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Analysis of Yeast 2μm Plasmid Segregation

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Submitted in partial fulfillment
of the requirements for the degree of
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at

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(Department of Biochemistry and Molecular Biology)
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FACULTY OF GRADUATE STUDIES

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by Arpita Sengupta

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dedication

This thesis is dedicated to my father,

Late Apurba Bhusan Sen.

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Abstract

The 2μm circle is an endogenous plasmid stably maintained in the nucleus of the budding yeast *Saccharomyces cerevisiae*. The aim of this research has been to gain an understanding of the maintenance of the 2μm circle, with particular focus on the 2μm-encoded Rep proteins Rep1 and Rep2 and a *cis*-acting site, *STB*, involved in mediating 2μm circle segregation. To analyze 2μm plasmid segregation, a 2μm-based plasmid (pAS4) which includes all the 2μm sequences required for segregation, was constructed. In addition, it carries the yeast *ADE2* gene and sequences that allow the plasmid to be propagated and selected in *E. coli*. Data show that this plasmid segregates as efficiently as the native 2μm, despite lacking the *FLP* gene required for plasmid amplification. pAS4 is the first artificial plasmid that is as stable as native 2μm circle. The presence of the *ADE2* gene has allowed segregation of this plasmid to be studied in an *ade2* mutant yeast host strain, using a pink/white colour assay system. The assay system was used to screen for mutant alleles of *REP1* and *REP2* generated by polymerase chain reaction (PCR) and introduced into the pAS4 plasmid by gap repair. pAS4 was also used to assess the ability of full-length and truncated *REP* genes to provide segregation function. A two-hybrid analysis was used to define the domains of interaction of the Rep proteins. The results indicated that the amino-terminal domains of the two Rep proteins are sufficient for their interaction, while *in vivo* complementation showed that both the amino and carboxy terminal regions of the Rep proteins are required for segregation function. The data from these studies have been used to develop a model that predicts how the interaction between the Rep proteins and their association with the *cis*-acting *STB* locus results in segregation of the 2μm circle.

List of Abbreviations

2μm	two micron
ARS	autonomous replication sequence
AT	3-aminotriazole
BSA	bovine serum albumin
Cbf	centromere-binding factor
CDE	centromere DNA element
CEN	centromere
CKC	centromere-kinetochore complex
DTT	dithiothretol
E. coli	*Escherichia coli*
EDTA	ethylene diamine-N, N, N', N'-tetraacetic acid
GFP	green fluorescent protein
GST	glutathione-S-transferase
IgG	immunoglobulin
IPTG	isopropyl-β-D-thiogalactoside
IR	inverted repeat
LB	Luria broth
NLS	nuclear localization signal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline

PMSF	phenylmethanesulphonyl fluoride
PVDF	polyvinylidene difluoride
PCR	polymerase chain reaction
SD	synthetic deficient
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TBE	Tris borate EDTA
Tris	Tris(hydroxymethyl)aminomethane
UAS	upstream activation sequence
U.S.E.	unique site elimination
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
YEPD	yeast extract-peptone-dextrose (rich) medium
YT	yeast tryptone

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I. Introduction

1. The 2μm circle of *Saccharomyces cerevisiae*

The 2μm circle is an endogenous plasmid found in the nucleus of the budding yeast *Saccharomyces cerevisiae* (Kielland-Brandt et al. 1980). The 2μm circle has evolved elegant mechanisms to maintain its copy number and to minimize the rate of plasmid loss (reviewed in Murray, 1987; Futcher, 1988). The high copy number and stability of the plasmid have made it the basis for all high-copy vectors that are used extensively in yeast molecular genetics and for biotechnology. The plasmid has been used effectively as a vector for propagation and expression of cloned genes in yeast. Apart from being an important technical tool, the 2μm circle itself is quite fascinating and has presented some novel and intriguing mechanisms for partitioning and maintenance of its copy number. Both these functions require plasmid-encoded proteins and *cis*-acting loci on the plasmid. The mechanism of plasmid partitioning is still unsolved and is the subject of this thesis. Interactions between the components of the 2μm partitioning system and the various constituents of the nucleus, once determined, will not only solve the mystery of 2μm partitioning but will also provide a better understanding of eukaryotic chromosome segregation. The structure and biology of the 2μm circle are discussed in the following sections.

A. Structure of the 2μm circle

The 2μm circle is a 6318-bp double-stranded circular DNA molecule and has been entirely sequenced (Hartley and Donelson, 1980). The physical map of the 2μm circle is shown in Figure 1. The plasmid consists of two unique regions separated by two 599-bp inverted repeat sequences (*IRS*s). The unique regions contain four open reading frames (ORFs), *REP1*, *REP2*, *RAF* and *FLP* (Hartley and Donelson, 1980), all of which are transcribed (Sutton and Broach, 1985). *FLP* encodes a site-specific recombinase (Sadowski, 1995) that recognizes a sequence called *FRT* within the inverted repeat sequence. Flp-mediated recombination between the two *FRT* sites produces the two forms of the 2μm circle A and B (Broach and Hicks, 1980), that are observed *in vivo*, and plays a role in amplifying the plasmid copy number, which is discussed in greater detail in a later section (see Section I 1D). The *REP1* and *REP2* genes encode proteins that are required for efficient partitioning of the 2μm circle between mother and daughter cell and may also act as transcriptional regulators, controlling expression of the *FLP* gene (see Section I 1H). The final ORF, *RAF1*, is less well studied but it is believed to play a role in relieving Rep protein-mediated repression of *FLP* gene expression (Murray et al. 1987).

In addition to the two *FRT* sequences, the 2μm circle has two other *cis*-acting sites. One is an autonomous replication sequence (*ARS*), a sequence that promotes autonomous replication of the plasmid on which it resides. The single *ARS* on the 2μm circle is present at the boundary of one of the inverted repeat sequences and functions as the plasmid's sole origin of DNA replication (see Section I 1C below). The

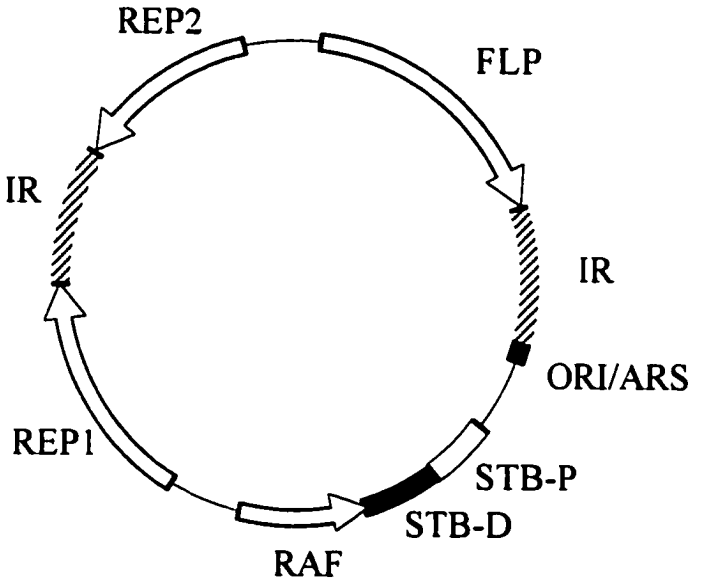

Figure 1

Figure 1. Map of the 2μm circle (B form). Open reading frames are represented by open arrows which also indicates the orientation of the gene. A small black box shows the origin of replication (*ORI/ARS*). Hatched boxes represent the inverted repeats (IR). STB-P refers to *STB*-proximal (open box) and STB-D refers to *STB*-distal (black box).

STB is the other *cis*-acting locus. Loss of this part of the plasmid results in loss of mitotic stability, suggesting that this *STB* (stability) locus is required for plasmid partitioning. The entire coding capacity of the 2μm circle is therefore dedicated to specifying components that function either in plasmid segregation or copy number control, and the role they play is discussed in more detail in the following sections.

B. Maintenance of 2μm circle in yeast

The 2μm circle is found in most strains of *S. cerevisiae* and these yeast strains are referred to as circle-bearing or [cir$^+$]. Yeast strains lacking 2μm plasmid are referred to as circle-free or [cir^0]. The plasmid does not seem to provide any selective advantage to the host; in fact [cir^0] cells grow about 1% faster than isogenic [cir$^+$] cells (Mead et al. 1986). However, despite this slight disadvantage, 2μm circles are stably maintained (loss rate of 10^{-4} per [cir$^+$] cell per generation), although prolonged logarithmic-phase growth can cause spontaneous loss of 2μm circle from [cir$^+$] strains (Futcher and Cox, 1983). It is also possible to cure strains of 2μm plasmid by introducing high copy number artificial plasmids which are derivatives of the 2μm circle (Dobson et al. 1980a). These results suggest that the plasmid is not essential for the yeast host and it can therefore be thought of as parasitic. The 2μm circle is not infectious; that is, it cannot be transmitted to a [cir^0] strain except by mating with a [cir$^+$] strain (Futcher and Cox, 1983).

C. Replication of 2μm circle

The 2μm circle contains a single *ARS* which coincides with its single origin of replication (Brewer and Fangman, 1987). Replication is bidirectional, probably the same as chromosomal replication and uses the host's chromosomal replication machinery. Replication of each copy of the 2μm circle is initiated only once per cell cycle at the *ARS* (Livingston and Kupfer, 1977) during S phase (Zakian et al. 1979).

