites on the 2 micron plasmid genome, and can be further divided into two sub-regions: *STB*-proximal and *STB*-distal, relative to the position of the plasmid replication origin (*ORI*). *STB*-proximal contains 5 tandem direct repeats of a 62 bp consensus AT-rich element, and is central to plasmid partitioning. *STB*-distal is important in keeping *STB*-proximal as a transcription-free zone. Two plasmid transcripts directed toward the *STB*-proximal are terminated within *STB*-distal. A third transcript with the opposite direction traverses the *STB*-distal region. The shaded box within *STB*-distal represents a "silencer sequence". The figure is adapted from Jayaram et al., (2004).

1.3.2 Copy number control of 2 micron plasmid: a well regulated DNA amplification system

Although the Rep1-Rep2-*STB* system is efficient in partitioning replicated plasmid copies equally to daughter cells, rare missegregation events do occur. The resulting drop in plasmid copy number is corrected by a Flp-mediated amplification reaction (Futcher, 1986; Volkert and Broach, 1986). Consistent with the robustness of the partitioning system, amplification events are quite infrequent. Density shift-equilibrium gradient centrifugation experiments have shown that, during steady state growth, over 95% of the plasmid molecules undergo only one round of replication during one cell generation (Zakian et al., 1979).

In a cell that receives less than the normal plasmid complement, restoration of copy number has to occur without violating the cell cycle rule that a plasmid origin can only fire once during a cell cycle. This is accomplished by a carefully timed Flp recombination event during bi-directional replication of the plasmid (Futcher, 1986; Volkert and Broach, 1986) (Fig. 1.15). Since *ORI* is located close to one *FRT* site and far away from the other, the proximal *FRT* is duplicated earlier than the distal one. Recombination between one of the duplicated *FRT*s and the unduplicated one would cause one replication fork to be inverted with respect to the other. The uni-directional forks can synthesize multiple tandem copies of the plasmid by a rolling circle mechanism. A second recombination event, which restores bidirectional replication, will terminate amplification. The concatemer product of amplification can be resolved into plasmid monomers by site-specific recombination mediated by Flp or by homologous recombination mediated by the host recombination machinery.

While the amplification system needs to be commissioned into action when there is a drop in plasmid copy number, it has to be controlled tightly as well in order to avoid run away increase in copy number (Murray et al., 1987; Reynolds et al., 1987; Som et al., 1988). This delicate balance is accomplished by an intricate regulatory network. The expression of the *FLP* gene is negatively regulated at the transcriptional level by the Rep1and Rep2 proteins acting in concert, presumably as a bipartite repressor. The repressor also turns down the expression of the *REP1* and *RAF1* genes; however, the *REP2* gene appears to be constitutively expressed. The Raf1 protein promotes *FLP* expression by antagonizing the action of the Rep1-Rep2 repressor. When plasmid copy number is low, the Rep levels drop causing the repressor to fall below a threshold, thus turning up *FLP* and *RAF1* gene expression. Under this condition, plasmid amplification will be triggered. When the copy number reaches the steady state value, Rep1 levels will have increased sufficiently to restore the critical concentration of the repressor required for establishing negative regulation of the plasmid genes. The positive and negative components of the regulatory system ensure that a quick amplification response can be mounted when required without the danger of unrestricted increase in plasmid population.

Recent evidence suggests that there is a second, post-translational level of regulation of Flp (Chen et al., 2005; Xiong et al., 2009). Flp is subject to modification by SUMO attachment, and mutations in the SUMO pathway (*siz1*Δ, *siz2*Δ) leads to aberrant amplification of the 2 micron plasmid. The hyper-amplified plasmid exists as a high molecular weight DNA form, which causes premature cell death. Sumoylation appears to signal subsequent ubiquitination and degradation of Flp. Thus, post-translational modification may be critical in controlling the steady state levels of Flp.

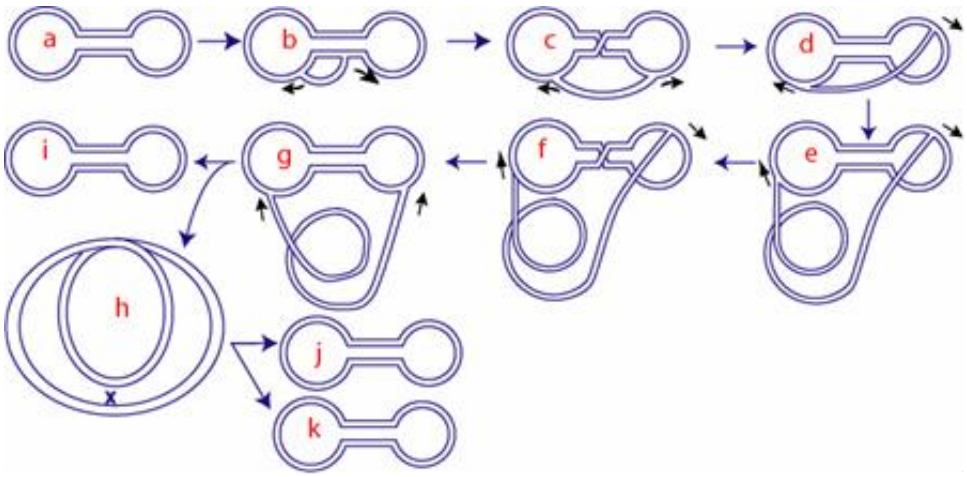


Figure 1.15 The Futcher model for 2 micron circle amplification. As the figures shows, during bidirectional replication initiated at the origin of the 2 micron circle (b), the *FRT* site proximal to *ORI* is replicated earlier than the *FRT* site distal to it. A recombination event between one of the replicated *FRT*s and the unreplicated distal *FRT* (c) results in the inversion of one replication fork with respect to the other (d). When these two replication forks chase each other on the circular template, multiple copies of the 2 micron DNA are spun out (e). A second recombination event restores the bidirectional replication fork (f-g). Termination of replication produces a concatemer containing multiple tandem plasmid copies (h) plus the unit copy plasmid template (i). The concatemer is then resolved into monomers by homologous recombination or Flp-mediated recombination (j and k). The figure is adapted from Velmurugan et al., (2003).

1.3.3 The partitioning system of the 2 micron circle

As noted above, the plasmid coded Rep1 and Rep2 proteins together with the *STB* locus constitute the partitioning system of the 2 micron plasmid. It is quite remarkable that such an apparently simple system is capable of achieving nearly chromosome-like segregation efficiency. At least superficially, the makeup of the 2 micron circle partitioning system (two proteins that interact with a *cis*-acting partitioning locus) is reminiscent of the bacterial plasmid partitioning systems. However, no NTPase activity has been shown to be associated with Rep1 or Rep2. Their primary amino acid sequences do not reveal the presence of active or defunct NTP binding motifs. It is possible the bacterial and yeast plasmid systems had a common evolutionary origin, but functionally diverged from each other in accordance with the physiological contexts of their respective hosts.

Early genetic and biochemical analyses suggested that the interactions among Rep1, Rep2 and *STB* are likely important to form a functional partitioning complex (Jayaram et al., 1983; Kikuchi, 1983). Further analysis showed that Rep1 and Rep2 exhibit self- and cross- interactions in vivo and in vitro (Ahn et al., 1997; Scott-Drew and Murray, 1998; Velmurugan et al., 1998), and their *STB*-binding ability in vivo is independent of each other (Mehta et al., 2002; Yang et al., 2004). Florescence tagging by the [LacO]n-[GFP-LacI] system (Straight et al., 1996), reveals a 2 micron circle derived reporter plasmid as three to five foci per nucleus in the majority of haploid cells (Velmurugan et al., 2000). A small fraction of cells contains fewer than three or greater than five foci. These plasmid foci are co-localized with the Rep1 and Rep2 proteins,

whereas a reporter plasmid lacking *STB* (*ARS* plasmid) does not show this co-localization (Fig. 1.16).

Earlier time-lapse studies using haploid cells suggested that the plasmid foci form a clustered unit, and segregates as a cluster during anaphase. Clustering would reduce the effective plasmid copy number to one, or at least close to one, justifying the need for an active partitioning system for a plasmid with as high a copy number as 40-60 per cell. More recent analysis of plasmid localization and segregation in diploid cells during mitosis and meiosis suggests that not all plasmid foci are tightly clustered. Some of the foci are localized close to each other while others are well separated in the nuclei. It is likely that individual foci act as independent entities during segregation. The initial impression of a single plasmid cluster was likely due to the small size of the haploid nucleus and the consequent limited spatial resolution during fluorescence microscopy. Assuming that the fluorescence tagged plasmid forms a common pool with endogenous 2 micron circles, the presence of 3-5 foci in a nucleus suggests that each focus consists of several plasmid molecules. The association of plasmid molecules into groups is not surprising since the Rep proteins associated with *STB* can interact with each other. Using a chromosome conformation capture assay, it has been demonstrated that *STB* loci have a clustered organization in vivo (Rodley et al., 2009).

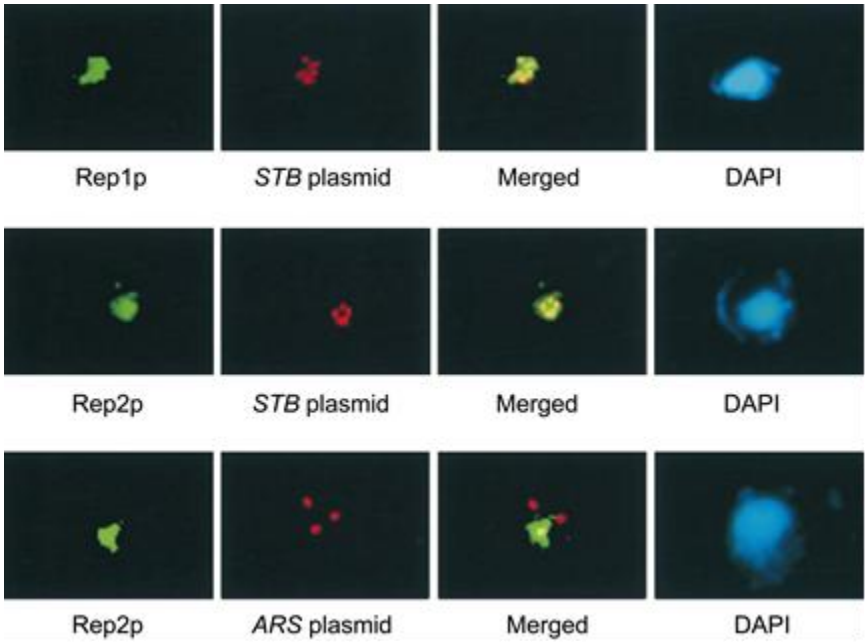


Figure 1.16 Colocalization of Rep1 and Rep2 with 2 micron-derived (*STB*) plasmid. In this assay, an *STB* reporter plasmid containing a Lac operator array is detected through its association with the GFP-Lac repressor expressed in the host strain. A commercially obtained antibody to the Lac repressor is employed in the immunofluorescence assay. Rep1 and Rep2 are detected by antibodies raised against the native proteins. Chromosomes are visualized by staining with DAPI. The figure is adapted from Velmurugan et al., (2000).

1.3.4 Chromosome segregation in *S. cerevisiae*: A brief overview

As is the case in all eukaryotes, precise one-to-one segregation of each chromosome during *S. cerevisiae* mitosis is achieved by a highly orchestrated series of molecular events including the operation of multiple checkpoints. As pointed out, the cell cycle control on replication initiation proscribes more than one doubling of each chromosome during S phase. Concomitant with replication, the resulting sister chromatids are held together by a multi-subunit protein complex called the cohesin complex. This phenomenon was first demonstrated in yeast and in *Xenopus*, and is conserved among eukaryotes (Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997). The bridging action of cohesin provides a simple mechanism for the binary counting of chromosomes, and, in the case of diploid eukaryotes, helps distinguish between chromosome sisters and chromosome homologues. The paired sister chromatids are attached through their kinetochores to microtubules emanating from opposite spindle poles (Santaguida and Musacchio, 2009). Bi-orientation of sister kinetochores on the mitotic spindle is accomplished with the help of the Aurora kinase (Ipl1) and its associated proteins by dissociating incorrect attachments and recognizing correct attachments through the resulting tension generated by the pulling force of the spindle (Biggins and Murray, 2001; Biggins et al., 1999; Cheeseman et al., 2002; Dewar et al., 2004). Sensing tension is also the mechanism utilized by the spindle checkpoint to ensure that chromosomes are properly aligned at the spindle before the subsequent steps of mitosis can proceed. Bi-orientation of sister chromatids at the metaphase plate is crucial in all eukaryotes to avoid aneuploidy. Even though the physical organization of the metaphase plate is a point of debate in *S. cerevisiae*, there is no question regarding the

operation of a functionally equivalent stage to preserve the integrity of chromosomes and the fidelity of their segregation. At the onset of anaphase, the cohesin bridge between sister chromatids is disassembled, and they are pulled apart towards opposite cell poles through spindle dynamics (Fig. 1.17).

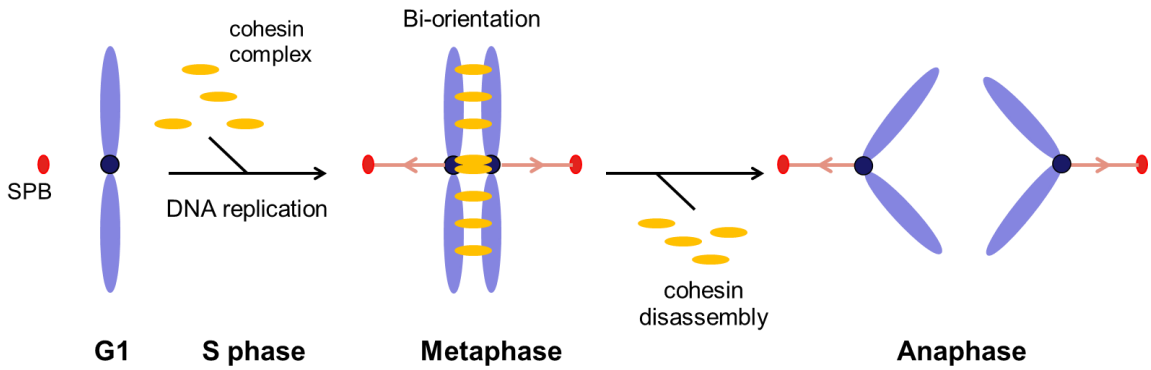


Figure 1.17 Replication, cohesion, bi-orientation and dissolution of cohesion during the segregation of sister chromosomes. The cohesin complex is assembled on chromosomes at the G1-S phase of the cell cycle. Concomitant with, or immediately following DNA replication, cohesion is established between sister chromatids. The cohesed sisters are bi-oriented on the mitotic spindle (pink line) by the attachment of sister kinetochores to microtubules from opposite spindle pole bodies (SPB). During anaphase, when cohesin is disassembled, sister chromatids separate and move to opposite cell poles. Correct spindle attachment is detected by the tension generated from the inward force provided by cohesion and the outward force generated by spindle dynamics.

1.3.5 2 micron circle segregation: analogies to chromosome segregation

Cell biological observations on fluorescence tagged reporter plasmids revealed that the dynamics and kinetics of the 2 micron plasmid during the cell cycle are quite similar to those of a chromosome (Velmurugan et al., 2000). Furthermore, conditional mutations that cause chromosome missegregation have a similar effect on the 2 micron plasmid as well. For example, when an *ipl1-1* host strain is shifted to the non-permissive temperature, the 2 micron plasmid missegregates in tandem with the bulk of the chromosomes. This coupled behavior between the plasmid and chromosomes has also been observed for several kinetochore mutations, including *ndc10, ctf7, ctf13* and *ndc80* (Mehta et al., 2002). Furthermore, this apparent coupling is absolutely dependent up on the Rep proteins and the presence of the *STB* locus on the reporter plasmid. It has thus been concluded that the 2 micron plasmid partitioning system achieves chromosome-like equal segregation either by exploiting the chromosome segregation machinery directly or doing so indirectly by tethering to chromosomes. Segregation of the 2 micron plasmid by bipolar attachment to the spindle is quite unlikely. For example, when a reporter plasmid harbors two copies of *STB,* plasmid instabilities analogous to those observed for a dicentric plasmid do not occur. Similarly, a reporter plasmid carrying one copy of *STB* and one copy of a centromere can be maintained stably for multiple generations. Thus, chromosome associated segregation of the 2 micron plasmid (the hitchhiking model) remains an attractive hypothesis (Fig. 1.18), unifying the logic of stable maintenance for yeast plasmids and viral episomes.

It is possible that eukaryotic extra-chromosomal selfish DNA elements have converged upon a common strategy of linking their stable propagation with that of their

host's chromosomes. While tethering to chromosomes protects viral episomes from being expelled from the nucleus into the cytoplasm during the disassembly and reassembly of the nuclear membrane during mitosis, the 2 micron circle does not require this protective function, as the closed mitosis of *S. cerevisiae* does not involve the breakdown of the nuclear membrane. It should be admitted that a segregation mechanism that has nearly the same efficiency as chromosome segregation, but is completely independent of the latter, cannot be ruled out for the 2 micron plasmid. The technical difficulties posed by the small size of the yeast nucleus and the inability to resolve individual chromosomes make the direct demonstration of plasmid-chromosome tethering an extremely challenging task.

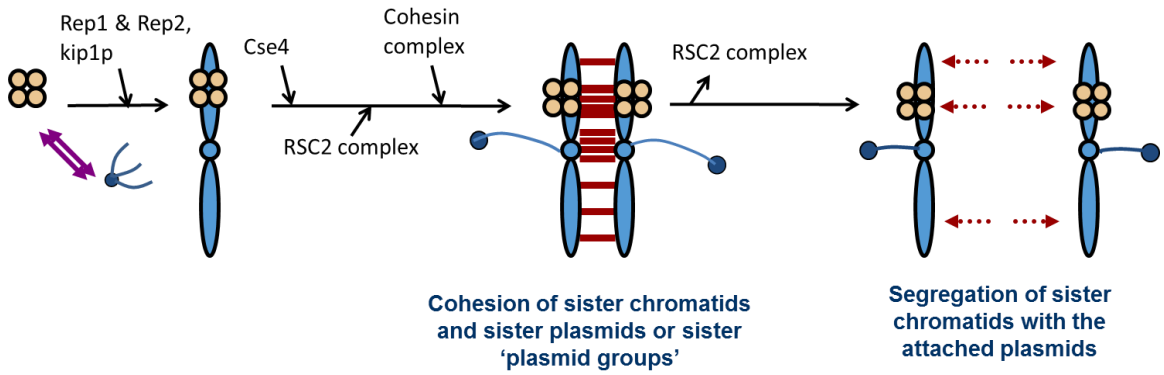


Figure 1.18 A plausible hitchhiking model for the segregation of the 2 micron plasmid. According to the hitchhiking model, the 2 micron plasmid associates with chromosomes with the help of the Rep proteins, the mitotic spindle and the spindle associated motor protein Kip1. The RSC2 chromatin remodeling complex interacts with *STB*, and is likely responsible for establishing its functional chromatin state. The histone H3 variant Cse4, highly characteristic of centromeric nucleosomes, also associates with *STB* (Hajra et al., 2006). Following DNA replication, sister chromatids are bridged by cohesin. Cohesin also bridges replicated plasmids (Mehta et al., 2002). Whether this bridging involves individual pairs of plasmid sisters or groups of replicated plasmids is not known. Experiments employing a nearly single copy *STB* reporter plasmid suggest that sister plasmids are paired by the cohesin complex (Ghosh et al., 2007). Sister chromatid cohesion and plasmid cohesion may act cooperatively to tether sister plasmids or sister 'plasmid groups' to sister chromatids. Dissolution of the cohesin bridge during anaphase would lead to plasmid segregation in association with sister chromatids.

1.3.6 Association of chromosome segregation factors with *STB*

Early evidence for functional interactions between host factors and the 2 micron circle partitioning system came from the finding that Rep1 and Rep2 proteins purified using bacterial expression systems could not bind to *STB* (Hadfield et al., 1995). However, binding occurs in the presence of a urea solubilized extract prepared from cells lacking 2 micron circles [Cir^0]. Consistent with the chromosome coupled segregation of the 2 micron plasmid, a number of host factors that play important roles in chromosome segregation have now been found to be associated with the *STB* locus. These include the cohesin complex, the RSC2 chromatin remodeling complex, the Kip1 nuclear motor and the centromere specific histone H3 variant Cse4 (CENP-A) (Cui et al., 2009; Ghosh et al., 2007; Hajra et al., 2006; Huang et al., 2011b; Mehta et al., 2002; Wong et al., 2002). Furthermore, their associations with *STB* are absolutely dependent on Rep1 and Rep2 proteins. In addition, the integrity of the mitotic spindle is also required for the equal segregation of the 2 micron plasmid, although not in its conventional role as the dynamic force generator for pulling apart plasmids into daughter cells (Mehta et al., 2005). It would appear that the success of the Rep-*STB* system as an efficient partitioning system is dependent on its ability to channel critical chromosome segregation factors towards plasmid segregation. We briefly discuss the potential functional significance of the recruitment of these factors at the *STB* locus in 2 micron circle stability.

1.3.6.1 The yeast cohesin complex

As briefly noted earlier, the cohesin complex plays a crucial rule in conferring high fidelity to chromosome segregation. In addition to this principal role, cohesin is also involved in other key cellular processes, such as DNA repair (Cortes-Ledesma and

Aguilera, 2006; Sjögren and Nasmyth, 2001) and establishment of transcriptionally silent DNA domains (Donze et al., 1999; Peric-Hupkes and van Steensel, 2008). The underlying mechanistic principle common to these apparently diverse DNA transactions is likely the ability of cohesin to bridge DNA segments.

The cohesin complex is composed of four subunits, Mcd1/Scc1, Scc3, Smc1 and Smc3 (Fig. 1.19). Smc1 and Smc3 are members of SMC (Structural maintenance of chromosome) family, with globular head domains harboring ATPase function separated from 'hinge' domains by long coiled coils. These proteins form a V-shaped heterodimer by interactions through their hinge domain (Haering et al., 2002). Mcd1, a member of kleisin family, is the binding partner of the SMC proteins (Schleiffer et al., 2003). Mcd1 interacts with Smc1 and Smc3 through its C-terminal and N-terminal domains respectively, and can thus form a tripartite polypeptide ring structure (Gruber et al., 2003; Haering et al., 2002). Scc3 interacts with the C-terminal domain of Mcd1, but does not directly contribute to the ring structure. The large diameter of the cohesin ring, 40 -50 nm, is sufficient to entrap two DNA duplexes and to allow the passage of an advancing replisome.

Cohesin is assembled at the centromere, pericentric regions and at fairly regular intervals along chromosome arms with the assistance of the loading complex, consisting of Scc2 and Scc4 (Ciosk et al., 2000). Furthermore, the establishment of sister chromatid cohesion requires the acetyl transferase activity of the establishment factor, Eco1/Ctf17 (Ivanov et al., 2002; Toth et al., 1999). It has been proposed that, together with the ATPase activity of Smc1/Smc3 and assistance from Scc2-Scc4, the hinge region of the cohesin complex opens and lets sister chromatids inside the cohesin ring (Arumugam et

al., 2003; Gruber et al., 2006). During the establishment of cohesion, the hinge closes, trapping sister chromatids inside the protein ring.

At the onset of anaphase, cohesin is disassembled from chromosomes to trigger sister chromatid separation and segregation. The activation of the anaphase promoting complex (APC) induces ubiquitin-mediated proteolytic degradation of the anaphase inhibitor securin (Pds1) (Morgan, 1999). The cysteine protease separase (Esp1), which is now freed from sequestration by securin, cleaves Mcd1 to open the cohesin ring and release sister chromatids (Ciosk et al., 1998; Uhlmann et al., 1999; Uhlmann et al., 2000). The proposed topological embrace mechanism for the entrapment of sister chromatids by the cohesin ring has received strong experimental support in *S. cerevisiae* (Haering et al., 2008; Ivanov and Nasmyth, 2005, 2007).

The first evidence for a potential role of the cohesin complex in 2 micron plasmid segregation came from the finding that the cohesin subunit Mcd1 associates specifically with *STB* in a Rep1-Rep2 as well as Scc2-Scc4 dependent manner (Mehta et al., 2002). The timings of cohesin assembly and disassembly at *STB* during the cell cycle are similar to those at chromosomal cohesin binding loci. When cohesin cleavage at the onset of anaphase is blocked with the help of a non-cleavable version of Mcd1, not only do sister chromatids fail to separate, but replicated 2 micron plasmid foci also behave similarly.

A subsequent study using a fluorescence tagged single-copy (or close to single copy) *STB* reporter plasmid showed that the association of cohesin with *STB* leads to the cohesion of sister plasmids (Ghosh et al., 2007). Furthermore, when a cell harbors two single copy *STB* reporter plasmids, tagged by green fluorescence and red fluorescence, respectively, they segregate in a sister-to-sister fashion, red from red and green from

green (Ghosh et al., 2007) (Fig. 1.20). Thus, the cohesin mediated binary counting mechanism appears to act on the 2 micron plasmid as well. It is possible that cohesin helps pair sister plasmid foci, and promotes their segregation to daughter cells (Fig. 1.18). A more recent analysis suggests that the interaction of cohesin at the *STB* locus also follows the topological mechanism, a monomeric ring of cohesin holding two sister plasmids in the form of a tri-linked DNA-protein catenane (Ghosh et al., 2010).

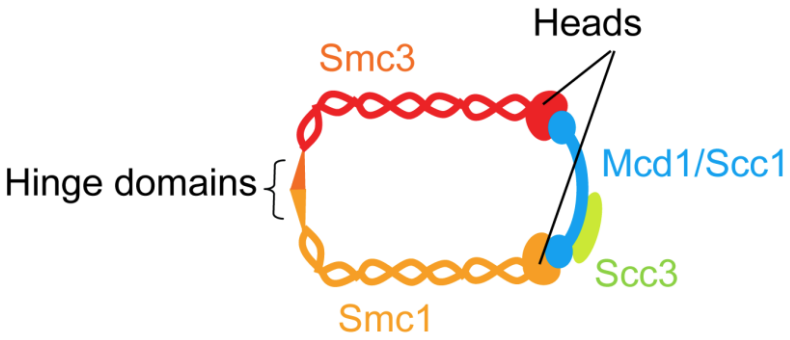


Figure 1.19 The ring structure of the yeast cohesin complex. The cohesin complex consists of four subunits; Smc1, Smc3, Mcd1/Scc1, and Scc3. Smc1 and Smc3 associate with each other through their hinge domains. Mcd1 connects to the global head domains of Smc1 and Smc3 through its C-terminal domain and N-terminal domain, respectively. Scc3 interacts with the C-terminal region of Mcd1. The interactions among Smc1, Smc3 and Mcd1 are responsible for the ring structure of the cohesin complex.

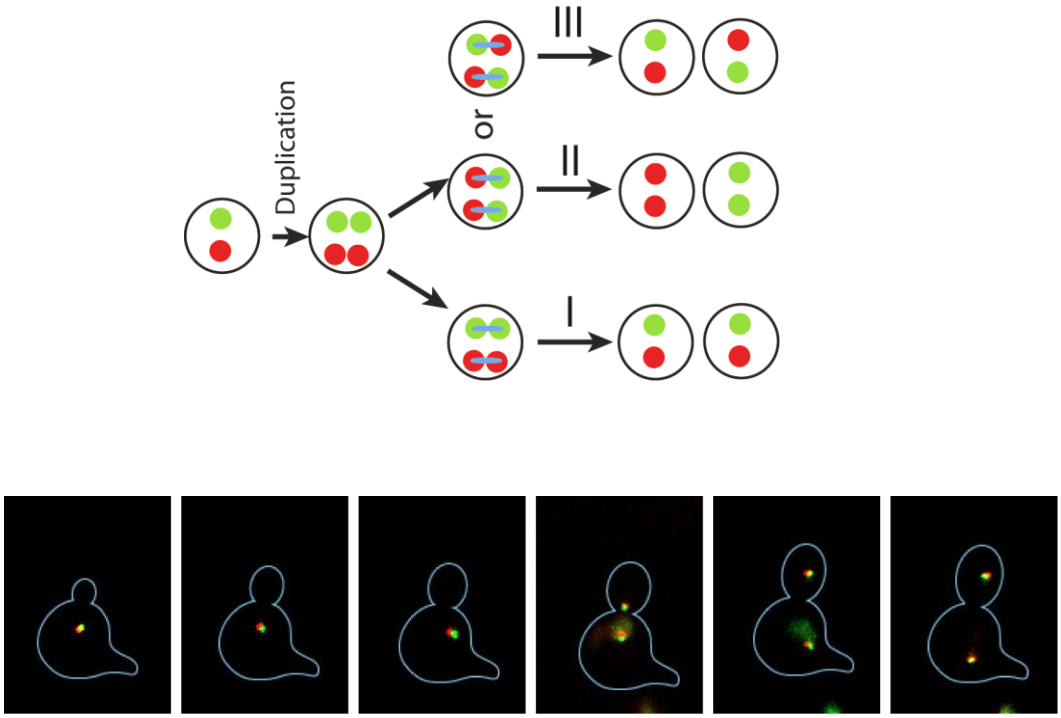


Figure 1.20 Segregation of two single-copy *STB* reporters differentially tagged by fluorescence. To address whether 2 micron plasmid segregation follows the sister-to-sister rule that applies to chromosomes, two single-copy *STB* reporter plasmids tagged by green and red fluorescence, respectively, were introduced into the experimental strain. The differential fluorescence tagging was accomplished by utilizing [GFP-LacI]-LacO interaction in one case and [RFP-TetR]-TetO interaction in the other. Sister-to-sister segregation predicts that each daughter cell will receive one green and one red plasmid (I). Equal segregation by random assortment of the replicated plasmids will have three equally likely outcomes (I, II, III), that is 33% of daughter cells will receive two green or two red plasmids. Time lapse analysis of individual cells and population assays are consistent with the sister-to-sister segregation mechanism, green-to-green and red-to-red.

1.3.6.2 The mitotic spindle and the spindle associated motor protein Kip1

The integrity of the mitotic spindle is essential for the proper nuclear localization of the 2 micron plasmid and for equal plasmid segregation. In nocodazole treated cells, the residence zone of the plasmid foci is widened, suggesting alterations in their normal localization (Velmurugan et al., 2000). Furthermore, chromosome spreads prepared from such cells do not show the association of plasmid, which occurs in spreads prepared from untreated cells (Mehta et al., 2005). Finally, disruption of spindle integrity blocks the recruitment of at least a subset of host factors at *STB*, including the cohesin complex and Cse4 (Hajra et al., 2006; Mehta et al., 2005). By contrast, most centromere functions, except for the physical separation of sister chromosomes, are not affected by the absence of the spindle. Kinetochore assembly, cohesin recruitment at centromeres and chromosome arms, and sister chromatid cohesion proceed normally in the absence of the spindle, although cells arrest in G2/M due to activation of the spindle checkpoint. When the spindle is allowed to reform at this stage, chromosome segregation proceeds normally; however the 2 micron plasmid missegregates (Mehta et al., 2005). Spindle restoration in G2/M arrested cells results in cohesin-*STB* association, although this late association is not functional in plasmid segregation.

The association of Kip1, which together with Cin8 forms one of the two spindle associated plus end directed motor proteins in *S. cerevisiae*, with *STB* occurs in a Rep1-Rep2 dependent but spindle independent fashion (Cui et al., 2009). In a *kip1*Δ strain, there is an increase in the unequal segregation of replicated plasmids to daughter cells. By contrast, *cin8*Δ has no such effect on plasmid segregation. Together with the other kinesin family motors (Cin8, Kip3 and Kar3), Kip1 promotes proper chromosome

segregation through its role in the assembly and dynamics of the mitotic spindle (Hildebrandt and Hoyt, 2000). In addition, Kip1 contributes to the typical bi-lobed organization of centromere clusters during metaphase, presumably by bundling kinetochore microtubules (Tytell and Sorger, 2006).

A single copy *STB* reporter plasmid is often seen in close proximity to the spindle pole body in wild type cells, and the absence of Kip1 disrupts this localization (Cui et al., 2009). It is possible that that the Kip1 motor acts in cooperation with the mitotic spindle to promote the specific nuclear localization of the 2 micron plasmid. The minus end directed Kar3 motor has been shown to promote the capture of kinetochores by the spindle, and promote chromosome transport towards the spindle pole body (Tanaka et al., 2007; Tanaka et al., 2005). It is puzzling how a plus end directed motor such as Kip1 would be able to transport the plasmid towards the spindle pole body. Recently, it has been demonstrated that Cin8, which is also a plus end directed motor redundant in function with Kip1, can reverse its direction of motion (Gerson-Gurwitz et al., 2011; Roostalu et al., 2011). Kip1 may also help organize 2 micron plasmid molecules into groups by its interaction with *STB*, which would be somewhat analogous to its role in clustering kinetochores through bundling of kinetochore microtubules (Tytell and Sorger, 2006). Although the precise role of Kip1 in 2 micron plasmid stability is not understood, it is likely that, in association with the spindle, Kip1 is involved in plasmid organization, dynamics and/or localization.

1.3.6.3 Centromere (*CEN*) -specific histone H3 variant Cse4

The histone H3 variant Cse4 of *S. cerevisiae* (more generally known as CENP-A) is the hallmark of the central histone core of nucleosomes assembled at centromeres (Choy et al., 2012). Cse4 replaces histone H3 in centromeric nucleosomes (Meluh et al., 1998), although a recent study suggests the presence of both histone H3 and Cse4 at yeast centromeres (Lochmann and Ivanov, 2012). Cse4 provides a common epigenetic mark in all eukaryotic centromeres, and is critical for kinetochore assembly and cohesin recruitment. The point centromere of *S. cerevisiae*, characteristic of budding yeasts but distinct from the regional centromeres of most eukaryotes, contains a well-positioned single Cse4 containing nucleosome (Furuyama and Biggins, 2007), although additional such nucleosomes may be present in the adjacent pericentric regions (Coffman et al., 2011; Lawrimore et al., 2011).

Surprisingly, Cse4 is also detected at the *STB* locus of the 2 micron plasmid, and is required for proper plasmid segregation (Hajra et al., 2006). Cse4-*STB* association, which requires the Rep1 and Rep2 proteins, occurs de novo at the G1-S transition stage of each cell cycle. This association lasts until late telophase, when spindle disassembly takes place. The cell cycle timing of Cse4-*STB* association is the same as that of Cse4-*CEN* association. However, Cse4 at the centromere lasts throughout the cell cycle, the dissociation-reassociation being restricted only to the G1-S window.

A recent topological analysis suggests that Cse4 induces non-standard positive supercoiling at the centromere in *S.* cerevisiae (Furuyama and Henikoff, 2009). Under conditions that prevent the recruitment of Cse4 at the centromere, the centromere chromatin is more negatively supercoiled than in a wild type strain. Based on the

quantitative aspects of the centromere topology, it has been argued that the Cse4 containing nucleosome, contrary to the standard H3 containing nucleosome, has a right handed DNA writhe. The *STB* chromatin, in its functional state, also engenders a net positive supercoiling, which is quantitatively almost equal to that of a functional centromere (Huang et al., 2011a). Furthermore, this unusual topology at *STB* is lost under conditions that block Cse4-*STB* association.

It is not clear at this time that the presence of a Cse4 containing nucleosome at *STB* is the cause of this positive supercoiling or that such a nucleosome induces a reversed handedness of the DNA wrap around it. Biochemical and structural evidence suggests that the CENP-A containing nucleosome has an octameric histone core with the DNA wrapped around it in a left handed fashion (Camahort et al., 2009; Kingston et al., 2011; Sekulic et al., 2010). It is likely that the overall supercoiling at the centromere is determined not by the topology of a single nucleosome but rather by the level of nucleosome occupancy of centromere proximal regions and/or by changes in DNA twist contributed by protein factors associated with the centromere. Notwithstanding the uncertainties regarding the precise topology of the Cse4 containing nucleosome or the exact source of positive supercoiling at the centromere, the topological similarities between *STB* and the centromere has interesting implications for their roles as efficient partitioning loci for the 2 micron plasmid and yeast chromosomes, respectively.

1.3.6.4 The RSC2 chromatin remodeling complex

Two forms of the SWI/SNF related RSC chromatin remodeling complex have been identified in *S. cerevisiae*, RSC1 and RSC2. They are composed of multiple shared components including the ATPase Sth1 and at least two unique components Rsc1 and

Rsc2, as indicated by their respective designations (Cairns et al., 1996; Cairns et al., 1999). Whereas deletion of either *RSC1* or *RSC2* is tolerated by the cell, their simultaneous deletion causes lethality. The RSC complex appears to regulate chromatin organization at *CEN* and *CEN*-proximal regions to promote normal chromosome segregation (Du et al., 1998; Hsu et al., 2003; Tsuchiya et al., 1998).

Previous work demonstrated that *rsc2*Δ, but not *rsc1*Δ, and point mutations in Rsc2 lead to increased loss of the 2 micron plasmid (Wong et al., 2002). Similarly, a T^s mutant allele of *STH1* (*sth1*(L1346A)) causes a marked reduction in the copy number of an *STB* reporter plasmid at the semi-permissive temperature (36°C), suggesting poor plasmid maintenance when Sth1 function is deficient (Huang et al., 2004). These findings are consistent with the lack of cohesin assembly at *STB* in the *rsc2*Δ background (Huang et al., 2004; Yang et al., 2004) or when Sth1 is inactivated (Huang et al., 2004). The association of Sth1 with the 2 micron plasmid has been shown to be restricted to the *STB* region (Huang et al., 2004). The recruitment of Rsc2 or Rsc8 at *STB* is blocked when a strain harboring a T^s mutant of Cse4 is shifted to the non-permissive temperature (Hajra et al., 2006). Furthermore, in the absence of Rsc2, Rep1 fails to associate with *STB* but Rep2 association is not abrogated (Yang et al., 2004). The micrococcal nuclease digestion pattern at *STB*-proximal, spanning the repeat elements of *STB*, is altered in an *rsc2*Δ strain (Wong et al., 2002). Cleavages at two strong sensitive sites present at its borders and a weaker site present internally are conspicuously diminished in the absence of Rsc2, while such changes are not observed in other regions of the 2 micron plasmid genome. In more recent unpublished studies, affinity purification analyses have revealed the interactions of several RSC subunits with Rep1 and Rep2. Consistent with these

interactions, there is an increased loss of the 2 micron plasmid in the *rsc8* and *rsc58* mutants at the non-permisisve temperature. Taken together these findings argue that the organization of the functional architecture of the *STB* chromatin is mediated by the RSC2 complex. Thus, the *STB* locus and the centromere depend on shared chromatin remodeling mechanisms for their respective partitioning activities.

The modular organization of the Rsc2 protein consists of two bromodomains, an AT-hook motif, a bromodomain-adjacent homology region and a C-terminal (CT) domain (Cairns et al., 1999). The AT-hook motif and the bromodomains of Rsc2 are interesting from the standpoint of the potential chromosome associated segregation of the 2 micron plasmid. As pointed out earlier, the partitioning protein EBNA-1 of EBV harbors AT-hook motifs, which might play a role in the attachment of the viral episomes to chromosomes (Sears et al., 2004). Similarly, the tethering of BPV episomes to chromosomes is mediated by the interaction of the viral E2 protein with the host Brd4 protein (You et al., 2004). At this time, though, the notion that Rsc2 might be involved in the tethering of the 2 micron plasmid to yeast chromosome is purely speculative.

1.3.7 Functional relevance of the association of host factors at *STB*: potential caveats

The localization of host factors involved in chromosome segregation at *STB* per se cannot be interpreted as signifying their direct roles in 2 micron plasmid segregation as well. Caution has to be exercised in analyzing the effects of the functional inactivation of such factors on plasmid segregation. If plasmid segregation is physically or functionally coupled to chromosome segregation, an adverse effect on chromosome segregation due to lack of a particular function will also have a corresponding effect on plasmid segregation.

The role of the RSC2 complex in organizing the *STB* chromatin and promoting plasmid stability is rather clear-cut. The effect of *rsc2*Δ is specific to the plasmid, and does not cause cell lethality. The potential role of the RSC2 complex in centromere function (and thus chromosome segregation) is likely covered by the functionally redundant RSC1 complex. The effect of *kip1*Δ on segregation is also predominantly plasmid specific, as Cin8 can almost fully replace Kip1 in satisfying its functions in spindle integrity, spindle dynamics and centromere organization. Assays using a multi-copy reporter plasmid indicate that *kip1*Δ, rather than causing gross plasmid missegregation, distorts normal segregation to be unequal between the daughter cells. This inequality can be accentuated by using a single copy reporter plasmid, in which case unequal segregation is visualized as 2:0.

Although the cohesin complex and Cse4 associate with *STB*, their association is highly sub-stoichiometric. It could be argued therefore that neither cohesin nor Cse4 is functionally relevant in plasmid segregation. However, the possibility that the plasmid may have evolved mechanisms to make do with limiting amounts of these host factors cannot be ruled out. For example, if multiple plasmid molecules are organized into a group, a single functional *STB* may direct the segregation of the entire group. Or, some of the host factors may act catalytically, and may turn over among the individual plasmid molecules within a group, conferring segregation competence on each one of them.

Although single copy (or close to single copy) *STB* plasmids harboring a conditional *CEN* are valuable for probing plasmid partitioning mechanisms (Cui et al., 2009; Ghosh et al., 2007), they are not free from potential drawbacks. The *CEN* sequence introduced into an *STB* plasmid to obtain this low copy number is inactivated, when

desired, by driving transcription through it, or is excised from the plasmid by site-specific recombination. The *CEN*-inactive or *CEN*-free *STB* plasmid thus obtained may not be strictly under Rep-*STB* control during a cell cycle that immediately follows *CEN* annulment. For example, the carryover effects of *CEN* mediated plasmid localization, host factor associations, kinetochore assembly and segregation from previous cell cycles may not be inconsequential. In a number of experiments performed in the present study, this potential problem is avoided by integrating the reporter plasmid into a haploid chromosome, and excising it as a single copy circle by site-specific recombination.

1.3.8 Possible evolutionary link between the budding yeast centromere and *STB*

The genetically defined point centromere typified by the budding yeasts is considered an evolutionary oddity (Malik and Henikoff, 2009). The emergence of the point centromere appears to have been contemporaneous with the loss of components of the RNAi and heterochromatin machineries required for the epigenetic establishment of regional centromeres (Aravind et al., 2000). Rather strikingly, the budding yeast lineage (Saccharomycetaceae) is unique in harboring plasmids similar in organization to the 2 micron plasmid. It is also noteworthy that that the scaffold proteins of the inner kinetochore complex such as Ndc10 and Ctf13 have no homologues outside of Saccharomycetaceae among fungi and other eukaryotes (Malik and Henikoff, 2009). So also, Rep1 and Rep2 homologues are confined to the family of 2 micron related plasmids. The Rep2 proteins have scant homology among them, perhaps signifying their rapid evolution as an adaptive response to their respective host environments. Based on these tantalizing observations, it has been speculated that the point centromere and *STB* might share a common evolutionary history (Malik and Henikoff, 2009).

The present day chromosome and plasmid segregation pathways might have been arrived at by divergence from a common pathway. Since there is a fitness cost, however small it may be, to the host in harboring the plasmid, the chromosome segregation mechanism would have evolved away from the plasmid segregation mechanism. The plasmid, in turn, would have counter-evolved by acquiring strategies to couple its segregation to chromosome segregation. The association of certain common protein factors with *CEN* and *STB*, in spite of their obvious functional differences, could denote an evolutionary relic of their common ancestry.

1.4 Specific aims of this study

The primary objective of the work presented in this thesis is to test the validity of the chromosome tethering mechanism in the segregation of the 2 micron plasmid. Since, direct demonstration of such tethering is extremely difficult in yeast, we have utilized indirect strategies to address this issue. A fundamental difference between mitotic cell division and the first meiotic cell division in yeast is in the manner in which sister chromatids segregate. During mitosis, sisters segregate to opposite poles whereas they cosegregate during meiosis I. If sister 2 micron plasmids hitchhike on sister chromatids, this distinction should apply to these plasmids as well. We have manipulated mitosis to mimic meiosis I, and followed the segregation of a single copy *STB* reporter plasmid under this artificial condition.

A second objective of this study is to assess the efficiency of chromosome tethering as a general strategy for the propagation of extra-chromosomal selfish DNA elements. For this purpose, we have employed fluorescence tagging and single copy

reporter plasmids to incisively analyze the segregation features of EBV and BPV based reporter plasmids using a reconstituted partitioning system in *S. cerevisiae*.

1.5 Chapters to follow

In **Chapter 2**, yeast strains, plasmids, and experimental protocols used in the studies embodied by this thesis are summarized. In **Chapter 3**, careful quantitative analyses of the segregation of a single copy *STB* reporter plasmid and that of a chromosome, when chromosome segregation is manipulated in specific ways, are presented. In **Chapter 4** and **Chapter 5**, sensitive cell biological assays are utilized to characterize the properties of the EBV and BPV partitioning systems reconstituted in *S. cerevisiae*. In **Chapter 6**, we summarize and critique the overall implications of the results from Chapters **3**-**5**, and pose interesting questions to be answered in the future.

CHAPTER 2

Materials and Methods

In this chapter, I have provided a summary of the materials and experimental procedures employed in this study. I have listed the plasmids and yeast strains constructed during the course of this work or those generously provided by other laboratories. I have included the relevant citations when referring to previously published strains, reagents and protocols.

2.1 [Cir$^+$] and [Cir^0] yeast strains

The yeast strains that contain native 2 micron circles are designated as [Cir$^+$], whereas the strains cured of 2 micron plasmids are designated as [Cir^0]. The plasmids that serve as reporters for the localization and segregation behavior of the 2 micron plasmid harbor the *ORI* and *STB* regions, and are referred to as *STB* reporter plasmids. Plasmids similar in overall organization but lacking *STB* are referred to as *ARS* (containing an **a**utonomously **r**eplicating **s**equence) reporter plasmids. These plasmids are capable of efficient replication in yeast strains but lack an active partitioning system. When an *STB* plasmid is present in a [Cir$^+$] strain, it receives a supply of the Rep1 and Rep2 proteins from the endogenous 2 micron plasmid. However, in a [Cir^0] background, that is, in the absence of Rep proteins, an *STB* plasmid would behave as an *ARS* plasmid.

For the studies described in **Chapters 4** and **5**, we utilized *ARS* plasmids harboring the partitioning loci derived from EBV (the FR region) (Kapoor et al., 2001 and this study) and from BPV (the LCR region) (Brannon et al., 2005 and this study), respectively. These are referred to as EBV and BPV reporter plasmids. As the Rep1 and

Rep2 proteins have no effect on these plasmids, there segregation properties are unaltered by whether a yeast strain harboring either of these plasmids is [Cir^0] or [Cir$^+$].

2.2 Experimental yeast strains and plasmids

Yeast strains and plasmids utilized in the present work are listed as Table 2.1 and Table 2.2. Plasmids harbored by individual strains are also indicated.

Table 2.1 Strains

Strains used for experiments described in Chapter 3		
Strain	**Genotype**	**Source/Ref.**
CMY383	MAT **a** *can1-100 trp1-1 ura3-1 leu2::(P_{GAL}-RecR::LEU2)X2 his3-11,15::pSTB::HIS3 ade2-1::P_{HIS3}-GFP-LacI::ADE2* [Cir^0]	This study
MJY7120	MAT **a** *can1-100 trp1-1 ura3-1 leu2::(P_{GAL}-RecR::LEU2)X2 his3-11,15::pARS::HIS3 ade2-1::P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study
MJY7122	MAT **a** *can1-100 trp1-1 ura3-1 leu2::(P_{GAL}-RecR::LEU2)X2 his3-11,15::pSTB::HIS3 ade2-1::P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study
MJY7124	MAT **a** *can1-100 trp1-1 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-3HA-MAM1::KanMX6 his3-11,15::pSTB::HIS3 ade2-1::P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study
MJY7127	MAT **a** *can1-100 trp1-1 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-3HA-MAM1::KanMX6 his3-11,15::pARS::HIS3 ade2-1::P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study
MJY7128	MAT **a** *can1-100 trp1-1 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-3HA-MAM1::KanMX6 his3-11,15::pSTB::HIS3 ade2-1::P_{URA3}-TetR-GFP::ADE2* [Cir$^+$]	This study

Table 2.1 (cont.)

MJY7136	MAT **a** *can1-100 trp1-1 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 his3-11,15::pSTB::HIS3 ade2-1::P_{URA3}-TetR-GFP::ADE2 ipl1-321* [Cir$^+$]	This study
MJY7137	MAT **a** *can1-100 trp1-1 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 his3-11,15::pSTB::HIS3 ade2-1:: P_{HIS3}-GFP-LacI::ADE2 ipl1-321* [Cir$^+$]	This study
MJY7144	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 P_{GAL}-SPO13::TRP1 his3-11,15::pSTB::HIS3 ade2-1::P_{URA3}-TetR-GFP::ADE2* [Cir$^+$]	This study
MJY7145	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 P_{GAL}-SPO13::TRP1 his3-11,15::pSTB::HIS3 ade2-1:: P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study
MJY7149	MAT **a** *can1-100 trp1-1 ura3-1 leu2::(P_{GAL}-RecR::LEU2)X2 his3-11,15::pARS::HIS3 ade2-1::P_{HIS3}-GFP-LacI::ADE2 ipl1-321* [Cir$^+$]	This study
MJY7150	MAT **a** *can1-100 trp1-1 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 his3-11,15::pARS::HIS3 ade2-1:: P_{HIS3}-GFP-LacI::ADE2 ipl1-321* [Cir$^+$]	This study
MJY7154	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 P_{GAL}-SPO13::TRP1 his3-11,15::pARS::HIS3 ade2-1:: P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study
MJY7157	MAT **a** *can1-100 trp1-1 ura3-1 leu2::(P_{GAL}-RecR::LEU2)X2 his3-11,15::pSTB::HIS3 ade2-1::P_{HIS3}-GFP-LacI::ADE2 ipl1-321* [Cir$^+$]	This study
MJY7162	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENV::TetO2x112::HIS3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 scc1::P_{MET}-SCC1-18MYC::TRP1 his3-11,15::pARS::HIS3 ade2-1:: P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study
MJY7163	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENV::TetO2x112::HIS3 scc1::P_{MET}-SCC1-18MYC::TRP1 his3-11,15::pARS::HIS3 ade2-1:: P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study
MJY7165	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENV::TetO2x112::HIS3 scc1::P_{MET}-SCC1-18MYC::TRP1 his3-11,15::pSTB::HIS3 ade2-1:: P_{HIS3}-GFP-LacI::ADE2* [Cir$^+$]	This study

Table 2.1 (cont.)

MJY7171	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENV::TetO2x112::HIS3 scc1::P_{MET}-SCC1-18MYC::TRP1 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir^+]	This study
MJY7172	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENV::TetO2x112::HIS3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 scc1::P_{MET}-SCC1-18MYC::TRP1 his3-11,15::pSTB::HIS3 ade2-1:: P_{HIS3}-GFP-LacI::ADE2* [Cir^+]	This study
MJY7174	MAT **a** *can1-100 leu2::(P_{GAL}-RecR::LEU2)X2 CENV::TetO2x112::HIS3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 scc1::P_{MET}-SCC1-18MYC::TRP1 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir^+]	This study
MJY7177	MAT **a** *can1-100 his3-11,15 leu2-3 CENIV::TetOx448::URA3 ura3-1:: P_{GAL}-3MYC-CDC5::URA3 P_{GAL}-MAM1::KanMX6 P_{GAL}-SPO13::TRP1 ade2-1:: P_{HIS3}-GFP-LacI::ADE2* [Cir^+]	This study

Strains utilized for experiments described in Chapter 4

Strain	Genotype	Source/Ref.
MJY124	MAT **a** *ade2-1 ura3-1 leu2-3,112 trp1 his3-11* [Cir^+]	(Mehta et al., 2002)
MJY3016	MAT **a** *ura3-1 leu2-3,112 trp1 his3-11 ade2-1::P_{HIS3}-GFP-LacI::ADE2* [Cir^+]	(Ghosh et al., 2007)
MJY3017	MAT **a** *ura3-1 leu2-3,112 trp1 his3-11 ade2-1::P_{HIS3}-GFP-LacI::ADE2* [Cir^0]	(Ghosh et al., 2007)
MJY7011	MAT **a** *ura3-1 leu2-3,112 trp1 his3-11 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir+]	This study
MJY7013	MAT **a** *ura3-1 trp1 his3-11 leu2-3,112::P_{PGK}-hEBP2::LEU2 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir+]	This study
MJY7014	MAT **a** *leu2-3,112 trp1 his3-11 ura3-1::P_{MET}-EBNA1::URA3 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir+]	This study

Table 2.1 (cont.)

MJY7049	MAT **a** *trp1 his3-11 ura3-1::P_{MET}-EBNA1::URA3 leu2-3,112::P_{PGK}-hEBP2::LEU2 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir+]	This study

Strains utilized for experiments described in Chapter 5		
Strain	**Genotype**	**Source/Ref.**
MJY124	MAT **a** *ade2-1 ura3-1 leu2-3,112 trp1 his3-11* [Cir^+]	(Mehta et al., 2002)
MJY7011	MAT **a** *ura3-1 leu2-3,112 trp1 his3-11 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir+]	This study
MJY7020	MAT **a** *leu2-3,112 trp1 his3-11 ura3-1::P_{CYC1}-E2::URA3 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir+]	This study
MJY7021	MAT **a** *ura3-1 trp1 his3-11 leu2-3,112::P_{ADH}-BRD4::LEU2 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir+]	This study
MJY7031	MAT **a** *trp1 his3-11 ura3-1::P_{CYC1}-E2::URA3 leu2-3,112::P_{ADH}-BRD4::LEU2 ade2-1:: P_{URA3}-TetR-GFP::ADE2* [Cir+]	This study

Table 2.2 Plasmids

Plasmids employed for the work presented in Chapter 3		
Plasmid	**Salient features**	**Source/Ref.**
pSV1	256 copies of Lac operator sequence cloned in YEpLac181 (*LEU2*)	(Velmurugan et al., 2000)
pCM218	*STB* plasmid excision cassette (RS-*ORI-STB*-$[LacO]_{256}$-RS)* cloned in pRS403 (*HIS3*)	This study
pTL29	*ARS* plasmid excision cassette (RS-*ORI*-$[LacO]_{256}$-RS)* cloned in pRS403 (*HIS3*)	This study
Plasmids employed for the work presented in Chapter 4		

Table 2.2 (cont.)

Plasmid	Salient features	Source/Ref.
pRS314	Centromere vector with *CEN6-ARSH4-TRP1* cloned in pRSS56 (*TRP1*)	ATCC
YRp7	*ARS* vector with *TRP1-ARS1* cloned in pBR322 (*TRP1*)	(Stinchcomb et al., 1979)
YRp7FR	EBV-derived vector with *FR* cloned in YRp7 (*TRP1*)	(Kapoor et al., 2001)
pR425/PGK	*PGK* promoter cloned in pRS425 (*LEU2*)	(Kapoor et al., 2001)
pR425/PGKEBP2	P_{PGK}-*hEBP2* cloned in pRS425 (*LEU2*)	(Kapoor et al., 2001)
p416MET	*MET25* promoter cloned in pRS416 (*URA3*)	(Kapoor et al., 2001)
p416MET/EBNA1	P_{MET25}-*EBNA1* cloned in pRS416 (*URA3*)	(Kapoor et al., 2001)
pSG1	P_{GAL1}-*CEN3-STB-ORI* and 256 copies of Lac operator sequence cloned in YEpLac112 (*TRP1*)	(Ghosh et al., 2007)
pTL24	P_{GAL1}-*CEN3-FR-ARS1* and 224 copies of Tet operator sequence cloned in pRS304 (*TRP1*)	This study
pTL26	P_{GAL1}-*CEN3-ARSH4* and 112 copies of Tet operator sequence cloned in pRS304 (*TRP1*)	This study
Plasmids employed for the work presented in Chapter 5		
Plasmid	**Salient features**	**Source/Ref.**
pRS314	Centromere vector with *CEN6-ARSH4-TRP1* cloned in pRSS56 (*TRP1*)	ATCC
YRp7	*ARS* vector with *TRP1-ARS1* cloned in pBR322 (*TRP1*)	(Stinchcomb et al., 1979)
1919 (YRP7LCR)	BPV-derived vector with *LCR* cloned in YRp7 (*TRP1*)	(Brannon et al., 2005)
2252 (p416CYC1-E2)	P_{CYC1}-*E2* cloned in pRS416 (*URA3*)	(Brannon et al., 2005)
2424 (pADNS-MCAP)	P_{ADH}-*MCAP* (Brd4) cloned in pRS425 (*LEU2*)	(Brannon et al., 2005)
pTL33	E2 ORF removed from p416CYC1-E2 (*URA3*)	This study
pTL34	MCAP (Brd4) ORF removed from pADNS-MCAP (*LEU2*)	This study
pTL35	P_{GAL1}-*CEN3-LCR-ARSH4* and 224 copies of Tet operator sequence cloned in pRS304 (*TRP1*)	This study
pTL26	P_{GAL1}-*CEN3-ARSH4* and 112 copies of Tet operator sequence cloned in pRS304 (*TRP1*)	This study

2.3 Antibodies used in this study

The monoclonal anti-HA (HA.11) antibody and anti-Myc (9E10) antibody used in this study were supplied by Covance, CA. They were used with 1:1000 and 1:100 dilutions in western blotting and ChIP assays, respectively. The monoclonal anti-GFP (LGB-1) antibody used for immunofluorescence assays was purchased from Abcam, and used at 1:500 dilution. Monoclonal anti-EBNA1 antibody supplied by ViroStat and monoclonal anti-E2 (1E2) supplied by Abcam were used in western blotting assays at 1:1000 dilutions. Polyclonal anti-EBP2 (N-13) antibody supplied by Santa Cruz Inc. and polyclonal anti-Brd4 antibody from Abnova were generated in goat and rabbit, respectively. They were used in western blotting assays at 1:1000 dilutions.

2.4 Culture conditions

Yeast strains were grown in complex medium or synthetic dropout medium at 30 ℃ unless they harbored a temperature sensitive (T^s) mutation. Cultures of the *ipl1-321* strain were normally grown in 26℃ (permissive temperature), and was then shifted to 34 ℃ (semi-permissive temperature) to partially inactivate Ipl1 function (Monje-Casas et al., 2007). For cohesin depletion experiments, the strains expressing the *MCD1* gene from the *MET3* promoter were maintained at 25℃ throughout the assays (Monje-Casas et al., 2007). The *MET3* promoter was repressed by the addition of methionine to the medium.

2.5 Synchronization of yeast cells

In order to arrest cells at the G1 stage, *MATa* cells in early log phase were treated with α-factor at a final concentration of 10 μg/ml for *BAR1* strains. The incubation time

varied from 2 to 3 hours, depending on individual strains, to obtain maximum arrest. Samples were examined under a light microscope to ensure that >90% of cells had the 'shmoo' form, typical of G1 arrest.

In order to arrest cells at the G2/M stage, nocodazole (Sigma) dissolved in DMSO was added to an exponentially growing culture at a final concentration 15 µg/ml. Depending on the individual strains, 2-3 hr treatment with nocodazole resulted in > 85% of the cells being arrested at G2/M with a large-budded phenotype. After staining with DAPI, the arrested cells showed a single DNA mess localized close to the bud neck in the mother compartment.

2.6 Single-copy derivatives of reporter plasmids

One of two strategies was employed to obtain single-copy derivatives of reporter plasmids. In one case, a *CEN* sequence that could be conditionally inactivated by driving transcription through it from the *GAL* promoter was cloned into the plasmid. In the other, the plasmid was integrated into a chromosome with the target sites for a site-specific recombinase placed at selected locations in a head-to-tail orientation. In the P_{GAL}-*CEN* system, *CEN* was inactivated at the appropriate time during an assay by adding galactose to the medium (final concentration of 2%). In the integrant plasmid system, the recombinase was expressed at the desired time to excise the plasmid from the chromosome. Whereas a *CEN*-containing plasmid maintained a copy number close to (and not precisely) one, a plasmid excised from its integrated state had a copy number of exactly one.

The P_{GAL}-*CEN* strategy described in published work (Ghosh et al., 2007) was employed in the construction and functional analyses of single copy EBV and BPV reporter plasmids described in **Chapters 4** and **5**, respectively. Briefly, a typical viral reporter plasmid was constructed by incorporating the viral partitioning sequence (FR for EBV and LCR for BPV) and a yeast centromere into an *ARS* vector. The inducible *GAL1* promoter was placed upstream of *CEN* to conditionally inactive it. The *CYC1* terminator sequence inserted immediately downstream of *CEN* prevented transcription from the *GAL* promoter from extending into other plasmid regions. When cells harboring a reporter plasmid were grown in glucose or raffinose containing medium, the plasmid-borne *CEN* was active in partitioning and copy number control. Upon shifting cells to galactose containing medium, the *CEN* function was inactivated, and the plasmids were dependent solely on the viral partitioning sequence for their segregation during the ensuing cell cycle. Additional descriptions concerning relevant aspects of the 'single' copy viral reporter plasmids can be found under sections 4.3.2 and 5.3.2 as well as under Figure 4.4 and Figure 5.3.

The plasmid excision strategy was employed for the experiments detailed in **Chapter 3** whose intent was to determine the correlation between a chromosome and an *STB* reporter plasmid in segregation behavior by perturbing normal mitosis in specific ways. In order to do this unambiguously, we needed to maintain the copy number of the plasmid the same as that of the haploid chromosome, namely, one. This was accomplished by integrating a cassette harboring the 2 micron circle replication origin, *STB* locus and a $[LacO]_{256}$ array flanked by two copies of the *Zygosaccharomyces rouxii* R recombinase target sites (*RRT*s) at the chromosomal *HIS3* locus. The plasmid

sequences comprising the origin, *STB*, the LacO array and one copy of *RRT* were excised in G1 arrested cells by inducing the expression of the R recombinase from the *GAL* promoter. Upon releasing the cells, the excised plasmid underwent replication to generate two sister copies, whose segregation was scored in anaphase cells. The experimental protocol for the segregation assay using the plasmid excision system is outlined in Figure 3.4 (**Chapter 3**).

2.7 Fluorescence tagging of a plasmid or a chromosome for visualization in live cells

In order to track the localization or segregation of a reporter plasmid or a chromosome, each was fluorescence tagged by the interaction between an operator array and its cognate repressor fused to a fluorescent protein (Straight et al., 1996). The Lac and Tet operator-repressor systems were used for DNA visualization in this study. A long array of operator sequences, $[LacO]_{256}$ or $[TetO]_{224}$/ $[TetO]_{112}$, was incorporated into the reporter plasmid or a chromosome by standard cloning or integrative transformation protocols, respectively. A strain harboring the tagged chromosome, or a strain into which the tagged plasmid, was introduced was engineered to express the GFP-repressor fusion protein (P_{HIS3}-*GFP-LacI* for the Lac system and P_{URA3}-*TetR-GFP* for the Tet system). A single copy reporter plasmid or a chromosome was visualized as a single sharp fluorescent focus formed by the concentration of the repressor protein at the operator locus. A multi-copy *STB* reporter plasmid revealed itself as a collection of 3-5 fluorescent foci (Velmurugan et al., 2000).

2.8 Fluorescence microscopy and fluorescence intensity measurements

Fluorescence signals were detected using an Olympus BX-60 microscope. Images captured at room temperature at 100x magnification (oil NA 1.30 objective) using a

Photometrics Quantix camera (Roper Scientific) were processed by MetaMorph (Universal Imaging Corporation) and PhotoShop CS (Adobe Systems, Inc.). Z-series sectioning of the yeast nucleus, deconvolution of the stacks, and their 2-D projections were performed as detailed previously (Mehta et al., 2005; Velmurugan et al., 2000). To measure the fluorescence intensity of a single focus, the highest signal intensities within individual pixels comprising that focus were measured from 2-D projections using the Metamorph software. The normalized intensity so obtained was further refined by subtracting the average intensity, similarly estimated, of an equal number of surrounding pixels.

2.9 Plasmid segregation assays

2.9.1 Segregation of close-to-single copy reporter plasmids

In **Chapters 4 and 5**, the reporter plasmids for segregation assays were designed using the P_{GAL}-*CEN* system described above. As such, their copy number was not exactly one in every individual cell, but a distribution with an average value of ~1 per cell. The yeast strains containing such 'single copy' reporter plasmids were grown to an OD_{600} = ~0.2 in 2% raffinose medium before treatment with α factor for 1 hr. Cells were harvested and subjected to continued treatment with α-factor in 2% glucose or galactose medium for an additional 2 hr before washing them and releasing them into pheromone-free glucose or galactose medium. Examination of cells just before release showed that nearly 80% of them contained a single focus of the fluorescence tagged reporter plasmid. The remaining cells contained predominantly two plasmid foci, while more than two were also encountered occasionally. A very small fraction did not contain the plasmid

focus. In assaying plasmid segregation after releasing cells from G1 arrest, we restricted ourselves to those cells containing two plasmid foci, signifying one focus prior to replication. Distribution of plasmids was scored in anaphase cells, that is, large-budded cells with two equally distributed DAPI masses in both mother and bud compartments. The general procedure is outlined in Figure 2.1 below.

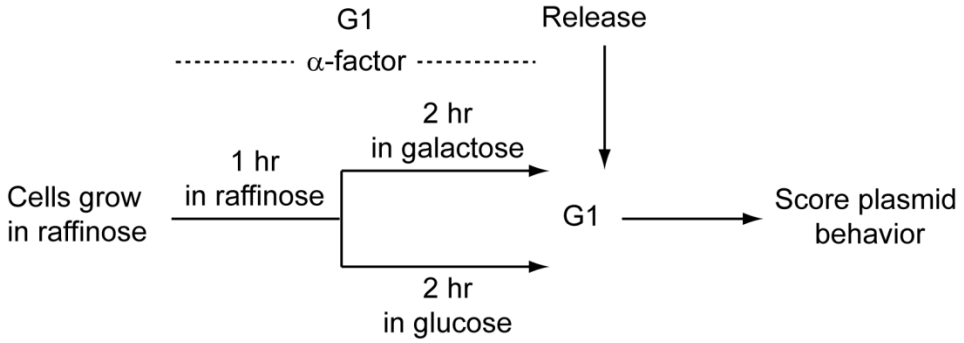


Figure 2.1 Experimental procedure for following the behavior of a single-copy viral reporter plasmid in cells released from G1 arrest. During growth in glucose medium, the centromere remains active; however, it is inactive during a cell cycle in galactose medium. The scheme is modified from Ghosh et al., (2007).

2.9.2 Segregation of single-copy plasmids obtained by excision from their integrated states

For the assays described in **Chapter 3**, a reporter plasmid, obtained by the plasmid excision strategy, was present as one copy per cell prior to replication. The yeast strains containing the integrated forms of reporter plasmids were grown in 2% raffinose media to OD_{600} = ~0.2 before arresting them in G1 using α factor. After a 2-3 hr

treatment with α factor, depending on individual assay conditions, over 98% of the cells were arrested in G1. Cells were then shifted to 2% galactose to induce the production of the R recombinase. After 3 hr in galactose, by which time a reporter plasmid was nearly quantitatively excised from its integrated state, cells were released into the cell cycle in glucose or galactose containing medium as desired. Fluorescence signals from the reporter plasmid were scored in anaphase/post-anaphase cells displaying two separated DAPI staining zones in the mother and bud compartments. The general procedure is outlined below (Fig. 2.2).

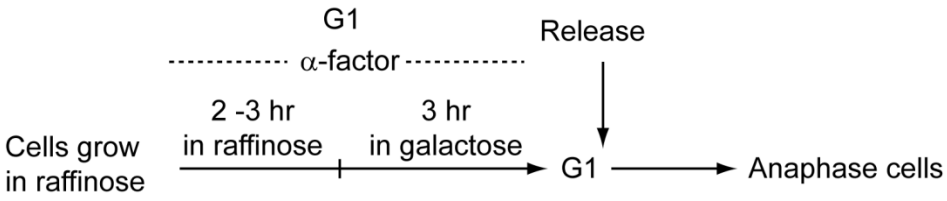


Figure 2.2 Experimental procedure for following the segregation behavior of an excised single-copy *STB* reporter plasmid during a cell cycle. A 3 hr incubation of G1 arrested cells in galactose medium, with the attendant induction of the R recombinase, was sufficient to excise the integrated single copy plasmid in nearly all of the cells. After releasing the cells from arrest, plasmid segregation was analyzed in anaphase/post-anaphase cells.

2.9.3 Segregation of multi-copy reporter plasmids

The segregation of multi-copy reporter plasmids was analyzed for one set of segregation assays described in **Chapter 3**. Yeast strains harboring the multi-copy *STB* reporter plasmid, grown to OD_{600} = ~0.2 in selective media to maintain the plasmid, were treated with α factor for 2hr to obtain >90% arrest of cells in G1. In assays where the

induction of *CDC5* and *MAM1* from the *GAL* promoter was required, the arrested cells were shifted to 2% galactose containing medium for 1 hr (Monje-Casas et al., 2007). After releasing cells from arrest in glucose or galactose containing medium as desired, plasmid distributions in the mother and bud compartments were scored in anaphase/post-anaphase cells.

2.9.4 Segregation during a cohesin-depleted cell cycle

In the experimental strain utilized for the cohesin depletion assays addressed in section 3.3.7 (**Chapter 3**), the native promoter of the gene for the cohesin subunit Mcd1/Scc1 was replaced by the methionine repressible promoter P_{MET}. During the 3 hr period of galactose induction for excising the single copy reporter plasmids in G1 arrested cells, 8 mM methionine was included in the medium. Furthermore, the medium in which the released cells resumed cell cycle also contained 8mM methionine (Monje-Casas et al., 2007).

2.10 DNA analysis by Southern blotting

Southern blot analysis was performed to assay the excision efficiency of the single-copy reporter plasmids. Yeast genomic DNA digested with diagnostic restriction enzymes was fractionated by agarose gel electrophoresis, and was transferred to Hybond-XL membrane according to the manufacture recommended protocol (GE Healthcare). After hybridization to a P^{32}-labeled DNA probe, bands were detected and quantitated by phosphorimaging using a Typhoon Trio phosphorimager and ImageQuant software (GE Healthcare). The excision efficiency was estimated from the intensities of the bands corresponding to the excised and integrated forms of the plasmid.

2.11 Protein analysis by Western blotting

Protein detection by Western blotting was performed by following the protocol described previously (Ghosh et al., 2010) with some modifications. Protein samples were prepared from the yeast whole cell extracts according to procedures described on the laboratory web page (see section 2.15 below). Proteins fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to PVDF membranes. After processing the membranes to block non-specific interactions, they were incubated sequentially with the primary antibody and HRP-conjugated secondary antibody. Positive antibody interaction signals were detected using Amersham™ ECL™ Western blotting detection system (GE Healthcare).

2.12 Chromatin immunoprecipitation (ChIP)

The ChIP analyses were performed by procedures described previously (Hajra et al., 2006) in a strain containing the native *MAM1* gene modified by 3HA epitope tagging. 20 OD_{600} units of metaphase cells were treated with 1% formaldehyde for 30 min at room temperature to mediate DNA-protein crosslinking. The crosslinking reaction was stopped by adding glycine to a final concentration of 125 mM. Following the wash (with 1x PBS) and centrifugation, pelleted cells were resuspended in lysis buffer and lysed by bead-beating (1 min ×4, 2 min interval on ice between successive operations of the beater) at 4℃ in the mini-bead-beater-8 (BioSpec Products, Inc.). DNA present in the supernatant was sheared into small fragments with an average length of 500 bp by sonication using a Sonics Vibra Cell sonicator. Immunoprecipitation was performed using antibodies to the HA epitope (Covance), with slow overnight rotation of samples in a rotary shaker at 4℃. DNA-protein complex was further purified from the procedure of pull-down (through protein A

beads) and followed by reversed crosslinking to separate DNA fragment from protein. DNA-protein crosslinks were reversed by heating samples at 65℃ for overnight. After isolation of DNA by phenol-chloroform extraction and ethanol precipitation, aliquots were subjected to PCR amplification. Primer pairs employed to probe for *STB*, *CEN3* and chromosome arm sequences have been described previously (Hajra et al., 2006; Rabitsch et al., 2003). The linear range of amplification in the PCR reactions was standardized by serial dilutions of the template DNA. In the 'input' reactions, the template DNA was diluted 1:200 with respect to the immunoprecipitated samples. Equal aliquots of PCR reactions were fractionated in agarose gels by electrophoresis, and stained with ethidium bromide. Quantity One software (Bio-Rad) was used to quantitate DNA band intensities. Signal intensities were corrected by subtracting the corresponding intensities from the lanes representing a mock immunoprecipitation (no antibody added). The ChIP efficiency of a particular DNA locus was estimated as the ratio of its corrected PCR signal normalized to the corrected signal for the input reaction, after factoring in the dilution factor. The ChIP efficiency of *CEN3* estimated by this method provided the positive reference for expressing the relative ChIP efficiencies of other DNA loci tested.

2.13 Mitotic stability of reporter plasmids over multiple generations

Under sections 4.3.1 (**Chapter 4**) and 5.3.1 (**Chapter 5**), we utilized reporter plasmids that were not tagged by fluorescence to assay their stabilities in the presence of reconstituted EBV and BPV partitioning systems, respectively. These assays measured the rate at which plasmids were lost from their host strains when the selection pressure to maintain them was removed. The reporter plasmids harbored the *TRP1* marker, while the plasmids supplying the two partitioning proteins contained *LEU2* or *URA3*. The

experimental yeast strains were grown in the appropriate drop-out medium (SC-Trp, Ura, Leu) until mid-log phase (T=0). Equal aliquots (containing approximately 200-300 cells) from each sample were plated out on either 'selective' (SC-Trp, Ura, Leu) or 'non-selective' (SC-Ura, Leu) medium. These cells provided the T=0 data set for the fraction of cells f0 harboring the reporter plasmid (containing the *TRP1* marker). Each T=0 culture was diluted 10-fold into the non-selective medium (SC-Ura, Leu), and grown for another 5 generations (T =10; approximately 10 hr growth). Again, equal aliquots from each sample were seeded on selective and non-selective plates to collect T=10 data set for the fraction of cells f10 containing the *TRP1* plasmid. The instability index 'i', that is, the plasmid loss rate per generation, was calculated according to the formula (Murray and Cesareni, 1986)

$$i = \frac{1}{N} \ln(f0/\, fn),$$

where N represents the number of generations of non-selective growth, and f0 and fn are the fractions of cells containing the reporter plasmid at time zero and after N generations of non-selective growth, respectively.

2.14 Chromosome spreads preparation & Immunofluorescence

Preparation of chromosome spreads from mitotic cells and the detection of plasmids in them by immunofluorescence were performed as described previously (Mehta et al., 2002; Velmurugan et al., 2000) with minor modifications. Yeast spheroplasts were obtained by zymolyase treatment (the protocol is presented on the laboratory web page, see section 2.15). For each spread, 20µl of the spheroplast preparation were transferred to a clean glass slide, and were gently mixed with 40µl of the fixing solution (4% paraformaldehyde/3.4% sucrose). 80µl of 1% lipsol were added

followed by an additional fixing step before spreading the sample uniformly throughout the slide. After overnight incubation at room temperature, slides were washed with 0.4% photoflo-200 (Kodak) and submerged in 1 × PBS before performing the steps of the immunofluorescence analysis. The chromosome spreads were blocked with 10 mg/ml BSA for 30 min before incubating them with the requisite antibody. The incubation time for the primary antibody was 2-3 hr, and that for the secondary antibody conjugated to a fluorescent dye was 1 hr. The DNA was stained using 1 μg/ml DAPI in 1× PBS. Slides were mounted with mounting media and cover slips, and examined by fluorescence microscopy. The chromosome spread analysis was performed in experiments described under section 4.3.3 (**Chapter 4**) and 5.3.3 (**Chapter 5**) in order to determine the association of a reporter plasmid with chromosomes. The relevant results are shown in Fig. 4.6, Fig. 4.8, Fig. 5.5, and Fig. 5.6.

2.15 General experimental procedures

General experiment protocols such as yeast and bacterial transformation, yeast plasmid DNA and genomic DNA preparation, yeast whole cell extract preparation, curing of native 2 micron circles from [Cir^+] strains to obtain [Cir^0] genetic backgrounds etc. are described on the laboratory web page (http://www.sbs.utexas.edu/jayaram/jayaramlab.htm).

CHAPTER3

2 micron plasmid segregation during forced co-segregation of sister chromatids by expression of the monopolin complex in mitosis is consistent with the hitchhiking model for plasmid segregation

3.1 Summary

We have challenged the predictions of the hitchhiking model for 2 micron plasmid segergation during mitosis. The rationale of the analysis is to follow the segregation of a fluorescence tagged single copy *STB* reporter plasmid *vis a vis* a fluorescence tagged chromosome under the forced co-segregation of sister chromatids by inappropriate expression of the monopolin complex. The key findings from this analysis are as follows. **1**. During the monopolin directed mitosis, there is a strong correlation between the increased co-segregation frequency of chromosome IV sisters and that of *STB* plasmid sisters. The co-segregation of both chromosome and plasmid sisters occurs with essentially no mother-daughter bias, or perhaps a very small mother bias **2**. When monopolin assembly is combined with Spo13 expression, there is a marked additional increase in both sister chromatid and sister plasmid co-segregation. The magnitude of the effect is somewhat stronger on the chromosome than on the plasmid. In both cases, the co-segregation is associated with a very small mother bias. **3**. When the Ipl1 (Aurora kinase) function is inactivated during a monopolin directed mitosis, there is a comparable increase in co-segregation of chromosome IV and the *STB* plasmid. Strikingly, the co-segregation now shows a small but distinct daughter bias. **4**. A delay in the assembly of

the mitotic spindle until G2/M or depletion of the cohesin complex uncouples *STB* plasmid segregation from the monopolin induced co-segregation of sister chromatids.

The sum of the results is accommodated by the tethering of plasmid to chromosomes and plasmid segregation by hitchhiking. Furthermore, sister plasmids must associate with sister chromatids in a one-to-one fashion.

3.2 Introduction

Coupling of plasmid partition and chromosome segregation in yeast

Several lines of evidence indicate that the faithful propagation of 2 micron plasmid is tightly linked to the segregation of host chromosomes. First, previous studies have shown that a number of host factors required for faithful chromosome segregation are co-opted by the plasmid for its own equal segregation with the assistance of the Rep-*STB* partitioning system (Cui et al., 2009; Ghosh et al., 2007; Hajra et al., 2006; Mehta et al., 2002; Mehta et al., 2005; Yang et al., 2004). Second, microscopy analyses revealed that the dynamics and kinetics of segregation of a fluorescence tagged chromosome and that of a fluorescence tagged 2 micron circle derived reporter plasmid are remarkably similar. Third, several mutations which induce chromosome missegregation, *ipl1* and *ndc10*, for example, also cause the plasmid to missegregate in tandem with the bulk of the chromosomes (Mehta et al., 2002; Velmurugan et al., 2000). A simple explanation that best fits the observations listed above posits that the plasmid segregates by tethering to chromosomes and hitchhiking on them. However, direct demonstration of plasmid-chromosome association in mitosis has been difficult due to the small size of the yeast nucleus and the inability to resolve individual chromosomes by fluorescence microscopy.

At this time, plasmid segregation mechanisms that do not involve direct physical association with chromosomes cannot be ruled out.