D. Copy number control of 2μm circle

The copy number of 2μm circle ranges from 20 to 100 copies per haploid cell depending on the host strain background. The high copy number of the 2μm plasmid suggests the existence of some mechanism for copy number amplification. It has been demonstrated by Sigurdson et al. (1981) that if a single molecule of 2μm circle is transferred to a [cir^0] strain by cytoduction, by the time the resulting yeast colony has grown large enough to be analyzed the 2μm circle has returned to its usual high copy number. Futcher (1986) proposed a model for copy number amplification of the 2μm circle. According to the "double rolling circle" model described by Futcher, replication initiates at the single origin of the plasmid which is located adjacent to one of the inverted repeats and proceeds bi-directionally until one of the inverted repeats is replicated. At this point Flp recombinase causes recombination between the two *FRT* sites, one at the newly replicated *IR* and the other located within the unreplicated *IR*. As a result of this recombination, the direction of the replication forks are flipped with respect to each other such that these are now moving in the same direction around the circular plasmid. A

second Flp-mediated recombination would restore the head-to-head orientation of the replication forks so that these can meet and terminate replication. The result is a multimer of 2μm plasmids which can be resolved into 2μm monomers by further Flp-mediated recombination events. Thus a single initiation of replication leads to several rounds of replication within a eukaryotic cell in which multiple initiations do not take place. This amplification of the plasmid is possible due to the geometry of the plasmid. The asymmetric location of the origin with respect to the inverted repeats and the inverted repeats being far apart from each other on opposite sides of the plasmid facilitate the process. Volkert and Broach (1986) have proved experimentally that the presence of a functional Flp protein, a pair of functional inverted *FRTs* and an appropriately placed *ARS* are the only requirements for 2μm circle amplification.

E. The partition system

The 2μm circle is efficiently partitioned during cell division. The products of the 2μm-encoded *REP1* and *REP2* genes and the *cis*-acting plasmid *STB* locus are required for this efficient partitioning of the plasmids (Kikuchi, 1983).

i. Loss of mitotic stability of *rep*$^-$ or *STB*$^-$ 2μm plasmids

Artificial plasmids that contain only an *ARS* can replicate in yeast but cannot be partitioned efficiently between the mother and daughter cells during mitosis. These *ARS* plasmids exhibit 'maternal bias in inheritance' or, in other words, during cell division the plasmids tend to remain within the mother cell instead of being distributed between the mother and the daughter cells. A 2μm circle lacking either *REP1* or *REP2* (often called

rep⁻ plasmids) or the *STB* locus behaves like a typical *ARS* plasmid, showing a loss rate of about 20% per generation (Murray and Szostak, 1983).

These *rep*⁻ plasmids have an average copy number per cell similar to *REP*⁺ plasmids, but since only 5-10% of the population contains plasmids, the copy number per plasmid-bearing cell is much higher (Futcher and Cox, 1984; Murray and Szostak, 1983; Kikuchi, 1983). This concentration of the plasmid into a small fraction of the cells would be expected if, in the absence of the *REP* genes, these 2μm-based plasmids show 'maternal bias in inheritance' as has been demonstrated for *ARS* plasmids. A defect in replication would reduce the copy number of plasmids, whereas defective segregation should not alter the total number of plasmid molecules but should change the distribution of these molecules. It has also been demonstrated that supplying the Rep proteins in *trans* by expressing these from heterologous promoters on non-2μm plasmids or form genes integrated into the chromosome could also stabilize plasmids containing only the *STB* locus and the origin of replication (Dobson et al. 1988; Cashmore et al. 1986). The importance of the *STB* locus in partitioning has been shown by Kikuchi (1983). The inclusion of the *STB* locus on an *ARS* plasmid was able to stabilize the plasmid, provided *REP1* and *REP2* were expressed in the cell either from the same plasmid or in *trans* (Kikuchi, 1983). Kikuchi demonstrated that replacing the 2μm origin of replication with any other chromosomal *ARS* (*ARS1* in this case) does not affect the stability of the plasmid, if *REP1* and *REP2* are expressed in the same cell and *STB* is included in the plasmid. He has also shown that there is no increase in the average copy number when unstable *ARS* plasmids are stabilized by adding *REP1*, *REP2* and *STB*. The average copy

number of these 2μm-based plasmids and the copy number per plasmid-bearing cell are similar, since approximately 80% of the cells contain plasmids (Kikuchi, 1983). The recovery of an equal distribution of plasmids between cells in the population when the *REP* genes and *STB* are incorporated on an *ARS* plasmid suggests that the *REP* gene products and the *cis*-acting *STB* locus of the 2μm plasmid are involved in segregation of the plasmid and not in replication. The mechanism of 2μm circle partitioning is still unknown.

ii. 2μm-based plasmids

Hybrid 2μm-based plasmids had been used to study the roles of the *REP* genes and the *STB* locus in partitioning of the 2μm circle (Kikuchi, 1983; Jayaram et al. 1983, 1985). None of these plasmids (especially in the absence of selection) have been as stable as the 2μm circle itself. Most 2μm-based vectors contain the *ARS* and the *STB* locus as their only 2μm circle sequences and are propagated in [cir^+] strains where Rep1 and Rep2 are supplied *in trans* (Kikuchi, 1983). However, these plasmids are not nearly as stable as 2μm circle in the absence of selection for the amino acid biosynthetic gene which is also included in the artificial plasmids. The 2μm-based plasmids are almost 100% stable when the strains are grown under selective conditions, since cells which do lose plasmid fail to propagate.

F. Components of the partition system

Various studies have been undertaken in attempts to determine the role of individual components of the 2μm circle segregation system (Jayaram et al, 1983, 1985;

Kikuchi, 1983). I describe these individual components in more detail below (see Sections I 1Fi to v).

i. Rep1

The Rep1 protein is predicted to be 373 amino acids in length with a molecular weight of 43 kDa. A protein with an apparent mobility of 48 kDa on SDS-PAGE has been precipitated by anti-Rep1 antibody and has been confirmed by microsequencing to be the 2μm-encoded Rep1 protein (Wu et al. 1987). The carboxy-terminal half of the Rep1 protein has been predicted to form an extended α-helical form (Wu et al. 1987). This predicted coiled-coil domain of Rep1 exhibits some limited similarity (approximately 60%) at the amino acid level to the fibrous proteins tubulin and myosin heavy chain, and to the intermediate filament protein vimentin. Based on these similarities Rep1 has been interpreted to have a globular amino-terminal head and a fibrous carboxy-terminal tail, the latter of which may associate with the nuclear matrix (Wu et al. 1987). Cell fractionation studies demonstrated that Rep1 does co-fractionate with an insoluble nuclear karyoskeletal fraction (Wu et al. 1987). Immunofluorescence studies demonstrated a punctate staining of the Rep1 protein within the nucleus, indicating that Rep1 is not a freely diffusible molecule but is present as dispersed aggregates (Broach and Volkert, 1991). Recent studies have demonstrated nuclear localization of Rep1 (Ahn et al. 1997; Scott-Drew and Murray, 1998). Based on these observations it has been suggested that Rep1 intercalates in the nuclear matrix by means of the carboxy-terminal domain. This would provide dispersed attachment sites for the plasmid and ensure proper partitioning during nuclear division (Wu et al. 1987).

ii. Rep2

Rep2 protein is predicted to be a 296 amino acid protein with a molecular weight of 33 kDa and a high predicted isoelectric point of 9.1. The amino acid sequence does not share significant similarity with that of any known protein. Immunofluorescence staining reveals Rep2 to be a nuclear protein (Broach and Volkert, 1991). Rep2 has been shown to be nuclear (Ahn et al. 1997) and it is localized to discrete nuclear foci (Scott-Drew and Murray, 1998).

iii. Rep1/Rep2 interaction

As previously mentioned, mutational studies demonstrated that both Rep1 and Rep2 are required for plasmid partitioning. Absence of either of the Rep proteins does not reduce the mitotic stability of the plasmid to any greater extent than when both Rep1 and Rep2 are missing or mutated. These genetic data, and the observation that the two proteins are required in stoichiometric amounts for partitioning to function efficiently, suggest that Rep1 and Rep2 may function as a complex to mediate plasmid partitioning (Dobson et al. 1988; Cashmore et al. 1986).

Using a two-hybrid assay, we have shown *in vivo* association of Rep1 and Rep2 as well as self-association of Rep2, which is presented in a following section (see Section III 7). Ahn et al. (1997) have shown direct association of Rep1 and Rep2 and self-association of Rep1 and Rep2 using an *in vitro* interaction assay and *E. coli* synthesized Rep proteins. These results have also been confirmed in our laboratory by *in vitro* baiting assays (Joyce Chew, unpublished results) (see Section II 5C). Direct interaction of Rep1 and Rep2 and their self-association *in vitro* have also been shown by Scott-Drew and Murray (1998).

To associate with each other the Rep proteins must co-localize at some point during the cell cycle at some site of action within the cell. Hybrid proteins in which either Rep1 or Rep2 are fused to green fluorescent protein (GFP) have been localized to the nucleus in [cir$^+$] strain (Ahn et al. 1997). The nuclear localization of GFP-Rep1 in the absence of Rep2, or of GFP-Rep2 in the absence of Rep1, is less efficient in a [cir^0] strain, suggesting that the nuclear transport of each Rep protein is facilitated by the other. This nuclear targeting can take place in the absence of either Flp or the product of the *RAF* gene (Ahn et al. 1997). Scott-Drew and Murray (1998) have shown that the Rep proteins localize to discrete nuclear foci that duplicate during the cell cycle and become segregated to both mother and daughter cells. They also demonstrated that the nuclear localization of Rep1 requires the presence of Rep2. Based on their observation that overexpression of one of the Rep proteins reduces its localization within the nucleus but enhances the localization of the other Rep protein to the foci, Scott-Drew and Murray (1998) suggested that this interdependence of Rep protein localization provides indirect evidence for *in vivo* Rep1-Rep2 interactions.

Recent studies have localized nuclear targeting sequences at the very carboxy-terminal ends of both the Rep1 and Rep2 proteins (Velmurugan et al. 1998). Small deletions of 25 and 20 amino acids from the carboxy ends of Rep1 and Rep2, respectively, were sufficient to disrupt both nuclear targeting and Rep protein function. Nuclear targeting and plasmid stabilizing properties of these deletion mutants could be restored by an exogenous nuclear localization signal (NLS) (Velmurugan et al. 1998) thereby verifying that this was the only requirement for this NLS.

iv. STB

The third important region required for partitioning is the *STB* locus. *STB* is the *cis*-acting locus of the 2μm circle required for efficient partitioning of the 2μm plasmid (Jayaram et al. 1983, 1985; Kikuchi, 1983). It consists of two functional domains, *STB* proximal (proximal to the origin of replication) and *STB* distal (Murray and Cesareni, 1986). The *STB*-proximal region consists of five and a half tandem direct repeats of 62 bp sharing about 90% identity (Hartley and Donelson, 1980). The *STB*-proximal region is sufficient to mediate proper partitioning (Jayaram et al, 1985; Murray and Cesareni, 1986). Functional dissection of the *STB* proximal region, carried out in hybrid plasmids, demonstrated that not all of the copies of the tandem repeats are necessary for plasmid stability (Kikuchi, 1983; Jayaram et al. 1985). Natural variants with deletions in the *STB* locus have also been reported (Cameron et al. 1977).