The monopolin complex and the mono-orientation of kinetochores during meiosis I

In budding yeast, as in most eukaryotes, diploid cells can undergo the process termed meiosis in which one round of DNA replication is followed by two contiguous cell division events (meiosis I and meiosis II, respectively) to form haploid gametes. In meiosis I and meiosis II, chromosomes are separated to opposite poles but do so by different rules. During meiosis I, homologous chromosomes are separated from each other, while sister chromosomes co-segregate to same pole (Fig. 3.1). During meiosis II, sister chromatids segregate to opposite poles. This important difference between the two meiotic division events arises from differences in how sister kinetochores are oriented on the spindle. When sister kinetochores are attached to microtubules emerging from opposite poles (meiosis II and mitosis), this arrangement is called bi-orientation. On the other hand, attachment of sister kinetochores to microtubules emerging from the same pole (meiosis I) is termed mono-orientation (or co-orientation). In order to achieve the unique reductional division event, sister kinetochores have to be appropriately configured for them to be co-oriented. This task is accomplished through the activity of the protein complex “monopolin” (Corbett et al., 2010; Petronczki et al., 2006; Rabitsch et al., 2003; Tóth et al., 2000).

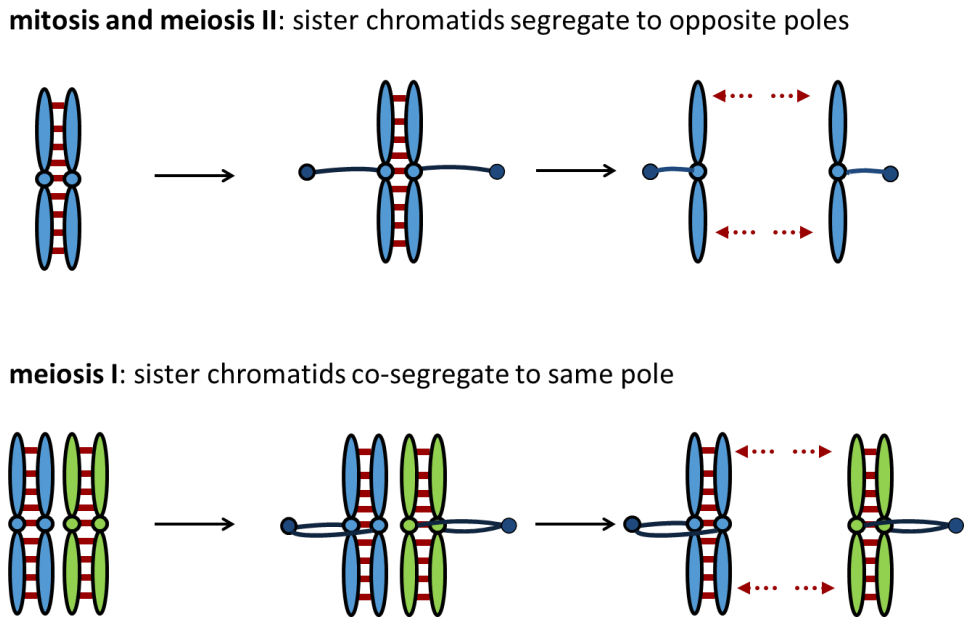


Figure 3.1 Sister chromosome orientation during mitosis and meiosis. During mitosis and meiosis II, sister chromosomes (kinetochores) are captured by microtubules emerging from opposite poles (bi-orientation). During anapahse, the sisters segregate away from each other. In meiosis I, a pair of sister chromatids (kinetochores) is captured by a microtubule emerging from the same pole (mono-orientation). As a result they cosegregate at the end of meiosis I, but are separated from the homologous sisters. A pair of homologous chromosomes is shown in separate colors, blue and green. A pair of sister chromatids is shown in the same color, blue or green.

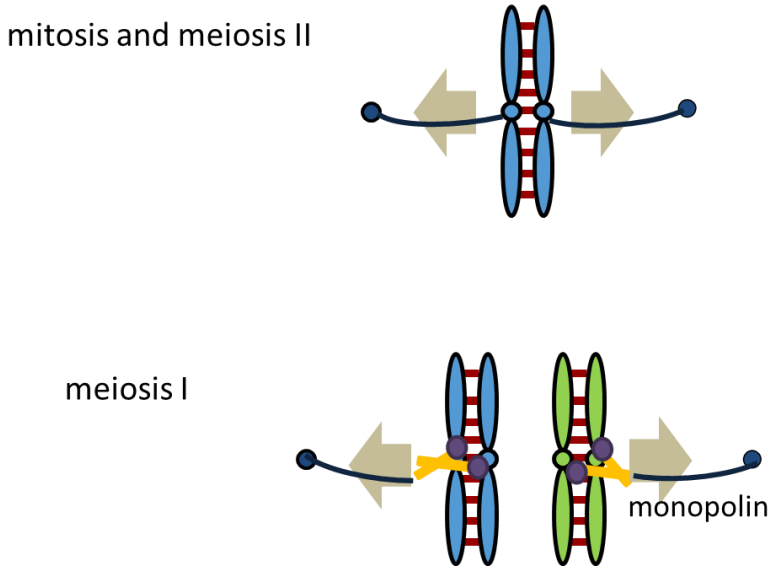


Figure 3.2 Model for mono-orientation of kinetochores mediated by the monopolin complex. In mitosis and meiosis II, outer kinetochores of sister chromatids form separate microtubule binding sites and are attached to spindle from the opposite poles. During meiosis I, monopolin complex binds to inner/linker kinetochores and fuses outer kinetochores of sister chromatids into one microtubule binding site, which cause the mono-orientation of sister kinetochores. The figure is modified from Corbett et al., (2010).

The monopolin complex is composed of four subunits: Mam1, Csm1, Lrs4 and Hrr25. Mam1 (monopolar microtubule attachment during meiosis I) is the only meiosis-specific protein of the complex, which localizes at kinetochores specifically in meiosis I, but not in meiosis II (Tóth et al., 2000). Csm1 (chromosome segregation in meiosis) and its interacting protein Lrs4 (loss of rDNA silencing) are localized in the nucleolus during most of the time in both mitosis and meiosis, and has been implicated in rDNA organization and function (Huang et al., 2006; Smith et al., 1999; Waples et al., 2009). During prophase of meiosis I, the Csm1-Lrs4 complex is temporarily released from the nucleolus, and binds to kinetochores together with Mam1 in an interdependent manner (Clyne et al., 2003; Lee and Amon, 2003; Rabitsch et al., 2003). The localization of Csm1-Lrs4 at kinetochores also takes place at the anaphase stage during mitosis, and is required for proper chromosome segregation (Brito et al., 2010a). By the strategy of tandem affinity purification, Hrr25, an ortholog of casein kinase 1 (CK1) which is involved in multiple cellular processes, has been shown to associate with Mam1 (Knippschild et al., 2005; Petronczki et al., 2006). Furthermore, the Hrr25 kinase activity and Mam1 binding are required for monopolar attachment of sister kinetochores (Petronczki et al., 2006). According to the current model, monopolin directs mono-orientation by clamping together the microtubule attachment sites on sister kinetochores into a single binding site (Corbett et al., 2010) (Fig. 3.2).

Several factors that regulate meiosis I progression act by regulating monopolin's association with kinetochores. Cdc5, the Polo-like kinase, is responsible for Csm1-Lrs4 release from the nucleolus and Mam1 binding to kinetochores. When Cdc5 is depleted, mono-orientation becomes severely defective in most of the cells. (Clyne et al., 2003; Lee

and Amon, 2003). Spo13, a meiosis I-specific protein, is thought to regulate monopolin function coordinately with Cdc5. In *spo13Δ* cells, monopolin complex localizes at kinetochores during prophase I but is lost during metaphase I, which suggests that Spo13 is required to maintain monopolin at kinetochores (Katis et al., 2004; Lee et al., 2004).

Reconstituting monopolin activity during mitosis

Reconstitution of monopolin activity in mitosis has helped define the minimal number of genes required for the mono-orientation of sister kinetochores (Monje-Casas et al., 2007). Expression of *MAM1* and *CDC5* from the inducible *GAL* promoter at the start of the cell cycle is sufficient to promote sister kinetochore coorientation in mitosis (Brito et al., 2010b; Monje-Casas et al., 2007). These manipulations are required since Mam1 is a meiotic-specific protein and *CDC5* is not normally expressed until the onset of anaphase for its role in the mitotic exit network (Segal, 2011; Song and Lee, 2001). Early expression of *CDC5* leads to corresponding early release of Lrs4 (and likely Csm1) from the nucleolus, followed by their localization to kinetochores along with the inappropriately expressed Mam1 (Monje-Casas et al., 2007). As mentioned earlier, Spo13 is required for maintenance of monopolin on kinetochores during meiosis. Co-expression of *SPO13* with *MAM1* and *CDC5* in mitosis increases the percentage of sister chromatid co-segregation promoted by *MAM1* and *CDC5* expression. However, the Spo13 effect on sister kinetochore co-orientation is apparently independent of Lrs4-Csm1. (Monje-Casas et al., 2007). Thus, the functional collaboration between Spo13 and the monopolin complex, which is characteristic of meiosis I, may not occur during mitosis. Instead, the overproduced Spo13 may independently interfere with kinetochore function and/or cohesin removal to promote co-orientation of sister kinetochores.

Characterization of the 2µm plasmid segregation during mitosis under conditions that promote sister-chromatid co-segregation: utility of a single copy reporter plasmid system

In spite of the technical challenges in probing the association of the 2 micron plasmid with yeast chromosomes, we wished to design experimental conditions that could still critically test predictions of the hitchhiking model for plasmid segregation.

One difficulty in following 2 micron plasmid segregation by cell biological methods is the organization of a fluorescence tagged *STB* reporter plasmid in the form of multiple foci (with a median number of 3-5 per nucleus). Presumably, each of these foci contains several plasmid molecules, and their distribution may not be uniform among the different foci. These uncertainties make quantitation of plasmid segregation imprecise, not to mention that there is an element of subjectivity in foci counting because of their close clustering. To overcome these impediments, we have performed the current analysis using a single-copy fluorescence tagged reporter plasmid, which is excised from its chromosomally integrated state just prior to the start of an experimental cell cycle. Plasmid excision is mediated by the action of the R site-specific recombinase on a pair of directly oriented recombination target sites. The single copy *STB* reporter plasmid contains the 2 micron circle replication origin, the *STB* locus and the $[LacO]_{256}$ array for fluorescence tagging by GFP-LacI association. An analogous control plasmid (the *ARS* reporter) lacks the *STB* locus. These reporter plasmids reveal equal segregation (1:1) or missegregation (2:0) without ambiguity. By including a fluorescence tagged chromosome ($[TetO]_{224}$-TetR-GFP) in the assay, the segregation behavior of a pair of plasmid sisters can be monitored with respect to that of a pair of sister chromatids.

The precision of the single copy plasmid reporter system permits us to address how plasmid segregation is affected when chromosome segregation is tampered with in distinct ways. In the present study, we have employed monopolin assembly as the basic strategy for inducing sister-chromatid co-segregation (same as chromosome missegregation). We have further introduced variations in this basic strategy by combining monopolin assembly with Spo13 expression or *ipl1* inactivation. Coupling between plasmid and chromosome segregation, predicted by the tethering-hitchhiking model, would be revealed as increased plasmid missegregation when chromosomes are forced to missegregate. In addition, near perfect correlation between chromosome and plasmid in their missegregation patterns would be strongly suggestive of sister plasmids being tethered to sister chromatids with high efficiency during hitchihiking.

Mother-daughter bias or lack of such bias associated with plasmid or chromosome missegregation under different conditions

The missegregation of an *STB* plasmid in presence of a functional Rep-*STB* system is almost free of bias towards the mother or the daughter. A marginal mother bias has been noted. By contrast, the missegregation of an *STB* plasmid in the absence of the Rep proteins (in a [Cir^+] strain) or that of an *ARS* plasmid in their presence or absence occurs with a large mother bias. Chromosome missegregation induced by monopolin or monopolin plus Spo13 is without bias whereas chromosome missegregation induced by Ipl1 inactivation shows a small but finite daughter bias. These characteristics of missegregation, namely, bias or lack of bias, the magnitude of bias and the direction of bias, are also useful indicators of whether plasmid and chromosome segregation events are coupled or not.

Strategies for uncoupling 2 micron plasmid segregation from chromosome segregation

The mitotic spindle is required for the normal segregation of the 2 micron plasmid, but not in the conventional way that it promotes chromosome segregation (Mehta et al., 2005). There is no reason to suspect that replicated plasmids are attached to the mitotic spindle in a bi-oriented fashion, and pulled apart to opposite poles. Kinetochore comoponents are not associated with the *STB* locus. The presence of more than one *STB* locus on a plasmid does not induce instabilities that mimic those induced by the presence of two copies of *CEN* in *cis*. When the spindle assembly is delayed until the G2/M phase of mitosis, chromosome segregation proceeds normally; however, 2 micron circle segregation is disrupted. The *STB* locus also associates with the cohesin complex, and this association requires a functional spindle. The timings of cohesin assembly and disassembly at *STB* during the cell cycle are important in the normal segregation of the 2 micron plasmid (Mehta et al., 2002). Since cohesin recruited to *CEN* and chromosome arms plays a crucial role in the one-to-one segregation of sister chromatids (Nasmyth, 2011; Onn et al., 2008), it is difficult to judge whether cohesin's effect on the plasmid is direct, or is mediated indirectly through cohesin's role in chromosome segregation. In the absence of cohesin, expression of monopolin during mitosis can mediate sister kinetochore association, although cohesion along sister chroamtid arms would be lost (Monje-Casas et al., 2007). However, this mode of pairing sister chromatids just at their kinetochores would still result in their co-segregation. We can utilize the single copy reporter system and monopolin directed mitosis to ask whether the postponement of

spindle assembly to G2/M or the depletion of cohesin during the cell cycle will uncouple the *STB* plasmid from the monopolin induced sister chromatid co-segregation.

The results of our experimental tests of the hitchhiking model are presented below.

3.3 Results

3.3.1 Single copy fluorescence tagged *STB* and *ARS* reporter plasmid systems for assaying monopolin directed cosegregation of sister plasmids

In previous studies, we employed *CEN-STB* reporter plasmids to characterize the segregation of single copy, or nearly single copy, derivatives of the 2 micron plasmid during mitosis (Ghosh et al., 2007). The plasmid-borne *CEN* sequence, responsible for lowering the copy number close to one, could be conditionally inactivated to bring plasmid segregation exclusively under the control of the Rep-*STB* system. Analyses performed on a single copy reporter plasmid tagged by green fluorescence or two such reporter plasmids tagged by green and red fluorescence, respectively, suggested that segregation occurs in a sister-to-sister fashion, green-to-green and red-to-red (Ghosh et al., 2007). Based on the association of the cohesin complex with *STB* (Mehta et al., 2002), it seemed likely that the cohesin mediated binary counting mechanism for distinguishing chromosome sisters from chromosome homologues, was being exploited by the 2 micron plasmid in an analogous fashion. In efforts to test the validity of the hitchhiking model for plasmid segregation, the present studies characterize the behavior of a pair of sister plasmids *vis a vis* that of a pair of sister chromatids during normal mitosis and a deviant 'meiosis I-like' version of mitosis induced by monopolin, when sister chromatids missegregate to the same cell pole.

The general experimental strategies to generate precisely two sister copies of a reporter plasmid and to institute monopolin directed chromosome segregation are schematically illustrated in Figure 3.3. The two reporter plasmids employed, containing the 2 micron circle replication origin and a $[LacO]_{256}$ array, were excised from their chromosomally integrated states during G1 by the R recombinase from *Zygosaccharomyces rouxii* (Araki et al., 1992). They differed from each other in that one contained the *STB* sequence (referred to as the *STB* reporter plasmid) and the other lacked this sequence (referred to as the *ARS* reporter plasmid) (Fig. 3.3A). A time course assay in G1 arrested cells showed that galactose induction for 3 hr resulted in nearly quantitative (93%) excision of the integrated *STB* plasmid. In cells maintained in glucose medium, the excised form of the plasmid was not detected (Fig. 3.3B). In all experiments described below, we employed the 3 hr galactose induction schedule to obtain 'complete' plasmid excision.

The individual reporters were resident in strains containing the native 2 micron plasmid (serving as a source of the Rep1 and Rep2 proteins; $[Cir^{+}]$) or lacking it (no supply of Rep1 and Rep2; $[Cir^{0}]$). The functional state of *STB* could be maintained in the $[Cir^{0}]$ strain by expressing the *REP1* and *REP2* genes from a chromosomally integrated expression cassette controlled by the bidirectional *GAL* promoter (which is inducible by galactose) or by the constitutive *ADH* promoter. GFP-LacI expressed from the *HIS3* promoter in the host strains conferred green fluorescence on the reporter plasmids they harbored. The positive control for segregation of sisters during normal mitosis and co-segregation of sisters during mitosis contrived by monopolin was provided by a strain in

which chromosome IV harbored a $[TetO]_{224}$ array proximal to the centromere and TetR-GFP was expressed from the *URA3* promoter (Monje-Casas et al., 2007).

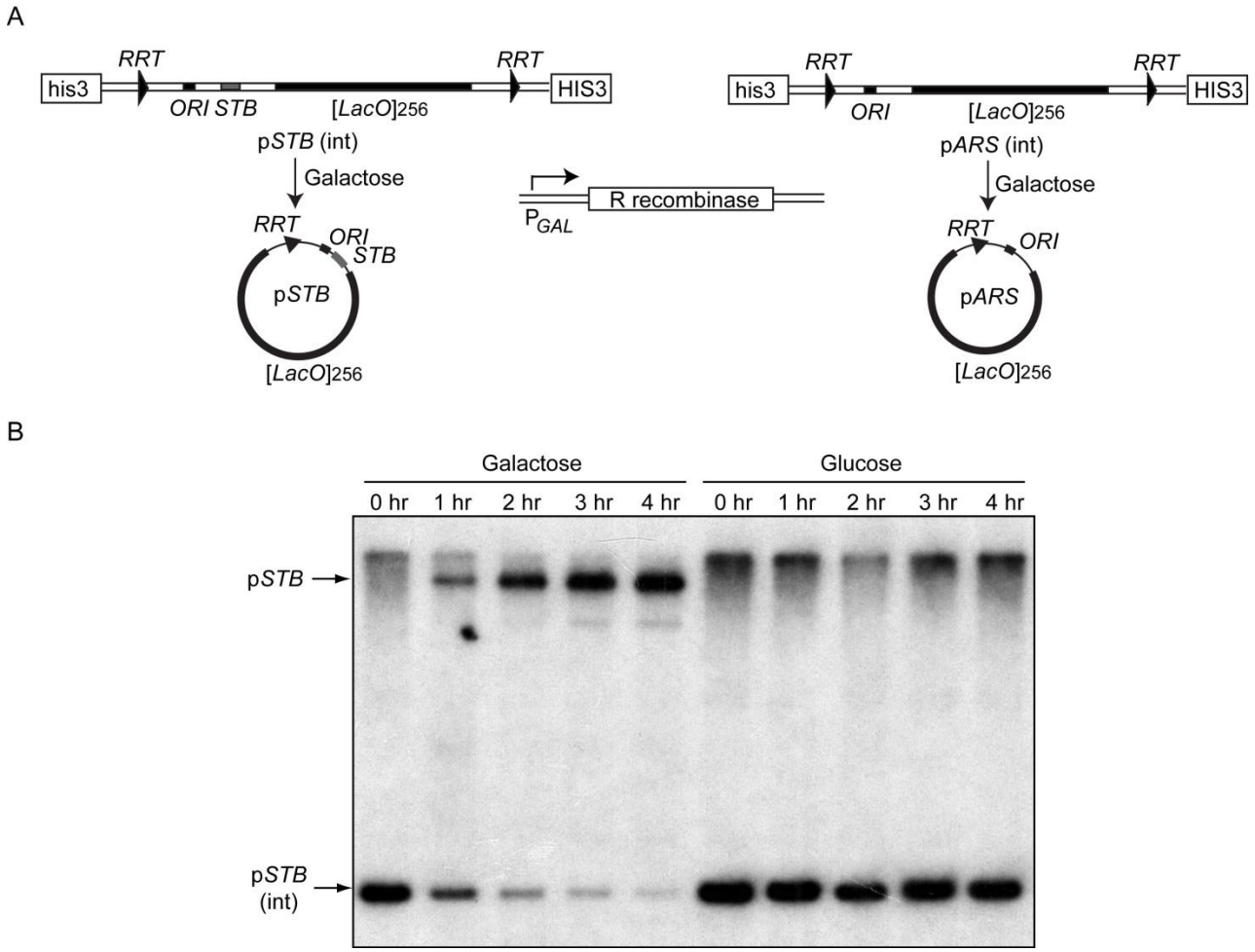


Figure 3.3 Single-copy plasmid reporters. (A) Generation of single copy reporter plasmids for analysis of the segregation of sister plasmids. The experimental set up for obtaining precisely one copy of the reporter plasmid, pSTB or pARS, by R recombianse mediated excision from chromosome XV is schematically diagrammed. (B) Time course of plasmid excision showed near completion of the reaction in 3 hr and no reaction in the absence of recombinase induction (glucose).

3.3.2 Analysis of the 2 micron plasmid segregation behavior using the single copy excision system

The segregation patterns of a reporter plasmid (excised in G1) or those of chromosome IV were assayed in anaphase cells following their release from G1 arrest (Fig. 3.4). The expected patterns of segregation are 1:1 (equal) or 2:0 (unequal). In order to indicate the mother-daughter bias in plasmid segregation, we have assigned the number at the left in these ratios to the mother and that at the right to the daughter. Thus, 2:0 denotes missegregation biased towards the mother, and 0:2 denotes that biased towards the daughter.

For the *STB* reporter plasmid, equal segregation during normal mitosis (absence of monopolin) was 63.7% in the presence of the Rep proteins ([Cir^+] host strain) and 27.5% in their absence ([Cir^0] host strain). The *ARS* reporter plasmid followed the 1:1 pattern in 23.4% of the cells analyzed (Fig. 3.5), and was unaffected by the presence or absence of the Rep proteins (data not shown). Chromosome IV, as expected, segregated 1:1 in every case (Fig. 3.5). These values provide a reference frame for the interpretation of the segregation results obtained in cells undergoing mitosis in the presence of monopolin.

The missegregation of the *ARS* reporter plasmid was biased strongly towards the mother (91.8%; 11.14) whereas the *STB* reporter missegregation occurred with a very small mother bias (57.3%; 1.34) (Fig. 3.5). These results were consistent with the mother-daughter bias during plasmid segregation documented previously (Murray and Szostak, 1983). The poor equal segregation efficiency and the strong mother bias of the *STB*

reporter plasmid in the [Cir^0] background indicates that, in the absence of the Rep-*STB* system, there is no functional difference between an *STB* plasmid and an *ARS* plasmid.

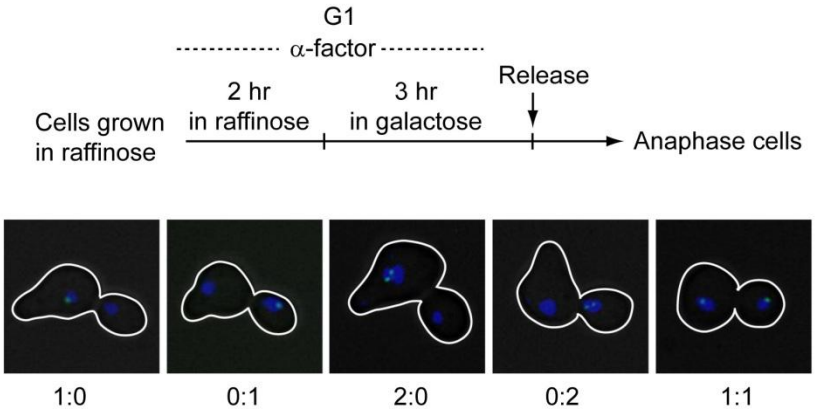
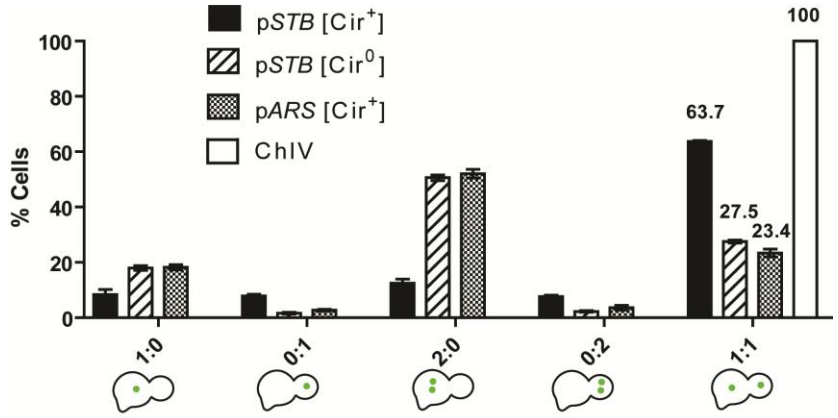


Figure 3.4 The experimental scheme for plasmid excision and subsequent analysis of plasmid segregation during mitosis. Cells grown in raffinose were arrested in G1 and shifted to galactose for 3 hr before releasing them into the cell cycle (in galactose medium). The shift to galactose in G1 resulted in the induction of the R recombinase and consequent excision of a reporter plasmid from its chromosomally integrated state. Plasmid segregation was assayed in cells released from arrest at the anaphase stage.



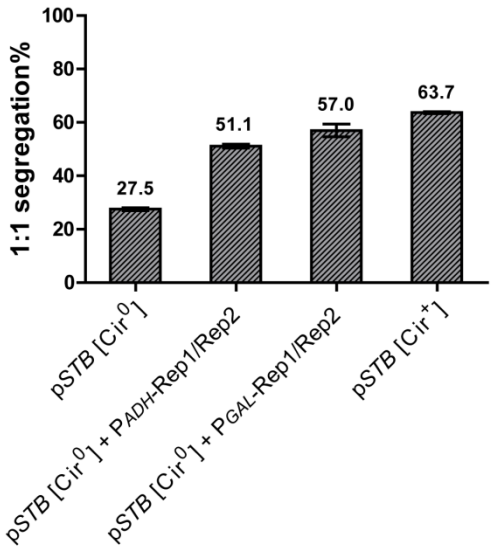
Bias in missegregation (%)		
	Mother	Daughter
p*STB* [Cir$^+$]	57.3	42.7
p*STB* [Cir^0]	94.8	5.2
p*ARS* [Cir$^+$]	91.8	8.2

Figure 3.5 Plasmid and chromosome segregation patterns during normal mitosis. Plasmid and chromosome segregation patterns were analyzed in anaphase cells by counting the number of fluorescent foci in the well separated daughter nuclei. The Table summarizes the mother or daughter bias within the populations showing plasmid missegregation. Results represented by individual histograms were obtained from scoring 100-200 cells in each case.

The equal segregation efficiency of the *STB* reporter plasmid was restored almost completely (57.0%) in the [Cir^0] strain by expressing *REP1* and *REP2* from the *GAL* promoter. The extent of complementation by expressing *REP1* and *REP2* from the *ADH* promoter was somewhat lower, 51.1% 1:1 segregation (Fig. 3.6). In either case, the mother bias was reduced to the low value (63.6%; 64.0%) characteristic of that for the [Cir^+] strain. These results establish an important point, namely, that the segregation properties of the single copy *STB* reporter plasmid supported by the Rep proteins are not altered by the absence or the presence of the native 2 micron plasmid. In other words, sister copies of the reporter plasmid are recognized as such, even when they are outnumbered by a large majority of *STB* containing plasmids that differ from them.

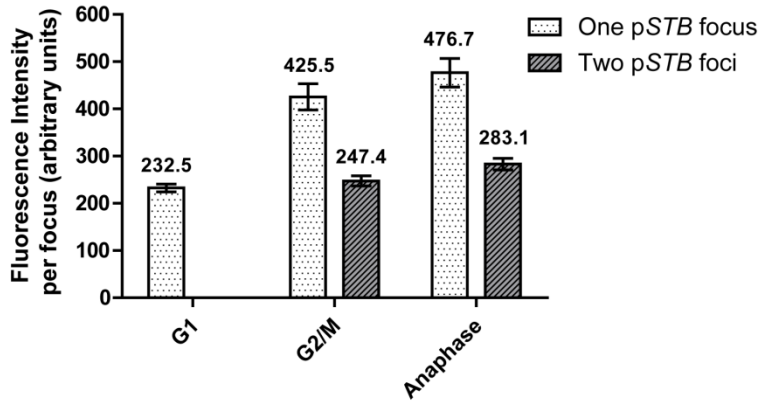
In our analysis, we noticed a subset of anaphase cells that revealed only a single fluorescent plasmid focus. This was the case for the *STB* reporter plasmid in the [Cir^0] or [Cir^+] host or the *ARS* plasmid in the [Cir^+] host (Fig. 3.5). In principle, the single focus could represent the segregation of an unreplicated plasmid or a missegregated pair of plasmid sisters that happened to be colocalized. Fluorescence intensity estimations revealed that the plasmid focus in anaphase cells containing a single focus was almost always twice as bright as each of the plasmid foci in cells containing two separate foci or the single plasmid focus in G1 cells (prior to plasmid replication) (Fig. 3.7). The intensity of a plasmid focus in single focus containing anaphase cells relative to that in two foci containing cells was 1.68 for the *STB* reporter plasmid and 1.86 for the *ARS* reporter plasmid. Similar ratios were estimated for metaphase cells also (Fig. 3.7). We conclude that 1:0 and 0:1 patterns denote missegregation of plasmid sisters (2:0 and 0:2) rather than a failure of plasmid replication.

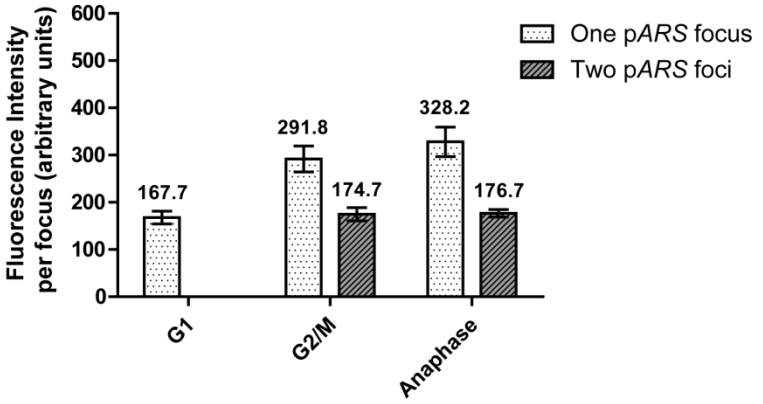
We do not have a good explanation for the colocalization of replicated plasmids during anaphase in a sizable fraction of cells. One possibility is that plasmid sisters might adhere to a common substratum in the nucleus. While this might be true for the *STB* plasmid, it is unlikely to be the case for the *ARS* plasmid. *ARS* plasmids have been shown to move rapidly and freely within the nucleus, and do not tend to associate to the nuclear periphery (Gehlen et al., 2011). Another possibility is that the colocalized plasmids have escaped decatanation by topoisomerase II. For our experiments, what is relevant is whether or not replicated plasmid copies segregate equally or unequally and not whether missegregated plasmids are colocalized or not.



	Bias in missegregation (%)	
	Mother	Daughter
p*STB* [Cir$^+$]	57.3	42.7
p*STB* [Cir^0]	94.8	5.2
p*STB* [Cir^0] + P*ADH*-R1/R2	64.0	36.0
p*STB* [Cir^0] + P*GAL*-R1/R2	63.6	36.4

Figure 3.6 Complementation of Rep proteins restores the segregation of *STB* reporter under [Cir^0] background. Expression of Rep1/Rep2 under *GAL* or *ADH* promoter restored the segregation and the bias status of *STB* reporter under the absence of native 2 micron circle.





	$I_{(1)} / I_{(2)}$	
	G2/M	Anaphase
pSTB	1.72	1.68
pARS	1.67	1.86

Figure 3.7 Fluorescence intensities of a single plasmid focus or two plasmid foci in G2/M and anaphase cells. Fluorescence intensities of the individual plasmid foci were measured and plotted as average intensity per focus. While G1 cells contained a single plasmid focus, G2/M cells contained either one focus or two foci. The anaphase population assayed was a subset containing one plasmid focus or two plasmid foci in one nucleus with the other nucleus being plasmid-free (missegregation). The ratios of the intensity of the single focus I(1) to the mean intensity of the two foci I(2) are shown.

3.3.3 Expression of the monopolin complex during mitosis causes missegregation of the 2 micron plasmid

In order to accomplish monopolin assembly at kinetochores during mitosis, *CDC5* and *MAM1* genes were placed under the control of the *GAL* promoter, *MAM1* at its native genomic locale and *CDC5* was the extra copy integrated at the *URA3* locus (Monje-Casas et al., 2007). These genes, along with the R recombinase gene, were expressed in G1 arrested cells by galactose induction prior to their release into the cell cycle in galactose containing medium. While Cdc5 can mediate the release of Lrs4 and Csm1 from the nucleolus, their recruitment to kinetochores is dependent on Mam1. The mitotic cell cycle is not perturbed significantly by monopolin, except for a brief delay in the degradation of the anaphase inhibitor securin due, presumably, to a transient activation of the spindle checkpoint (Monje-Casas et al., 2007). All of the assays for probing the effect of monopolin on plasmid segregation were carried out in [Cir^+] host strains with the single copy *STB* and *ARS* plasmids as reporters.

During mitosis in the presence of monopolin, equal segregation of the *STB* reporter plasmid dropped to 39.2% (from 63.7% in the absence of monopolin; Fig. 3.8A), and that of chromosome IV to 65.8% (from 100% in the absence of monopolin; Fig. 3.8B). The relative decrease in equal segregation, normalized to mitosis without monopolin expression, was 38.5% for the *STB* plasmid ([63.7 – 39.2] ÷ 63.7), nearly identical to that of chromosome IV (34.2%). By contrast, monopolin did not make any difference in the equal segregation frequency of the *ARS* reporter plasmid (Fig. 3.8C). The frequency of monopolin induced chromosome IV missegregation observed in our assays agrees well with that reported previously (Monje-Casas et al., 2007).

The missegregation of the *STB* plasmid sisters as a consequence of monopolin expression showed only a very small mother bias (54.4%; Fig. 3.8D). This near lack of bias was strikingly different from the strong mother bias (94.8%; Fig. 3.5) observed when missegregation was imposed by the lack of Rep proteins (in a [Cir^0] strain). The *ARS* plasmid sisters missegregated with a clear-cut mother bias regardless of the presence or absence of monopolin during mitosis. However, there was a reproducible decrease in the magnitude of this bias (from 91.8% to 69.3%; Fig. 3.8D) in cells expressing monopolin. Reasons for this modulation in segregation bias are not known. As was reported previously (Monje-Casas et al., 2007), chromosome IV missegregation due to monopolin was essentially unbiased (54.1%; Fig. 3.8D).

Taken together, our data suggest that the tendency of a pair of *STB* sister plasmids and that of a pair of sister chromatids to co-segregate under the influence of monopolin are strongly correlated. Furthermore, the co-segregation of sisters is not significantly biased towards mother or daughter in either case. These features of the *STB* reporter plasmid distinguishes it from an *ARS* reporter plasmid whose missegregation frequency is unaffected by monopolin. Furthermore, at least qualitatively, the mother bias of the *ARS* plasmid is also unaltered in the presence of monopolin.

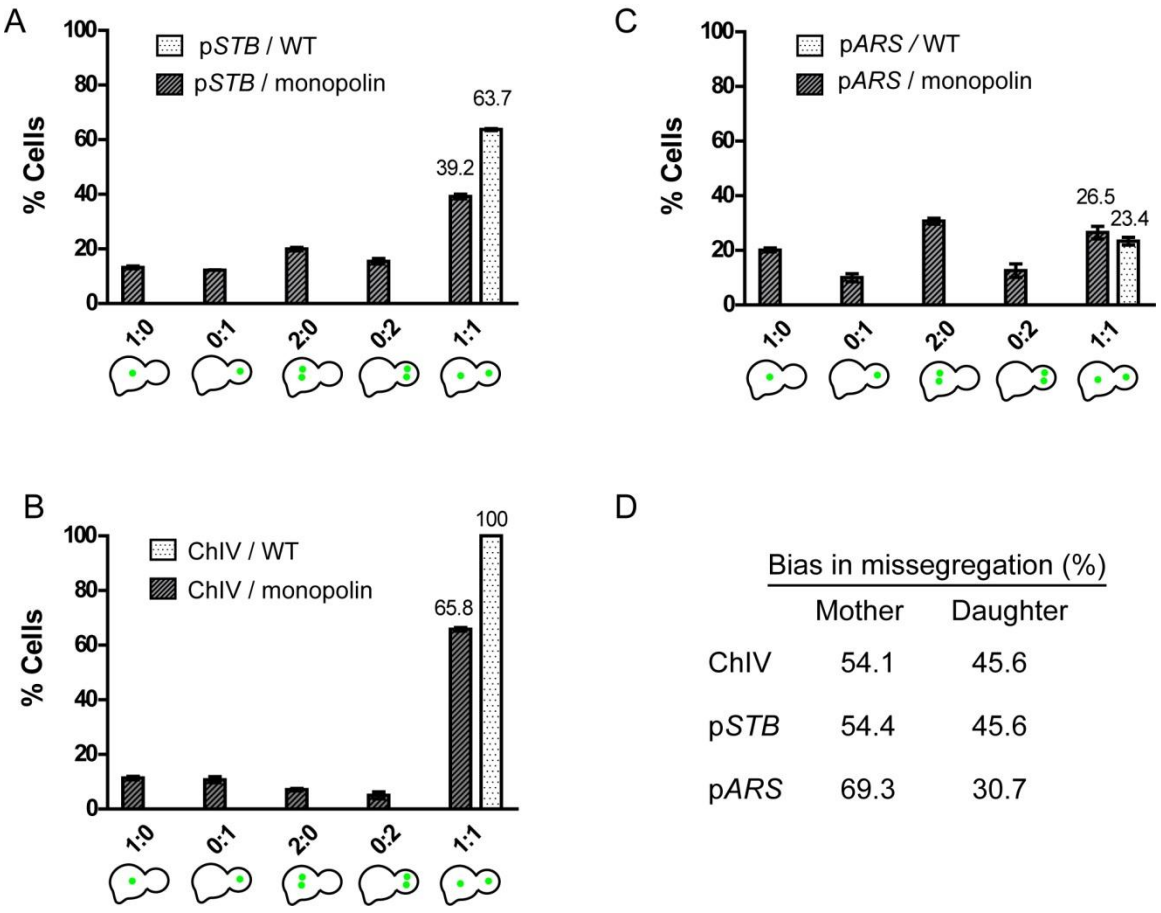


Figure 3.8 Segregation of reporter plasmids and chromosome IV during a mitotic cell cycle with the assembly of the monopolin complex. For monopolin assembly, *MAM1* and *CDC5* expression was induced in the experimental strain from the *GAL* promoter when G1 arrested cells were shifted to galactose medium (following the procedure described in Fig. 3.4). All segregation assays were performed in [Cir$^+$] strains. The segregation results and the mother or daughter bias during missegregation are displayed as under Fig. 3.5. Each segregation value is derived from 100-200 cells.