The *STB*-distal region contains the 3' end of the *RAF* gene and a transcription terminator, sequences that signal termination of transcription (Sutton and Broach, 1985). Murray and Cesareni (1986) demonstrated that this terminator is essential for normal functioning of the *STB* locus as well as for prevention of replication inhibition, probably by protecting the *STB*-proximal repeats and the origin of replication from readthrough transcription from the adjacent *RAF* gene. In addition, the *STB*-distal contains a silencer-like element which represses transcription from upstream promoters (Murray and Cesareni, 1986). The exact role of this silencer is unclear.

v. Protein binding at the *STB* locus

In analogy with bacterial plasmid partition loci, the *STB*-proximal repeats would appear to be obvious sites at which proteins involved in partitioning the plasmid could bind. The lack of an obvious nucleosomal protection pattern over these repeats (Veit and Fangman, 1985), and absence of transcription (Murray and Cesareni, 1986), indicate that proteins other than histones may associate with this region *in vivo*. Since the *STB*-proximal region containing the repeat sequences and the Rep1 and Rep2 proteins are required for partitioning of the 2μm circle it has been suggested that Rep1 and/or Rep2 might bind to the *STB*-proximal region. Gel retardation assays using urea-solubilized yeast extracts demonstrated protein binding to *STB*-proximal. This binding activity has been observed only in [cir^+] but not in [cir^0] yeast extracts (Hadfield et al. 1995), suggesting that one or both Rep proteins are involved. Although there is no evidence for direct interaction of the Rep proteins with *STB*, binding activity to *STB*-proximal has been shown with extracts from [cir^0] yeast containing chromosomally integrated *REP1* and *REP2* genes (Hadfield et al, 1995). This binding activity was performed using a bio-sensor assay which can detect transient protein/DNA associations. However, this binding occurs only in association with an unidentified host factor (Hadfield et al. 1995). The 2μm Raf protein, encoded by the smallest 2μm circle open reading frame, has also been shown to possess *STB*-binding activity using this same detection system (Hadfield et al. 1995). Thus three out of the four 2μm-encoded proteins show binding activity to *STB*-proximal and may regulate the binding of the host factor (Hadfield et al. 1995). Recent studies using a one-hybrid genetic screen have suggested that Rep1, Rep2 and an uncharacterized

host protein Shf1 (for *STB* binding host factor) may interact with the *STB*-proximal locus (Velmurugan et al. 1998). However, no direct interaction between the Shf1 and the Rep proteins has been demonstrated. The Shf1 protein, which is encoded by an uncharacterized yeast gene YILO36W, has been identified independently in our lab using a mono-hybrid assay (K. Blomqvist, unpublished data) where it has been shown to specifically interact with the *STB*-distal region, but not the *STB*-proximal region (Dobson, unpublished results).

The *STB* region thus seems to be a multiple protein-binding site. The interaction of the Rep proteins with themselves and with *STB* further shows that these three components of the plasmid may form a DNA/protein complex that mediates efficient partitioning of the 2μm circle. Further studies to show the interaction of the host factors with *STB* and with the Rep proteins will help to elucidate the mechanism of partitioning of the 2μm circle.

G. *RAF*, the smallest ORF of the 2μm circle

Raf is a protein predicted to be encoded by *RAF*, the smallest and the least studied of the 2μm circle ORFs. Overexpression of *RAF* alleviates Rep protein-mediated repression of the *FLP* gene described in more detail below (Murray et al. 1987), but disruption of *RAF* has little effect on the stability of the plasmid (Veit and Fangman, 1985). Hadfield et al. (1995) have suggested that the *STB*-binding activity of Raf and the derepression of *FLP* by this protein provide additional fine-tuning functions to the Rep1 and Rep2 proteins, which comprise the main components of the stability system.

H. Models for Rep protein function

The Rep1 and the Rep2 proteins seem to play multiple roles in the maintenance of the 2μm plasmid. The two proteins somehow interact with the *cis*-acting *STB* locus, either directly or indirectly, and cause equal partitioning of the plasmids during cell division. The Rep proteins may also control expression of the *FLP* gene and are thus indirectly influencing the copy number amplification system. The evidence for Rep protein control of the *FLP* gene comes from several different types of experiments. Sensitivity to micrococcal nuclease has demonstrated that disruption of either the *REP1* or the *REP2* gene alters the chromatin organization of the 5' end the *FLP* gene (Veit and Fangman, 1985). Similarly, overexpression of both Rep1 and Rep2 together from *GAL1* and *GAL10* promoters has been shown to downregulate the *FLP* promoter as measured by β-galactosidase activity produced from a *FLP-lacZ* fusion (Reynolds et al. 1987). This same study showed that when the strain containing this integrated *FLP-lacZ* fusion gene was mated with [cir^0] and [cir^+] strains, β-galactosidase activity was lower in the [cir^+] diploid than the [cir^0] diploid. Thus normal levels of the Rep1 and Rep2 proteins supplied by the [cir^+] strain could repress the *FLP-lacZ* fusion gene. In contrast, the same authors reported that co-expression of *REP1* and *REP2* from their own promoters at low copy number failed to downregulate the *FLP-lacZ* fusion gene. Som et al. (1988) have shown that expression of both endogenous *FLP* and *REP1* but not *REP2* are repressed by 2μm-encoded products, as indicated by altered transcript levels directed by the promoters of these genes in [cir^+] strains. The repression is absent in a [cir^0] strain (Som et al. 1988). This latter study also demonstrated that expression of *FLP* and *REP1*

is downregulated by the combined expression of Rep1 and Rep2. Som and co-workers (1988) measured the β-galactosidase activity produced from either *FLP-lacZ* or *REP1-lacZ* promoter fusions in strains over-expressing different 2μm circle genes under the control of inducible promoters and integrated into the chromosome. They concluded that expression of both *REP1* and *REP2* is necessary for repression of transcription of either the *FLP* or the *REP1* gene. No other combination of 2μm-encoded gene products affects the expression of these genes (Som et al. 1988).

Simultaneous overexpression of Rep1 and Rep2 has also been shown to reduce the level of *RAF* mRNA (Murray et al. 1987). The same study has shown that overexpression of *RAF* alleviates Rep1-Rep2 mediated repression of the *FLP* gene. However, overexpression of *RAF* had no additional effect on the level of *FLP* transcripts when *REP1* or *REP2* were absent. Thus it has been suggested that *RAF* antagonizes the Rep1-Rep2 mediated repression of *FLP* rather than acting as an activator of *FLP* expression (Murray et al. 1987).

Taken together, the data from these studies indicate that there are complicated regulatory circuits controlling gene expression on the 2μm circle. Interpretations of the data are complicated by the fact that most of the studies involved non-physiological gene dosages where the 2μm genes were overexpressed from inducible promoters. Nonetheless, models for the control of 2μm circle copy number have been proposed based on these studies. According to Som et al. (1988), regulation of *FLP* by the Rep proteins is based on plasmid copy number. In cells with high plasmid copy number, the concentrations of Rep1 and Rep2 are sufficient to repress *FLP* and inhibit further plasmid

amplification. Conversely, when Rep protein levels fall, due to a drop in plasmid copy level, repression of *FLP* is relieved and amplification is induced. The autoregulation of *REP1* has been described as a feedback loop on copy number increase (Som et al. 1988). At low plasmid copy number, *REP1* is also derepressed along with *FLP*, which promotes amplification and a subsequent increase in the concentration of the repressor proteins Rep1 and Rep2 (Som et al. 1988). In this model, the Rep1 and Rep2 proteins play a key role in regulating plasmid copy number that appears to be distinct from their role in mediating plasmid partitioning. It is the existence of these two distinct but interdependent mechanisms that contributes to the stable maintenance of the 2μm plasmid: the segregation machinery comprising of Rep1, Rep2 and the *STB* locus and the amplification system using the site-specific recombinase Flp. However, the actual mechanism involving the Rep proteins in segregation and regulation of 2μm genes is still unclear and is the subject of this thesis.

I. Other 2μm-like plasmids

Plasmids with any resemblance to 2μm circle are only found in a small number of yeast species that are fairly closely related to *Saccharomyces cerevisiae*. Circular double-stranded DNA plasmids have been isolated from the yeasts *Kluyveromyces drosophilarum* (Falcone et al. 1986; Chen et al. 1986), *Kluyveromyces waltii* (Chen et al. 1992), *Zygosaccharomyces rouxii* (Toh-e et al. 1982), *Zygosaccharomyces bisporus* (Toh-e et al. 1984), *Zygosaccharomyces fermentati*, *Zygosacchaomyces bailii* (Utatsu et al. 1987) and *Torulaspora delbrueckii* (Blaisonneau et al. 1997). Although there is very

little sequence similarity among these plasmids, one striking feature is the similarity of their genome organization to that of the 2μm circle. All these plasmids contain a large inverted repeat sequence separating two unique regions containing at least three ORFs. For all of these plasmids, one of the ORFs encodes a site-specific recombinase which is a recognizable homolog of the *S. cerevisiae* 2μm Flp recombinase and which, like the 2μm Flp, promotes recombination between the inverted repeats as part of the amplification process. As a result of this recombination, the plasmids exist in two equimolar forms, similar to the 2μm circle. All the plasmids contain one or more *ARS* elements and proteins encoded by two of the remaining ORFs, like Rep1 and Rep2, are required in *trans* for mitotic stability of the plasmids. A *cis*-acting locus required for mitotic stability is also present (Utatsu et al. 1987). The existence of a similar molecular structure in all these plasmids may indicate a common ancestor or suggests that this structure is critical for plasmid maintenance.

Although the plasmids are quite similar in architecture, they do not show any similarity at the DNA sequence level. A comparison of the predicted amino acid sequences of the encoded proteins reveals some significant differences. The predicted amino acid sequences of the respective Flp proteins from these plasmids are quite similar and there is a limited degree of similarity between the *S. cerevisiae* Rep1 and the Rep1-equivalent proteins of these plasmids. In contrast, the Rep2-like proteins are quite dissimilar, while the *cis*-acting *STB*-like loci in these plasmids are also dissimilar in sequence (Murray et al. 1988). These plasmids are also not mitotically stable when transformed to one of the other yeast strains acting as host. This could be due to the

dissimilarities in the *STB* sequences, which may not be recognized by proteins in the new host (Hartley and Donelson, 1980). Also, the equivalent Rep proteins of one plasmid do not function with the *STB* of another, and a *rep* mutation in one cannot be complemented by the equivalent Rep protein from a heterologous plasmid (Toh-E and Utatsu, 1985; Araki et al. 1985; Chen et al. 1986). Thus the divergence of the plasmid proteins between different 2μm-like plasmids may reflect their co-evolution with the *cis*-acting DNA sequence that comprises the stability locus with which these proteins interact (Murray et al. 1988).

2. Bacterial plasmids

To understand the segregation and copy number control of the yeast 2μm plasmid, it is useful to review what is known about the more intensively studied bacterial plasmids. Bacterial plasmids are considered as cellular parasites which are dispensable for cell viability. They replicate independently of the bacterial chromosome and may be present at low copy numbers, as in the case of the P1 and F plasmids, or as multiple copies such as is found for ColE1 (Scott, 1984). The maintenance of a plasmid in a bacterial cell depends on two processes: one is replication to increase the number, and the other is an efficient partitioning mechanism to ensure accurate distribution of plasmids between daughter cells during cell division. Regulation of replication is important for stable maintenance of plasmids and determines the characteristic copy number (reviewed in Scott, 1984). Large plasmids are usually present at low copy numbers, which reduces the metabolic burden on the host, whereas small plasmids are maintained at high copy

number (Williams and Thomas, 1992). Bacterial plasmids have adopted strategies for stable persistence in the host cell. Site-specific recombination systems act as helper elements that increase the efficiency of random distribution of replicated molecules (Nordstrom and Austin, 1989) and control copy number. Post-segregational killing is also a mechanism that ensures stable maintenance of plasmids. Low copy number plasmids have developed partitioning mechanisms. The following sections provide a brief review of these strategies.

A. Site-specific recombination systems in bacterial plasmids

In a cell, all copies of a given plasmid (being homologous to each other) can recombine and form multimers, thereby reducing the number of segregating units during cell division. *E. coli* cells transformed with small multicopy plasmids can grow into a population which contains about 50% of the plasmid DNA as multimers (James et al. 1982). Resolution of plasmid multimers is particularly important for efficient partitioning of high copy number plasmids, which depend on random distribution. Multimer formation can also reduce the partitioning efficiency of low copy number plasmids, in which case some cells will contain a single multimeric plasmid making it difficult to distribute plasmids between daughter cells (Nordstrom and Austin, 1989). Various site-specific recombination systems exist to resolve multmeric plasmids into monomers to facilitate the partitioning process. The *cer* site and the host *xer*-encoded recombinase of ColE1 (Summers and Sherratt, 1984; Stirling et al. 1988), the *rsfF* site and the product of the D gene of the F plasmid (O'Connor and Malamy, 1984; Lane et al. 1986) and the loxP site and Cre recombinase of the P1 plasmid (Austin et al. 1981) are

examples of such site-specific recombination systems. A similar mechanism is used by the yeast 2μm circle to resolve multimers by the *FRT*- Flp site-specific recombination system which is used as part of the mechanism for amplification of plasmid copy number (see Section I 1D).

B. Plasmid stability mechanisms

Two processes that determine the stability of a plasmid are replication, which increases the number, and partition, which distributes the replicated molecules to the daughter cells at cell division. The stable maintenance of low copy number plasmids relies on the presence of several plasmid-borne gene systems which prevent the formation of plasmid-free cells.

One strategy developed by prokaryotic plasmids for stable maintenance in a bacterial cell population is the killing of plasmid-free cells, also termed as post-segregational killing (Jensen and Gerdes, 1995). All these killer systems are based on a toxin-antidote principle, both being encoded by genes carried by the plasmids. Each of these systems consists of two components, a stable toxin and an unstable antidote to block the activity of the toxin. When the plasmid is lost the antidote decays and the toxin, which is still present, kills the plasmid-free cell. In one system, both the toxin and the antidote are proteins (reviewed in Jensen and Gerdes, 1995) and in another system they are antisense RNAs (reviewed in Gerdes et al. 1997). A well-studied killer system of the first category is the *ccd* system of the F plasmid (Jaffe et al. 1985). In this system the proteins CcdA and CcdB, the antidote and the toxin, respectively, are encoded by genes

that form an operon. Two identical systems, the parD and pem systems of plasmids R1 and R100, respectively, also contain genes arranged in operons (Bravo et al, 1987; Tsuchimoto et al. 1988). In the *pem* system, the toxin PemK is neutralized by the blocking agent PemI probably by the formation of a tight complex (Ruiz-Echevarria et al. 1995). The phd/doc system of prophage P1 is another example of stabilization by killing of plasmid-free cells where the protein Phd antagonizes the toxic effect of the Doc protein (Lehnherr et al. 1993). An example of a killer system with an antisense RNA exists in the plasmid R1, where the killer is a messenger RNA for a toxic protein and the antidote is a small antisense RNA that blocks expression of the mRNA (Rasmussen et al. 1987). In contrast to these killer-systems which stabilize plasmids by killing plasmid-free cells, true partitioning systems (discussed in the following section) confer stability to plasmids by actively distributing the molecules to the daughter cells (reviewed by Nordstrom and Austin, 1989; Hiraga, 1992).

C. Partitioning of bacterial plasmids

Efficient partitioning of bacterial plasmids ensures that each daughter cell receives copies of the plasmids during cell division. Small, high copy number plasmids are usually inherited based on random segregation alone (Williams and Thomas, 1992), with the plasmid ColE1 being representative of the plasmids that use this strategy. In the case of large, low copy number plasmids partitioning is not random, and there is evidence for the existence of active partitioning systems (reviewed by Hiraga, 1992; Nordstrom and Austin, 1989; Williams and Thomas, 1992). These systems require a *cis*-acting locus and plasmid-encoded proteins. The proteins may be either host or plasmid-encoded

depending on the system. The *par* locus of the intermediate copy number plasmid pSC101 (15 copies per cell) is an example of a *cis*-acting site that is required for partitioning (Meacock and Cohen, 1980). The *par* locus is required for stability and is relatively simple. It does not interact with plasmid-encoded proteins but rather induces negative supercoiling in adjacent DNA by acting as a binding site for the host-encoded DNA gyrase (Wahle and Kornberg, 1988). Since mutations in *par* that affect partitioning also affect supercoiling, it has been predicted that an increase in negative superhelical density facilitates random partitioning (Conley and Cohen, 1995).

Among the low copy number plasmids, the most extensively studied active partitioning systems are *sop* of F and *par* of P1. The F plasmid, which is present at one or two copies per cell, has a partition system comprised of two *trans*-acting proteins encoded by the plasmid genes *sopA* and *sopB*, and a *cis*-acting locus *sopC* (Ogura and Hiraga, 1983). The *sopC* region consists of 12 direct repeats of a 43-bp motif, and a pair of 7-bp inverted repeats exists within each of the direct repeats (Mori et al. 1986; Helsberg and Eichenlaub, 1986). The SopB protein recognizes the 7-bp inverted repeats and binds to them (Mori et al. 1989). According to the model suggested by Ogura and Hiraga (1983), the SopB protein may be the link between the plasmid DNA and other cellular components through its interaction with the *sopC* locus. The SopB protein was also found to sediment with host membrane fraction in the presence of Mg^{+2} ions, indicating that the SopB protein may provide a link between the plasmid and a membrane-bound host site involved in partition (Watanabe et al. 1989). The SopA protein does not directly interact with *sopC* but instead regulates the expression of the *sopA* and *sopB* genes by binding to their promoter as an autorepressor. This binding activity is enhanced by the addition of SopB protein (Mori et al.

1989), suggesting a cooperative action of the SopA and SopB proteins is the regulation of the *sopAB* operon (Hiraga, 1992). This autoregulation is analogous to that of the yeast 2μm circle, where the *REP1* gene is repressed by the combined effect of the Rep1 and Rep2 proteins. The P1 plasmid maintained at one copy per cell has a partitioning system similar to the *sop* of F, except that the two proteins are called ParA and ParB and the *cis*-acting locus is called *parS* (Friedman and Austin, 1988).

Plasmid R1, also a low copy number plasmid, resembles F and P1 in terms of organization of its partition system, which consists of two open reading frames *parM* and *parR* and a *cis*-acting locus *parC* (Dam and Gerdes, 1994). The ParR protein binds to the *parC* site (Jensen et al. 1994; Breuner et al. 1996) and ParM possesses an ATPase activity which is required for plasmid partitioning and interacts with ParR bound to *parC* (Jensen and Gerdes, 1997). It has been shown by electron microscopy that two *parC*-containing DNA molecules are paired by the partitioning proteins *in vitro*. Binding of only ParR to *parC* is required for this pairing, but this binding is stimulated by ParM in the presence of ATP (Jensen and Gerdes, 1998). Thus this ParR- and ParM-mediated pairing of two *parC*-containing DNA molecules is probably a requirement for the partitioning process. It has been proposed that this association between a pair of plasmids provides directionality, which may be necessary for partitioning where the two plasmids are recognized by some apparatus that pulls the two plasmids towards opposite directions in a dividing cell (Jensen and Gerdes, 1998). Alternatively, the plasmid pairs could be attached to a cellular structure that positions the two plasmids on opposite sides of the septum formed at the mid-cell (Jensen and Gerdes, 1998). Interestingly, the ParM-GFP fusion protein and a plasmid containing the *parA* system

have been shown to co-localize at defined sites near cell poles and at mid-cell (Jensen and Gerdes, 1999). From the above results, Jensen and Gerdes (1999) have extended their model for the partitioning of plasmid R1. They have proposed that after replication the daughter plasmids are paired at the mid-cell, followed by active movement of the molecules towards the poles before cell division. This movement, caused by the partition apparatus, probably involves some host factors as well.