3.3.4 Co-segregation of *STB* sister plasmids in the presence of monopolin is compounded by expression of *SPO13*

The *SPO13* gene, expressed exclusively during meiosis (Buckingham et al., 1990), is required for the maintenance of centromeric cohesion and kinetochore co-orientation during meiosis I, and for progression of meiosis through the second cell division without an additional round of DNA synthesis (Katis et al., 2004; Lee et al., 2004). In the absence of Spo13, cells go through only the first meiotic division, giving rise to two spores containing a mixture of equationally segregated (sisters separated) and reductionally segregated (homologues separated) chromosomes (Hugerat and Simchen, 1993; Klapholz and Esposito, 1980a, b). Overexpression of *SPO13* during meiosis and mitosis causes a transient delay in securin destruction, and leads to metaphase cell cycle arrest by inhibiting the cleavage of the Rec8 and Mcd1 subunits of cohesin, respectively (Lee et al., 2002; McCarroll and Esposito, 1994). When *SPO13* is overexpressed along with *CDC5* and *MAM1* during mitosis, no cell cycle arrest occurs; however, sister chromatid missegregation is elevated dramatically (Monje-Casas et al., 2007). The Spo13 effect appears not to be mediated through the monopolin complex, but rather by interfering with kinetochore function and/or cohesin disassembly at centromeres (Monje-Casas et al., 2007). Nevertheless, the strikingly high incidence of sister chromatid co-orientation brought about by the two together provides the opportunity to test whether the co-segregation of the *STB* plasmid sisters is also elevated by their combined action. We induced the expression of *SPO13*, controlled by the *GAL* promoter, along with that of *CDC5* and *MAM1* in G1 arrested cells, released them in presence of galactose, and scored plasmid segregation in anaphase cells.

The 1:1 segregation of the *STB* plasmid sisters was reduced to 17.8% under monopolin plus Spo13 (Fig. 3.9A), an additional drop of 21.4% from that observed under monopolin alone (39.2%; Fig. 3.8A). Chromosome IV segregated 1:1 in only 2.2% of the cells during the monopolin plus Spo13 mitosis (Fig. 3.9B), signifying a 63.6% decrease from that conferred by monopolin alone (Fig. 3.8B). In our assays, the extent of chromosome missegregation due to monopolin and Spo13 combined was larger than the ~90% value reported in an earlier study (Monje-Casas et al., 2007). When normalized to the data from regular mitosis, monopolin, together with Spo13, decreased the equal segregation frequency of the *STB* plasmid by 72.1% ([63.7 – 17.8] ÷ 63.7) and that of chromosome IV by 97.8%. Unlike monopolin alone, monopolin plus Spo13 caused a decrease in the 1:1 segregation of the *ARS* plasmid sisters as well (from 23.4% to 11%; Fig. 3.9C). However, in contrast to the *STB* plasmid and chromosome IV, whose missegregation showed little or no bias (56.8% for the *STB* plasmid; 51.7% for chromosome IV), the missegregation of the *ARS* plasmid was still strongly biased towards the mother (82.2%; Fig. 3.9D).

The cosegregation patterns of sister *STB* plasmids and sister chromatids due to monopolin plus Spo13 are qualitatively consistent with those due to monopolin alone. Although, the additional effect of Spo13 was more pronounced for sister chromatids than the plasmid, they both missegregated without strong mother or daughter bias.

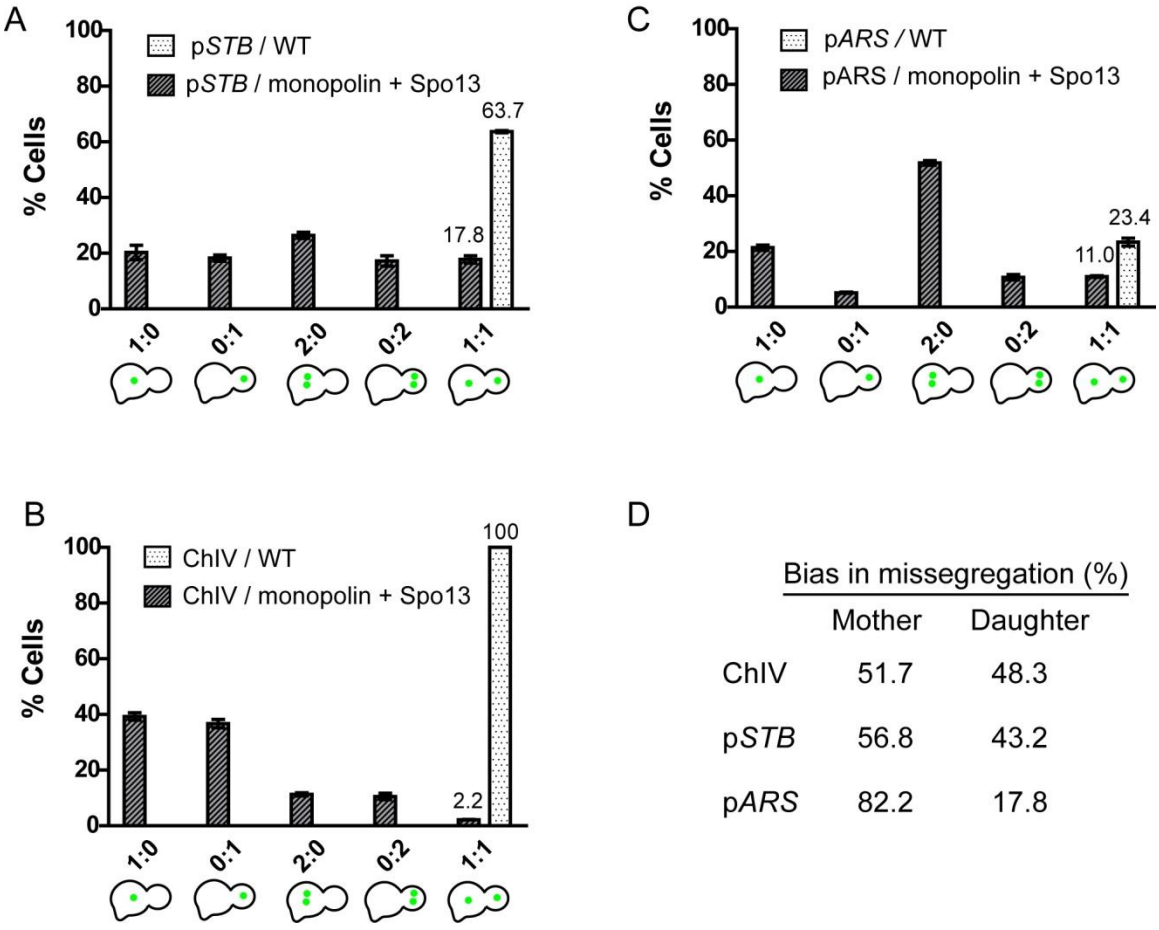


Figure 3.9 Mitotic segregation of reporter plasmids and chromosome IV under the influence of monopolin plus Spo13. The only difference in the experimental protocol from that described under Fig. 3.8 is that *SPO13*, *CDC5*, and *MAM1* were co-induced in the G1 arrested cells prior to their release.

3.3.5 The combined effects of monopolin and *ipl1-321* on plasmid segregation

Ipl1 (Aurora B kinase), in association with Sli15 (INCENP) and Bir1 (Survivin), regulates several aspects of chromosome segregation during mitosis and meiosis in *S. cerevisiae* (Biggins et al., 1999; Kim et al., 1999; Makrantoni and Stark, 2009). These include kinetochore microtubule attachment and orientation (Monje-Casas et al., 2007; Tanaka et al., 2002), spindle assembly and stability (Ducat and Zheng, 2004) and coordination of chromosome segregation and cytokinesis (Norden et al., 2006). In the absence of Ipl1 function during the mitotic cell cycle, chromosomes missegregate because co-oriented sister kinetochores fail to detach from the spindle and establish bi-orientation. Furthermore, these kinetochores preferentially attach to microtubules emanating from the old spindle body, which migrates to the bud compartment (Tanaka et al., 2002). As a result, sister chromatid missegregation caused by the lack of Ipl1 function has a distinct daughter bias (Tanaka et al., 2002). The reasons for the biased co-orientation of sisters are not entirely clear, but might arise from the completion of kinetochore assembly prior to the maturation of the new spindle pole body. If the *STB* plasmid does hitchhike on chromosomes, the daughter bias imparted by the absence of Ipl1 on chromosomes should also apply to the plasmid. We tested this prediction by following plasmid segregation in *ipl1-321* (T^s) mutant cells going through mitosis at the semi-permissive temperature (34℃) in presence of the monopolin complex.

The relative decrease in the 1: 1 segregation frequency due to monopolin plus the *ipl1-321* mutation was similar between chromosome IV (51.3%; Fig. 3.10B) and the *STB* plasmid (58.7% = [63.7 – 26.3] ÷ 63.7; Fig. 3.10A); and so was the daughter bias in missegregation (64.0% for chromosome IV and 60.0% for the *STB* plasmid; Fig. 3.10D).

The *ARS* plasmid also showed an increase in missegregation under these conditions (Fig. 10C). However, unlike the *STB* plasmid, the direction of the bias was opposite to that of chromosome IV (65.8% towards mother; Fig. 3.10D). Similar values for the decrease in equal segregation frequencies (54.1% and 54% for chromosome IV and the *STB* plasmid, respectively) and segregation bias (61% for chromosome IV; 58.2% for the *STB* plasmid) were observed when the cell cycle proceeded at 34℃ even without the induction of *CDC5* and *MAM1* (Fig. 3.11). The dominance of the *ipl-321* mutation over the Mam1 complex with respect to the magnitude of chromosome missegregation and the direction of its bias were also observed previously (Monje-Casas et al., 2007).

Thus, the effects of the *ipl1-321* mutation, analogous to those of monopolin or monopolin plus Spo13, further highlight the correlation between the *STB* plasmid and a chromosome, and the lack of it between an *ARS* plasmid and a chromosome, when sister chromatids are forced to missegregate.

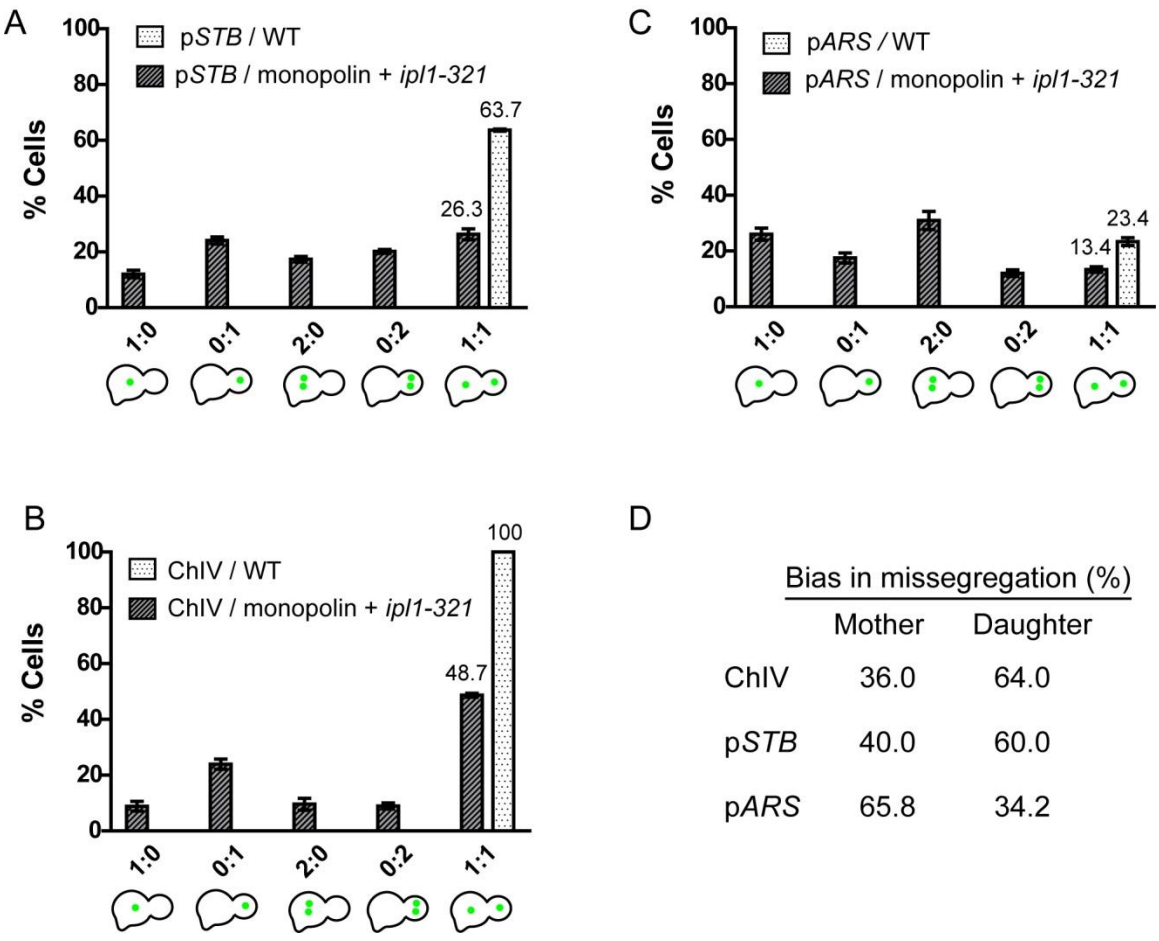


Figure 3.10 Segregation of reporter plasmids and chromosome IV under monopolin assembly combined with partial inactivation of Ipl1. The analysis was performed at 34℃, which is semi-permissive for Ipl1 function.

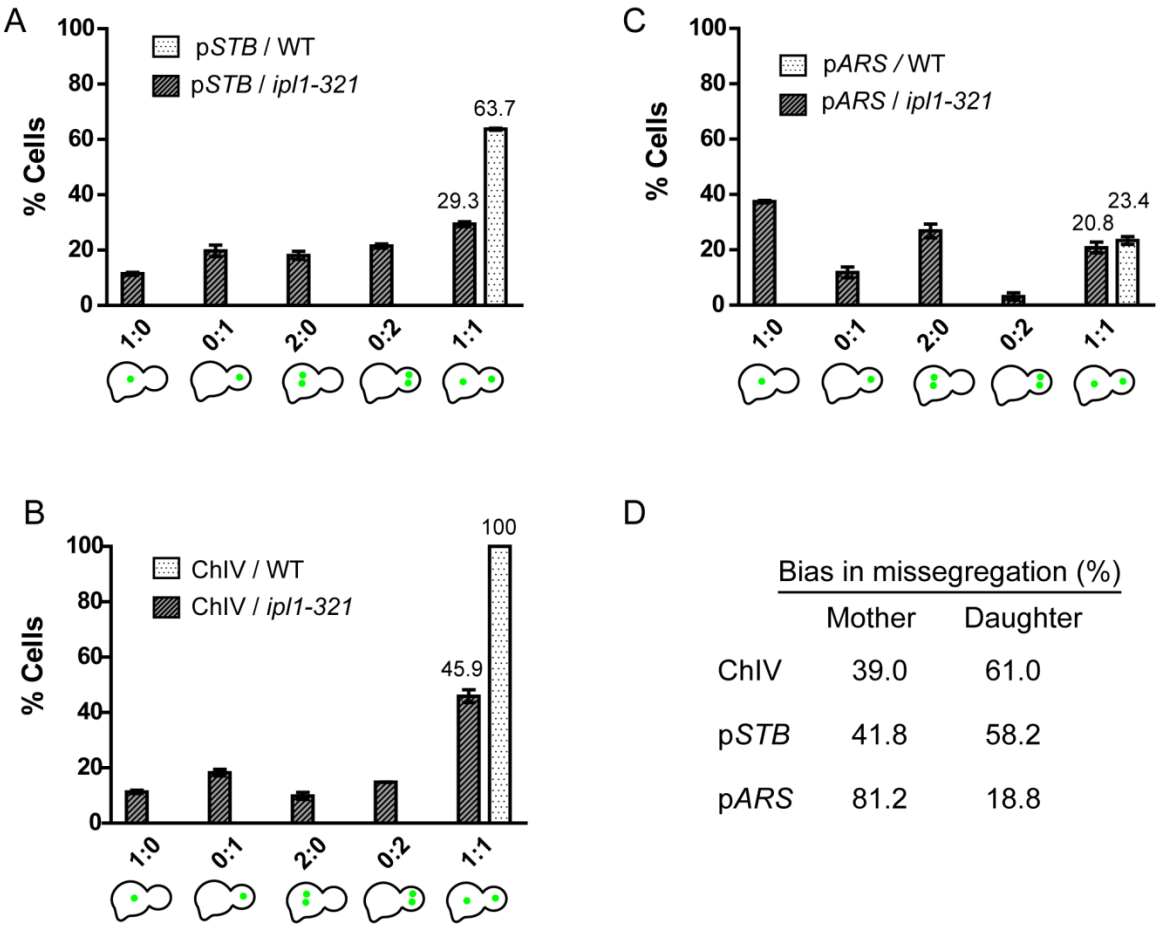


Figure 3.11 Plasmid segregation during a mitotic cell cycle under partial inactivation of Ipl1. Plasmid segregation analysis was carried out in *ipl1-321* strains that were engineered for generating the single copy reporter plasmids by site-specific excision, but not for the expression of *MAM1* or *CDC5*. Assays were done at the semi-permissive temperature of 34°C.

3.3.6 Effect of microtubule depolymerization on the monopolin induced missegregation of the *STB* reporter plasmid

The integrity of the mitotic spindle is an essential requirement for equal segregation of the 2 micron plasmid. When microtubules are depolymerized by treatment with nocodazole, normal plasmid localization close to the spindle pole body and plasmid association with chromosome spreads are disrupted (Mehta et al., 2005). Furthermore, the interactions of Cse4 and cohesin with *STB*, which are normally substoichiometric (Ghosh et al., 2010; Huang et al., 2011), become undetectable in the absence of the spindle (Mehta et al., 2005). Current evidence suggests that the plasmid utilizes the spindle and the spindle associated Kip1 motor to transport itself to its nuclear address (Cui et al., 2009), where the subsequent steps of plasmid partitioning are facilitated. When cells treated with nocodazole and arrested in G2/M are allowed to reassemble the spindle and are released from arrest, chromosomes segregate normally. However, a multi-copy *STB* reporter plasmid missegregates (Mehta et al., 2005). So far, spindle disassembly is the only means, other than inactivating the Rep-*STB* system, by which it has been possible to fully uncouple plasmid segregation from chromosome segregation. We wished to test the prediction then that the lack of spindle until G2/M and spindle assembly in G2/M would impact sister chromatid segregation and *STB* plasmid segregation distinctly during mitosis in the presence of monopolin.

During a cell cycle that progressed up to G2/M in the absence of the spindle and then continued with a restored spindle, the *STB* sister plasmids showed a reduction in 1:1 segregation from 63.7% to 39.0% (Fig. 3.12A). However, this diminished equal segregation was not as severe as that recorded for the *STB* reporter plasmid in the absence

of Rep1 and Rep2 ([Cir^0]; 27.5%) or for the *ARS* reporter plasmid (lacking *STB*; 23.4%) (Fig. 3.5). Furthermore, unlike the strong mother bias in segregation induced by the lack of the REP-*STB* system, the mother bias was absent or quite weak (53.1%) under the spindle disassembly and reassembly regimen (Fig. 3.12D). Clearly, the mechanisms by which the *STB* plasmid is uncoupled from chromosomes due to the absence of the partitioning system or due to the pre-G2/M absence of the spindle are not the same. Spindle restoration in G2/M fully supported 1:1 segregation of sister chromatids (Fig. 3.12B). Manipulation of spindle integrity during the cell cycle did not affect the segregation of the *ARS* reporter plasmid (Fig. 3.12C) or its pronounced mother bias (90.7%; Fig. 3.12D).

When monopolin expression was superposed on spindle disassembly-reassembly, the co-segregation of chromosome IV increased dramatically. The relative decrease in 1:1 segregation was 72.4% ([99.7 – 27.5] ÷ 99.7) (Fig. 3.12B). The reason why the monopolin effect is exacerbated by delaying spindle assembly until G2/M is not clear. Perhaps, the extra time increases the fraction of sister kinetochores stably clamped by monopolin before they are captured by the spindle, facilitating their co-orientation. The significant result is that, in comparison to chromosome IV, the *STB* plasmid showed only a modest increase in co-segregation of sisters by monopolin when a functional spindle was absent until G2/M. The relative reduction in 1:1 plasmid segregation was 32.1% ([39.0 - 26.5] ÷ 39).

Thus, the correlation between sister chromatids and *STB* plasmid sisters in their monopolin imposed co-segregation is strongly diminished when the spindle contribution towards plasmid segregation (but not chromosome segregation) is ablated.

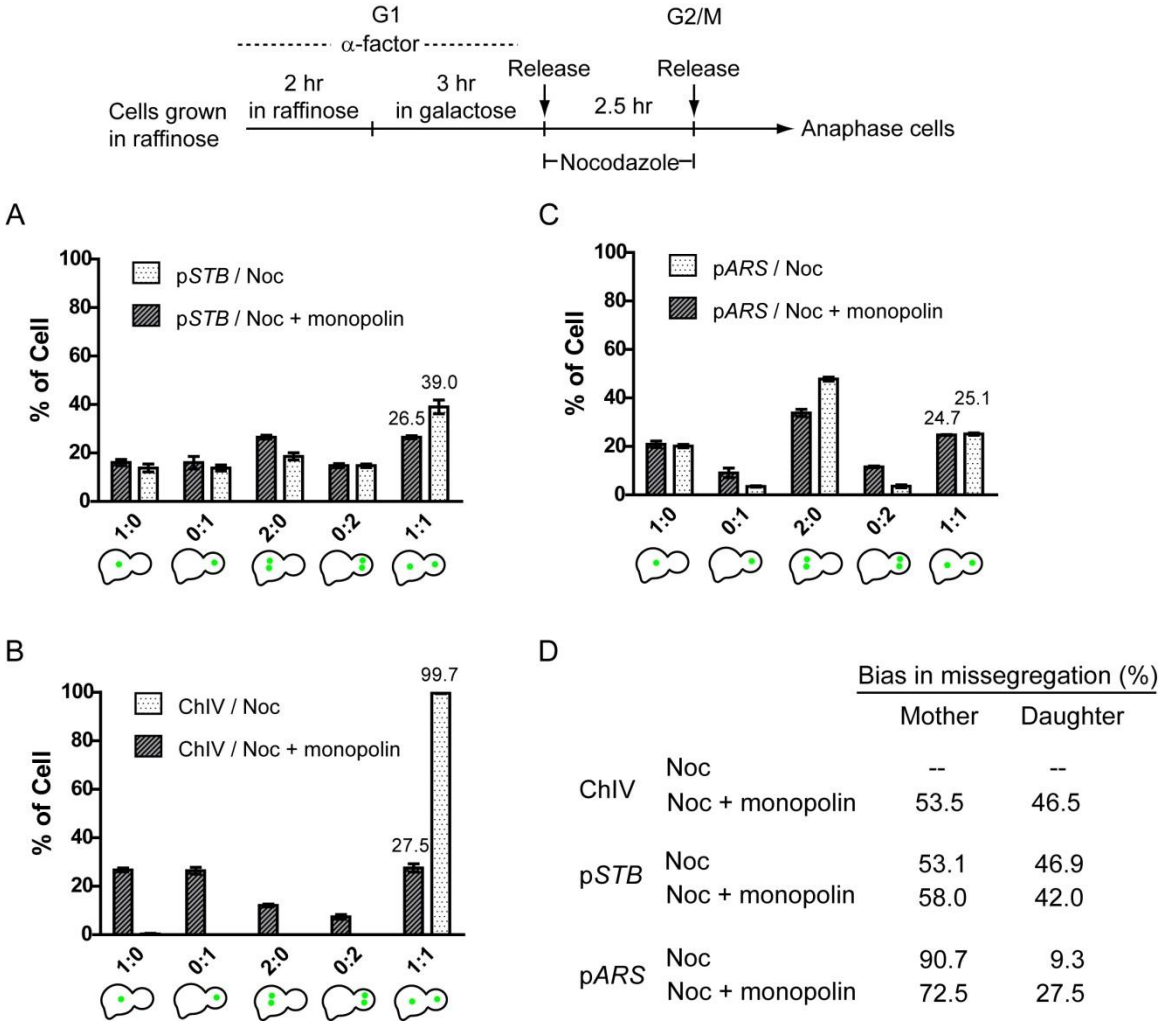


Figure 3.12 Effects of delaying spindle assembly or delaying spindle assembly in the presence of monopolin on the segregation of reporter plasmids and chromosome IV. The experimental scheme is outlined. The cells were released from G1 in the presence of nocodazole so as to implement cell cycle progression till G2/M without the mitotic spindle. Removal of nocodazole resulted in restoration of the spindle and resumption of the cell cycle.

3.3.7 Segregation of chromosome V sisters and *STB* plasmid sisters during cohesin depleted or cohesin depleted but monopolin supplemented mitosis

The cohesin complex, assembled at the centromere and along chromosome arms at roughly regular intervals, holds sister chromatids together from S phase through the onset of anaphase (Nasmyth, 2011; Onn et al., 2008). The cohesin bridge provides sufficient time for sister kinetochores to biorient themselves on the mitotic spindle before the anaphase promoting complex (APC) is activated. The destruction of securin, the release of separase and separase mediated cleavage of the cohesin subunit Mcd1 result in the 1:1 segregation of sister chromatids. The cohesin complex also associates with the *STB* locus in a Rep1 and Rep2 dependent manner (Mehta et al., 2002). Furthermore, the timing of this association and its life-time during the cell cycle are matched to cohesin-chromosome association (Huang et al., 2004; Mehta et al., 2002). However, there is the caveat that only a small fraction of the plasmid molecules are occupied by cohesin (Ghosh et al., 2010). The equal segregation of close to single copy *STB* plasmids (Ghosh et al., 2007) or a truly single copy *STB* plasmid (this study) by the hitchhiking mechanism could, in principle, be facilitated by cohesin. The proximity of a pair of sister plasmids, bridged by the cohesin complex, is likely to increase their chances of associating with symmetrically disposed tethering sites on sister chromatids, which are also bridged by cohesin. If this is true, lack of cohesin assembly during a cell cycle should uncouple *STB* plasmid segregation from sister chromatid segregation and from sister chromatid co-segregation promoted by monopolin. The tests of these predictions are summarized below. The reference chromosome for these assays was chromosome V, not IV.

In order to deplete cohesin during the mitotic cell cycle, cells from the experimental strain expressing the *MCD1* gene from the *MET3* promoter were arrested in G1, conditioned with methionine and released into medium supplemented with methionine (Fig. 3.13). Under these conditions, chromosome V showed 65.4% 1:1 segregation (Fig. 3.14A). Under the absence of cohesin, but with monopolin expressed, the 1:1 segregation of chromosome V was further decreased to 49% (Fig. 3.14A), or an additional relative drop of 25.1% ([65.4 – 49] ÷ 65.4) imposed by monopolin. The 1:1 segregation frequencies of the *STB* plasmid sisters were almost identical (36.2% and 36.6%) in the absence of cohesin and in the absence of cohesin but the presence of monopolin, respectively (Fig. 3.14B). The corresponding values for the *ARS* plasmid were 26.8% and 26.2%, respectively (Fig. 3.14C).

The correlated co-segregation of *STB* plasmid sisters and chromosome IV (chromosome V) sisters that occurs during mitosis in the presence of monopolin is terminated if this mitosis is also cohesin deprived. According to Monje-Casa et al. (Monje-Casas et al., 2007), when sister chromatids carrying a centromere proximal fluorescence tag co-segregate under the influence of monopolin and cohesin depletion, the two fluorescence signals are most often tightly associated into a single fluorescent dot. Missegregation resulting from the absence of cohesin only is frequently signified by two separate fluorescent dots. Based on this distinction, it is argued that monopolin mediated chromosome co-segregation occurs independently of cohesin. In this case, uncoupling of plasmid segregation, due to lack of cohesin, from monopolin induced sister chromatid co-segregation can only occur through cohesin's effect on the plasmid (see also 'Discussion').

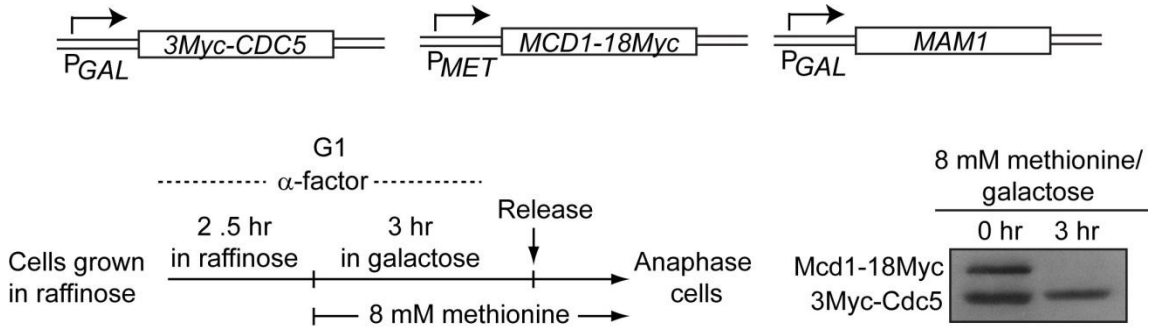


Figure 3.13 The scheme for implementing cohesin depletion during the cell cycle. Cohesin was depleted by turning off the expression of *MCD1*-18Myc from the *MET* promoter by addition of 8 mM methionine. The absence of the Mcd1 protein under this condition was verified by western blot analysis. In assays requiring monopolin assembly, *MAM1* and *CDC5* were induced from the *GAL* promoter by shifting cells to galactose containing medium. The inclusion of methionine in the medium did not affect the induction of the *GAL* promoter, as shown by the western blot analysis for Cdc5. For assays without monopolin assembly, the medium contained glucose as the carbon source.

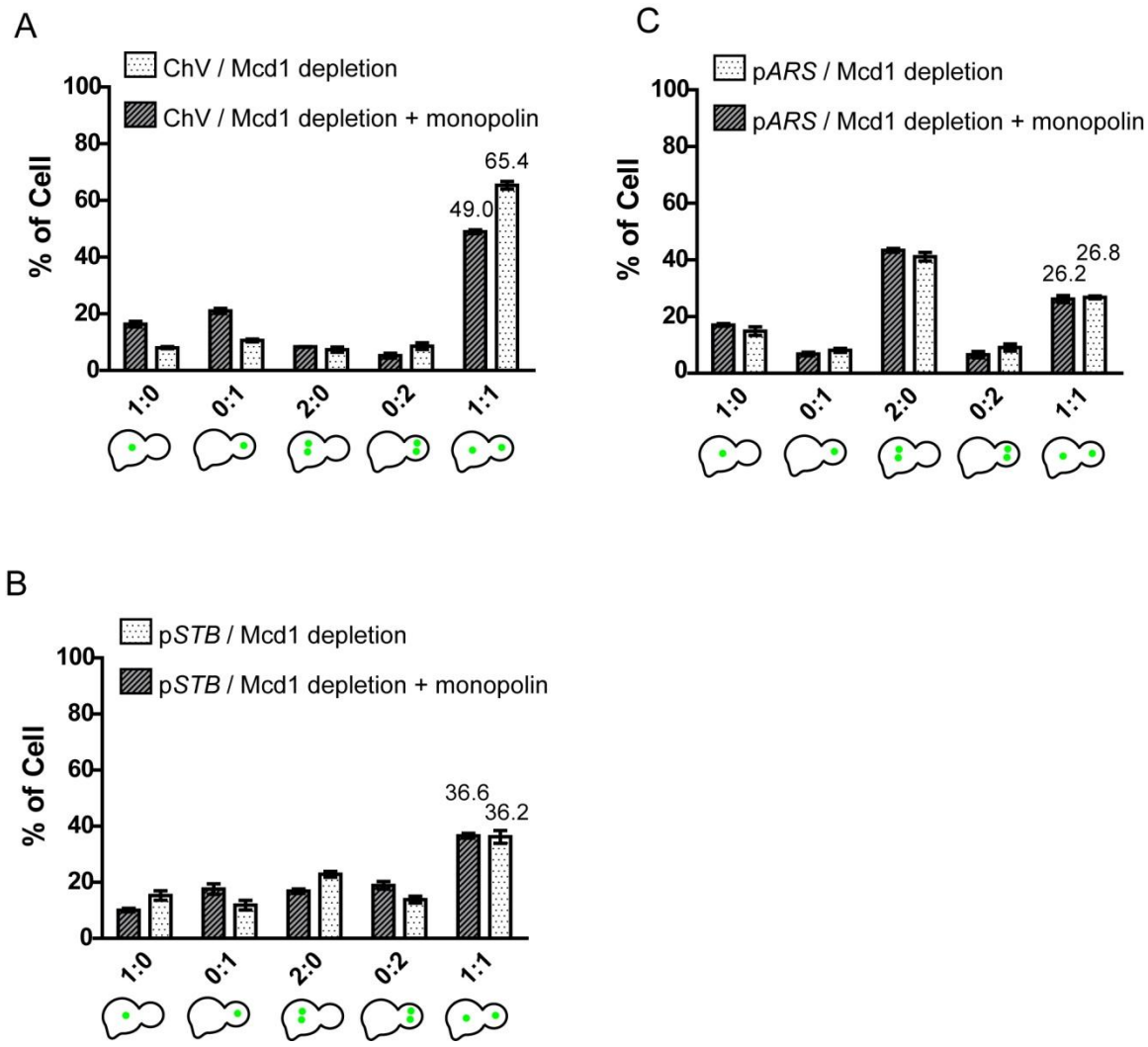


Figure 3.14 Effects of cohesin depletion or cohesin depletion and monopolin assembly on reporter plasmid and chromosome segregation. Cohesin was depleted during the cell cycle by turning off the *MET* promoter from which *MCD1*-18Myc was expressed (see Fig. 3.13). In this set of experiments, the reference chromosome was V (and not IV utilized in all the other analyses). This chromosome was also fluorescence tagged near the centromere by TetR-GFP and $[TetO]_{224}$. The segregation data are depicted in A-C.

3.3.8 The effect of monopolin on *STB* plasmid sisters in promoting their co-segregation with sister chromatids is indirect

Mam1 associates with kinetochores from late prophase I to the onset of anaphase I during meiosis (Rabitsch et al., 2003; Tóth et al., 2000). Non-centromeric chromosomal loci are not sites of Mam1 localization (Rabitsch et al., 2003). Furthermore, Mam1 and Lrs4 colocalize within the nucleus in metaphase when *MAM1* and *CDC5* are overexpressed during mitosis (Monje-Casas et al., 2007). Since several centromere associated proteins also interact with *STB*, Kip1 and cohesin, for example, it is formally possible that monopolin affects *STB* plasmid segregation directly rather than through chromosome segregation. For example, analogous to clamping down sister kinetochores (Corbett et al., 2010), monopolin might also hand-cuff sister *STB*s by interacting with the plasmid partitioning complex. Under this scenario, the mitotic co-segregation of sister *STB* plasmids in presence of monopolin need not necessarily be dependent on sister chromatid co-segregation. In order to address the possibility of direct monopolin action on the plasmid, we have queried the presence of Mam1 at *STB* by chromatin immunoprecipitation (ChIP).

An antibody to HA-tagged Mam1 immunoprecipitated centromere DNA from cells induced for the expression of 3HA-*MAM1* and *CDC5* from the *GAL* promoter. No *CEN* DNA was detectable in the immunoprecipitate from uninduced cells (Fig. 3.15). The signals for *STB* were within the range of those for two arm loci from chromosomes III and V, with no significant difference between cells induced or uninduced for *MAM1* expression (Fig. 3.15). We conclude that Mam1, and monopolin by inference, is not recruited at *STB*.

As far as we know, the action of monopolin is restricted to centromeres, where it promotes the co-orientation of sister kinetochores and, consequently, co-segregation of sister chromatids. Monopolin brings about a similar effect on *STB* sister plasmids, apparently vicariously. This seeming anomaly is easily resolved if the plasmid sisters are tethered to a pair of sister chromatids in a one-to-one fashion.

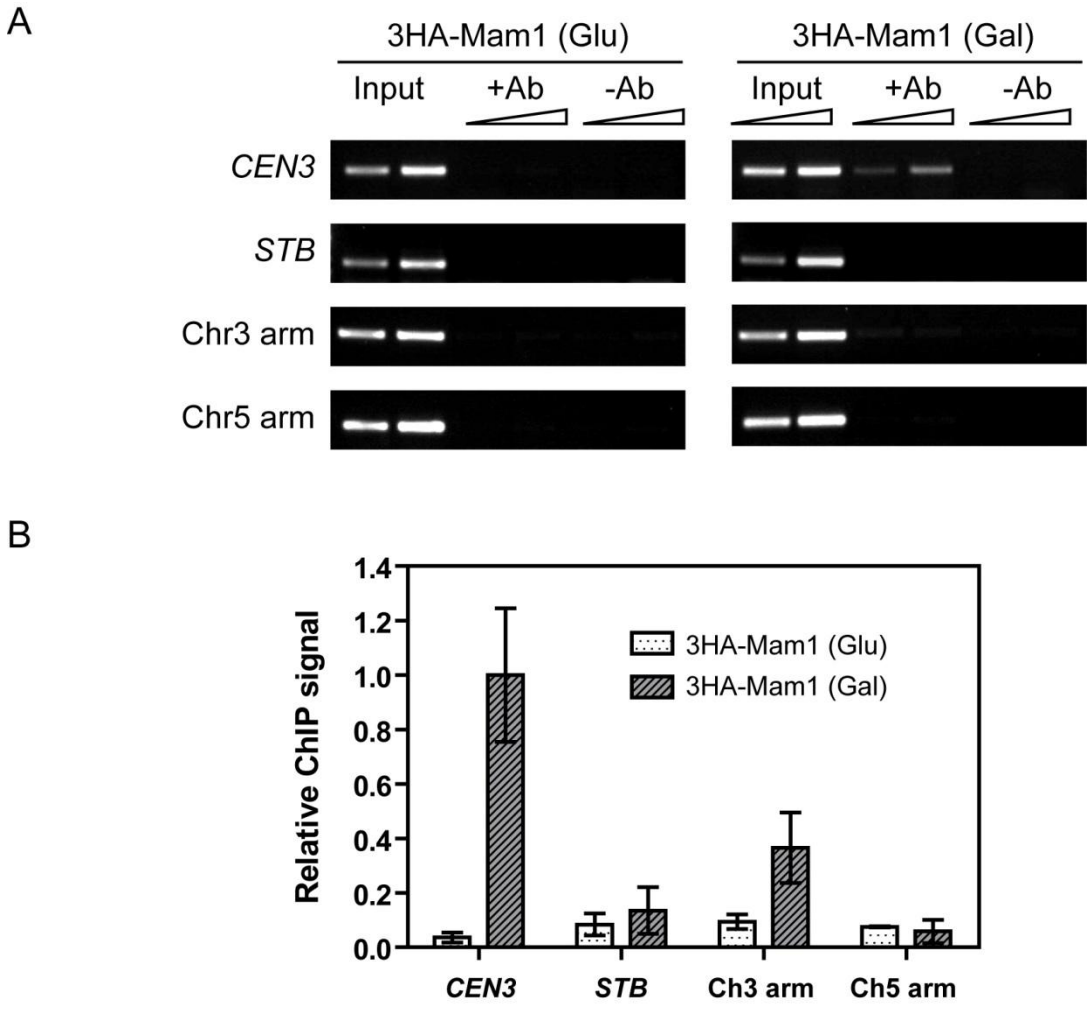


Figure 3.15 ChIP assays to test Mam1-*STB* association. (A) The associations of Mam1, tagged by 3-HA, with *CEN3* and *STB* was probed by ChIP using an antibody to the HA epitope. *MAM1*, placed under the control of the *GAL* promoter, was not expressed in glucose grown cells. Two loci from chromosomes III and V were utilized as controls. (B) The relative ChIP signals from the analysis were plotted based on the intensities of the PCR amplified DNA bands after normalizing them to the input signals.

3.4 Discussion

Integration into the host DNA confers on a selfish element the same stability as its host genome. In the absence of integration, non-covalent association with chromosomes provides perhaps the next most reliable means of propagation for such an element. Papilloma and gammaherpes family viruses exemplify the utility of tethering to chromosomes in the long-term maintenance of viral episomes (Cotter Ii and Robertson, 1999; Harris et al., 1985; Ilves et al., 1999; Lehman and Botchan, 1998). The partitioning systems of Epstein-Barr virus (EBV) and bovine papilloma virus (BPV) have been reconstituted in *S. cerevisiae* (Brannon et al., 2005; Kapoor et al., 2001). However, direct demonstration that segregation follows the chromosome tethering mechanism under this non-native context is lacking. The 2 micron plasmid, a highly successful selfish DNA element inhabiting a lower eukaryote, resembles the viral episomes in that its stable segregation is apparently coupled to that of the host chromosomes (Mehta et al., 2002; Velmurugan et al., 2000). However, there is no evidence that the plasmid hitchhikes on chromosomes.

A serious impediment to verifying the hitchhiking model is the inability to resolve individual yeast chromosomes by cytology. An additional complexity is the potential existence of multiple plasmid tethering loci distributed on more than one chromosome. Forced missegregation of individual chromosomes during a cell cycle by conditional *CEN* inactivation does not result in the corresponding missegregation of an *STB* reporter plasmid (unpublished data). If the plasmid does hitchhike, it does not do so on any one chromosome. Standard 2 micron circle reporter plasmids tagged by fluorescence are present in several copies and form closely clustered multiple foci, which makes it

difficult to score their segregation during a cell cycle accurately. Segregation behavior of close to single copy *STB* reporter plasmids observed previously (Ghosh et al., 2007) suggests that sister plasmids split apart from each other and move to opposite poles. This one-to-one segregation can be accommodated by the hitchhiking model only if sister copies of a plasmid are tethered to sister chromatids. The present study challenges the model by utilizing a high-precision reporter system in which the plasmid copy number (prior to replication) is kept strictly as one. By characterizing the mitotic segregation of *STB* plasmid sisters under forced co-segregation of sister chromatids, we conclude that the hitchhiking model with sister plasmids tethered to sister chromatids best accounts for the mechanism of 2 micron plasmid partitioning.

3.4.1 *STB* plasmid sisters co-segregate when co-segregation is imposed on sister chromatids

The nearly bias-free relative increases in the co-segregation of *STB* plasmid sisters and of sister chromatids promoted by monopolin (34.2% for chromosome; 38.5% for plasmid) and monopolin plus Spo13 (72.1% for plasmid; 97.8% for chromosome) are well correlated. The correlation is consistent with sister plasmids being tethered to sister chromatids in a one-to-one fashion. This mode of plasmid association with chromosomes may be referred to as ‘symmetric tethering’ to distinguish it from the association of plasmid sisters to the same chromosome (asymmetric tethering) or to distinct chromosomes (random tethering). Asymmetric tethering, which will missegregate plasmid sisters, is inconsistent with the equal segregation frequency of ~64% during normal mitosis under a functional Rep-*STB* system. Random tethering is also untenable,

as it can only achieve 50% equal segregation due to independent assortment of individual chromosomes.

The lack of perfect correlation between plasmid and chromosome (72.1% versus 97.8% 2:0 segregation, respectively) (This is decrease of 1:1 but not increase of 2:0) when missegregation is effected by monopolin plus Spo13 may indicate an innate upper limit to the efficiency of the plasmid partitioning system. Note that the equal segregation frequency of the *STB* reporter plasmid under a functional Rep-*STB* system (63.7%) is significantly lower than that of the chromosomes (~100%). In general, fluorescence tagged single copy *STB* reporter plasmids show 70-80% equal segregation in the single cell cycle assay. The stabilities of 2 micron plasmid derived vectors under non-selective growth are well below that of the native plasmid (lower by two to three orders of magnitude). Disrupting the genetic organization of the plasmid or insertion of extraneous sequences may have a significant deleterious effect on plasmid stability. Also note that the analysis of correlation between plasmid and chromosome is only with respect to chromosome IV. The monopolin plus Spo13 effect on individual chromosomes may not be the same with respect to co-segregation. The differences could arise from differences in chromosome sizes or other aspects of chromosome organization. If the *STB* plasmid can associate with any of the chromosomes (as inferred from previous observations), the monopolin plus Spo13 effect on the plasmid would be averaged over the effects on the entire chromosome set.

3.4.2 Imparting daughter bias to *STB* plasmid missegregation by inducing sister chromatids to co-segregate to the daughter

Disrupting the bi-orientation of sister kinetochores by inactivating Ipl1 leads to nearly the same relative decrease in the 1:1 segregation frequency for the *STB* plasmid (54.0%) as for chromosome IV (54.1%). A similar correspondence is also seen when mono-orientation is promoted by monopolin together with Ipl1 inactivation (51.3% for chromosome; 58.7% for plasmid). Unlike the bias-free missegregation induced by monopolin, the *ipl1-321* induced missegregation is distinctly biased towards the daughter for both the chromosome (61%) and the plasmid (58.2%). The daughter bias is also true for monopolin plus *ipl1-321* induced missegregation (64.0% for chromosome; 60.0% for plasmid). Note that the normal small mother bias in *STB* plasmid missegregation (57.3%) during normal mitosis is reversed towards the daughter when such a bias is imparted to chromosomes through Ipl1 inactivation. The correspondence between sister chromatids and *STB* plasmid sisters not only in the extent of missegregation but also the bias in missegregation is readily accommodated by the physical association between plasmid and chromosome.

3.4.3 Uncoupling 2 micron plasmid segregation from monopolin induced sister chromatid co-segregation

Segregation of the *STB* plasmid sisters can be unlinked from monopolin induced co-segregation of sister chromatids either by delaying the assembly of the mitotic spindle until G2/M or by running the cell cycle under depletion of the cohesin complex. Association of the 2 micron plasmid with chromosome spreads is dependent on the

mitotic spindle but not on cohesin (Mehta et al., 2005). The presence of the plasmid in these spreads is suggestive of, but does not prove, physical association between plasmid and chromosomes. As the spreads display unresolved chromosome masses, the possibility that the plasmid is associated with a subnuclear entity that is fortuitously included in the spreads cannot be ruled out. As already pointed out, spindle integrity is also a pre-requisite for the association of cohesin with *STB* (Mehta et al., 2005). Furthermore, the cell cycle timing of cohesin-*STB* association is critical in the equal segregation of the 2 micron plasmid (Mehta et al., 2002). When the spindle is assembled in G2/M, the plasmid localizes in chromosome spreads and cohesin associates with *STB*. Yet, these associations are futile with respect to plasmid partitioning.

There is a plausible common explanation for how cohesin depletion or lack of the spindle till late in the cell cycle might uncouple *STB* plasmid segregation from chromosome segregation. The spindle dependent assembly of cohesin at *STB*, concomitant with plasmid replication, may spatially confine plasmid sisters to promote their attachment to sister chromatids. In the absence of such containment, tethering to chromosomes would be random, reducing the theoretical efficiency of equal segregation of plasmid sisters to 50%. Since sister chromatids are also confined by cohesin, the probability of symmetric tethering of plasmid sisters to sister chromatids would be high. In the absence of cohesin, constraining chromosome sisters by monopolin will not help symmetric tethering, unless monopolin exerts a similar effect on plasmids as well. Indeed, Mam1 is not detected at *STB* but associates with *CEN*, as expected. This finding is in line with earlier observations that there is no assembly of kinetochore components at *STB* (unpublished data).

3.5 Summary and Perspectives

The present studies, in conjunction with prior observations, are in agreement with the hitchhiking model for 2 micron plasmid segregation. One-to-one segregation of sister plasmids during normal mitosis and co-segregation of plasmid sisters during aberrant versions of mitosis that co-segregate sister chromatids can only be accommodated by symmetric tethering. This type of plasmid-chromosome association may be promoted by cohesin assisted concurrent bridging of plasmid sisters and of sister chromatids. An important question is how the inferences from the segregation behavior of a single copy reporter plasmid translate to the native multi-copy 2 micron plasmid.

A fluorescence tagged multi-copy *STB* reporter plasmid exists as a group of 3 to 4 dynamic foci, and appear to segregate as a single clustered entity during mitosis (Velmurugan et al., 2000). The apparent segregation of the plasmid effectively as 'unit copy' (one cluster) is likely due to the small size of the yeast nucleus and the resolution limits of fluorescence microscopy. During mitosis under monopolin expression, all plasmid foci do not missegregate in unison (Fig. 3.16), suggesting that each focus, likely harboring several plasmid copies, is an independent unit in segregation. The requirement of cohesin, as suggested by the present studies, for symmetric tethering of sister plasmids or sister plasmid foci (in the multi-copy context) to sister chromatids would seem to be at odds with the highly substoichiometric association of cohesin with *STB* (Ghosh et al., 2010). As noted earlier, the association of Cse4 with *STB* is also highly substoichiometric (Huang et al., 2011). Furthermore, the steady state level of Cse4 is not significantly different between [Cir^0] and [Cir^+] yeast strains (Coffman et al., 2011; Lawrimore et al., 2011; our unpublished results). It is possible that these host factors need act on one or a

limited number of plasmid molecules to confer segregation competence on all molecules belonging to a group. The organization of multiple *STB*s into one functional unit by the Rep proteins, perhaps with assistance from host proteins, might provide a mechanism for limiting cohesin and Cse4 utilization by the plasmid.

The 2 micron plasmid is propagated efficiently to all four spores during meiosis (Brewer and Fangman, 1980). The mechanisms by which plasmid molecules segregate during the reductional and equational divisions of meiosis are unknown. If mitosis under monopolin expression mimics meiosis I, the prediction is that sister plasmids would co-segregate with sister chromatids during meiosis I. Single copy *STB* reporter plasmids would be valuable in characterizing the meiotic segregation of the 2 micron plasmid.

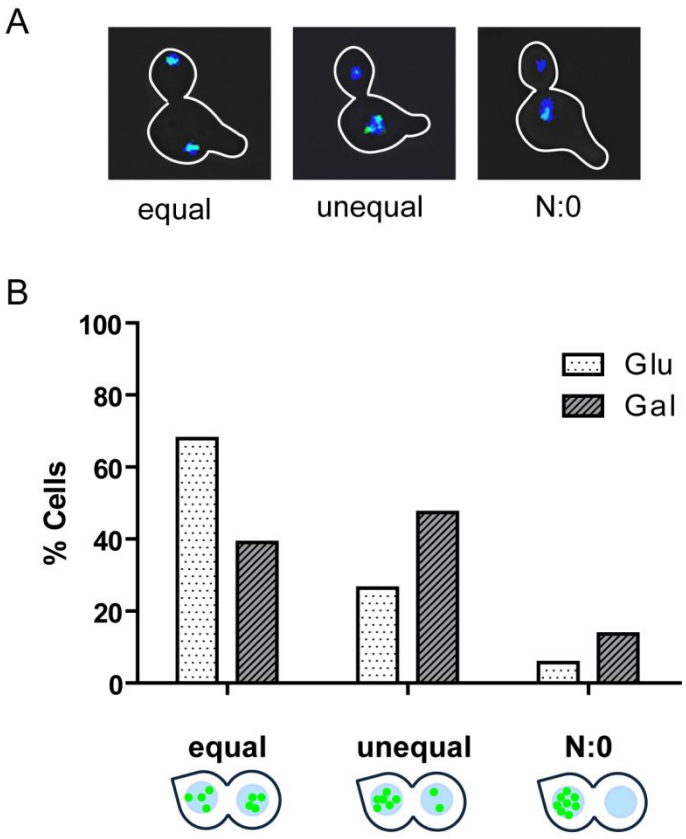


Figure 3.16 The segregation of a fluorescence tagged multi-copy reporter plasmid during a cell cycle under monopolin assembly along with *SPO13* expression was assayed in anaphase cells. (A) The segregation types are classified as equal (N:N) or unequal (N: ≠N) or failed segregation (N:0). (B) The values for the three segregation types in the presence (galactose) and absence (glucose) of monopolin plus Spo13 are plotted as histograms. The low level of N:0 class argues against the segregation of the plasmid foci as a single clustered unit. Otherwise the monoploin effect on the single copy and multi-copy *STB* reporter plasmids should have been the same.

Appendix A

A quantitative analysis of the data from Chapter 3 to test the correlation between chromosome and 2 micron plasmid segregation

We incorporate currently available data into the hitchhiking model for 2 micron plasmid segregation by making the following proposals. Replicated 2 micron sister plasmids become tethered to sister chromatids, and the cohesin complex promotes this event in two ways. First, cohesin bridges sister chromatids (Nasmyth, 2011; Onn et al., 2008), providing plasmid tethering sites as symmetric pairs. Second, cohesin holds sister plasmid copies to keep them in close proximity (Ghosh et al., 2007; Ghosh et al., 2010), facilitating their symmetric tethering to sister chromatids. Since cohesin association with plasmids is much less than stoichiometric (Ghosh et al., 2010), cohesin likely only initiates sister plasmid pairing, which is then stabilized by other interactions involving the Rep proteins and perhaps host proteins. In the case of several plasmid copies organized into a group, cohesin turnover among plasmid pairs may help organize replicated molecules into two sister groups that can split apart from each other in association with sister chromatids. The association of cohesin with *STB* is dependent on spindle integrity (Mehta et al., 2005) whereas cohesin's association with chromosomes and sister chromatid cohesion proceed normally in the absence of the spindle. Thus, depleting cohesin during the mitotic cell cycle or dismantling the spindle during the relevant cell cycle stage should disrupt the association of sister plasmids with sister chromatids. As a result, plasmid segregation should become uncoupled from chromosome segregation.

As we have demonstrated experimentally conditions that result in co-segregation of sister chromatids also produce well correlated co-segregation of the single copy *STB* reporter plasmid, but not of the single copy *ARS* reporter plasmid. Furthermore, the absence of cohesin assembly or delayed spindle organization during a cell cycle uncouples *STB* plasmid segregation from chromosome segregation. This general validation of the hitchhiking model is further reinforced by several features of the chromosome and plasmid segregation events that we consider in more quantitative detail below.

Rationale for the quantitative analyses

Our data for each experimental set are composed of five categories of segregation, equal (1:1) and unequal (2:0 and 0:2; 1:0 and 0:1). However, since the 1:0 and 0:1 classes represent missegregated, but coalesced, plasmid sisters, they are merged into the 2:0 and 0:2 classes respectively. We thus have three variables, Ve, Vm and Vd, denoting equal segregation, missegregation biased towards the mother and missegregation biased towards the daughter, respectively. In a particular assay, the sum of Ve, Vm and Vd for a reporter chromosome or for a reporter plasmid will be 100%. For example, during normal mitosis, when the chromosome shows no missegregation, Ve = 100% and Vm and Vd are each = 0%. For characterizing segregation quantitatively during an experimentally manipulated mitosis, the relevant parameter is the difference in the value of each variable under this condition from the corresponding value under a reference condition (ΔV). Furthermore, the appropriate sign (+ or -) assigned to ΔV indicates the direction of this change. For example a negative ΔVe means that there is a decrease in equal segregation which would result in positive ΔVm plus ΔVd, such that ΔVm + ΔVd + ΔVe = 0. These

values for the chromosome and the *STB* or the *ARS* reporter plasmid are assembled in Table A-1. Note that the Ve values (denoting equal segregation) for the chromosome and the reporter plasmids under a given reference condition are not same. For example, during normal mitosis, the chromosome, the *STB* plasmid and the *ARS* plasmid show 100%, 63.7% and 23.4% equal segregation, respectively. In order to take into account these innate differences, we normalized the ΔV values in Table A-1 to the corresponding reference Ve values. The ΔV' values thus obtained are listed in Table A-2. We have used these data (ΔV' values) to estimate the strengths of correlations between the *STB* plasmid or the *ARS* plasmid and the chromosome with respect to their segregation features. It needs to be pointed out that the correlation estimates will retain similar qualitative features, whether or not they are based on ΔV or ΔV' values. The quantitative differences arising from the two methods will not be large enough to affect the interpretations of the cumulative data.

	monopolin			monopolin+SPO13			monopolin+ *ipl1-321*			*ipl1-321*		
	ChIV	p*STB*	p*ARS*	ChIV	p*STB*	p*ARS*	ChIV	p*STB*	p*ARS*	ChIV	p*STB*	p*ARS*
ΔVe	-34.2%	-24.5%	3.1%	-97.8%	-45.9%	-12.4%	-51.4%	-37.4%	-10.0%	-54.1%	-34.3%	-2.6%
ΔVm	18.5%	12.3%	-19.4%	50.6%	25.9%	2.9%	18.5%	8.6%	-13.3%	21.1%	8.7%	-6.0%
ΔVd	15.7%	12.2%	16.3%	47.2%	20.0%	9.5%	32.9%	28.8%	23.3%	33.0%	25.6%	8.6%

Table A-1. Differences between normal mitosis and altered forms of mitosis in the segregation of a chromosome, an *STB* reporter plasmid and an *ARS* reporter plasmid. Equal segregation, missegregation biased towards the mother and missegregation biased towards the daughter are symbolized by Ve, Vm and Vd, respectively. The ΔV values represent the differences between individual variables for mitosis altered in a particular way from those for normal mitosis. The + and – signs indicate whether a given condition causes an increase or decrease, respectively, in V.

	monopolin			monopolin+SPO13			monopolin+ *ipl1-321*			*ipl1-321*		
	ChIV	p*STB*	p*ARS*	ChIV	p*STB*	p*ARS*	ChIV	p*STB*	p*ARS*	ChIV	p*STB*	p*ARS*
ΔV'e	-34.2%	-38.5%	13.2%	-97.8%	-72.1%	-53.0%	-51.4%	-58.7%	-42.7%	-54.1%	-53.9%	-11.1%
ΔV'm	18.5%	19.3%	-82.9%	50.6%	40.7%	12.4%	18.5%	13.5%	-56.8%	21.1%	13.7%	-25.6%
ΔV'd	15.7%	19.2%	69.7%	47.2%	31.4%	40.6%	32.9%	45.2%	99.5%	33.0%	40.2%	36.7%

Table A-2. Normalized differences between normal mitosis and altered forms of mitosis in chromosome and plasmid segregation. The ΔV values listed in Table A-1 for chromosome and plasmids were normalized to their respective equal segregation frequencies during normal mitosis: 100% for chromosome, 63.7% for the *STB* plasmid and 23.4% for the *ARS* plasmid.

Coupling between chromosome and the *STB* plasmid under conditions that co-segregate sister chromatids

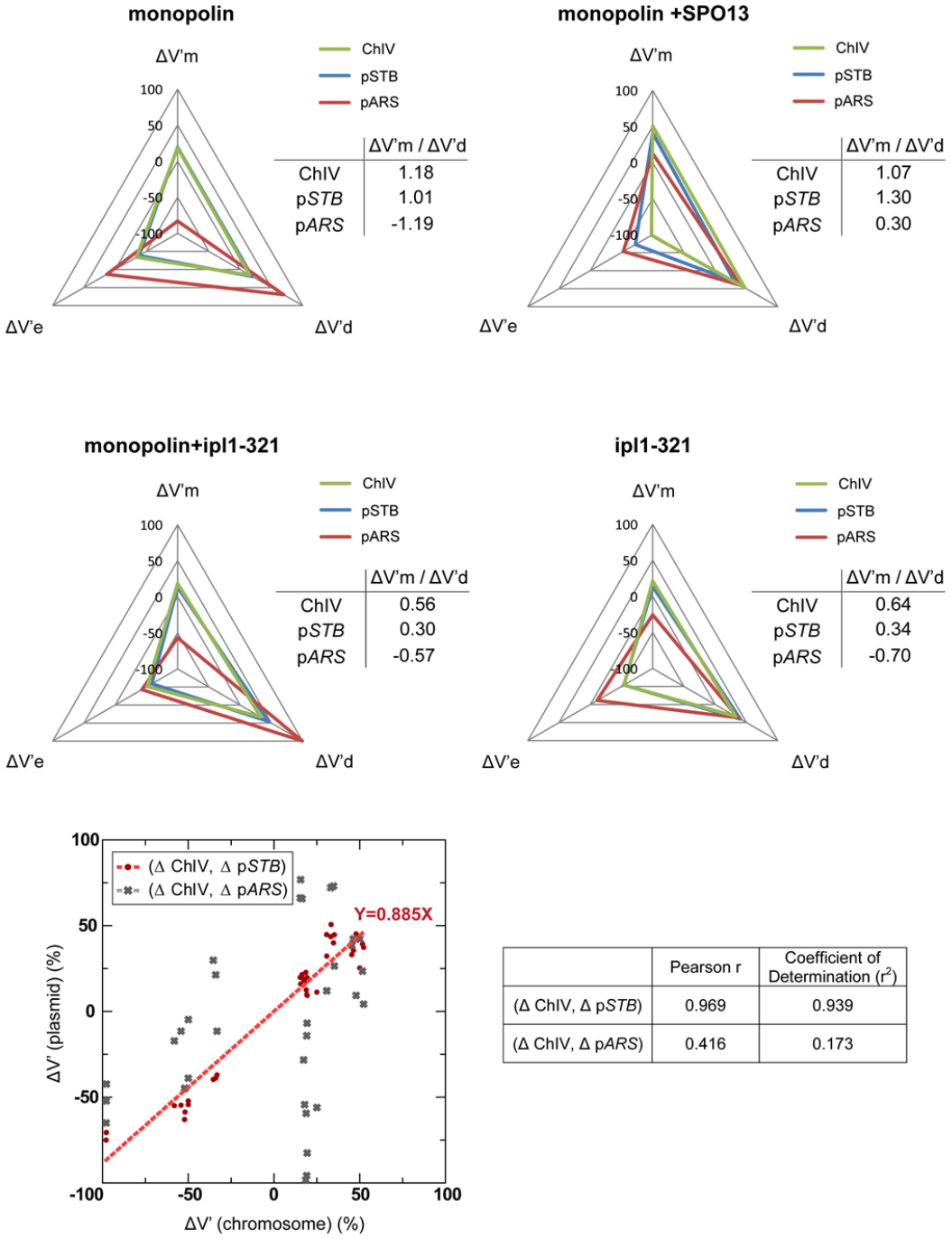
In Figure A-1, the normalized ΔV' values (ΔV'e, ΔV'm and ΔV'd from Table A-2) are represented graphically. These plots highlight the strong coupling between chromosome co-segregation and the *STB* plasmid missegregation when normal chromosome segregation is distorted by monopolin assembly, monopolin assembly plus Spo13 expression and by monopolin assembly combined with partial inactivation of Ipl1. The effects of partial inactivation of Ipl1 alone are similar to those obtained when monopolin is also assembled during such inactivation.

We have assembled the ratios of ΔV'm to ΔV'd in order to appreciate how the nature of missegregation events is affected (bias-free, mother biased or daughter biased) by a particular condition that causes mitosis to deviate from normalcy (Fig. A-1). When there is no change in the bias from the reference mitosis, ΔV'm/ΔV'd will be = 1, that is, the normalized change in mother segregation is the same as that in daughter segregation. ΔV'm/ΔV'd values >1 and <1 will indicate changes in the direction of the mother and daughter, respectively. An important point to note from Figure A-1 is that the daughter bias imparted to chromosome missegregation by the impairment of Ipl1 function is also manifested in *STB* plasmid missegregation (0< ΔV'm/ΔV'd < 1). This is significant because missegregation of the *STB* plasmid imposed by conditions of chromosome missegregation that do not involve Ipl1 inactivation is either free of bias or displays a small mother bias (ΔV'm/ΔV'd close to 1). Also to be noted from Figure A-1 is the clear distinction of the *ARS* plasmid from either the chromosome or the *STB* plasmid. For three of the conditions (monopolin, monopolin plus *ipl1-321* and *ipli-321*), the ΔV'm/ΔV'd

values for the *ARS* plasmid are negative; and for the fourth (monopolin plus Spo13), the value is positive but quite smaller than that for the chromosome or the *STB* plasmid. Although altered conditions of mitosis do modulate the extent of bias of the *ARS* plasmid, the overall strong mother bias is retained by it under all four conditions.

A quantitative estimate of the correlation between a chromosome and the *STB* plasmid in their segregation phenotypes

In order to derive a better quantitative estimate of the coupling between a chromosome and the *STB* plasmid in their segregation behavior, we made use of the ΔV' values from Table A-2 to estimate the Pearson's linear correlation coefficient (Fig. A-1). The X-Y scatter plot showed a very strong positive correlation between the *STB* plasmid and chromosome IV, with r = 0.969 (r^2 = 0.939). The linear regression line for the data intersects the origin, with a slope of 0.885. The biological meaning of this slope is that within the 63.7% *STB* plasmid population that segregates equally during normal mitosis (against which the ΔV values were normalized), 88.5% shows sister plasmid co-segregation coupled to sister chromatid co-segregation, when conditions of mitosis are altered to intentionally missegregate chromosomes. When applied to the entire plasmid population, the strength of this coupling is 56.4% (63. 7% x 88.5%). By contrast, the scatter plot for the *ARS* plasmid and chromosome IV showed poor correlation (r = 0.416 or r^2 = 0.173).



	Pearson r	Coefficient of Determination (r^2)
(Δ ChIV, Δ p*STB*)	0.969	0.939
(Δ ChIV, Δ p*ARS*)	0.416	0.173

Figure A-1. Correlations between the segregation patterns of a chromosome and an *STB* plasmid or an *ARS* plasmid under different conditions. The normalized values for ΔV (ΔV') from Table A-2 are represented as radar plots to convey the correlation (or lack thereof) between a chromosome and a plasmid in segregation. The ratios of ΔV'm to ΔV'd are tabulated to denote how the mother-daughter bias in missegregation was affected by different manipulations of mitosis. The combined data from Table A-2 were transformed into a scatter plot to derive Pearson's linear correlation coefficients.

The lack of perfect coupling between the *STB* plasmid and chromosomes suggests an inherent limit to the efficiency with which sister plasmids can be tethered to sister chromosomes. This limitation is also reflected in the 63.7% equal segregation of this plasmid during normal mitosis. It is possible that the reporter plasmid suffers at least some loss in function compared to the native 2 micron plasmid. However, the observed strong linear correlation between plasmid and chromosome is entirely consistent with the hitchhiking model of 2 micron plasmid.

Uncoupling 2 micron plasmid segregation from monopolin induced sister chromatid co-segregation

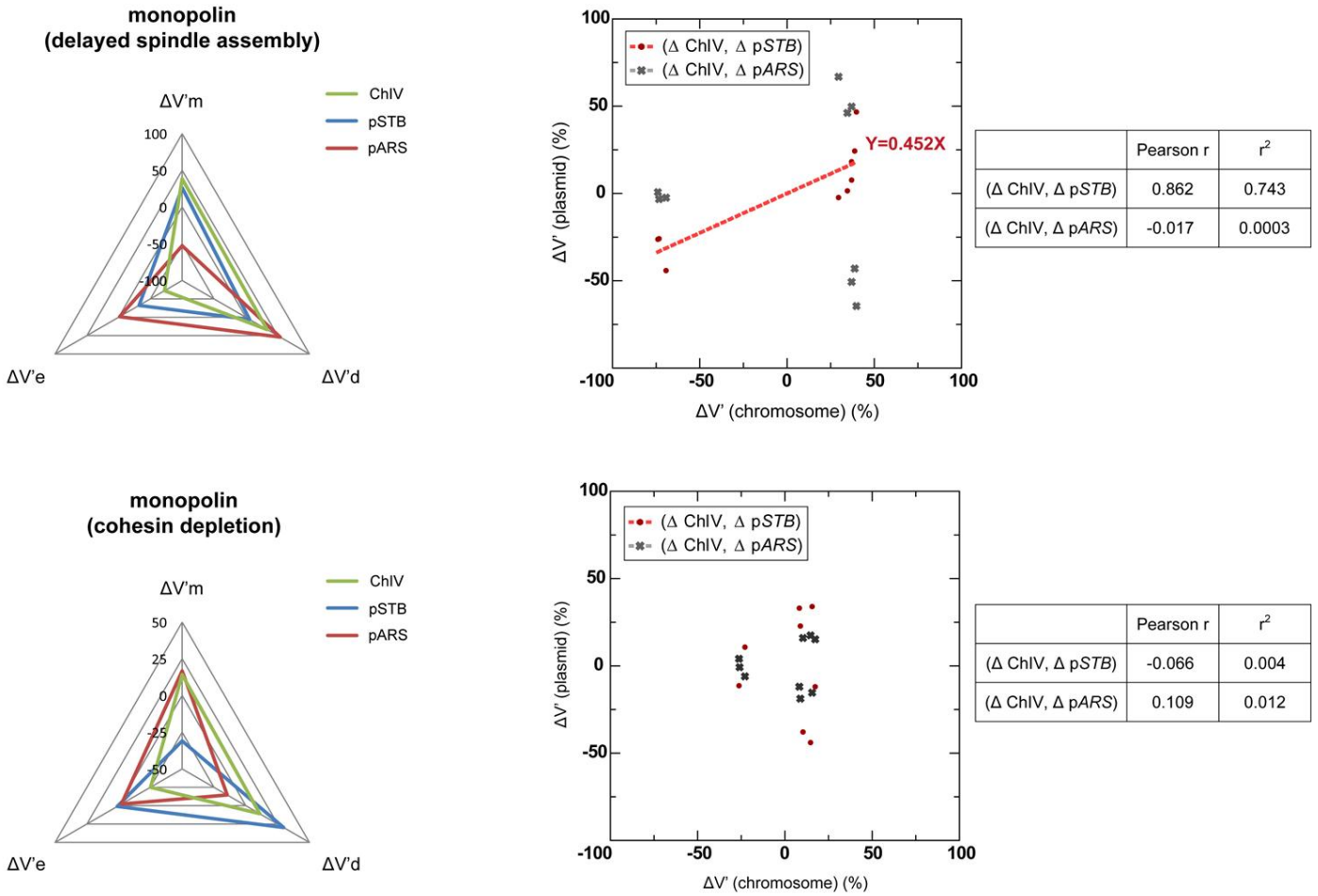
We now consider two situations that are expected to disrupt the hitchhiking of sister plasmids on sister chromatids, namely, the delay of spindle assembly until G2/M or the lack of cohesin assembly during a cell cycle. The pertinent ΔV and $\Delta V'$ values signifying the monopolin effects under these conditions on a chromosome (either IV or V) or the *STB* and *ARS* plasmids are summarized in Table A-3 and A-4. The $\Delta V'$ plots in Figure A-2 indicate that the *STB* plasmid segregation is uncoupled from chromosome segregation under these conditions. Consistent with this uncoupling, the linear correlation estimated from the scatter plot (Fig. A-2) is weakened under the nocodazole regimen ($r = 0.862$; $r^2 = 0.743$), and the slope for the linear regression line drops to 0.452 compared to 0.885 for the scatter plot in Figure A-1B. At the same time, the correlation between *STB* plasmid and chromosome is totally abolished under cohesin depletion ($r = -0.066$ or $r^2 = 0.004$).

	monopolin (delayed spindle assembly)			monopolin (cohesin depletion)		
	ChIV	p*STB*	p*ARS*	ChV	p*STB*	p*ARS*
ΔVe	-72.2%	-12.5%	-0.4%	-16.4%	0.4%	-0.6%
ΔVm	38.5%	10.2%	-13.2%	9.2%	-11.2%	4.5%
ΔVd	33.7%	2.3%	13.6%	7.2%	10.8%	-3.9%

Table A-3. Differences in segregation patterns signifying the monopolin effects on chromosome and plasmid segregation during mitosis under delayed spindle assembly or mitosis under cohesin depletion. The ΔV values denoting monopolin effects were estimated with mitosis under delayed spindle assembly or mitosis under cohesin depletion as the relevant reference. In the left panel, V values for mitosis under delayed spindle assembly were subtracted from corresponding V values for mitosis under monopolin plus delayed spindle assembly. In the right panel, V values for mitosis under cohesin depletion were subtracted from corresponding V values under monopolin plus cohesin depletion.

	monopolin (delayed spindle assembly)			monopolin (cohesin depletion)		
	ChIV	p*STB*	p*ARS*	ChV	p*STB*	p*ARS*
ΔV'e	-72.2%	-32.1%	-1.6%	-25.1%	1.1%	-2.2%
ΔV'm	38.5%	26.2%	-52.6%	14.1%	-30.9%	16.8%
ΔV'd	33.7%	5.9%	54.2%	11.0%	29.8%	-14.6%

Table A-4. Normalized differences in segregation properties of chromosome and plasmids due to monopolin when mitosis occurs under delayed spindle assembly or under cohesin depletion. The ΔV values from Table A-3 were normalized with respect to the appropriate equal segregation frequency (Ve). For the left panel, Ve values for mitosis under delayed spindle assembly (but without monopolin) for the chromosome, the *STB* plasmid and the *ARS* plasmid were: 100%, 39.0% and 25.1%, respectively. For the right panel, the corresponding Ve values for mitosis under cohesin depletion (but without monopolin) were: 65.4%, 36.2% and 26.8%, respectively.



	Pearson r	r^2
(Δ ChIV, Δ p*STB*)	0.862	0.743
(Δ ChIV, Δ p*ARS*)	-0.017	0.0003

	Pearson r	r^2
(Δ ChIV, Δ p*STB*)	-0.066	0.004
(Δ ChIV, Δ p*ARS*)	0.109	0.012

Figure A-2. Delayed spindle assembly or lack of the cohesin complex uncouples the *STB* plasmid from chromosome in mitotic segregation. The radar graphs and the scatter plots are assembled as described under Figure A-1 using the values from Table A-4.

Obviously, delaying spindle assembly until G2/M does not completely uncouple *STB* plasmid segregation from chromosome segregation. Earlier experiments suggested that once the spindle is restored, replicated plasmids can associate with chromosomes (Mehta et al., 2005), as suggested by chromosome spread assays. If the effect of nocodazole treatment followed by subsequent spindle restoration is to disrupt the association of sister plasmids to sister chromatids without blocking random association of plasmids with chromosomes, complete uncoupling will not occur.

We do not have a good explanation for why cohesin depletion causes stronger uncoupling of *STB* plasmid segregation from chromosome segregation than delayed spindle assembly. Whereas cohesin association with *STB* is blocked by nocodazole, cohesin assembly at centromeres and other chromosomal loci occurs normally to bridge sister chromatids in pairs. Perhaps such cohesed, and locally constrained, chromosomes provide better targets for plasmid tethering than unpaired individual chromosomes by effectively increasing the concentration of tethering sites.

Conclusions

The segregation properties of sister copies of the *STB* reporter plasmid are strongly correlated with those of a pair of sister chromatids. The behavior of the *ARS* sister plasmids shows no such correlation. The quantitative estimates of the correlations for the *STB* plasmid and a chromosome are in good agreement with the hitchhiking model for 2 micron plasmid segregation.

CHAPTER4

Characterization of the EBV partitioning system reconstituted in *Saccharomyces cerevisiae*

4.1 Summary

We have functionally characterized the EBV partitioning system reconstituted in *S. cerevisiae* using a fluorescence tagged single copy plasmid as the reporter. The salient findings are as follows. **1**. Consistent with the results from a multi-copy multi-generation plasmid loss assay, the equal segregation frequency of the single copy reporter plasmid is increased in a single generation assay when EBNA-1 and hEBP2 proteins are expressed in host cells. **2**. The association of the reporter plasmid with yeast chromosome spreads from G1 arrested cells is increased by the presence of the EBV partitioning proteins. This finding is consistent with the ability of these proteins to tether the EBV plasmid to chromosomes. **3**. The percentages of anaphase chromosome spreads displaying one fluorescent plasmid dot under different conditions are consistent with EBNA-1 and hEBP2 mediated plasmid segregation occurring in a chromosome associated manner. Furthermore, the ratio of one plasmid dot versus two dots in anaphase spreads suggest that tethering of sister plasmids to sister chromatids (non-random tethering) makes a significant contribution to equal segregation. **4**. Analysis of metaphase cells reveal an enhancement in sister plasmid colocalization in the presence of the partitioning system. **5**. Cumulatively, these findings suggest that the reconstituted EBV partitioning system in *S. cerevisiae* retains the fundamental attributes of the native partitioning system, however, with reduced efficiency.

4.2 Introduction

The genome of EBV, one of the well characterized members of the gammaherpesvirus family, is a highly successful parasite genome in mammalian cells. A particularly efficient replicon, it establishes persistent, lifelong infection in 90% of the world's population (Evans et al., 1968). Primary infection of EBV causes mononucleosis in some of the individuals, especially adolescents, whereas the latent virus life cycle that follows is normally asymptomatic. However, in a small number of cases, EBV can contribute to different types of carcinomas, including T-cell lymphomas, Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma and gastric carcinomas (Lindner and Sugden, 2007).

Replication of the EBV genome

The latent life style of EBV is quite distinct from its lytic life style. During the latent phase, the circularized viral episomes replicate once per cell cycle, and the replicated copies are efficiently partitioned into daughter cells. This behavior is reminiscent of that of the 2 micron plasmid, with one round of replication of individual plasmid molecules per S phase in *S. cerevisiae* followed by equal or near equal plasmid distribution to daughter cells during anaphase. Viral replication is dependent on the *cis*-acting *oriP* sequence as well as the viral protein EBNA-1 (Lupton and Levine, 1985; Yates et al., 1984). The *oriP* region can be divided into two functional units- the FR (family of repeats) locus and the DS (dyad symmetry) locus (Fig. 1.5B). There are 21 copies of an imperfect 30 bp sequence within FR, with 20 high affinity binding sites for EBNA-1. A set of four EBNA-1 binding elements, with lower affinity than those of FR,

are arranged as two pairs within DS. Each pair contains the binding elements in head-to head orientation (Rawlins et al., 1985). The spacing between the two pairs will place the two EBNA-1 dimers bound to them on the same face of the DNA double helix. Flanking the EBNA-1 binding sites of DS, there are three nonamer sequences that can bind cellular factors that associate with telomeres, including the TTAGGG-repeat binding factors, TRF1 and TRF2 (Deng et al., 2002; Yates et al., 2000). Replication is initiated at DS by the recruitment of the host replication machinery by the bound EBNA-1. TRF2 association with the nonamers stimulates replication initiation, by what appears to be a bipartite mechanism- one by increasing the affinity of EBNA-1 for DS, and the other by the direct interaction of TRF2 with the BAH (bromo adjacent homology) domain of ORC1 (Deng et al., 2002). A secondary origin called Rep*, containing a pair of EBNA-1 binding sites can also be utilized during latency, although Rep* is less efficient than DS (Kirchmaier and Sugden, 1998). The FR sequence enhances viral transcription, but also stimulates replication initiated at DS.

In contrast to the strictly regulated replication during latency, lytic replication is initiated at a separate origin (*oriLyt*), and multiple viral gene products are dedicated for assembling the basic replication machinery. The product of lytic replication is a concatemer of head-to-tail copies of the viral genome, which are resolved into unit length copies during encapsidation (Challberg, 1996). The details regarding the mechanism of lytic replication are sketchy. However, it is interesting to note that replicative amplification of the 2 micron plasmid also produces a concatemer containing multiple tandem copies of the plasmid genome.

Maintenance of EBV episomes during latency

The FR sequence and EBNA-1 also play a critical role in the partitioning of replicated episome copies to daughter cells. The FR sequence constitutes the *cis*-acting partitioning locus, and EBNA-1 serves as the partitioning protein (Wu et al., 2002). Of the 20 binding sites for EBNA-1 within FR, only six to eight are necessary and sufficient for episome maintenance (Wysokenski and Yates, 1989). Like other members of the gammaherpes family, the EBV partitioning system promotes episome tethering to chromosomes (Harris et al., 1985; Simpson et al., 1996). There is some debate as to whether EBNA-1 is directly responsible for such tethering, or whether it performs this function with the help of a partner host protein-hEBP2. According to one model, EBNA-1 may associate with AT-rich sequences in chromatin through its AT-hook motif (Sears et al., 2004). The two mechanisms for chromosome tethering of episomes need not be mutually exclusive. EBP2 is highly conserved among eukaryotes, and evidence from *S. cerevisiae* suggests that it is a nucleolar protein involved in ribosome biogenesis (Tsujii et al., 2000). An Arg/Gly-rich peptide region within EBNA-1, spanning amino acids 325-376, is responsible for its interaction with EBP2 (Ceccarelli and Frappier, 2000) (Fig. 1.8). A deletion derivative of EBNA-1 lacking this region can support replication initiation at *oriP*, but is defective in its segregation function. Although the deletion variant is localized to the nucleus, it is not associated with chromosomes. The ability of the 325-376 region to interact with metaphase chromosomes has been reported as well (Kanda et al., 2001; Marechal et al., 1999). The potential role of EBP2 in EBV-chromosome tethering would fit into the general theme of the interaction between a viral partitioning protein and a chromatin binding host protein being responsible for localizing episomes of the gammaherpes and papilloma families to chromosomes (Shire et al., 1999;

Wu, 2000) (Fig. 4.1). However, evidence contradicting the requirement of hEBP2 for episome stability has also been reported. According to these experiments, EBNA-1 derivatives that cannot interact with EBP2, but harbor AT-hook activity, are normal in episome replication and segregation (Sears et al., 2004).