The SopB partitioning protein of F plasmid has also been found positioned at mid-cell and one quarter of the way through the cell length in predivisional cells (Kim and Wang, 1998). The F plasmid itself has also been shown to be localized at mid-cell in newly divided cells, but later migrates to positions one-fourth or three-fourths of the cell length (Niki and Hiraga, 1997; Gordon et al. 1997). It seems likely that SopB is responsible for positioning plasmids containing the *sopC* site in specific cellular locations. The role played by these proteins in mediating the positioning of plasmid molecules in partitioning is probably one of tethering the replicated DNA molecules in areas away from each other until the mid-cell septum is formed (Kim and Wang, 1998; Jensen and Gerdes, 1999).

In spite of all the studies performed regarding the stable maintenance of bacterial plasmids the actual mechanisms of partitioning are still unclear. It is likely that more than one mechanism exists for the various prokaryotic plasmids. However, through tight control of replication, copy number, and active partitioning, plasmid persistence in the host cells is ensured.

3. Centromeres and associated proteins

Probably the best understood partitioning system is that involved in segregating eukaryotic chromosomes, which involves the attachment of the chromosomes through sequences called centromeres to the mitotic spindle apparatus. Centromeres were originally described as the constricted regions in the chromosomes where the two sister chromatids are attached. During mitosis and meiosis the cell builds up a specialized microtubule structure called the mitotic apparatus. It is through the centomeres that the microtubules connect the chromosomes to this mitotic apparatus. The region of attachment of microtubule to the centromere is called the kinetochore. The kinetochore may be defined as the region of the centromere which is associated with proteins (reviewed by Hyman and Sorger, 1995; Lechner and Ortiz, 1996) and together this DNA-protein complex is referred to as centromere-kinetochore-complex (CKC). The next section gives a brief description of chromosome movement and microtubule attachment. This is followed by a section on centromeres and associated proteins known to be part of the kinetochore.

A. Chromosome movement and microtubule attachment

Eukaryotic cells are equipped with a very efficient mechanism for chromosome segregation which ensures accurate transmission of genetic information during cell division. Chromosome segregation has been studied extensively, particularly in the budding yeast *Saccharomyces cerevisiae* (reviewed by Page and Snyder, 1993). Mitosis in yeast involves several steps. It begins with duplication of the spindle pole body (SPB), followed by separation of the SPBs and establishment of the mitotic spindle apparatus. In yeast the

nuclear membrane does not break down during mitosis and thus the spindle pole bodies remain embedded in the nuclear membrane. The next critical step is positioning of the mitotic spindle followed by chromosome attachment to microtubules of the spindle, and chromosome movement towards the poles. Chromosome movement involves three stages (Inoue, 1981). It begins with monopolar attachment during prometaphase when only one in a pair of sister kinetochores is bound to microtubules from a single spindle pole (Rieder et al. 1990). At metaphase the kinetochores capture microtubules from the other pole to establish bipolar attachment (Skibbens et al. 1993). In the third stage, during anaphase the chromosomes move towards the opposite poles. This separation of the chromosomes has two parts: during anaphase A, chromosomes move towards the poles and during anaphase B, the poles themselves separate. In budding yeast, there are sixteen pairs of sister chromatids and thirty two non-core microtubules, suggesting that each kinetochore binds to a single microtubule (Peterson and Ris, 1976). In addition, there are microtubules in the midzone that form a central core. Microtubules also extend from the pole into the cytoplasm. As a result the spindle pole bodies which remain associated with the nuclear membrane may have contact with the cytoplasmic microtubules emanating from the nucleas. Unlike most animal cells, in budding yeast, chromosomes are distributed along the length of the spindle and do not cluster near the centre of the spindle to form the metaphase plate (Straight et al. 1997). In yeast the majority of the chromosome movement occurs at anaphase B and is due to the separation of the poles. In late anaphase B, one set of sister chromatids is pulled into the bud, followed by nuclear division (Palmer et al. 1989).

B. Centromeres and Kinetochores

In yeast, the proper segregation of chromosomes during cell division requires three *cis*-acting DNA sequences. These are autonomously replicating sequences (*ARS*s) (Hsiao and Carbon, 1979; Stinchcomb et al. 1979) which function as origins of replication, telomeric repeats (Szostak and Blackburn, 1982) found as tandem arrays at the ends of all linear eukaryotic chromosomes, and centromeres (*CEN*) (Clarke and Carbon, 1980). The telomeric repeats bind a number of proteins to form a specialized structure called the telomere that is required for stabilizing the ends of the linear eukaryotic chromosomes. Following replication of chromosomes, centromeres form the final point of attachment between the sister chromatids prior to their separation at anaphase. Apart from being the bivalent connectors of the paired chromatids, centromeres and proteins associated with them are also essential for the attachment of chromosomes to the mitotic spindle and directing chromosome movement and segregation. To understand the mechanism by which the centromere interacts with the microtubules of the spindle it is appropriate to review what is known about the organization of the DNA and proteins in the centromere region.

Due to the small size of the centromere, the *S. cerevisiae CEN* DNA has been studied extensively. The relatively simple structure of the yeast kinetochore compared to that of higher eukaryotes has enabled the use of both genetic and biochemical methods for analyzing the centromere DNA and the associated proteins (reviewed by Fitzgerald-Hayes, 1987; Lechner and Ortiz, 1996; Bloom et al. 1989).

i. *CEN* DNA

The centromere DNA (*CEN* DNA) of *S. cerevisiae* consists of three domains, CDE I (centromere DNA element), CDE II and CDE III, which cover a total length of 125-bp (Hegemann and Fleig, 1993). CDE I consists of a conserved 8-bp sequence with a 6-bp palindrome between positions 3 and 8. The 26-bp CDE III has a highly conserved core sequence between positions 11 and 17. In between CDE I and CDE III is the 78- to 86-bp long highly AT-rich DNA of CDE II. Mutational analysis has shown that CDE III and at least part of CDE II are essential for centromere function; in fact, single point mutations in CDE III can inactivate the centromere (McGrew et al. 1986). However, deletion of CDE I causes only slight reduction in centromere function, increasing chromosome missegregation frequency by a factor of 10 (Niedenthal et al. 1991), suggesting that CDE I is not essential for mitosis. CDE I seems to be more important for meiosis than mitosis, since deleting CDE I causes nondisjunction in meiosis II (Sears et al. 1995).

ii. Centromere-associated proteins

When yeast chromatin was first isolated and analyzed, a 220- to 250-bp region of DNA that includes the conserved centromere sequences CDE I, CDE II and CDE III was found to be nuclease-resistant, suggesting that proteins were binding to the centromere DNA *in vivo* (Bloom and Carbon, 1982).

It has now been established that CDE I is the binding site for the protein Cbf1 (also known as Cpf1 or Cp1) (Jiang and Philippsen, 1989; Baker and Masison, 1990; Mellor et al. 1990; Cai and Davis, 1990). Cbf1 is a member of the helix-loop-helix family of DNA-binding proteins and it binds as a homodimer to the 6-bp palidrome in CDE I (Wilmen et al.

1994). Loss of Cbf1, like deletions of CDE I, results in a ten-fold increase in chromosome missegregation, suggesting that it is important but not essential for kinetochore function. Cbf1 is also involved in transcriptional regulation of methionine biosynthetic genes (Kuras and Thomas, 1995).

A multisubunit protein complex, Cbf3, binds to CDE III (Lechner and Carbon, 1991). Point mutations in CDE III that interfere with chromosome segregation also abolish the binding of Cbf3. The Cbf3 complex consists of subunits Cbf3a (Ndc10/Ctf14), Cbf3b (Cep3) and Cbf3c (Ctf13). A fourth component Cbf3d (Skp1) has been identified by co-purification with Cbf3c (Stemmann and Lechner, 1996). The cooperation of all four components of Cbf3 is necessary to form the active complex that interacts with CDE III. Cbf3 makes sequence-specific and non-specific contacts with DNA and three of the subunits, Cbf3a, Cbf3b and Cbf3c, are in direct contact with *CEN* DNA (Espelin et al. 1997). Affinity-purified Cbf3 has been implicated in binding to microtubules (Hyman et al. 1992), but while it is absolutely necessary for binding it is not sufficient (Sorger et al. 1994). All four components of Cbf3 are essential for cell growth.

Mif2, which interacts with CDE III (Meluah and Koshland, 1997), is an essential protein which shares limited homology with CENP-C, a mammalian kinetochore protein (Saitoh et al. 1992). Mif2 has been shown to interact directly with Cbf1 and Cbf3a, and all these proteins are associated with the centromere *in vivo* (Meluah and Koshland, 1997).

A non-essential protein, Ctf19, has been implicated as a kinetochore protein and has been suggested to be a link between the kinetochore and the mitotic spindle (Hyland et al. 1999). Although genetic analysis such as synthetic lethality (SL) studies, and synthetic dosage lethality (SDL) studies, or both, detected interactions between Ctf19 and all four

subunits of Cbf3 as well as with Mif2 (Hyland et al. 1999), it is not a subunit of Cbf3 (Stemmann and Lechner, 1996). Interactions between Ctf19 and Cbf3a and Cbf3b have also been established using a two-hybrid assay (Ortiz et al. 1999). The same studies also showed interaction of Ctf19 with two other centromere proteins, Mcm21 and Okp1, as well as interaction of Mcm21 and Mif2, suggesting a putative protein complex consisting of Ctf19, Mcm21 and Okp1which associates with Cbf3 via Ctf19 and also interacts with Mif2 (Ortiz et al. 1999). Ctf19 has also been found associated with centromeric DNA, although its interactions may be indirect as a consequence of its interactions with Cbf3 or a larger macromolecular complex whose assembly begins with the recruitment of Cbf3 to CDE III (Hyland et al. 1999). Ctf19 has also been shown to interact genetically with Ndc80, a spindle- and pole-associated protein. On the basis of interactions of Ctf19 with the centromere and with Ndc80, it has been proposed that Ctf19 provides a link between the mitotic spindle and the kinetochore in budding yeast (Hyland et al. 1999).