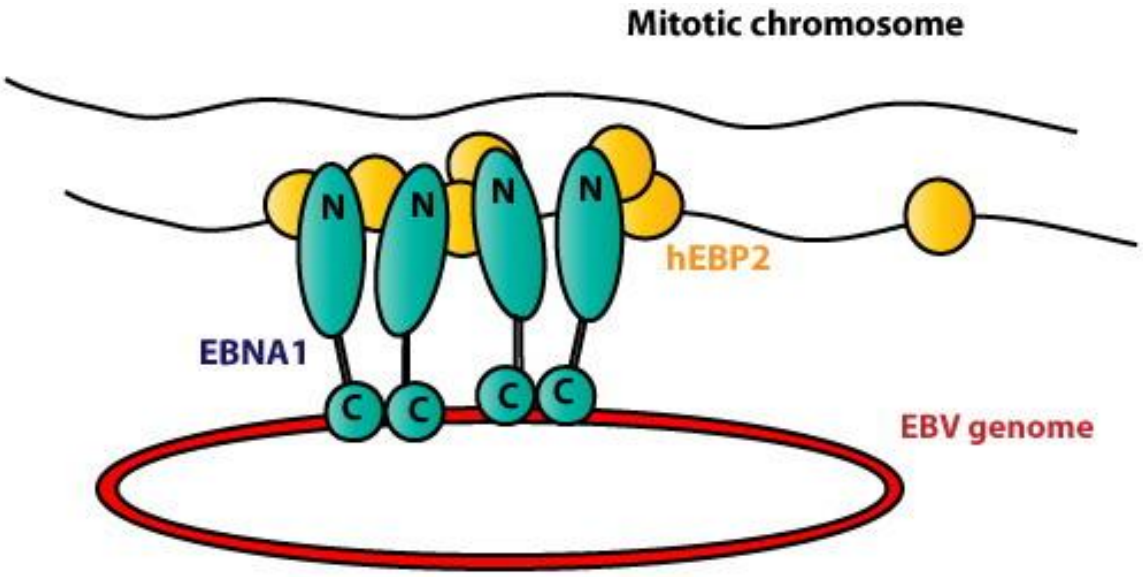


Figure 4.1 Tethering of EBV genome to mitotic chromosomes. The C-terminal (C) region of the viral protein EBNA1 binds to the FR elements within the EBV genome. hEBP2, the host chromosomal binding protein, interacts with N-terminal (N) region of EBNA1, and is responsible for episome tethering to a chromosome. The figure is modified from McBride et al., (2004).

Reconstitution of the EBV partitioning system in yeast

The (EBNA1-FR)-hEBP2 based partitioning system has been successfully reconstituted in *S. cerevisiae* (Kapoor et al., 2001). The stability of an *ARS* plasmid (capable of autonomous replication but lacking a partitioning system) harboring the FR locus can be increased substantially in a yeast strain expressing EBNA-1 and hEBP2. Furthermore, the interaction between EBNA-1 and hEBP2 is also critical for efficient plasmid maintenance. Based on these findings, it has been proposed that the reconstituted system faithfully recapitulates the native system in mediating chromosome associated segregation of FR containing plasmids.

Functional analogies between EBV and the 2 micron circle in segregation

As pointed out earlier, the currently available data for the equal segregation of the 2 micron plasmid are readily accommodated by a model in which the plasmid hitchhikes on chromosomes. Previous work from our laboratory showed that sister molecules of a single copy *STB* reporter plasmid formed by replication segregate from each other in a one-to-one fashion (Ghosh et al., 2007). According to the hitchhiking model, this mode of segregation strongly suggests that the sister plasmids are tethered to sister chromatids. Since the yeast cohesin complex assembled at the *STB* locus keeps the sister plasmids paired, and sister chromatids are also paired by cohesin, it is conceivable that sister plasmids may attach to symmetrically disposed tethering sites on sister chromatids. Strikingly, EBV segregation in mammalian cells is also non-random. Replicated episomes exist predominantly as pairs, and are symmetrically arranged on sister chromatids in chromosome spreads prepared from metaphase cells (Kanda et al., 2007).

Further characterization of the EBV partitioning system in *S. cerevisiae*

We wished to characterize the reconstituted EBV partitioning system in *S. cerevisiae* more critically using a fluorescence tagged reporter plasmid, so that segregation events during a single cell cycle could be directly monitored. In particular, we wished to reduce the copy number of the reporter plasmid to one (or nearly one) for the following reasons. Equal segregation of this reporter (with a starting copy number of one) during a synchronous cell cycle will be seen as 1:1 distribution of fluorescent foci in mother and daughter; missegregation as 2:0 distribution. If sister plasmids are tethered to sister chromatids the result will be 1:1 plasmid segregation. If sister plasmids are tethered to chromosomes in a random fashion, 1:1 and 2:0 segregation events will occur with equal probability. Furthermore, if the presumed plasmid-chromosome association occurs by sister plasmids tethering symmetrically to sister chromatids, which are paired by the cohesin complex, the two fluorescent foci are expected to be colocalized in metaphase cells. If plasmid-chromosome association is random, co-localization events should be rare. The results of our analyses are presented below.

4.3 Results

4.3.1 Verification of the functionality of the reconstituted EBV system in *S. cerevisiae*

To verify the reconstituted EBV partitioning system in *S. cerevisiae*, an initial plasmid loss assay was performed using the multi-copy reporter plasmid, YRP7FR, containing the *TRP1* marker and the associated autonomously replicating sequence (*ARS*) plus the FR locus from EBV (Fig. 4.2A). The viral EBNA-1 protein was expressed in the

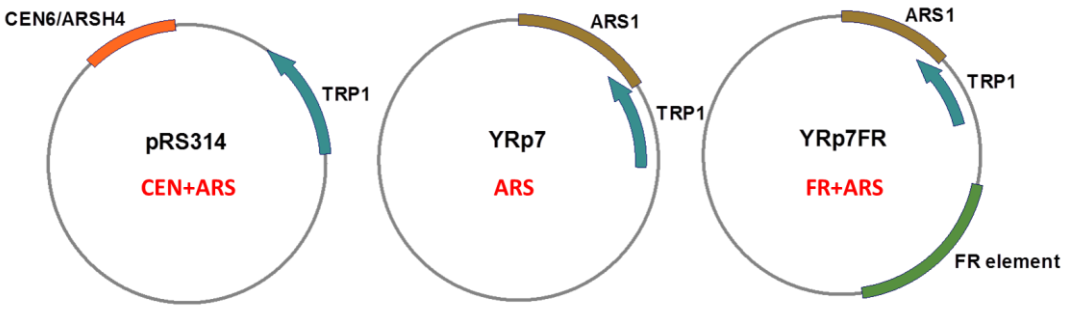
host strain from a *CEN* based plasmid under the *MET25* promoter. The human protein partner of EBNA-1, hEBP2, was expressed from a 2 micron plasmid based multi-copy plasmid. Our assay conditions followed closely those employed by Kapoor et al. (Kapoor et al., 2001). We found that EBNA-1 expression under the inducible condition (absence of methionine) was toxic to the cells. Hence, the protein was supplied through leaky expression in the absence of induction (Fig. 4.2B).

After growth in selective medium until mid-log phase (T = 0), followed by further growth in non-selection medium for five generations (T = ~10 hr), the fractions of cells harboring plasmid (%) at T0 and T10, f0 and f10, respectively, were measured. Based on these fractions, plasmid loss rates per generation (%) were computed. Under simultaneous expression of EBNA-1 and hEBP2, an FR reporter plasmid showed intermediate plasmid stability, inferior to a *CEN* plasmid but superior to an *ARS* plasmid, both of which lacked the FR partition locus (Fig. 4.3). The results conform to the view held by one camp that the interaction between the two proteins is responsible for the chromosome associated maintenance of the EBV plasmid in mammalian cells (Kapoor et al., 2001; Nayyar et al., 2009). However, the opposite notion that EBV partitioning can occur in the absence of hEBP2 in the native host system cannot be ruled out (Sears et al., 2004). The critical dependence on hEBP2 for EBNA-1 mediated EBV segregation in yeast may be a particular feature of the non-native host system.

In the first set of published results on the reconstituted EBV system by Frappier and colleagues, the estimated stability of an FR reporter plasmid in *S. cerevisiae* in the presence of EBNA-1 and hEBP2 was almost as high as that of a *CEN* plasmid (Kapoor et al., 2001). In our analysis, carried out under similar conditions, the efficiency of the EBV

system expressed in *S. cerevisiae* was clearly lower than that of the *CEN* based partitioning system (Fig. 4.3). In a subsequent publication, Frappier and colleagues reported lower stability of the FR plasmid under EBV partitioning control compared to a *CEN* plasmid (Lin et al., 2008). The reasons for the observed variability are not understood. Differences in strain backgrounds or in the expression levels of EBNA-1 and hEBP2 under different experimental conditions may account for the different outcomes. Nevertheless, our present results are consistent with previous reports that the EBV partitioning is functional in *S. cerevisiae*, even if the efficiency is not optimal.

A.



B.

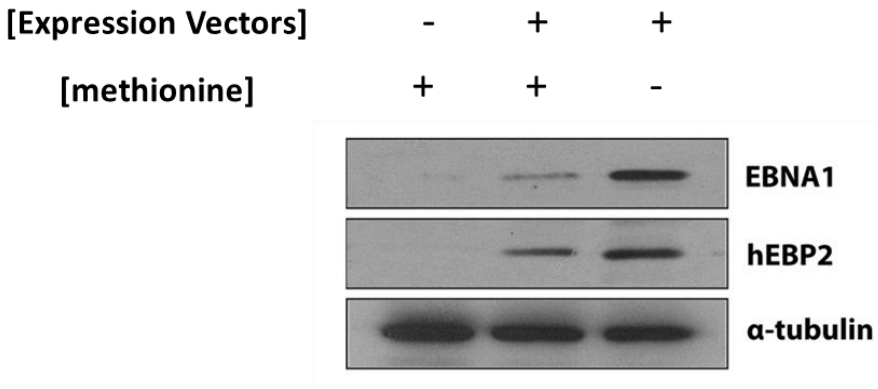


Figure 4.2 The EBV partitioning system reconstituted in *S. cerevisiae* (Kapoor et al., 2001). (A) The three reporter plasmids used for stability assays are schematically diagrammed. They are the same as those described by Kapoor et al (2001). All three harbor *TRP1* as the selectable marker in addition to an *ARS* element, which supports plasmid replication. In addition, YRp7FR contains the FR locus from EBV and pRS314 contains the centromere sequence form chromosome VI (*CEN6*). (B) The partitioning proteins EBNA-1 and hEBP2 were expressed in the experimental strains by transforming them with the appropriate expression plasmids. Cell lysates prepared from strains containing (+) or lacking (-) the expression cassette were assayed by western blotting. (methionine -) denotes the absence of methionine in the medium. The antibodies used were to native EBNA-1 and to native hEBP2. The loading control was provided by α-tubulin.

A.

Reporter plasmid	Partitioning Locus	EBNA1/hEBP2 expression	Cells harboring plasmid (%)	
			T0	T10
YRP7 (ARS)	–	+	38.9%	27.2%
		-	35.8%	20.7%
pRS314 (CEN+ARS)	*CEN*	+	78.8%	70.6%
		-	81.6%	75.4%
YRP7FR (FR+ARS)	FR	+	58.5%	48.1%
		-	38.1%	22.2%

B.

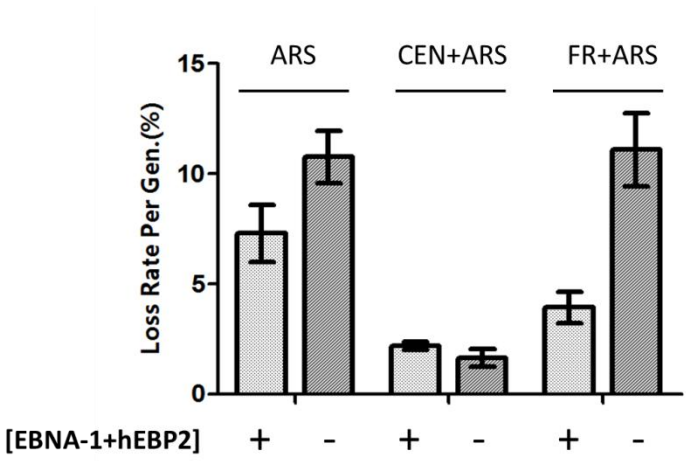


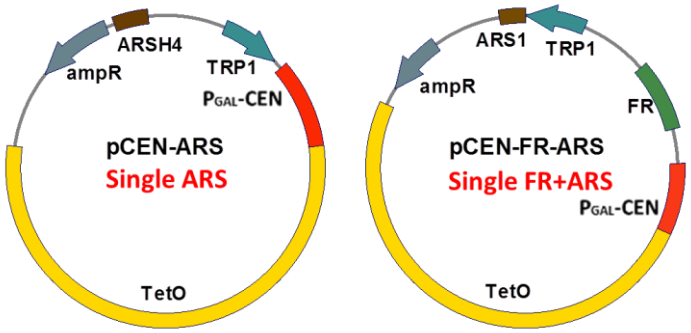
Figure 4.3 The reconstituted EBV partitioning system stabilizes an EBV reporter plasmid in *S. cerevisiae*. (A) The fractions of cells in the population containing the reporter plasmid at time zero (T0; under selective growth) and at time T10 (after ~10 hr; five generations of non-selective growth) under the indicated experimental conditions are tabulated. (B) The Instability index I, or the loss rate per generation (%), was calculated by the equation = 1/n × [ln (f0/fn)], where n = number of generations; f0 = fraction of plasmid bearing cells at time zero; fn = fraction of plasmid bearing cells after n generations. The values for the three reporter plasmids (see Fig. 4.2) in the presence (+) and absence (-) of EBNA-1 and hEBP2 are plotted as histograms.

4.3.2 The reconstituted EBV system improves equal segregation of a cognate single-copy reporter plasmid

In the native mammalian model, the EBV episomes are proposed to segregate in a non-random fashion (Kanda et al., 2007; Nanbo et al., 2007). Cell biological assays suggest that fluorescence tagged episomes frequently associate with chromosomes in pairs, and segregate from each other in a one-to-one fashion. The reconstituted EBV system in *S. cerevisiae* with the multi-copy reporter plasmid does not reveal whether plasmid stability is achieved by random tethering of replicated plasmids to chromosomes or by tethering of sister plasmids to sister chromatids. Strictly, our results cannot rule out plasmid stability by a mechanism that is independent of chromosome association.

In order to better address the features of plasmid segregation promoted by the EBV system in *S. cerevisiae*, we generated a derivative of the EBV reporter plasmid that is present at one or close to one copy per cell (Fig. 4.4A). Following the rationale used in constructing analogous derivatives of 2 micron circle reporter plasmids (Ghosh et al., 2007), the *CEN3* sequence, transcriptionally controlled by the *GAL* promoter, was incorporated into the EBV reporter. The presence of a functional *CEN* sequence has the dominant effect of reducing the copy number of an otherwise multi-copy plasmid to a value close to one. The 'very low copy' FR containing plasmid is hereafter referred to as a 'single copy' reporter plasmid. An array of $TetO_{224}$ was incorporated into the plasmid, so that it could be visualized by fluorescence microscopy in cells expressing TetR-GFP. In glucose grown cells, with the *CEN* being active, the plasmid was seen as a single fluorescent focus per nucleus in the vast majority of cells (>80%). In interpreting the experimental results, the small fraction of cells containing more than one plasmid focus

A.



B.

Plasmid	glucose		Galactose	
	[EBNA-1+hEBP2]		[EBNA-1+hEBP2]	
	-	+	-	+
pCEN-ARS (single copy ARS)	CEN+	CEN+	CEN-	CEN-
pCEN-viral-ARS (single copy FR+ARS)	CEN+ FR-	CEN+ FR+	CEN- FR-	CEN- FR+

C.

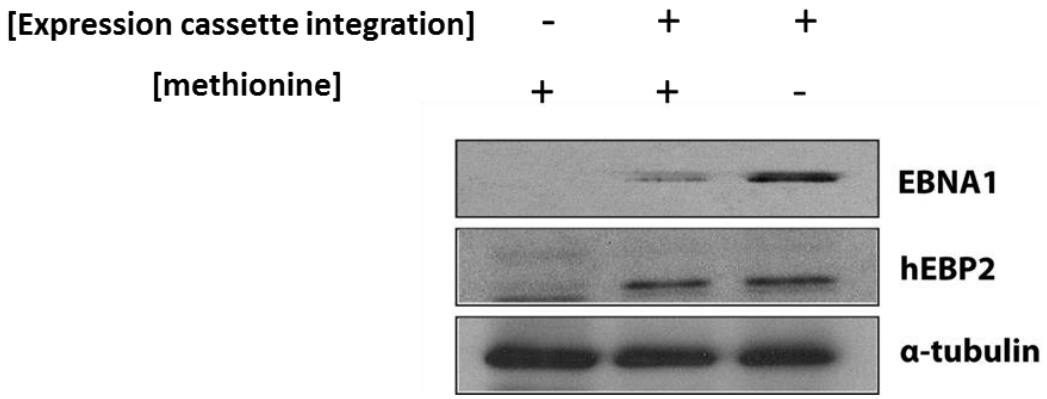


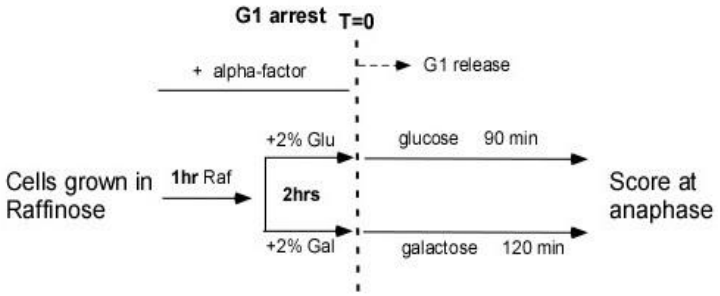
Figure 4.4 Analysis of the EBV partitioning system in *S. cerevisiae* using single copy reporter plasmids. (A) The organizational features of the single copy reporter plasmids employed in the one generation segregation assays are schematically diagrammed. The important functional difference between pCEN-ARS and pCEN-FR-ARS is the presence of the FR locus from EBV in the latter. Both these plasmids harbor a [TetO] array in order to visualize them by fluorescence in a host strain expressing TetR-GFP. In addition, they harbor the yeast *CEN3* sequence under the control of the *GAL* promoter. The *CEN* is active in the absence of transcription through it. It can be inactivated during a cell cycle by galactose induced transcription. (B) The functional states of *CEN* or FR in the two reporter plasmids under the experimental conditions employed are tabulated. (C) The EBNA-1 and hEBP2 proteins were supplied by expression cassettes integrated in the host strain at the chromosomal *URA3* and *LEU2* loci, respectively. Protein expressions were verified by western blot analysis performed as described under Fig. 4.2.

in G1 or more than two foci following replication (in metaphase or anaphase cells) was omitted.

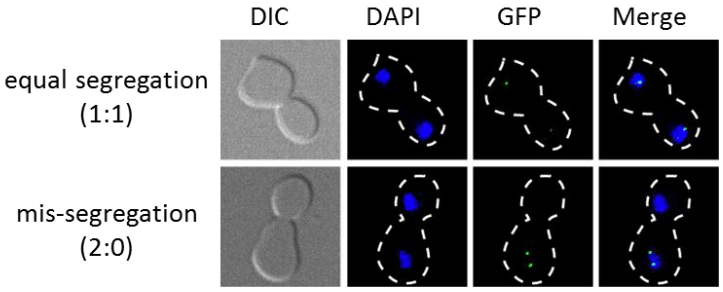
By shifting G1 arrested cells to galactose medium, the *CEN* function could be conditionally inactivated as a result of *GAL* promoter driven transcription. Upon releasing cells from G1 arrest in the presence of galactose, the plasmid would behave as an EBV reporter plasmid (Fig. 4.5A). The experimental strain was designed to express hEBP2 and EBNA-1 from chromosomally integrated expression cassettes (Fig. 4.4C). The advantage of the single copy reporter plasmid is that it can be induced to behave as a *CEN* plasmid (in glucose) or an FR plasmid (in galactose) or an *ARS* plasmid (lacking a partitioning system; in galactose in the absence of the partitioning proteins) (Fig. 4.4B). In some of the control assays, a 'single copy' *CEN-ARS* plasmid lacking the FR sequence was employed as the reporter.

The results of a single generation segregation assay performed on the single copy reporter plasmid are shown in Fig. 4.5. When *CEN* was active, over 95% of anaphase cells revealed equal (1:1) segregation (Fig. 4.5C). When *CEN* was inactivated, the percentage of equal segregation was approximately 42% in the presence of EBNA-1 and hEBP2, compared to roughly 16% in their absence (Fig. 4.5C). In the control *ARS* state of the plasmid, there was a small but reproducible increase in 1:1 segregation to ~23% in the presence of EBNA-1 and hEBP2 from the basal level of ~16%.

A.



B.



C.

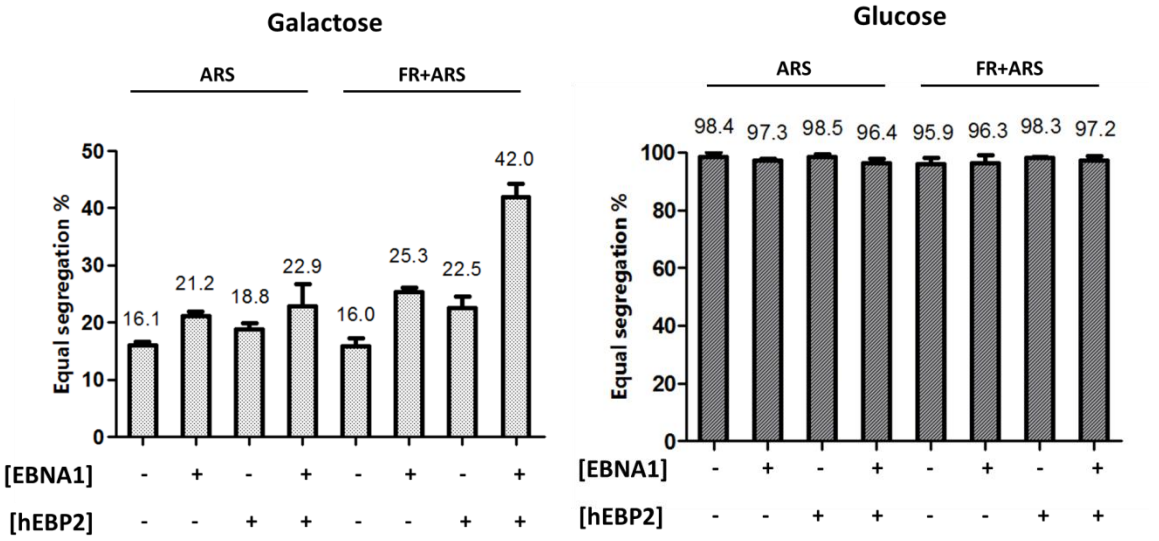


Figure 4.5 Increase in the equal segregation frequencies of the single-copy EBV plasmids in the reconstituted system. (A) The protocol for assaying plasmid partition in anaphase cells is schematically indicated. (B) The anaphase cells were scored for 1:1 and 2:0 patterns of plasmid segregation. (C) The fractions of anaphase cells with 1:1 segregation of reporter plasmid are shown in the presence or absence of the EBV partitioning proteins.

Clearly, the reconstituted EBV system causes a two to three fold improvement in equal segregation of the single copy FR reporter plasmid (from 16% to 42%). Yet, it is much less effective than the *CEN* partitioning system (42% versus >95%). It is also less effective than the 2 micron plasmid partitioning system. In a similar assay, Rep-*STB* mediated 1:1 segregation of a single copy reporter plasmid is 70-80% (Ghosh et al., 2007). The apparent inefficiency of the EBV system could be at one of two steps, either plasmid tethering to chromosomes in general, or tethering of replicated plasmid copies to sister chromatids, or a combination of the two. Even if plasmid association with chromosomes were entirely random, the predicted equal segregation frequency for a single copy plasmid would be 50% (due to independent assortment of chromosomes). The 1:1 segregation frequency of a single copy FR containing reporter plasmid, when deprived of EBNA-1 and hEBP2, is less than that predicted by a random diffusion model. This is because of the strong tendency of plasmids lacking a partitioning system to stay in the mother compartment (Murray and Szostak, 1983). In our assays, the observed mother bias for the 2:0 segregation events was >90%.

The EBV partitioning system appears to have a small positive effect even on the segregation of a plasmid lacking the FR partitioning locus. This could perhaps be due to some non-specific DNA binding activity of the foreign proteins expressed in the reconstituted system, which alleviates the mother bias. This non-specific effect appears to be due primarily to EBNA-1. Two human proteins, the bromodomain containing Brd2 and the methyl-CpG binding protein MeCp2, expressed in *S. cerevisiae* have been reported to non-specifically increase the stability of *CEN, ARS* and FR containing plasmids (Lin et al., 2008).

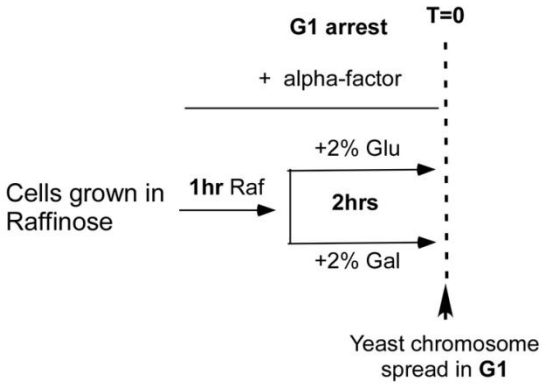
4.3.3 The single copy EBV reporter plasmid localizes in yeast chromosome spreads in the reconstituted system

Although the reconstituted EBV system provides increased plasmid stability, the evidence that this is mediated via plasmid-chromosome tethering is missing. To address this issue, the association of the single-copy EBV reporter plasmid with yeast chromosome spreads was assayed.

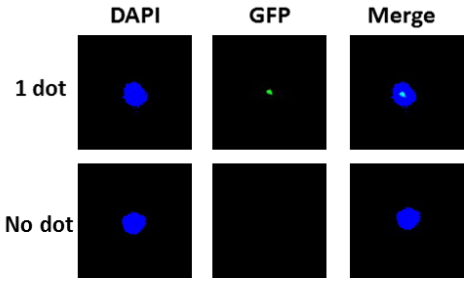
Chromosome spreads analyzed from G1 arrested cells (Fig. 4.6A) revealed the presence of the single copy FR plasmid in a little more than one third of the population (36.8%) in the absence of EBANA-1 and hEBP2. A similar result was obtained for the reporter plasmid lacking FR (34.4%; Fig. 4.6C). The localization of the FR plasmid in chromosome spreads increased to ~70% when EBNA-1 and hEBP2 were provided. Rather surprisingly, a somewhat smaller increase (nevertheless quite striking) was also observed for the *ARS* plasmid without FR (~61%). When *CEN* was active in the FR plasmid, its association with chromosome spreads was around 85% to 90% (Fig. 4.6C).

The chromosome spread data are consistent with the notion that EBNA-1 and hEBP2 proteins promote the tethering of the FR reporter plasmid to yeast chromosomes. In a separate study, a similar analysis using a single copy *STB* reporter plasmid revealed its frequency of association with G1 chromosome spreads to be ~80% (Fig. 4.7).

A.



B.



C.

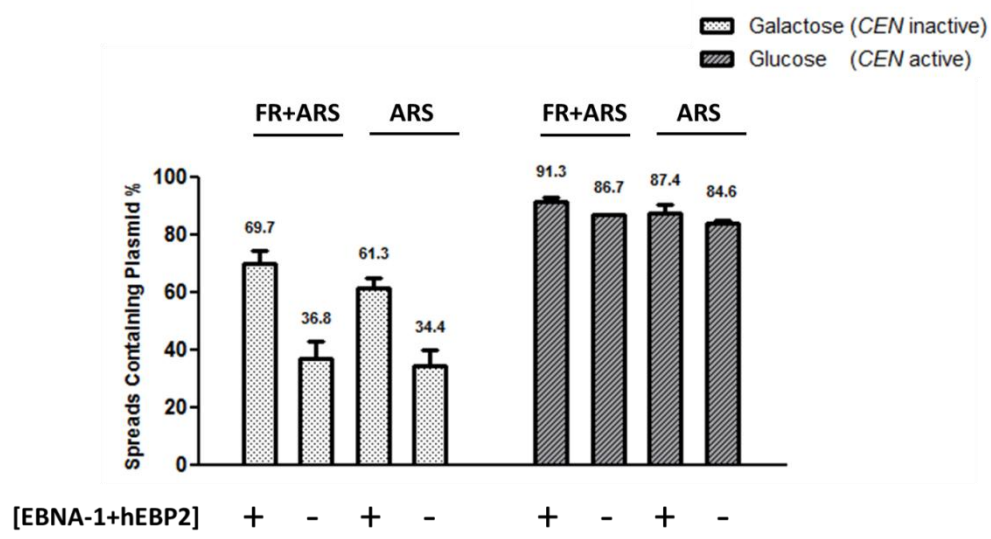


Figure 4.6 Association of single copy EBV plasmids in chromosome spreads from G1 arrested cells. (A) The experimental protocol is outlined at the top. Cells grown in raffinose were arrested in G1 using α factor. They were maintained in the presence of α factor in either glucose or galactose for 2 hours before processing them for chromosome spread assays. In glucose, the *CEN* harbored by the plasmids was functional; in galactose, it was non-functional. (B) Localization of a single copy EBV reporter plasmid in yeast chromosome spreads is shown. The spreads, stained with DAPI, were scored for two classes: containing one plasmid dot or lacking plasmid. The small fraction of cells containing two (or more) plasmid dots was omitted. (C) The results of the chromosome spread analysis are plotted as bar graphs.

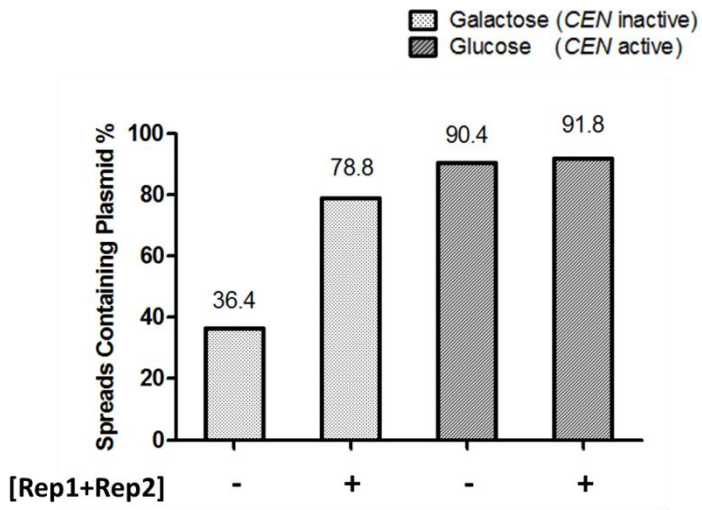


Figure 4.7 Association of single copy *STB* plasmids in chromosome spreads from G1 arrested cells. A chromosome spread analysis similar to Fig. 4.6 was performed using a single derivative *STB* plasmid (Ghosh et al., 2007). The experimental protocol shown in Fig. 4.6A was followed. Note that the *CEN* harbored by the plasmids was active in the presence of glucose and inactive in the presence of galactose. The availability of partitioning Rep proteins was based on the presence of the endogenous 2 micron plasmid [Cir^+] or not [Cir^0].

Plasmid distributions in chromosome spreads prepared from anaphase cells

Although chromosome spreads from G1 cells suggest that an FR containing plasmid may associate with chromosomes more efficiently in the presence of EBNA-1 and hEBP2, we know little regarding plasmid dynamics during the progression of the cell cycle. For example, do the plasmids detach from chromosomes during replication and reassociate with them? How efficient is such reassociation? What fraction of sister plasmids associate with sister chromatids and what fraction with non-sister chromosomes? Regardless of these issues, chromosome associated segregation of a single copy reporter plasmid can lead to only two outcomes. If the sister plasmids are tethered to sister chromatids, the result will be equal segregation (1:1). If they are tethered to non-sister chromosomes, the probability of equal segregation will be the same as that of unequal segregation (2:0) (since two non-sister chromosomes have a 50-50 chance of staying in the same cell compartment or segregating to opposite cell compartments). Assuming that chromosome associated plasmids remain so following segregation, a single plasmid dot in an anaphase chromosome spread would represent an equal segregation event mediated either by sister chromatids (non-random tethering) or by two non-sister chromosomes segregating to opposite cell poles (random tethering). The presence of two plasmid dots in a spread would signify unequal segregation resulting from random tethering. Thus the relative fractions of one plasmid dot to two plasmid dots in anaphase spreads would indicate the trend towards non-random versus random tethering. Because of the rather high background association of the control *ARS* plasmid (lacking FR) with chromosome spreads, which is further increased in the presence of EBNA1 and hEBP2, the anaphase patterns can only be interpreted in a qualitative sense.

In order to verify that our premises were valid, we first examined anaphase spreads (Fig. 4.8A) under conditions that maintained *CEN* active. Consistent with the high incidence of *CEN* mediated equal plasmid segregation; the observed fraction of spreads containing a single plasmid dot was also high (85-90%; Fig. 4.8C).

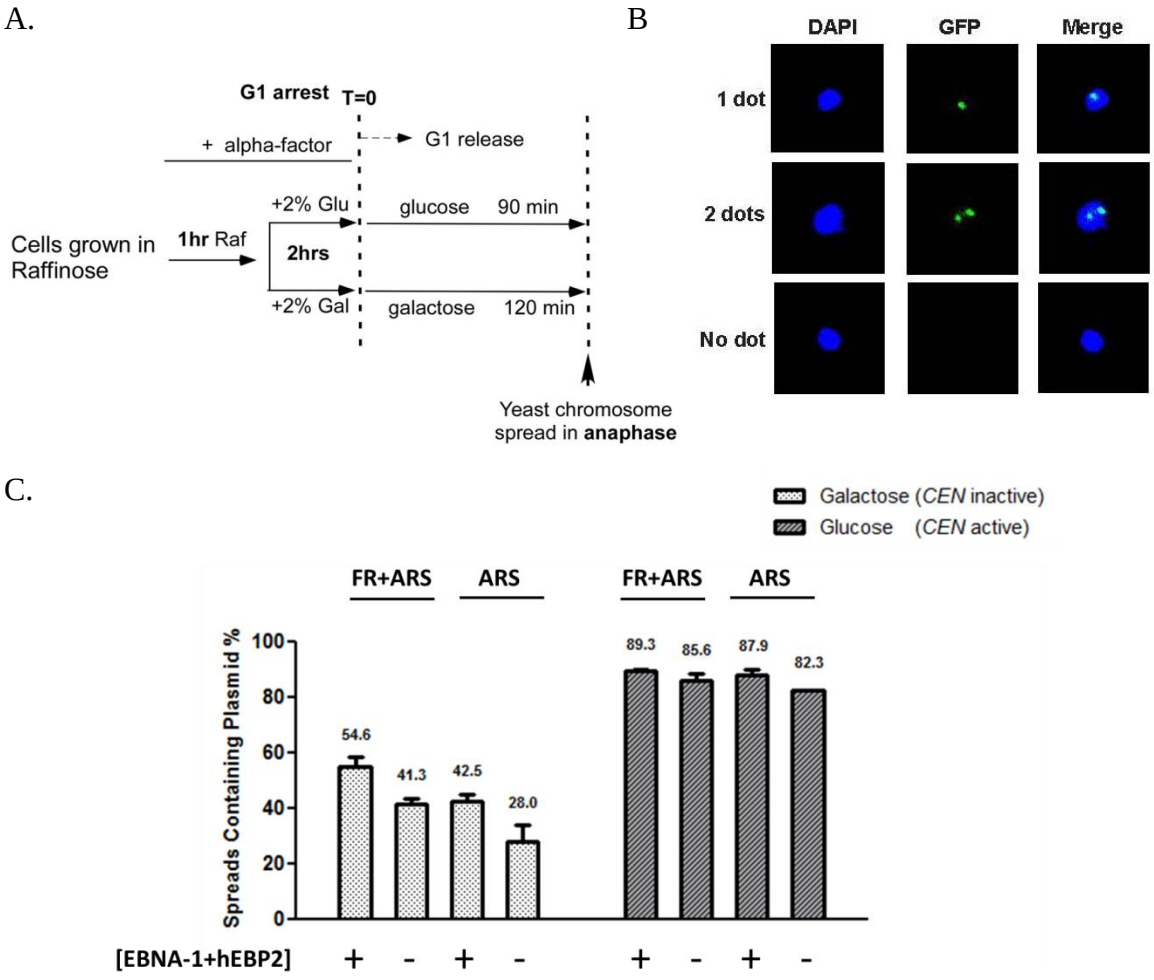


Figure 4.8 Association of EBV reporter plasmids with chromosome spreads prepared from anaphase cells. (A) The experimental scheme is outlined. Cells arrested in G1 were maintained in glucose or galactose for 2 hours before being released into the cell cycle. Chromosome spreads were prepared from cells growing in glucose at 90 min and from cells growing in galactose at 120 min after release into the cell cycle. At these time points, > 80% of the cells had the typical anaphase phenotype – large budded with two clearly segregated nuclei in the mother and daughter compartments. (B) The spreads were analyzed for three patterns: those containing no plasmid dot, one plasmid dot and two plasmid dots. The small fraction of spreads with more than two dots was ignored. (C) The percentages of spreads showing plasmid presence are graphed as bar diagrams.

Next we classified the plasmid containing anaphase spreads into those with a single plasmid dot or with two plasmid dots under *CEN* inactive conditions. In situations without the complete EBV partitioning system, the ratios of one dot to two dots were approximately 1:1 (Fig. 4.9B). There is no reason to suspect that plasmid segregation events under these conditions occur by chromosome tethering. Plasmid presence in spreads likely indicates chance association following plasmid segregation independent of chromosomes. When the EBV partitioning system was functional, the ratio of one dot to two dots increased to 3:1, which theoretically would reflect the occurrence of three equal segregation events for every two unequal ones (Fig. 4.9B and 4.10). This distribution would be consistent with non-random and random tethering of sister plasmids to chromosomes promoted by the EBV partitioning system being equally likely. Note, again, that random tethering has a 50% chance of resulting in equal plasmid segregation.

The segregation results, in conjunction with chromosome spread data, suggest that the EBNA-1 and hEBP2 proteins promote the tethering of the FR reporter plasmid to chromosomes. This tethering, random and non-random, will increase the proportion of 1:1 plasmid segregation events with 100% and 50% efficiencies, respectively. However, it should be noted that the overall efficiency of the EBV system is not high; yielding only ~42% equal plasmid segregation (Fig. 4.5). The majority still consists of 2:0 segregation events. Their strong mother bias would suggest that a large fraction of them are not due to EBV mediated random tethering of plasmids to chromosomes. If some of the EBV independent missegregation events fortuitously contribute to 2 plasmid dots in anaphase chromosome spreads, we could be underestimating the contribution of non-random plasmid tethering to equal segregation.

A.

B.

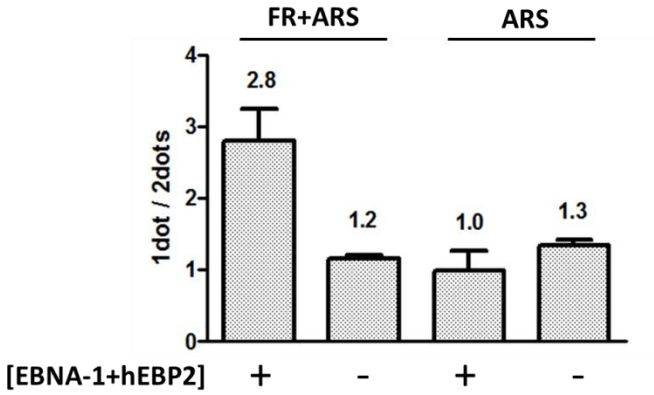


Figure 4.9 Segregation of sister plasmids by random versus non-random tethering to chromosomes. (A) Assuming that the EBV partitioning system drives the segregation of sister copies of the FR containing reporter plasmid by tethering them to chromosomes, the possible outcomes are schematically diagrammed. Equal segregation (1:1) of sister copies of the reporter plasmid will be indicated by the presence of a single plasmid dot in a chromosome spread. Unequal (2:0) segregation will be indicated by the presence of two plasmid dots. A higher incidence of 1:1 segregation compared to 2:0 indicates a bias towards non-random tethering. (B) The ratios of anaphase spreads containing one plasmid dot to two plasmid dots are plotted. The higher value when the EBV partitioning system is functional, suggests that at least a subset of the segregation events must be non-random.

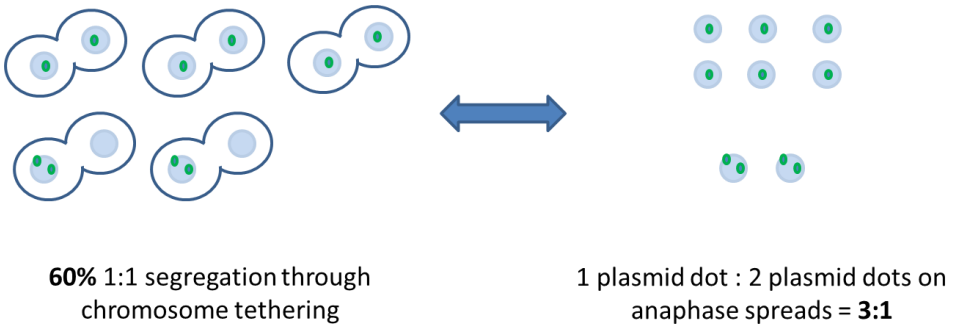


Figure 4.10 The percentage of equal plasmid segregation contributed by chromosome tethering as inferred from plasmid distributions in anaphase spreads. For the purpose of illustration, consider a case where there are three equal (1:1) for every two unequal (2:0) segregation events. When chromosome spreads from anaphase cells are scored, six of these will contain one plasmid focus each, and two will contain two plasmid foci each.

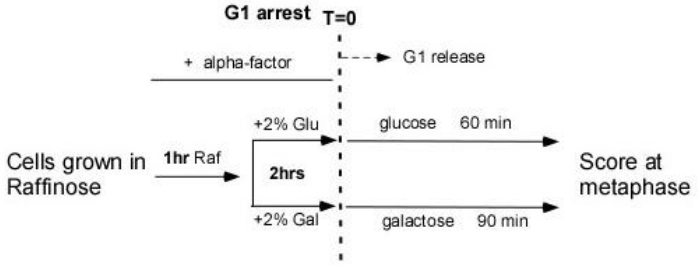
4.3.4 Colocalization of replicated plasmids in metaphase cells in the presence or absence of the mitotic spindle

In mammalian cells, replicated copies of sister EBV plasmids are colocalized as pairs with a high frequency which is consistent to symmetrical tethering on sister chromatids (Nanbo et al., 2007). This characteristic colocalization was also true for replicated viral episomes, even though the frequency of such events was reduced (Kanda et al., 2007). As discussed above, plasmid distributions in anaphase chromosome spreads suggest that a significant fraction of the FR sister plasmids is tethered to sister chromatids by EBNA-1 and hEBP2 in the *S. cerevisiae* system. A relevant question is whether the reconstituted system mimics the native system in the localization of sister plasmids prior to segregation. If the distance between sister plasmids is within ~ 15 kb or if they are localized symmetrically on cohesed sister chromatids, they should appear as a coalesced fluorescent dot. If they are associated with separate chromosomes or their spacing is >15 kb, they are likely to form two distinct foci.

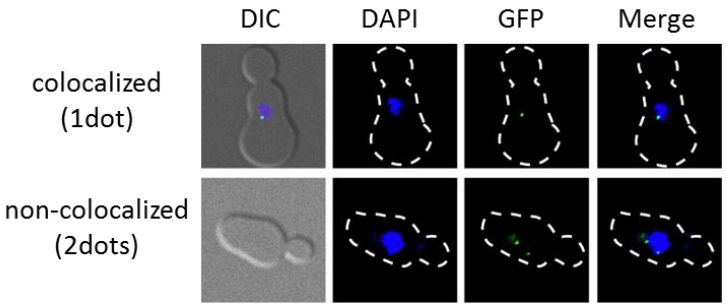
Based on this logic, we released cells from G1 arrest, and assayed the pattern of plasmid fluorescence in metaphase cells (Fig. 4.11A). These cells harbored an enlarged bud with the nucleus (identified by DAPI staining) located entirely within the mother near the bud neck. Approximately 50% of such cells showed colocalization of replicated plasmids when the EBV partitioning system was functional. In the absence of the partitioning system, the corresponding value was roughly 34% (Fig. 4.11C). When the plasmid borne *CEN* was kept active (in the presence of glucose), the vast majority of cells (~80%) revealed a single fluorescent dot (Fig. 4.11C).

It is the cohesin complex assembled at *CEN* that interlocks the sister plasmid copies (Ivanov and Nasmyth, 2005). Cohesin mediated pairing has also been observed for the yeast 2 micron plasmid, whose partitioning locus *STB* is a site of cohesin assembly (Ghosh et al., 2010; Mehta et al., 2002). A special feature of the establishment of cohesion at *STB* is its dependence on the integrity of the mitotic spindle. Spindle integrity is also critical for the localization of the 2 micron plasmid to its specific nuclear address in the proximity of the spindle pole body and the centromere cluster (Mehta et al., 2005). As far as we know, there is no evidence for the involvement of the spindle in the tethering of EBV to mammalian chromosomes. Consistent with this notion, colocalization of the FR plasmid sisters was not affected in cells released from G1 in presence of nocodazole and arrested in G2/M (Fig. 4.11D).

A.



B.



C.

D.

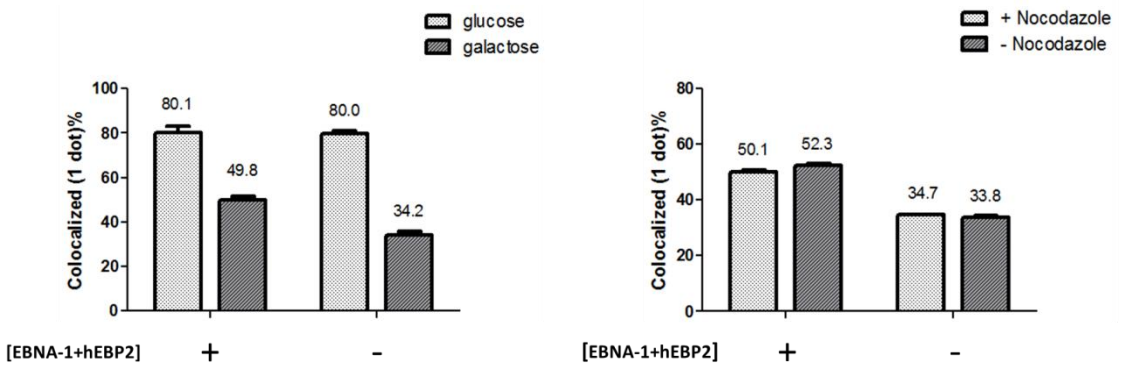


Figure 4.11 Replicated copies of the EBV reporter plasmids show improved colocalization in presence of the EBV partitioning systems in metaphase nuclei. (A) The assay procedures are schematically indicated. After release from G1 arrest, cells growing in glucose and galactose media were fixed at 60min and 90min, respectively, for visualizing the patterns of plasmid localization by fluorescence microscopy. Metaphase cells (with large buds and a single nucleus near the bud neck) were predominant (>80%) in the population at these time points. (B) Replicated plasmids were assayed for colocalization in metaphase cells. (C) The fractions of metaphase cells with colocalization of replicated EBV plasmids are shown under the reconstituted system. (D) The effect of microtubule integrity on plasmid sister colocalization were assayed in cells arrested in G2/M by nocodazole treatment.

4.4 Discussion

The partitioning system of EBV has been successfully reconstituted in *S. cerevisiae* by Kapoor et al. (Kapoor and Frappier, 2003; Kapoor et al., 2005; Kapoor et al., 2001). They showed that this artificial system retains several attributes of the native system. Partitioning is dependent on the presence of the FR sequence in *cis* on the reporter plasmid and the simultaneous presence of the EBNA-1 and hEBP2 proteins. They concluded that the efficient segregation of the FR containing plasmid is the result of its attachment to chromosomes with the assistance of the EBNA-1 and hEBP2 proteins.

In the present study, we have addressed the mechanism of the reconstituted EBV system in *S. cerevisiae* more systematically. Our analyses using the single copy fluorescence tagged reporter plasmid verify and extend previous conclusions that plasmid segregation is mediated via hitchhiking on chromosomes. Our results suggest both 'non-random' and 'random' tethering of the reporter plasmid to chromosomes, resulting in its improved equal segregation frequency. However, plasmid stability conferred by the EBV system in *S. cerevisiae* is much less than that conferred by the *CEN* system or the 2 micron plasmid partitioning system.

4.4.1 Role of hEBP2 in EBV partitioning in *S. cerevisiae*

The findings of Kapoor et al. implied that the hEBP2 homologue in *S. cerevisiae*, yEBP2, is not capable of substituting for the former in plasmid partitioning (Kapoor and Frappier, 2003). There is no interaction between yEBP2 and EBNA-1, as judged by a two-hybrid assay. Amino acids 325-276 of EBNA-1 and the C-terminal 86 amino acids of hEBP2 are responsible for the interaction between the two proteins (Fig. 1.8). They

share 36% identity and 54% similarity in their C-terminal domains, with divergent residues distributed throughout these regions. It is not clear which of these differences are responsible for their differential interactions with EBNA-1.

Human EBP2, in its native context, is localized to the nucleolus during interphase, and is redistributed throughout chromosomes during mitosis (Kapoor et al., 2005). This behavior is consistent with what is known about other nucleolar proteins involved in rRNA processing (Ford et al., 2006; Straight et al., 1999), which is thought to be the functional role of EBP2 as well. During open mitosis, the nucleolar organization breaks down, and some of the nucleolar proteins associate with chromosomes as a means for equal partitioning into daughter cells (De Souza and Osmani, 2009). hEBP2 expressed in *S. cerevisiae* also exhibits an analogous behavior, being confined to the nucleolus during G1 and spreading throughout chromosomes during mitosis. This cell cycle dependent relocalization of hEBP2 in *S. cerevisiae* is in contrast to yEBP2, which remains in the nucleolous during mitosis (Kapoor and Frappier, 2003).

The domainal organization of hEBP2 consists of an N-terminal and a C-terminal region separated by a coiled-coil middle domain (Shire et al., 1999) (Fig. 1.8). In human cells, the association of EBP2 with chromatin is mediated through the middle domain. A fusion protein in which this domain is joined to the FR binding/dimerization domain of EBNA-1, with an included nuclear localization signal (NLS), functions as a partitioning protein in yeast for the FR containing reporter plasmid (Kapoor and Frappier, 2003). Thus, the cumulative evidence suggests that the chromosomal hitchhiking mechanism for EBV episome stability in mammalian cells can be reproduced, at least qualitatively, in yeast.

4.4.2 Partitioning functions of other gammaherpesviruses

In addition to EBV, the segregation mechanisms of two gammaherpesviruses, the human Kaposi's sarcoma associated herpesvirus (KHSV) and the monkey herpesvirus saimiri (HSV), have been explored to varying degrees of depth (Ballestas et al., 1999; Ballestas and Kaye, 2001; Calderwood et al., 2004; Verma and Robertson, 2003). In KHSV, the viral LANA protein fulfills the partitioning function, and also regulates transcription of viral genes. LANA interacts with histone H2A and H2B as well as the methyl-CpG binding protein MeCP2 through its extreme N-terminal region (Barbera et al., 2006; Krithivas et al., 2002). It also interacts with the bromodomain proteins Brd2 and Brd4 as well as DEK, an abundant chromatin associated protein that preferentially binds to cruciform DNA, through its extreme C-terminal region (Krithivas et al., 2002; Ottinger et al., 2006; Viejo-Borbolla et al., 2005). MeCP2, DEK and Brd4 have been shown to promote the localization of LANA to chromosomes. Furthermore, the colocalization of LANA, Brd4 and KSHV episomes on mitotic chromosomes has been demonstrated (Krithivas et al., 2002; You et al., 2006). The LANA homologue ORF73, the partitioning protein of HSV, binds to the terminal repeats of the virus and to mitotic chromosomes. ORF73 interacts with MeCP2, but not DEK, and depletion of MeCP2 leads to higher loss of HSV based plasmids in human cells (Griffiths and Whitehouse, 2007).

4.4.3 Plasmid stabilization in *S. cerevisiae* by non-native chromatin binding proteins

Interestingly, if somewhat unexpectedly, Brd4, Brd2 and MeCP2 have been shown to promote EBNA-1 assisted maintenance of an FR reporter plasmid in *S.*

cerevisiae (Lin et al., 2008). Whereas the Brd4 effect is specific to an FR containing plasmid, Brd2 and MeCP2 are capable of stabilizing plasmids lacking FR as well. The mechanism of this general enhancement of plasmid maintenance is not known. However, this finding suggests that chromosome tethering may not be the exclusive means by which the reconstituted system brings about plasmid segregation. EBNA-1 interacts with Brd4 in both human and yeast cells. However, this interaction is mediated through the N-terminal sequences of EBNA-1 required for activation of viral transcription but not viral episome partitioning. Consistent with this feature, in latently infected human cells, Brd4 is preferentially enriched at the FR enhancer element regulated by EBNA1 over other viral genomic locales.

In our analysis using the multi-copy and the single copy fluorescence tagged FR reporter plasmids, we see a significant increase in segregation efficiency conferred by coexpression of EBNA-1 and hEBP2. We also notice a small but consistent increase in the equal segregation of an *ARS* plasmid in the presence of EBNA-1 and hEBP2.

4.4.4 Plasmid association with yeast chromosomes promoted by the EBV partitioning system

There is no easy method to derive the efficiency for the association of a reporter plasmid to yeast chromosomes, unless the association sites on chromosomes have been defined. Therefore, we had to rely on yeast chromosome spreads for assaying potential plasmid localization on chromosomes. Since these spreads do not permit resolution at the single chromosome level, a low level of spurious association by the interaction of the plasmid with a subnuclear structure that is retained in the spreads cannot be ruled out.

However, even with these caveats, the appropriate control assays in the absence of the partitioning proteins, or employing a reporter plasmid lacking FR, permit meaningful conclusions to be drawn from this analysis. In G1 arrested cells, the fraction of cells harboring the FR plasmid in chromosome spreads is substantially increased (~ two fold) in the presence of EBNA-1 and hEBP2 over that in their absence. This increase correlates reasonably with the increase in plasmid stability (~ 3 fold) observed under corresponding conditions. Interestingly, the association of even the *ARS* reporter plasmid with chromosome spreads is increased by EBNA-1 and hEBP2, though to a smaller extent, presumably through non-specific DNA binding. As pointed out already, these proteins also confer a small, but reproducible, increase in stability on the *ARS* plasmid.

4.4.5 Random versus non-random tethering of plasmids to chromosomes by the EBV partitioning system

Analysis of the plasmid fluorescence patterns in anaphase chromosome spreads suggest that tethering of the FR plasmid to chromosomes in the presence of EBNA-1 and hEBP2 is unlikely to be completely random. The three fold higher incidence of one plasmid over two per nucleus in anaphase spreads is indicative of a finite subset of non-random tethering events, that is, tethering of sister plasmids to sister chromatids. A reasonable estimate of non-random tethering as a fraction of total tethering is around 50%, perhaps higher.

4.4.6 Symmetric tethering of sister plasmids to sister chromatids by the EBV partitioning system

The non-random mode of plasmid tethering could be of two types: symmetric or asymmetric. In symmetric tethering, sister plasmids are positioned symmetrically on sister chromatids by attaching to identical tethering sites. In asymmetric tethering, the plasmids are tethered to separate tethering sites on sister chromatids. Since sister chromatids remain paired by cohesin until the onset of anaphase, symmetric tethering would display the colocalized plasmid pair as a single fluorescent dot or closely overlapping two dots. Asymmetric tethering, within the resolution of fluorescence microscopy, would more likely result in two separated dots. The increase in the fraction of metaphase nuclei with a single plasmid dot overt that with two dots when EBNA-1 and hEBP2 proteins are supplied suggests that at least a subset of the tethering events they promote are symmetric. Our assay cannot distinguish between a pair of sister plasmids tethered symmetrically to sister chromatids from a pair tethered to closely proximal sites (within ~15 kbp) on the same chromosome. This is a limitation of the *S. cerevisiae* system, in which individual mitotic chromosomes cannot be resolved by chromosome spreading.

4.4.7 Spindle integrity is not a pre-requisite for symmetric plasmid tethering by the EBV system

We have noticed no adverse effect of depolymerizing the mitotic spindle on the colocalization (single fluorescent dot) of sister FR plasmids in the nuclei from G2/M arrested cells obtained by nocodazole treatment. This clear difference from an *STB* reporter plasmid is consistent with the spindle dependence of the specific nuclear localization of the 2 micron plasmid as well as a similar dependence of the Rep1 and

Rep2 assisted cohesin assembly at *STB* and consequent sister plasmid cohesion (Ghosh et al., 2007; Mehta et al., 2005). Reporter plasmids containing human papilloma virus (HPV) origin have been shown to associate with the mitotic spindle with the help of the origin binding protein (Van Tine et al., 2004). The N-terminal domain of E2, required for activation of viral replication and transcription by interacting with viral and host coded proteins, as well as the C-terminal dimerization and ori-binding domain are capable of independently associating with the mitotic spindle. Thus, viral episomes have evolved two distinct strategies to exploit the host chromosome segregation machinery for their stable propagation: they either directly attach to the spindle or indirectly utilize the spindle by tethering to chromosomes. The EBV partitioning system follows the latter path.

4.5 Perspective

We have so far demonstrated that the action of the reconstituted EBV partitioning system in *S. cerevisiae*, though not efficient, is functionally analogous to that in the native mammalian system. The increased plasmid stability of the FR reporter plasmid can be accounted for, at least to a significant extent, by the EBNA-1 and hEBP2 mediated association of sister plasmids to sister chromatids. In the next chapter, we use the analytical tools based on the fluorescence tagged single copy reporter plasmid to examine the behavior of the bovine papilloma virus (BPV) partitioning system, which has also been reconstituted in *S. cerevisiae* (Brannon et al., 2005). Evidence from studies in mammalian cell lines suggest that, in contrast to EBV, BPV episomes associate with chromosomes in a more random fashion (McBride et al., 2006). We are also curious to

know whether the stability of an autonomously replicating plasmid in yeast can be enhanced through artificial means of tethering replicated plasmid copies to sister chromatids. If successful, such methodologies may find general applications across eukaryotic cell systems in the stable maintenance of beneficial extra-chromosomal elements.

CHAPTER5

Characterization of the BPV partitioning system reconstituted in *Saccharomyces cerevisiae*

5.1 Summary

Utilizing strategies analogous to those described in **Chapter 4**, we have analyzed the BPV partitioning system reconstituted in *S. cerevisiae*. **1**. The maintenance of a multi-copy *ARS* plasmid harboring the BPV partitioning locus is improved upon co-expressing the viral partitioning protein E2 and its mammalian partner protein Brd4 in the host strain. **2**. Consistent with this result, the equal segregation frequency of a fluorescence tagged single copy reporter plasmid in a single generation segregation assay is also increased in the presence of E2 and Brd4. **3**. Analyses of chromosome spreads prepared from G1 and anaphase cells suggest that the partitioning proteins promote the tethering of the reporter plasmid containing the BPV partitioning locus to chromosomes. **4**. The ratio of one plasmid focus to two foci in anaphase spreads is distinct between the BPV and EBV reporter plasmids. Unlike the EBV system, which display a 3:1 bias in favor of spreads containing one plasmid focus, the BPV system shows no obvious difference in the fractions of spreads containing one focus or two foci. **5**. The extent of co-localization of replicated copies of the BPV reporter plasmid in metaphase cells shows a modest (but significant) increase in the presence of E2 and Brd4. However, the increase is less than that conferred on the EBV reporter plasmid by the EBNA1 and hEBP2 proteins. **6**. Taken together, our results suggest that the increased stability of the BPV reporter plasmids in *S. cerevisiae* in the reconstituted system is due to E2 and Brd4 mediated

tethering of the plasmid to chromosomes. However, the efficiency of the system in the heterologous host is low. Finally, the BPV system is less competent than the EBV system in promoting the tethering of sister plasmids to sister chromatids.

5.2 Introduction

The papillomaviruses constitute a closely related group that infects higher vertebrates, including humans, and normally induces benign lesions. However, certain human papilloma viruses, HPV16 and 18, for example, have been associated with human cervical cancers and other tumors (zur Hausen, 2002). The 'high-risk' nature of a particular virus and its capacity for long-term latent persistence in the host are strongly correlated. However, it is not clear whether high-risk is associated with the specific mechanisms for stable viral maintenance, the capacity of a virus to infect multipotent stem cells in the susceptible tissue zone or its proficiency in evading the immune system.

Mechanisms for maintenance of bovine papilloma virus episomes

Several members of the papilloma virus family follow a strategy analogous to that of EBV for their long-term persistence in infected cells, namely, segregation in association with chromosomes. The most extensively characterized system among PVs for chromosome tethering is the type 1 bovine papilloma virus (BPV1). As mentioned earlier in **Chapter 1**, the virus coded E2 protein, which binds to its cognate sequences present within the viral genome, mediates viral tethering to chromosomes through its interaction with the host coded double bromodomain protein Brd4 (You et al., 2004). The E2 protein is composed of three domains, with a flexible hinge region connecting the C-terminal and N-terminal domains (Fig. 1.9). The C-terminal region of E2, which is

mainly responsible for E2 dimerization and DNA binding activity, recognizes the $ACCN_6GGT$ motif distributed within the viral genome at transcriptional enhancer regions and at the replication origin. The N-terminal region of E2 is responsible for interaction with Brd4, the viral E1 protein and other host proteins, and plays important roles in chromosome tethering, viral genome replication and transcriptional regulation (Fig. 1.9). On the other hand, Brd4 binds to acetylated chromatin through its bromodomains during interphase and mitosis (Dey et al., 2003), and is also involved in transcriptional regulation by association with P-TEFB (Jang et al., 2005; Wu and Chiang, 2007) (Fig. 1.10). As the primary cellular protein that promotes viral attachment to chromosomes, Brd4 interacts with E2 through its C-terminal domain (Fig. 1.10). Overexpression of this domain has a dominant negative effect on the tethering of BPV1 episomes to chromosomes, viral maintenance and BPV1 mediated cellular transformation. In cells expressing E2, the Brd4-E2 complex forms localized puncta along chromosomes. This E2 induced pattern contrasts with the diffused localization of Brd4 seen in the absence of E2. E2-Brd4 interaction is also important for E2 mediated transcriptional regulation.

It is important to note there are potentially several mechanisms for genome partitioning among PVs. Though the strategy of chromosome association appears to be shared by most papilloma viruses, Brd4 is not required for chromosome tethering of E2 under all circumstances. However, the interaction of E2 and Brd4 is a pre-requisite for E2-mediated transcription regulation among all PVs which have been tested. Furthermore, the patterns of E2 localization on chromosomes are not uniform among different papilloma viruses. In contrast to the punctuate localization of BPV1 E2 along chromosome arms, E2 proteins from a human papilloma virus (HPV8) and alpha

papillomaviruses are enriched in pericentric regions adjacent to kinetochores (Oliveira et al., 2006). Evidence has been presented for the association of E2 proteins form three common human papilloma virus types, HPV-11, HPV-16 and HPV-18, with spindle fibers (Van Tine et al., 2004). These E2 proteins appear to be excluded from mitotic chromosomes. Furthermore, E2 dependent localization of an HPV origin containing plasmid to the spindle has also been demonstrated. Taken together, these results argue for a direct spindle mediated segregation mechanism for at least a subset of papilloma viruses. Thus, papilloma viruses may have evolved capabilities for exploiting the chromosome segregation machineries of their hosts in more ways than one. Chromosome tethering and spindle attachment need not be mutually exclusive, and it is possible that some viruses may engender these redundant mechanisms to ensure their maintenance in host cells.

Reconstitution of the BPV 1 partitioning system in yeast

The BPV 1 partitioning system, like the EBV system described in the previous chapter, has also been reconstituted in *S. cerevisiae* (Brannon et al., 2005). The stability of an *ARS* reporter plasmid (multi-copy) containing iterated E2 binding sites can be increased when E2 and Brd4 are coexpressed in the host strain harboring the plasmid. Expression of E2 alone is not effective in improving plasmid maintenance. A reasonable interpretation of these results is that E2 and Brd4 act collaboratively to tether the plasmid to chromosomes, thus yielding more efficient plasmid segregation. Chromosome association would free the *ARS* plasmid from the diffusion constraints that cause a strong bias in plasmid retention by the mother cell (Murray and Szostak, 1983).

Further characterization of the reconstituted BPV partitioning system in *S. cerevisiae*

Based on the results presented in **Chapter 4** with the EBV partitioning system, we concluded that the segregation efficiency conferred by the EBNA1and hEBP2 proteins on a reporter plasmid in *S. cerevisiae* is modest. However, the mechanism of segregation is consistent with these proteins promoting the tethering of the plasmid to chromosomes. The BPV system offers the opportunity to analyze the efficiency and mechanism of a second heterologous chromosome tethering system in plasmid maintenance in *S. cerevisiae*. We have therefore characterized reporter plasmid segregation in *S. cerevisiae* under the control of the BPV system. The rationale and methodologies are analogous to those employed for the study of the EBV system (**Chapter 4**). As such, we will be brief in our discussion of the results and their interpretations.

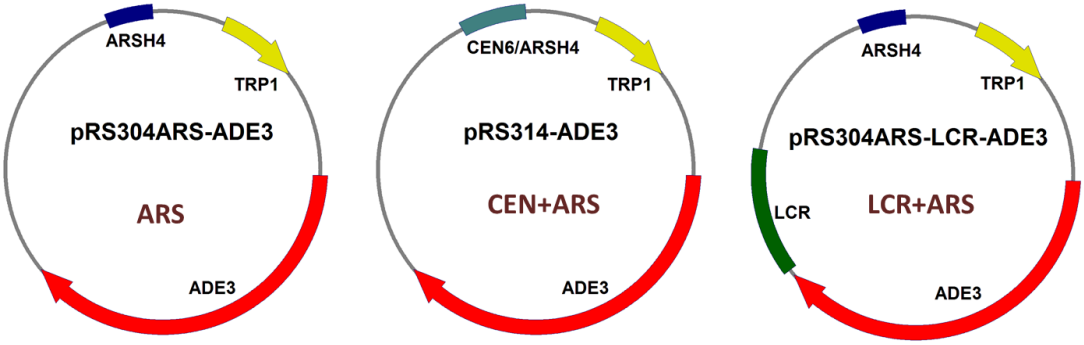
5.3 Results

5.3.1 Action of the reconstituted BPV system on a multi-copy reporter plasmid

As in the case of the EBV reconstituted system, the functionality of the reconstituted BPV system in yeast (Brannon et al., 2005) was verified through a plasmid loss assay. A multi-copy BPV reporter plasmid containing an autonomously replicating sequence (*ARS*), the partition locus of the BPV episome (LCR) and the *TRP1* and *ADE3* marker genes was employed in the assay (Fig. 5.1A). The host strain also harbored a *CEN* based plasmid expressing the viral E2 protein and a 2 micron circle based plasmid expressing the Brd4 protein (Brannon et al., 2005) (Fig. 5.1B). The plasmid loss assay

was followed as outlined earlier in **Chapter 4.3.1**. The loss rate per generation (%) of the reporter plasmid was calculated from the fractions of plasmid containing cells in the population immediately prior to and following growth for a fixed number of generations under non-selective conditions. The BPV reporter plasmid showed increased plasmid stability in the presence of E2 and Brd4 compared to that in the absence of these proteins. The plasmid stability conferred by the BPV partitioning system was higher than that of an *ARS* reporter plasmid but lower than that of a *CEN* reporter plasmid (Fig. 5.2). The results were generally consistent with previously reported data (Brannon et al., 2005), and verify that the reconstituted BPV system is at least partially functional in yeast.

A.



B.

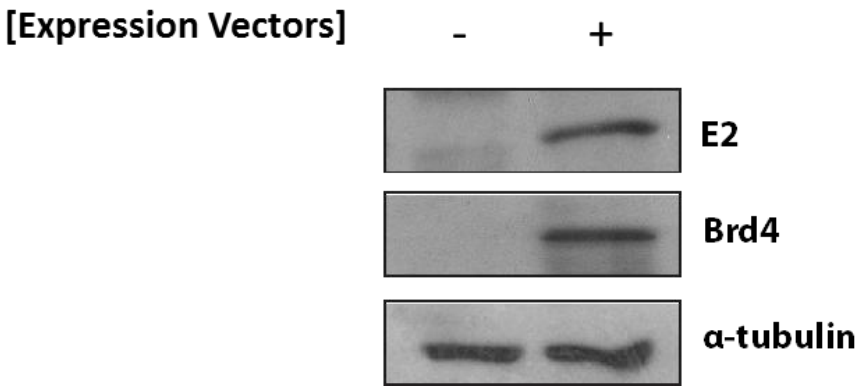


Figure 5.1 The reconstituted BPV partitioning system in *S. cerevisiae*. (A) The BPV, *CEN* and *ARS* reporter plasmids used for stability assays are the same as those described by Brannon et al. (2005), and are schematically diagrammed. All three harbor the *TRP1* and *ADE3* as the selectable markers in addition to an *ARS* element. The BPV reporter plasmid contains the LCR region from BPV. The partitioning proteins E2 and Brd4 were expressed in the experimental strains by transforming them with the appropriate expression plasmids. Cell lysates prepared from strains containing (+) or lacking (-) the expression plasmids were assayed by western blotting. The antibodies used were to native E2 and to native Brd4. The loading control was provided by α-tubulin.

A.

Reporter plasmid	Partitioning Locus	E2/Brd4 expression	Cells harboring plasmid (%)	
			T0	T10
pRS304ARS-ADE3 (ARS)	–	+	26.0%	12.0%
		-	10.0%	4.6%
pRS314-ADE3 (CEN+ARS)	*CEN*	+	70.7%	60.7%
		-	73.8%	61.7%
pRS304ARS-LCR-ADE3 (LCR+ARS)	LCR	+	53.5%	39.8%
		-	10.0%	4.6%

B.

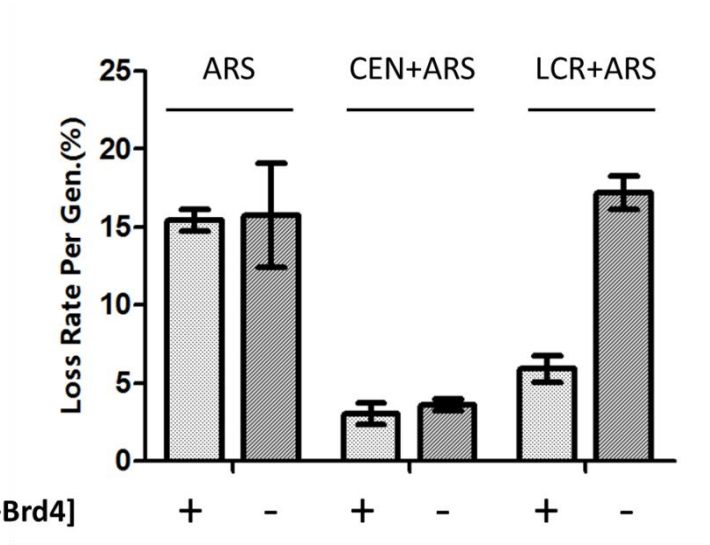


Figure 5.2 The reconstituted BPV partitioning system stabilizes a BPV reporter plasmid in *S. cerevisiae*. (A) The fractions of cells in the population containing the reporter plasmid at T0 (under selective growth) and at T10 (10 hours under non-selective growth, equal to **5** generations) with or without the expression of E2 and Brd4 are tabulated. (B) The Instability index or the loss rate per generation (%) for the three reporter plasmids (see Fig. 5.1) in the presence (+) and absence (-) of E2 and Brd4 are plotted as bar graphs.

5.3.2 Segregation of a single copy reporter plasmid promoted by the reconstituted BPV partitioning system

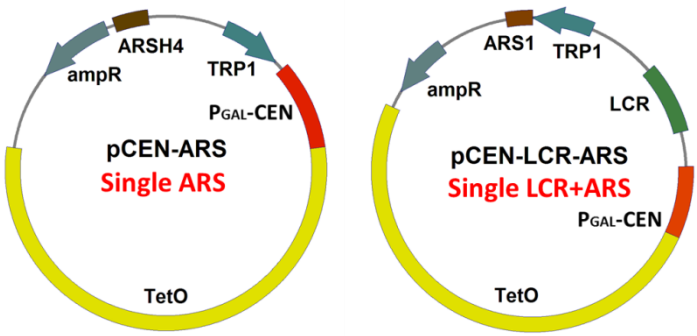
Unlike the EBV system, there is no evidence to show that BPV episomes segregate in a non-random (sister-to-sister or symmetric) fashion in their native hosts. It is likely that replicated sister plasmid copies associate randomly with chromosomes rather than specifically with sister chromatids. The plasmid loss assay using the multi-copy BPV reporter plasmid demonstrates the ability of the E2 and Brd4 proteins to promote partitioning in the non-native *S. cerevisiae* system. In order to characterize the reconstituted BPV system in further detail and to evaluate its efficiency more critically, we followed the segregation of a single copy derivative of the BPV plasmid during a single cell division cycle. As described in the previous chapter for the EBV system, the *CEN3* sequence, whose function could be transcriptionally controlled by the *GAL* promoter, was incorporated into the EBV reporter plasmid to lower its copy number to nearly one. In addition, a TetO array was also introduced into the plasmid, enabling it to be visualized by fluorescence in host cells expressing TetR-GFP (Fig. 5.3A). The plasmid would behave as a single copy BPV reporter when *CEN* is conditionally inactivated in presence of galactose (Fig. 5.3B). A single copy *CEN-ARS* plasmid mentioned in **Chapter 4** served as a control in these assays. The experimental strain was engineered to harbor chromosomally integrated expression cassettes for the E2 and Brd4 proteins. The authenticity of the integrations was ascertained by PCR, and by sequencing the amplified DNA. Expression of the proteins was verified by western blotting (Fig. 5.3C).

We followed the segregation of the single copy BPV reporter plasmid by first arresting cells in G1, and assaying 1:1 and 2:0 distributions of fluorescent plasmid foci in

cells released from arrest at the anaphase stage (Fig. 5.4A). By maintaining cells in glucose medium or shifting them to galactose medium, plasmid segregation could be placed under the control of the *CEN* system or the EBV system, respectively. In the absence of a functional *CEN*, the percentage of equal segregation of the BPV plasmid increased from ~14% in the absence of E2 and Brd4 to ~34% in their presence. This increase in 1:1 segregation was not observed when either E2 or Br4 alone was expressed. Thus, the single copy plasmid segregation results during a single cell cycle recapitulate, at least qualitatively, the observed stability of the multi-copy reporter plasmid over several generations (Fig. 5.4C).

Our results suggest that the reconstituted BPV system is less efficient than the reconstituted EBV system in promoting equal plasmid segregation in comparable assays. It is possible that the BPV system is less competent in mediating plasmid-chromosome association in *S. cerevisiae*. Alternatively, the BPV system may promote random association of plasmids with chromosomes rather than the association of sister plasmids to sister chromatids.

A.



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B.

Plasmid	glucose		Galactose	
	[E2+Brd4]		[E2+Brd4]	
	-	+	-	+
pCEN-ARS (single copy ARS)	CEN+	CEN+	CEN-	CEN-
pCEN-LCR-ARS (single copy LCR+ARS)	CEN+ LCR-	CEN+ LCR+	CEN- LCR-	CEN- LCR+

C.

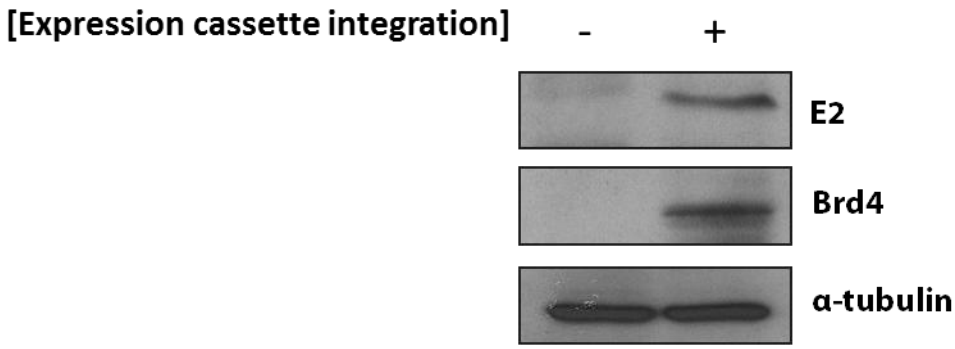
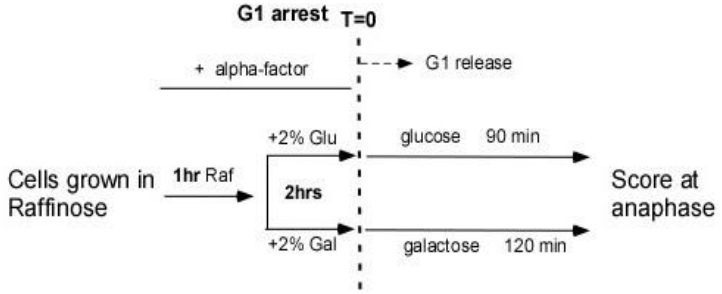
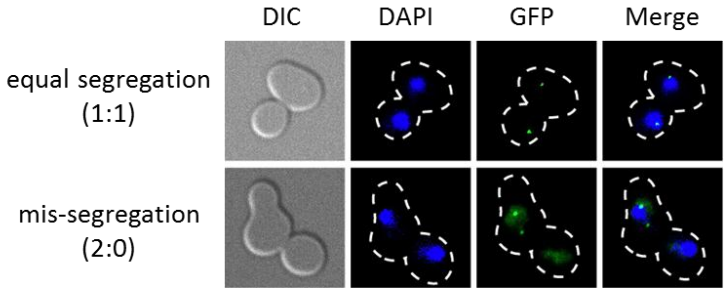


Figure 5.3 Single-copy reporter plasmids for segregation assays in the yeast strain harboring the reconstituted BPV partitioning system. (A) The BPV reporter plasmid contained a replication origin (*ARS*), the viral partition sequence (LCR), a [TetO] array and the yeast *CEN3* sequence controlled by the *GAL* promoter. The control plasmid lacked the LCR sequence but included all the other aforementioned features, and was also used for assays described in **Chapter 4**. (B) The functional states of *CEN* or *LCR* in the two reporter plasmids under the experimental conditions employed are tabulated. (C) The E2 and Brd4 proteins were expressed from chromosomally integrated cassettes at the *URA3* locus and *LEU2* locus respectively. Protein expressions were verified by western blot analysis performed as described under Fig. 5.1.