Available data suggest that Cbf3-*CEN* DNA complex formation is necessary for kinetochore-microtubule interaction and that CDE III is essential. The proximity of CDE II to CDE III stabilizes this interaction (Sorger et al. 1994). Current models of kinetochore assembly suggest that the Cbf3 complex, and possibly Mif2, are recruited to CDE III to initiate centromere-kinetochore assembly and this subcomplex then forms a nucleation site for the formation of a functional kinetochore (Meluah and Koshland, 1997). It is not clear how Ctf19 connects this complex to the spindle. Detection of more proteins of this large complex will provide the missing links and reveal how all these proteins function together in mitosis.

4. Centromere and *STB*

Efficient partitioning of 2μm plasmid requires the plasmid-encoded proteins Rep1 and Rep2 as well as the *cis*-acting *STB* locus. As mentioned above, yeast plasmids carrying only an *ARS* element can replicate but are extremely unstable and have a tendency to be retained by the mother cell during mitosis. Addition of the *STB* sequence stabilizes such a plasmid provided the Rep1 and Rep2 proteins are supplied in *trans* in the same cell. *ARS* plasmids can also be stabilized by incorporating a functional centromere. These *ARS/CEN* plasmids are maintained at single copy per cell (reviewed in Roth and Simpson, 1991). The stability and low copy number of the *CEN* plasmids make them ideal as cloning vectors. Addition of a centromere probably leads to the formation of a functional kinetochore, which involves interactions with host proteins that allow the plasmid to be segregated as if it were a new host chromosome. However, it is still unclear how including the *STB* locus can make an unstable *ARS* plasmid stable. Although the *STB* locus of the 2μm plasmid functions like a *CEN* sequence, there is no sequence similarity between the two. Therefore none of the proteins that directly contacts CDE I, CDE II and CDE III are expected to be at the *STB* locus. Instead, it is more likely that 2μm circle uses the same segregation machinery that contacts the kinetochore but may use different proteins to form the attachment. Understanding the proteins that mediate plasmid segregation will provide information on how the host's segregation machinery is used for segregation of this plasmid. Revealing potential interaction between the 2μm circle and the host segregation machinery will be important in solving the mystery of partitioning of this cryptic plasmid.

II. MATERIALS AND METHODS

1. Strains, media, transformation and DNA preparation

A. Yeast and bacterial strains

Escherichia coli strain DH5α (*supE*44, Δ*lac*U169 [F80*lacZ*DM15], *hsdR*17, *recA*1, *endA*1, *gyrA*96, thi-1, *relA*1) was used for most bacterial transformations. The repair-defective *E. coli* strain NM522 mutS (*thi*, *supE*, Δ[*hsd*MS-*mcr*B]5, Δ[*lac-proAB*][*mut*S::Tn10]F'[*pro*AB$^-$ lacI^q *lacZ*ΔM15]) was used for propagation of mutated plasmids for the site-directed mutagenesis experiment. *E. coli* strain BL21 [DE3] was used for the propagation of pET plasmids used in the *in vitro* baiting assay.

Saccharomyces cerevisiae strains used were isogenic [cir$^+$] and [cir^0] DBY746 (*MAT*a, *his3*Δ1, *ura3-52*, *leu2-3*, *leu2-112*, *trp1-289*), isogenic [cir$^+$] and [cir^0] AS3 (*MAT*a, *his3*Δ1, *ura3-52*, *leu2-3*, *leu2-112*, *trp1-289*, *ade2*Δ::*URA3*) and isogenic [cir$^+$] and [cir^0] MD40/4c (*MATα*, *leu2-3*, *leu2-112*, *his3-11*, *his3-15*, *trp1*, *ura2*). The yeast strain Y153 (*MAT*a, *gal4*, *gal80*, *his3*, *trp1-901*, *ade2-101*, *ura3-52*, *leu2-3*, *leu2-112*, *URA*::*gal-lacZ*, *LYS2*::*gal-HIS3*) was used as the host for two-hybrid genetic screen.

B. Media

E. coli were grown in 2X YT or LB at 37°C as described by Sambrook et al. (1989). Yeast were grown in YEPD (1% yeast extract, 2% bactopeptone, 2% glucose) or

with appropriate amino acids as described by Rose et al. (1990) at 30°C unless otherwise indicated. Selection for plasmids in AS3 was maintained by adding histidine, leucine and tryptophan to SD medium. Selection for plasmids in Y153 was maintained by supplementing SD medium with all amino acids except either leucine or tryptophan or both as required. For selection of two-hybrid interacting clones in Y153, 3-aminotriazole was included in solid medium at a concentration of 50 mM and histidine was omitted. Media were solidified with 2% agar. All media reagents were obtained from DIFCO Laboratories or Sigma Chemical Co.

C. Transformation

Bacterial transformations were performed either by electroporation, using a Bio-Rad Gene Pulser electroporator with cells made competent according to the manufacturer's instructions, or by using cells made competent by incubation with divalent cations (Sambrook et al. 1989).

Yeast transformations were performed by using the LiOAc/ssDNA/PEG method described by Gietz et al. (1995).

D. DNA preparation

Yeast DNA was prepared as described by Rose et al. (1990). *E. coli* DNA was prepared as described by Sambrook et al. (1989).

2. Development of a colour assay for plasmid stability

The construction of the plasmids and strains used in this study and the methods adopted are described in the following sections.

A. Plasmids

i. *ADE2* disruption plasmid (pAS3)

The vector pBR322 and the yeast *ADE2* gene-containing shuttle vector pL909 (Gottschling et al. 1990) were digested with restriction enzymes *Eco*RI and *Bam*HI. The 4.0-kbp fragment from pBR322 and the 3.5-kbp fragment of pL909 were gel-purified and ligated to produce pAS1 (Figure 2). To construct plasmid pAS3 (Figure 3), pAS1 was digested with *Hind*III and the 5.3-kbp fragment retaining the *ADE2* gene non-coding flanking regions was gel-purified and ligated to a 1.1-kbp yeast genomic *Hind* III fragment containing the *URA3* gene from plasmid pRB58 (Carlson and Botstein, 1982). See Table A1 in appendix.

ii. 2μm Stability vector (pAS4)

The 2μm stability vector (pAS4) was derived from the 2μm-based vector pJDB219B (Beggs, 1978). pJDB219B is a complete 2μm plasmid in the B form (Hartley

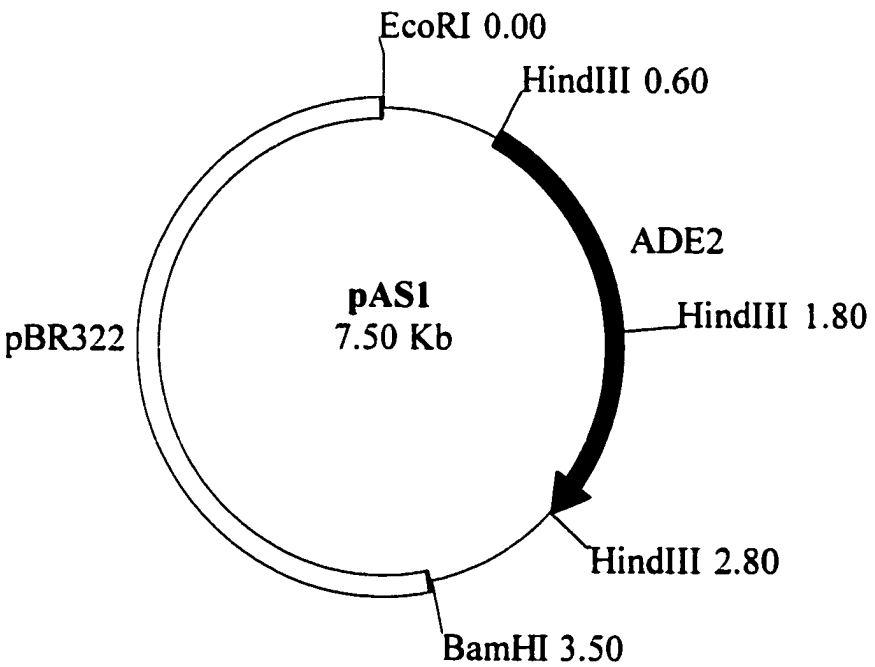
EcoRI 0.00
HindIII 0.60
ADE2
pAS1
7.50 Kb
HindIII 1.80
pBR322
HindIII 2.80
BamHI 3.50

Figure 2

Figure 2. Restriction Map of pAS1. The *ADE2* ORF is indicated by black arrow. Thin lines represent genome sequences flanking the *ADE2* gene. *E. coli* sequences are indicated in white. Selected restriction sites are shown.

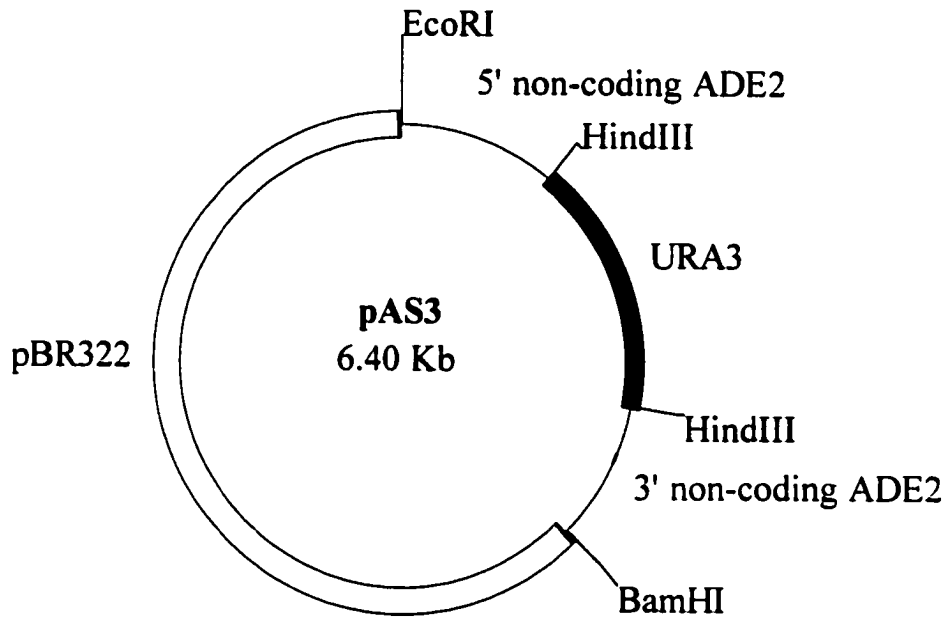
EcoRI
5' non-coding ADE2
HindIII
URA3
pAS3
6.40 Kb
pBR322
HindIII
3' non-coding ADE2
BamHI

Figure 3

Figure 3. Restriction Map of pAS3. Thick black line is a 1.1-kbp genomic fragment containing the yeast *URA3* gene. Thin lines indicate 5' and 3' non-coding regions of the *ADE2* gene. *E. coli* sequences are indicated in white. Selected restriction sites are shown.

and Donelson, 1980) with insertions at two positions. The pMB9 vector is inserted at the *Eco*RI site within the *FLP* gene and a sheared genomic DNA fragment containing the *LEU2* gene was GC-tailed into the unique *Pst*I site in the *STB* locus (Beggs, 1978). pAS4 was constructed by excising the pMB9 vector sequences using *Eco*RI and then inserting *Bam*HI-digested *E. coli* vector pTZ18R (Pharmacia) at a unique *Bam*HI site in the 2μm *REP1/REP2* intergenic region within the inverted repeat sequences (IRS). This *Bam*HI site was created by a brief BAL31 digestion at the *Xba*I site within the *IRS* followed by closure on *Bam*HI oligonucleotide linkers (Dobson et al. 1988). The *FLP* gene was then disrupted by flush-ending the internal *Eco*RI site with Klenow and inserting a filled-in 3.5-kbp genomic *Eco*RI/*Bam*HI fragment containing the yeast *ADE2* gene from pAS1. The 2μm *Eco*RI fragment spanning the *Pst*I site in *STB* was purified from another 2μm-based yeast plasmid pRB58 (Carlson and Botstein, 1982) and used to replace the equivalent *LEU2*-disrupted fragment derived from pJDB219B so that the vector would now contain an intact *STB* locus. The result was the 2μm stability vector pAS4 (see Table A1), which is an *ADE2 flp*$^-$ 2μm plasmid that can be propagated in yeast and *E. coli* (Figure 4).

iii. pAS4 derivatives

To construct pAS4ΔX, the 9.3-kbp *Bgl*II-*Hpa*I fragment from pAS4 was ligated with a 3.1-kbp *Bgl*II-*Ava*I fragment also from pAS4. The ligated DNAs were incubated with Klenow large fragment of DNA polymerase and nucleotides to allow fill-in of the *Ava*I ends. The plasmid was then circularized by self-ligation in the presence of an

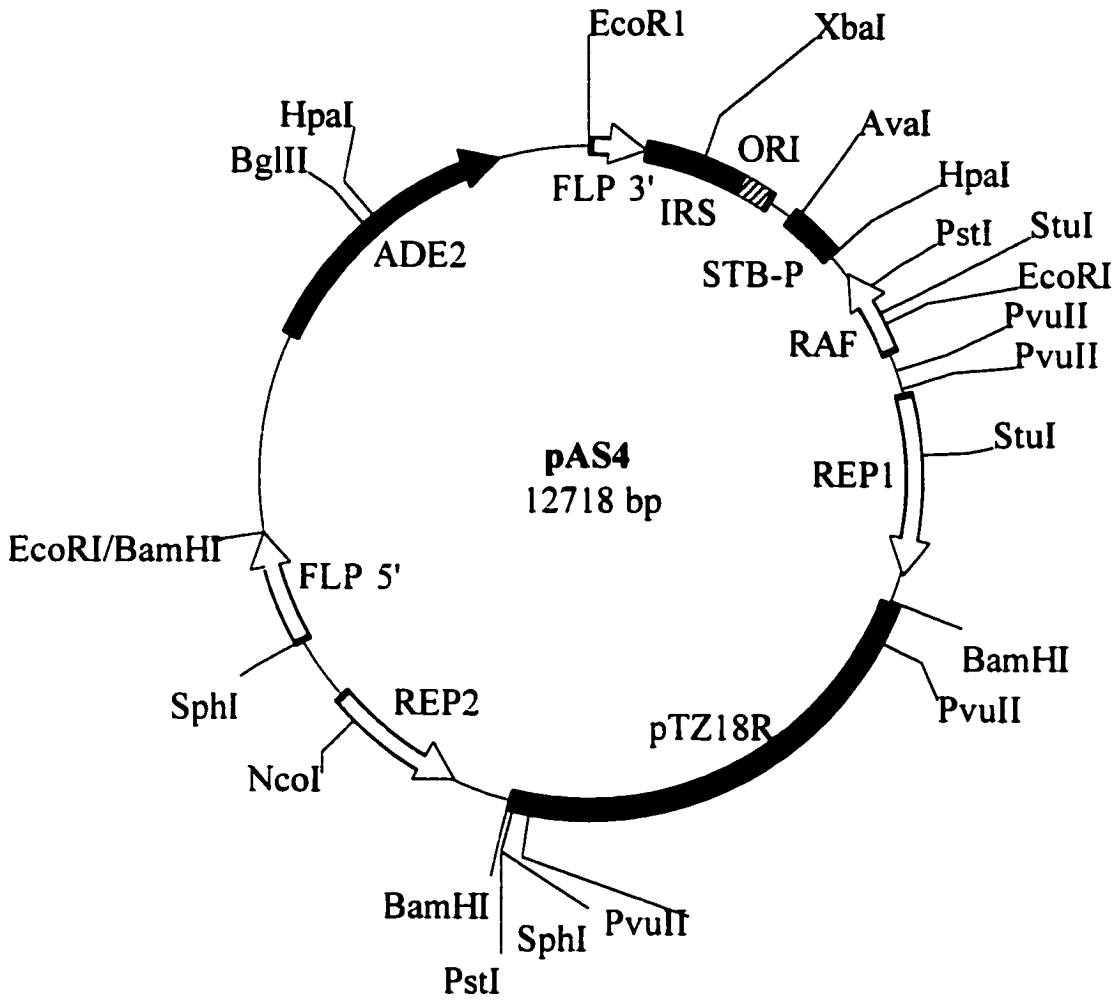
EcoR1
XbaI
HpaI
BglII
ORI
AvaI
FLP 3'
IRS
HpaI
PstI
StuI
ADE2
STB-P
EcoRI
PvuII
RAF
PvuII
pAS4
12718 bp
StuI
REP1
EcoRI/BamHI
FLP 5'
BamHI
PvuII
SphI
REP2
pTZ18R
NcoI
BamHI
SphI
PvuII
PstI

Figure 4

Figure 4. Map of the 2μm stability vector pAS4, showing selected relevant restriction enzyme sites. 2μm ORFs are represented by open arrow. Thick filled black arrow indicates the yeast *ADE2* gene. *STB*-proximal (STB-P) is represented by black box and gray box indicates one of the inverted repeats (*IR*). ORI is indicated in hatched box and thin lines represent 2μm sequences. *E. coli* sequences from vector pTZ18R are shown in black. The positions of relevant restriction enzyme sites starting from *Eco*RI at the 3' end of the *FLP* gene are as follows: *Eco*RI (0), *Xba*I (703), *Ava*I (1390), *Hpa*I (1684), *Pst*I (1996), *Stu*I (2159), *Eco*RI (2241), *Pvu*II (2515), *Pvu*II (2619), *Stu*I (3029), *Bam*HI (3945), *Pvu*II (4150), *Pvu*II (6750), *Sph*I (6830), *Pst*I (6835), *Bam*HI (6845), *Nco*I (7923), *Sph*I (8467), *Eco*RI/*Bam*HI (9218), *Bgl*II (10,968), *Hpa*I (10,974).

excess of *Xho*I linkers. This removed *STB* proximal from pAS4 and added an XhoI site at that junction (see Table A2).

The *rep1*-deleted plasmid pAS4Δrep1 was constructed by digesting pAS4 with *Pvu*II which removed the entire *REP1* coding region, including 126 bp of the 5' flanking sequence and the 3' flanking sequence up to the *Xba*I site, and self-ligating the 8.4-kbp fragment. The *rep2*-deleted plasmid pAS4Δrep2 was similarly constructed by self-ligation of the 11.0-kbp fragment obtained by digesting pAS4 with *Sph*I, which removed all of *REP2* coding region, including 376 bp of 5' flanking sequence and the 3' flanking sequence up to the *Xba*I site. Unlike the *rep1*-deleted plasmid which has all but 215 bp of pTZ18R removed, the *rep2*-deleted plasmid retains most of pTZ18R except for 25-bp from the polylinker region. Another derivative of pAS4, pAS4B, was created by digesting pAS4 with *Bam*HI and self-ligating to remove the *E. coli* vector sequences (see Table A2).

B. Creation of AS3 yeast strains

Targeted gene replacement was used to remove the *ADE2* coding region from the genome of yeast strains used for this study. The isogenic *ADE2* [cir^+] and [cir^0] DBY746 yeast strains were transformed with *Eco*RI/ *Bam*HI digested pAS3. The 2.4-kbp *Eco*RI/ *Bam*HI fragment in plasmid pAS3 contains the yeast *URA3* gene flanked by the 3' and 5' non-coding regions of the yeast *ADE2* gene and when integrated into the chromosome by homologous recombination will remove the *ADE2* gene. Transformants with the deleted *ADE2* gene were selected for uracil prototrophy. Since *ADE2* yeast form white colonies, and *ade2* yeast form red colonies, due to accumulation of a pigmented intermediate in the

adenine biosynthetic pathway (Silver and Eaton, 1969), the red colour of the uracil prototrophic transformants on rich YEPD medium confirmed the loss of the *ADE2* open reading frame. The resulting *URA3*$^{+}$ *ade2*$^{-}$ isogenic [cir^{+}] and [cir^{0}] strains were named AS3. Introduction of the of the *ADE2* 2μm stability vector pAS4 into these *ade2Δ* yeast strains by transformation to adenine prototrophy gives colonies that can be scored visually because they are white rather than red (Figure 5).