A.



B.



C.

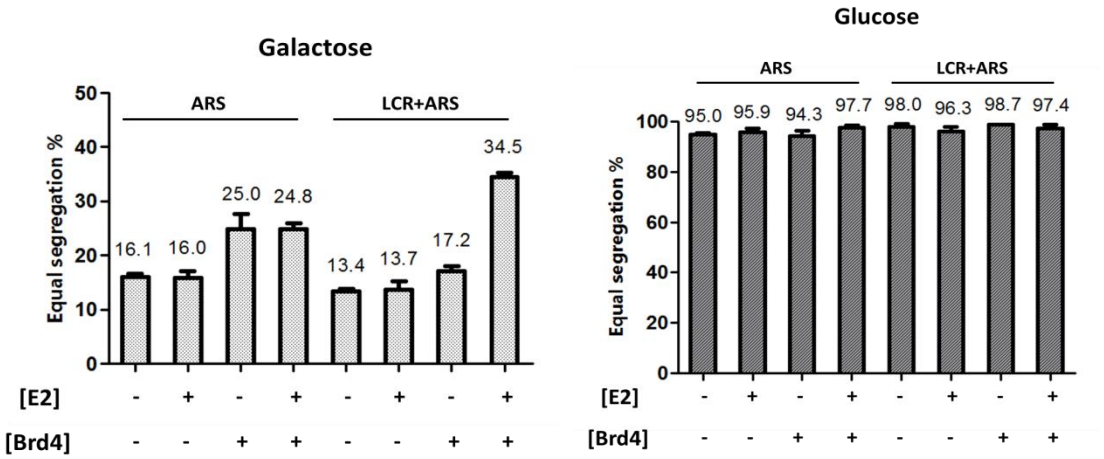


Figure 5.4 Segregation of single-copy reporter plasmids in the presence and absence of the BPV partitioning system. (A) The protocol for assaying plasmid partitioning in anaphase cells is that followed in similar experiments of the previous chapter, and is schematically outlined here. (B) The plasmid segregation events were classified as equal (1:1) and unequal (2:0) by fluorescence microscopy. (C) The fractions of anaphase cells displaying 1:1 Segregation frequencies of the reporter plasmids in the presence and absence of the partitioning proteins are graphically displayed.

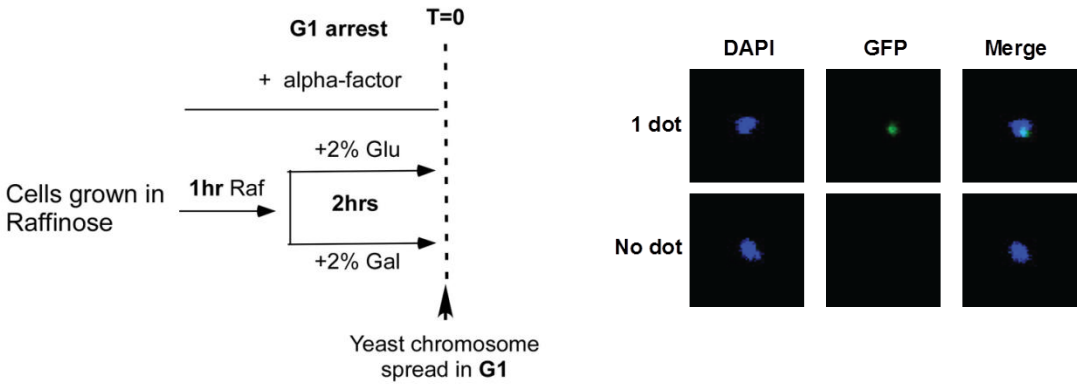
5.3.3 Localization of the single copy reporter plasmids in yeast chromosome spreads in the absence and presence of the BPV partitioning system

In order to test the notion that the increase in equal segregation of the BPV reporter plasmid in the presence of the partitioning proteins is mediated via plasmid-chromosome tethering, the association of the plasmid with yeast chromosome spreads was assayed under different conditions as detailed below.

5.3.3.1 Chromosome spreads prepared from G1-arrested cells

As was done in earlier experiments with the reconstituted EBV system, chromosome spreads were first assayed for the presence of the single copy BPV reporter plasmid in G1 arrested cells (Fig. 5.5A). The fraction of plasmid containing spreads increased from 38.6% to 57.2% in the presence of E2 and Brd4 compared to their absence. The presence of the partitioning proteins did not make a difference in the association of the *ARS* plasmid with the spreads (38.5% in the presence of E2 and Brd4 versus 34.7% in their absence). In the control assays, with the *CEN* maintained in its active state, both the *BPV* and *ARS* reporters showed high percentage of association with chromosome spreads (~85%) (Fig. 5.5C). The results are consistent with the partitioning proteins promoting the tethering of the BPV plasmid to chromosomes, but with lower efficiency in comparison to the reconstituted EBV system (Fig. 4.6C), at least during the G1 stage of the cell cycle.

A. B.



C.

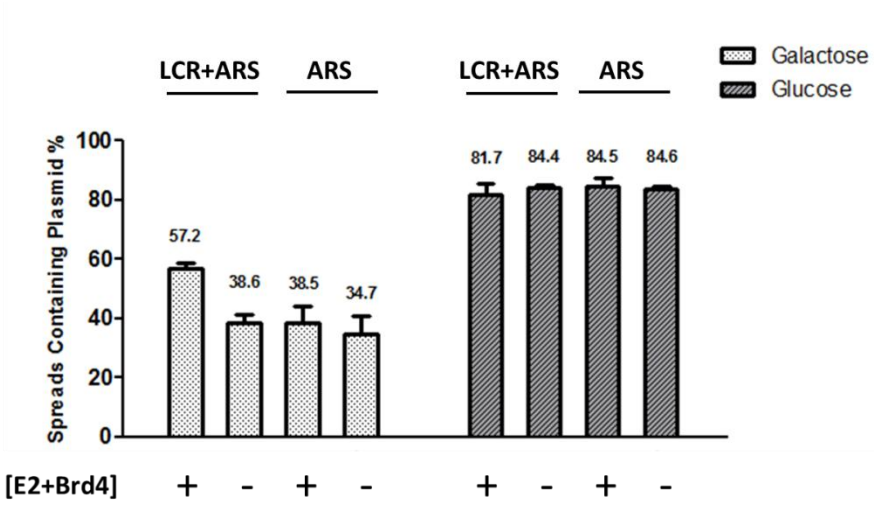


Figure 5.5 A single copy BPV reporter plasmid associates with chromosome spreads prepared from G1 arrested cells. (A) The experimental protocols for the chromosome spread assays presented in the previous chapter were followed here as well, and are schematically outlined. (B) Association of a single copy BPV reporter plasmid in yeast chromosome spreads is shown. The small fraction of cells containing two (or more) plasmid dots was omitted from data. (C) The results of the chromosome spread analysis are plotted as bar graphs.

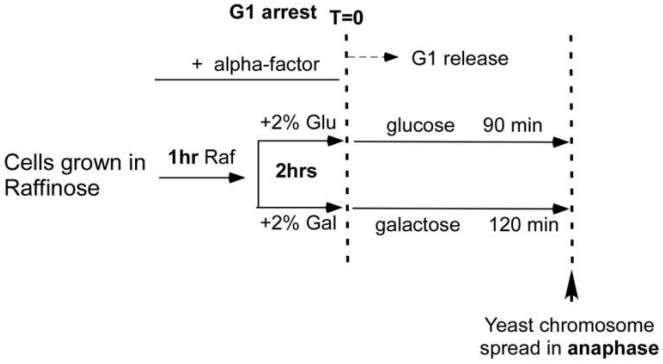
5.3.3.2 Plasmid distributions in chromosome spreads prepared from anaphase cells

Next, the presence of the single-copy BPV plasmid in chromosome spreads was assayed during anaphase in cells first arrested in G1 and then released into the cell cycle (Fig. 5.6A). Under the expression of E2 and Brd4, 49.5% of chromosome spreads revealed the presence of the single-copy BPV reporter plasmid. The number was 32.3% when partition proteins were absent. For the single-copy *ARS* reporter, the percentage of plasmid association with chromosome spreads increased slightly, from 28% to 35.6%, in the presence of the partitioning proteins compared to their absence (Fig. 5.6C). The results suggest that the extents of plasmid association with chromosomes during anaphase are comparable between the reconstituted BPV and EBV systems.

We further analyzed the relative fractions of one plasmid dot to two plasmid dots in anaphase spreads. Based on the rationale developed in the previous chapter, a single dot in anaphase spreads indicates equal segregation (1:1); two dots denote unequal segregation (2:0). Although the percentage of plasmid retention in the spreads was enhanced by the simultaneous presence of E2 and Brd4, the ratio of one dot to two dots remained essentially unchanged, approximately 1:1, in the presence or absence of these proteins. The two plasmid dot and one plasmid dot containing spreads were roughly equal in number for the *ARS* plasmid, and this approximate equality was not altered by the presence or absence of E2 plus Brd4 (Fig. 5.7). In contrast to the BPV system, the two plasmid dot to one plasmid dot ratio in anaphase spreads for the EBV system was nearly 3:1 (Fig. 4.9). A reasonable interpretation of this difference is that the BPV system promotes plasmid-chromosome association in a random fashion whereas the EBV system skews plasmid-chromosome association towards sister chromatids. Since sister

chromatids split away from each other, and each one occupies an individual daughter nucleus, a pair of sister plasmids associated with them in a one-to-one fashion would exhibit a similar behavior. By contrast, two sister plasmids associated with separate chromosomes will have a 50% probability of occupying the same daughter nucleus.

A.



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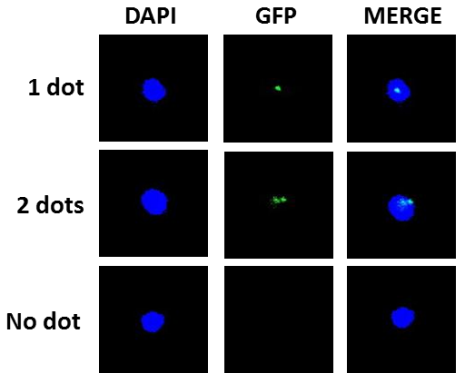


C.

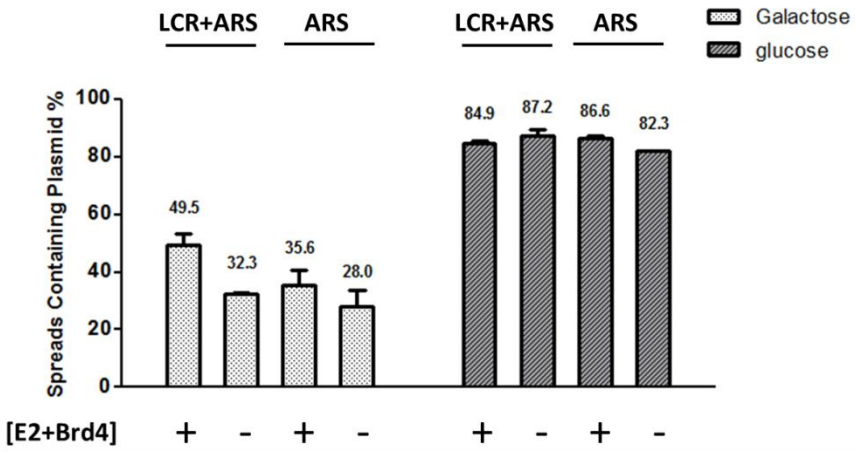


Figure 5.6 Association of the single copy BPV reporter plasmid with chromosome spreads prepared from anaphase cells. (A) The experimental scheme outlined is the same as that employed for analogous assays described in **Chapter 4**. (B) The spreads were grouped into three patterns: no plasmid dot, one plasmid dot and two plasmid dots. The small fraction of spreads with more than two dots was ignored. (C) The percentages of spreads showing plasmid presence (1 dot as well as 2 dots) are graphed as bar diagrams.

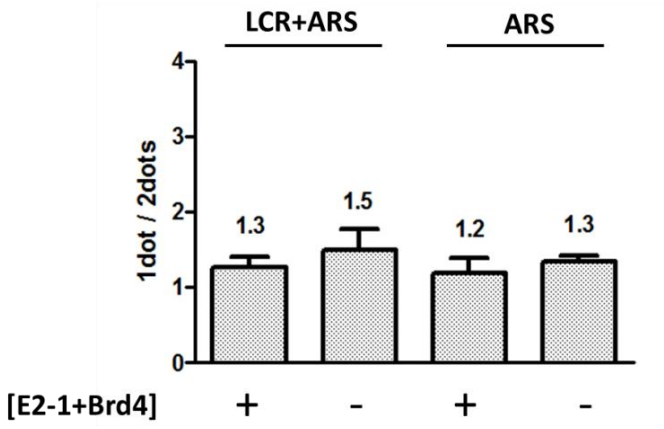


Figure 5.7 The ratios of anaphase spreads containing one plasmid dot and two plasmid dots under reconstituted BPV system. In the chromosome tethering model, equal segregation (1:1) will contribute to a single plasmid dot in a chromosome spread prepared from anaphase cells. Unequal segregation (2:0) will show two dots associated with a spread. A ratio that is biased towards one plasmid dot, which would be suggestive of sister chromatid-specific tethering of the plasmid, was not observed.

5.3.4 Localization of sister copies of the BPV reporter plasmid in metaphase cells under the BPV reconstituted system

In the chromosome tethering model for plasmid segregation, maximal efficiency of equal plasmid segregation would require the association of replicated sister plasmids with sister chromatids. While the segregation of EBV episomes in mammalian cells shows preferential 1:1 segregation, there is no evidence to suggest the operation of a similar symmetric segregation mechanism for HPV episomes. The E2 protein of BPV-1 is randomly localized along the chromosome arms during the mitotic stage (Oliveira et al., 2006).

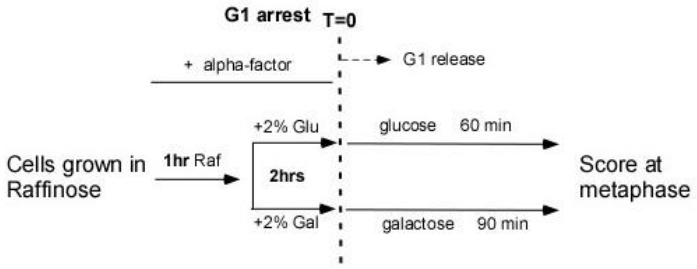
As was argued in the previous chapter, sister plasmids tethered to symmetrically disposed sites on sister chromatids would be colocalized during metaphase in *S. cerevisiae*. At this cell cycle stage, sister chromatids are still bridged by the cohesin complex assembled along their arms. The likelihood of sister plasmids tethered randomly to chromosomes being colocalized in metaphase cells would be quite low. However, two plasmids tethered to the same chromosome within distances that cannot be resolved by standard fluorescence microscopy (~15 kbp) would appear as colocalized. Conversely, sister plasmids tethered to distal sites on sister chromatids will not appear as coalesced fluorescent dots.

In order to assess the propensity of BPV sister plasmids to tether to sister chromatids, we assayed the pattern of plasmid fluorescence in metaphase cells (Fig. 5.8A). Around 42% of metaphase cells showed colocalization of replicated plasmid sisters in the BPV reconstituted system, compared to 31% in the absence of the

partitioning proteins. In the control assay, when the plasmid borne *CEN* was kept functional, over 80% of the cells showed a single florescence dot, as would be consistent with cohesion between plasmids established at *CEN* (Fig. 5.8C). We next tested if colocalization of BPV sister plasmids is affected by microtubule integrity, as this is the case for the 2 micron plasmid (Ghosh et al., 2007). Not surprisingly, nocodazole treatment did not affect sister plasmid colocalization in the BPV system (Fig. 5.8D).

In summary, the overall results suggest that the BPV partitioning system is functional in *S. cerevisiae*, and promotes plasmid stability by the chromosome tethering mechanism. In this respect, the BPV system resembles the EBV system. However, the BPV system is inferior to the EBV system in its ability to tether sister plasmids to sister chromatids. The EBV based partitioning system in *S. cerevisiae* likely functions by promoting plasmid association with chromosomes in a random fashion.

A.



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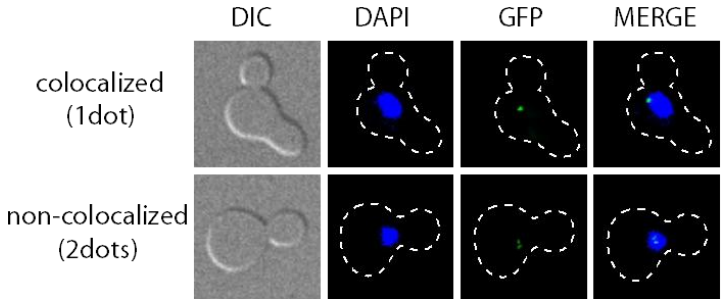

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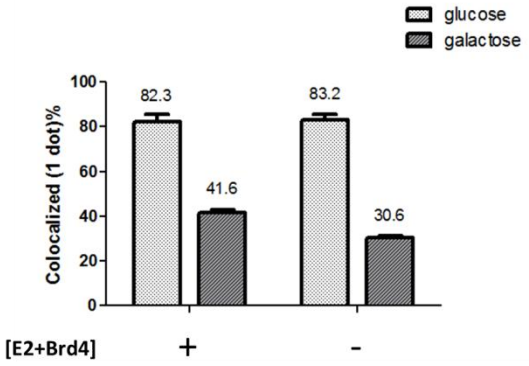

D.

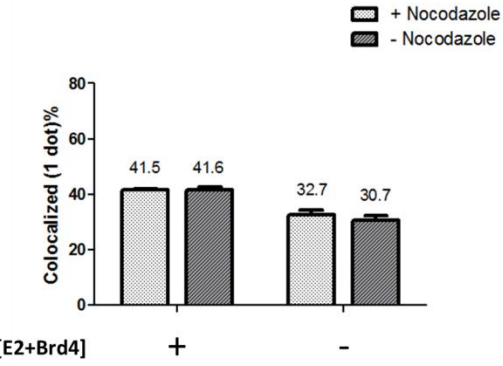

Figure 5.8 Replicated copies of the BPV reporter plasmids show improved colocalization in presence of the BPV partitioning systems in metaphase cells. (A) The protocol for assaying the colocalization of sister plasmids in metaphase cells (see also **Chapter 4**) is schematically indicated. (B) Colocalized sister plasmids appeared as a single fluorescent dot whereas non-colocalized sister plasmids were seen as two separate dots. (C) The fractions of metaphase cells in which sister plasmids were colocalized in the presence and absence of the BPV partitioning system are shown as histograms. The corresponding results when the plasmid harbored an active *CEN* (glucose) are also graphed for comparison. (D) The effect of microtubule integrity on plasmid sister colocalization was assayed in cells treated with nocodazole and arrested in G2/M.

5.4 Discussion

5.4.1 Comparison between the reconstituted EBV and BPV systems

The analyses comprising **Chapters 4** and **5** are consistent with the conclusion that the underlying common mechanism by which EBV and BPV partitioning systems reconstituted in *S. cerevisiae* stabilize their cognate reporter plasmids is the tethering of replicated plasmids to chromosomes. Although the EBV system is apparently more efficient than the BPV system as judged by the stabilities of the multi-copy reporter plasmids, this difference could be due to the size difference of the respective reporter plasmids. The BPV reporter is larger by approximately 3 kbp due to the presence of the *ADE3* gene. Consistent with this possibility, the stabilities of the *CEN* and *ARS* reporter plasmids are also decreased by the presence of the extra *ADE3* marker.

The efficiency of plasmid segregation by physical association with chromosomes will depend on three factors: (1) the efficiency of plasmid tethering to chromosomes; (2) random association of plasmids with chromosomes versus the association of sister plasmids with sister chromatids; and (3) the average copy number of the plasmid. For a single copy plasmid, the association of sister plasmids with sister chromatids will bring about 100% efficiency in segregation; random chromosome association can only be 50% efficient in segregation as chromosomes assort independently during mitosis. However, as the copy number increases, random tethering will tend to increase plasmid stability correspondingly. For example, if the plasmid copy number is two, and the replicated four molecules associate with three separate chromosomes, the probability of plasmid loss

during a cell division will only be 12.5%. However, there is a 50% chance that the segregation will be unequal (3:1) and only a 37.5% chance that it will be equal 2:2.

The assays using the single copy reporter plasmids in a single generation also show that the EBV system is better at promoting 1:1 segregation than the BPV system. Although chromosome spreads from G1 arrested cells indicate a higher frequency of plasmid-chromosome association under the EBV system than the BPV system, the difference between the two is quite small for chromosome spreads obtained from anaphase cells. However, the anaphase spreads reveal a clear-cut distinction in the ratio of the one plasmid containing nuclei to two plasmid containing nuclei between the EBV and BPV partitioning systems. The three fold increase observed for the EBV system over the BPV system in favor of the one plasmid containing spreads would be consistent with the tethering of sister plasmids to sister chromatids in a one-to-one fashion being more prevalent for the EBV system. Random tethering of plasmids to chromosomes by the BPV system would account for the lower efficiency with which it segregates the single copy reporter plasmid equally between mother and daughter cells when compared to the EBV system. It is interesting to note that the EBV and BPV systems acting in a completely artificial host such as *S. cerevisiae* still appear to recapitulate their individual attributes in mediating the association of cognate viral episomes with host chromosomes in their native mammalian environments. Whereas the EBV partitioning system tends to mediate the attachment of replicated episome sisters to symmetric loci on sister chromatids, the BPV partitioning system does not display this symmetry in tethering episomes to chromosomes (Oliveira et al., 2006).

5.4.2 Sister chromatid tethering and non-random segregation of the yeast plasmid and the EBV episome

We demonstrated in **Chapter 3** that sister copies of a 2 micron reporter plasmid segregate in association with sister chromatids. An analogous mode of segregation has been proposed for the EBV episomes in mammalian cells (Kanda et al., 2007; Nanbo et al., 2007). Because of the inability of current methods to resolve individual mitotic chromosomes of *S. cerevisiae*, we could only address symmetric tethering of sister plasmids to sister chromatids indirectly by assaying the co-localization of sister plasmids during the G2/M phase of the cell cycle. Despite this limitation, the higher levels of colocalization of sister plasmids in metaphase cells by the EBV system than the BPV system is in agreement with their inferred propensities to facilitate sister chromatid specific versus random chromosome association of plasmids. Earlier studies on the 2 micron plasmid partitioning system revealed that 70 to 80% of replicated plasmid sisters remain coalesced in metaphase cells, when the partitioning system is active, in a cohesin dependent fashion (Ghosh et al., 2007). The cohesin complex can promote the one-to-one segregation of sister plasmids by two mutually cooperative mechanisms: first by keeping sister plasmids in proximity to each other; second by holding sister chromatids together. The net result would be a high probability of the association of sister plasmids with sister chromatids. In the absence of cohesion between sister plasmids, their tendency would be to associate with chromosomes in a random fashion rather than tether to sister chromatids. This in turn would result in a drop in equal segregation, and lead to plasmid instability, especially if the copy number of the plasmid is low.

5.5 Perspective: Further verification of the modes of chromosome association of the viral plasmids under the reconstituted systems

As discussed above, the differences in the segregation behaviors of the single copy EBV and BPV plasmids in *S. cereviciae* can be accounted for by whether plasmid association with chromosomes is random, or specific to sister chromatids. Whereas BPV appears to follow the former type of chromosome tethering, EBV conforms to the latter mode. However, since the overall efficiencies of the reconstituted viral partitioning systems in yeast are relatively low, it is not possible to draw quantitative conclusions with respect to the extents of each type of chromosomal association for the two systems.

We have considered two potential experiments to further probe into the role of potential symmetric tethering to sister chromatids in the segregation of sister plasmids, at least in the case of the EBV system. If replication of a plasmid occurs in a state of dissociation from the chromosome, the association of the duplicated plasmid copies to sister chromatids would likely be dependent on their being bridged by the cohesin complex. By placing the gene for the Mcd1 subunit of cohesin under the *MET3* promoter, it is possible to repress *MCD1* expression by supplementing the growth medium with methionine. When G1 arrested cells are allowed to resume cell cycle in methionine containing medium, sister chromatids will not be paired by cohesin during S phase. One could then test whether, under this condition, metaphase cells show a higher percentage of non-coalesced sister plasmids and chromosome spreads from anaphase cells contain a higher proportion of two plasmid foci per spread. Alternatively, as described in **Chapter 3**, one may follow the segregation of the single copy reporter plasmid during monopolin directed mitosis. As sister chromatids co-segregate at high frequencies during this deviant

mitosis, the unequal segregation (2:0) frequency of a plasmid that hitchhikes on sister chromatids should be elevated. We have not performed the experiments to test the predictions for cohesin-free or monopolin directed mitosis primarily because the performance of the viral partitioning systems in *S. cerevisiae* has been less robust than we had hoped for.

CHAPTER 6

Summary and perspectives

6.1 Major Conclusions from this study

1. The central part of this study is the work presented in **Chapter 3**. By manipulating mitosis in a number of ways to effect chromosome missegregation, we have demonstrated that the partitioning of the 2 micron plasmid, but not that of an *ARS* plasmid, is intimately coupled to that of chromosomes.

2. A quantitative analysis of the results from **Chapter 3** has established a strong linear correlation (r=0.969) between the segregation behaviors of a chromosome and those of the 2 micron plasmid (**Appendix A**).

3. The findings from **Chapter 3** are most simply explained by the hitchhiking model for plasmid segregation, that is, physical linkage between plasmids and chromosomes during segregation. The equal segregation frequency of a single copy reporter plasmid argues that replicated plasmid copies cannot be randomly tethered to chromosomes. The observed equal segregation efficiency of such a reporter plasmid is greater than the maximum 50% efficiency expected for the random tethering mechanism.

4. In order to address the overall efficiencies of distinct chromosome associated partitioning mechanisms, we have utilized in **Chapters 4** and **5** the EBV and HPV partitioning systems reconstituted in *S. cerevisiae* (Brannon et al., 2005; Kapoor et al., 2001). The single copy reporter plasmid assays have revealed a significant increase in equal plasmid segregation under the action of the viral partitioning systems. However, the

efficiencies are inferior to that of a 2 micron reporter plasmid segregating under the control of the Rep-*STB* system. The underperformance of the viral systems could be due to their limited capability to effect chromosome tethering under the non-native circumstances or due to their inability to tether plasmid sisters to sister chromatids.

5. In Figure 6.1A, we have diagrammed the potential hitchhiking mechanisms for the 2 micron and viral partitioning systems acting on their respective single copy reporter plasmids. In the natural multi-copy situation, we suspect that the 2 micron plasmid segregates in groups as shown in Figure 6.1B. This is because fluorescence tagging reveals the nearly 40-60 copies of the plasmid as four to five fluorescent foci. Even random tethering to chromosomes with three or more segregating entities would decrease the plasmid loss during cell division to a very low value. For a single unit of segregation, the loss rate followed one segregation would be 50%, for two and three units, the values would be 12.5% and 3.125%, respectively. However, such a mechanism appears to be unlikely in the case of the 2 micron plasmid. Under this scenario, the copy number will have to be continuously corrected by the amplification system. Experimental evidence argues against the constant operation of plasmid amplification (Zakian et al., 1979).

For 2 micron circle, cohesion of replicated sister followed the replication facilitate the tethering of sister plasmids to sister chromatid. On the other hand, without the plasmid cohesion, replicated viral sisters perform sister chromatid tethering only because of close proximity of sister chromatid held by the cohesin complex (proximity effect), which is less efficient compared to the one of 2 micron circle. The potential translocation

of plasmid before the onset of anaphase will further weaken the sister chromatid tethering of viral episome but not to 2 micron circle.

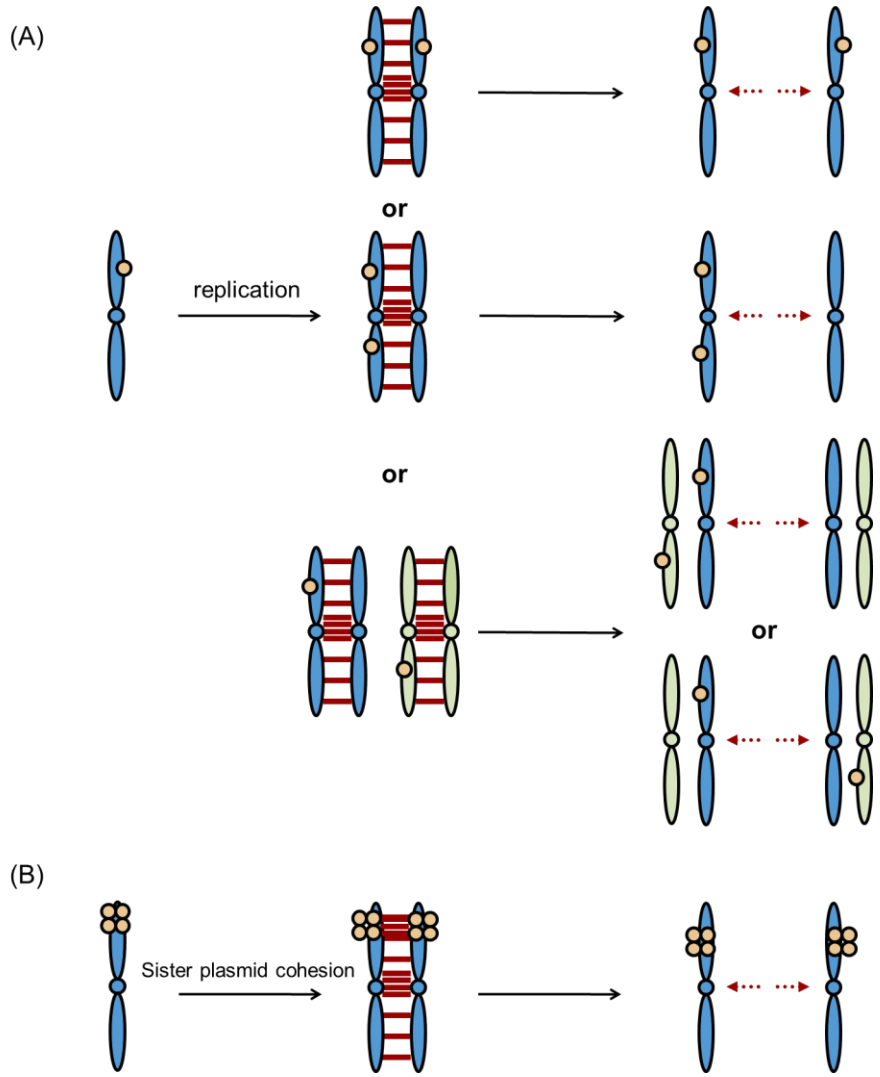

Figure 6.1 Chromosome associated segregation of the 2 micron circle and EBV or HPV viral episomes. A. Following replication, sisters a single copy reporter plasmid may tether to sister chromatids or to one sister of a pair of sister chroamtids or to two distinct chromosomes. The superior efficiency the 2 micron reporter plasmid in one-to-one segregation is likely due to the ability of sister plasmids to tether efficiently to sister chromatids. **B**. In the multi-copy situation of the native 2 micron plasmid, a single plasmid focus consisting of several plasmid copies or even a cluster of such plasmid foci may tether to a chromosomal locus. Following duplication, sister plasmid foci or sister plasmid clusters attach to sister chromatids, and thus accomplish efficient chromosome-like segregation.

6.2 A self-critique: Potential mechanisms for 2 micron plasmid partitioning other than chromosome association?

Even though the experimental evidence provided by this study favors the hitchhiking model for 2 micron plasmid segregation, this interpretation is not unequivocal. The uncertainty arises from the fact that current methodologies are not quite sufficient to verify direct association of the plasmid with chromosomes. The problem would be even more complicated if the plasmid does not tether to a specific chromosome, and if there are multiple tethering sites on a single chromosome. From unpublished results we know that the plasmid does not hitchhike on a particular chromosome. We have followed plasmid segregation in host strains in which each chromosome is forced to missegregate by conditional inactivation of its centromere by *GAL* promoter driven transcription through it. No elevation in plasmid loss is observed in any of the sixteen host strains.

The possible models for hitchhiking of the 2 micron plasmid on distinct nuclear components to achieve faithful segregation are diagrammed in Figure 6.2. In principle, association with the spindle pole body (SPB) could provide a mechanism for equal segregation of the 2 micron plasmid. If replicated plasmid molecules are symmetrically associated with duplicated spindle pole bodies, plasmid segregation would be efficient, and would at least superficially mimic chromosome segregation. Single copy *STB* reporter plasmids carrying a *CEN* sequence that can be conditionally inactivated is most often localized close to the spindle pole body (Cui et al., 2009). However, this particular localization could have been influenced by the proclivity of *CEN* to be proximal to the spindle pole body. Early observations using a multi-copy 2 micron reporter plasmid present in a haploid strain also suggested that the cluster of plasmid foci is present at a

shorter distance from the spindle pole body than foci formed by a multi-copy *ARS* reporter plasmid (Mehta et al., 2005). The tightly clustered appearance of the 3-5 plasmid foci of the 2 micron reporter plasmid is likely due to the small size of the nucleus and the consequent resolution limits to fluorescence microscopy. In diploid cells, with a larger nucleus, the plasmid foci are better resolved. Although, one sees one or two foci in individual cells to be associated with the spindle pole body, there are also other foci that are well separated from it. Since the spindle pole body is embedded in the nuclear membrane, the question whether the 2 micron plasmid is associated with the membrane (for example, to the nuclear pores), and whether such a localization is important in its segregation, has to be considered.

Recent experiments suggest that the preferential retention of *ARS* plasmids in the mother cell, the reason for their missegregation, may be explained in terms of the overall shape and geometry of the nucleus and the short duration of the closed mitosis of *S. cerevisiae* (Gehlen et al., 2011). The smaller volume of the nuclear compartment in the bud and the nuclear constriction at the bud neck pose diffusion barriers that prevent plasmid molecules from equilibrating between mother and daughter within the time interval in which nuclear elongation and division are completed. Apparently diffusion along the nuclear membrane can counteract the diffusion barrier due to the nuclear geometry to a large extent. By artificially tethering *ARS* plasmids to the nuclear envelope or to the nuclear pore complex, it has been possible to reduce their normal mother bias in segregation (Gehlen et al., 2011; Khmelinskii et al., 2011). The *STB* locus and replication origin of 2 micron plasmid have been reported to act as silencing loci that are dependent on Sir proteins for this activity (Grünweller and Ehrenhofer-Murray, 2002; Papacs et al.,

2004), it is therefore possible that anchoring of the 2 micron plasmid to Sir protein-enriched regions, such as the nuclear periphery or telomeres or the rDNA locus, may be important for their equal segregation.

A very early study suggested that the Rep proteins are enriched in nuclear matrix preparations (Wu et al., 1987). It is not clear whether this denotes an authentic association or whether the tendency of these proteins to aggregate causes them to be present in the insoluble fractions during the high salt washes employed for the matrix preparation. Very little is known about the dynamics of the matrix during cell division in *S. cerevisiae*. As far as we know, processes by which the matrix is duplicated or reorganized in the mother and daughter cell compartments are not understood. A symmetric redistribution of the matrix between mother and daughter could mediate the equal segregation of an entity that is associated with it, such as an extra-chromosomal DNA element.

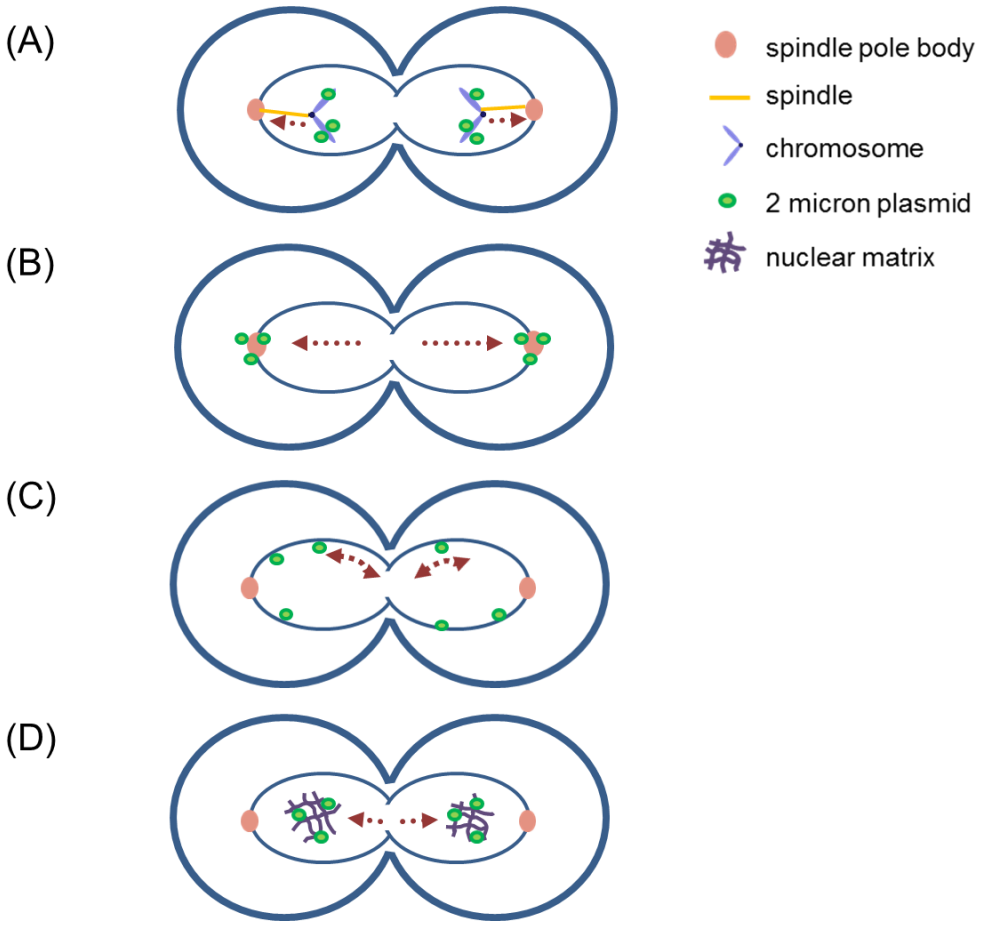

Figure 6.2 Models for hitchhiking of the 2 micron plasmid on distinct nuclear components. The possible models for faithful 2 micron plasmid segregation by associating with distinct nuclear components are schematically diagrammed. Association with sister chromatids (**A**) or with duplicated spindle pole bodies (**B**) would be highly efficient means for equal segregation. Less efficient perhaps, although still competent, mechanisms of segregation may involve association with the nuclear membrane (**C**) or the nuclear matrix (**D**).

6.3 Summary

We acknowledge that mechanisms other than direct attachment to chromosomes can give rise to a segregation behavior of the plasmid that resembles that of chromosomes. Nevertheless, the plausible extra-chromosomal segregation mechanisms considered here should not be directly blocked by interfering with chromosome segregation. For example, it is hard to imagine that misdirecting sister chromatids by monopolin expression would have any effect on segregation of a small genome that is associated with the spindle pole body or the nuclear membrane or the nuclear matrix. As a result, based on the results of the present study, in conjunction with evidence from earlier work, we suggest that chromosome association of the 2 micron plasmid proves the most satisfactory explanation for its chromosome coupled segregation.

Our interpretations are intended to suggest that the hitchhiking model signifies the sole mechanism for 2 micron plasmid propagation. While the plasmid may use chromosome tethering as the principal means for its segregation, it may also utilize, to lesser and perhaps varying extents, other means such as nuclear membrane association or matrix association. Since plasmid segregation involves more than one focus comprising several plasmid molecules, not each focus may conform to the same segregation mode during a particular cell cycle. Having backup or secondary mechanisms for segregation to complement hitchhiking on chromosomes may be important contributing factors to the evolutionary fitness of the 2 micron plasmid.

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