C. Plasmid stability assays

To calculate the rate of loss of plasmid per generation, the AS3 [cir^{0}] strain containing the *ADE2* plasmid pAS4 or its derivatives were initially grown in SD medium lacking adenine to select for cells containing plasmid. To assess the percentage of cells containing plasmids at this initial point, cells were plated on SD medium either containing or lacking adenine. The initial percentage of cells containing plasmid was obtained from the ratio of the number of colonies growing on medium lacking adenine to those growing on adenine-containing medium. The same culture was also used to inoculate rich (YEPD) medium and cells were grown for approximately ten to fourteen generations. Cells that lose plasmid during growth in YEPD as well as those that retain plasmid will grow. To establish the percentage of cells that did lose plasmid during this non-selective growth period, cells were again plated on SD medium with or without adenine as before and the final percentage of plasmid-bearing cells was obtained from the ratio of the two numbers. The rate of loss of plasmid per generation during non-selective growth was calculated from the values obtained for initial and final percentage of plasmid bearing cells (Dobson et al. 1988). Experiments were repeated with four different

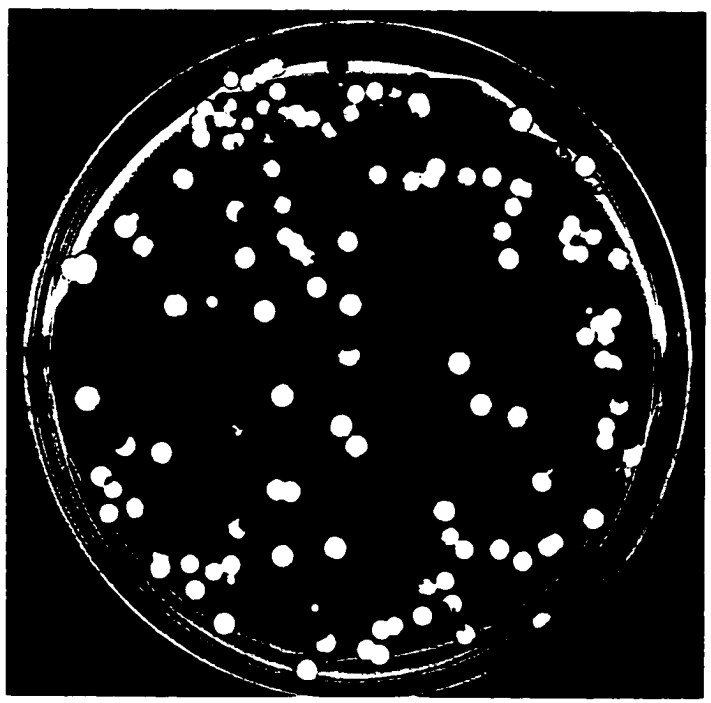

Figure 5.

Figure 5. Presence of pAS4 plasmid determines colony colour. The strain AS3 [cir^0][pAS4] was grown non-selectively in YEPD for several generations and plated on YEPD medium to illustrate that colony colour reflects presence (white colonies) versus absence (red colonies) of the *ADE2*-containing pAS4 plasmid. White colonies are formed by cells containing the *ADE2* plasmid pAS4. Cells that had lost the plasmid prior to plating form red colonies, and sectored colonies represent lineages within a plasmid-containing colony that lost plasmid while the colony was growing.

transformants for each plasmid. For the stable plasmids like pAS4 and pAS4B, there was occasionally a slightly smaller number of colonies on the non-selective versus selective plate, most likely due to variability in pipetting or sampling. These colonies were scored as 100% of the cells being plasmid-containing for the purposes of this assay. Stability assays were performed for all yeast transformants containing pAS4 or its mutated derivatives.

D. Steady-state levels of Rep1 and Rep2 proteins

i. Antibodies

Anti-Rep1 and Anti- Rep2 polyclonal antisera were raised by injecting rabbits with purified *E. coli*-expressed glutathione-S-transferase-Rep fusion proteins (Melanie Dobson, Ying Zhang, unpublished data). To express glutathione-S-transferase (GST)-Rep fusion proteins in *E. coli*, *Bam*HI fragments containing the *REP1* and *REP2* ORFs were generated by polymerase chain reaction (PCR), flush-ended by a fill-in reaction using Klenow large fragment of DNA polymerase I, and cloned into *Sma*I-digested pGEX-2T (Pharmacia) in the correct orientation to give pGEX-REP1 and pGEX-REP2. GST-Rep1 and GST-Rep2 fusion proteins were expressed in and extracted from *E. coli* as described by Koerner et al. (1991). The GST-Rep fusion proteins could not be purified by adsorption to glutathione-linked agarose due to their insolubility and therefore were purified by electroelution from SDS-PAGE gels (Harlow and Lane, 1988). Polyclonal antisera were generated by subcutaneous injection of rabbits with purified fusion protein at monthly and then bi-weekly intervals until a sufficient titre of anti-Rep1 and anti-Rep2

antibodies were obtained as determined by Western blot analysis. The antibodies were affinity-purified from the crude antisera by the method of Pringle et al. (1991). Trans-Blot 0.2 micron PVDF membrane (Bio-Rad) was used for immobilization of gel-purified GST-Rep1 and GST-Rep2 fusion proteins and GEG buffer (0.2 M glycine, 1 mM EGTA, pH 2.5) was used for antibody elution. The eluted antibodies were neutralized and used for Western blot analysis at a dilution of 1:1000 for anti-Rep1 and 1:500 for anti-Rep2.

ii. Western blot analysis

Western blot analysis of whole-cell protein extracts prepared from yeast transformed with pAS4 and its mutant derivatives, and also from [cir$^+$] and [cir^0] yeast strains, was used to determine the steady-state levels of the 2μm Rep proteins. Proteins were separated either on 10% or 12% SDS-polyacrylamide gels and transferred to PVDF membranes essentially as described by Towbin et al. (1979), except that an additional 0.037% SDS was included in the transfer buffer to achieve efficient transfer of Rep2 and its fusion proteins (Pickett, 1998). Primary antibodies used in this study were affinity-purified polyclonal anti-Rep1, anti-Rep2 or rabbit anti-Gal4 $_{AD}$ (Santa Cruz) as necessary. The anti-Gal4$_{AD}$ antibody was used at a dilution of 1:100. The secondary antibody was horseradish peroxidase-labeled goat anti-rabbit IgG (Kierkegaard and Perry Co.) which was detected by chemiluminescence using a LumiGLO kit (Kierkegaard and Perry Co.).

E. Plasmid copy number determination

Total yeast DNA preparations were digested with *Eco*RI and *Bam*HI, then separated in 1% agarose gels containing 1mg/ml ethidium bromide and using 1x TBE (0.9 M Tris-borate, 2 mM EDTA) as buffer. Four different dilutions of each of the restricted genomic DNAs were separated. Gels were subjected to depurination in 0.25 N HCl for 15 minutes followed by denaturation in 1.5 M NaCl, 0.4 N NaOH for 15 minutes. DNA was transferred to Zeta-Probe GT nylon membrane (Bio-Rad) by capillary transfer method (Southern, 1975) in 0.4 N NaOH. The membranes were neutralized in 0.2 M Tris-Cl pH 7.5, 2xSSC. A 1.4-kbp *XbaI/StuI* fragment gel-purified from plasmid pAS4 containing the 2μm origin of replication, part of one of the inverted repeats (IRS) and the *STB* region was used as a plasmid-specific probe. This probe detects a 2.2-kbp *Eco*RI fragment that is common to all pAS4-based plasmids used in this study and is also present in the B form of the native 2μm plasmid. It also recognizes a 3.8-kbp *Eco*RI fragment from the A form of the 2μm plasmid (see Figure 10 panel B). The second probe was a 2.4-kbp *Eco*RI/*Bam*HI fragment purified from the plasmid pAS3 containing the yeast *URA3* gene flanked by the 5' and 3' non-coding regions of the yeast *ADE2* gene. This probe recognizes the 3.5-kbp genomic *URA3* locus and also a 5.8-kbp band from plasmids pAS4, pAS4B, pAS4Δrep1 and a 7.1-kbp band from plasmid pAS4Δrep2 due to the presence of the *ADE2* gene in these plasmids (see Figure 10 panels A and C). A 6.2-kbp *Eco*RI/*Bam*HI fragment is also recognized by this probe, which is the size expected for the *URA3*-disrupted *ADE2* locus. Hybridization was carried out as described for the colony hybridization in section 4C, except SDS was included at 1% rather than 0.1%. The amount of hybridization to plasmid-specific restriction fragments (indicated in

Figure 10 as 2μm-vector in panel A and B and as 2μm/2μm-vector in panel B) and to the genomic *URA3* band was detected by phosphorimaging and quantified using Molecular Analyst software (Bio-Rad). Plasmid copy numbers were determined from the ratios of the counts obtained for the plasmid relative to the counts for the single copy chromosomal *URA3* band. Prior to calculation, the counts were corrected for differences in the amount of homology between the probes and their respective target sequences and for differences in specific activity between the probes. The specific activity of probes was determined by liquid Scintillation counting. The copy numbers for plasmids pAS4 and pAS4B were determined from the Southern blot shown in panel A of Figure 10. The copy numbers for plasmids pAS4Δrep1 and pAS4Δrep2 were determined from the Southern blot shown in panel C. To calculate copy number of native 2μm in strain AS3 [cir^+], loading of AS3 [cir^+] and AS3[cir^0][pAS4] DNA was determined by comparing counts obtained from the genomic *URA3* band between these two samples in the blot shown in panel A (Figure 10), since 2μm has no homology to this probe. In the blot shown in panel B, hybridization to the 2μm band relative to pAS4 bands was used to compare their relative amounts in the two DNA samples. Then the ratios of the *URA3* counts between the two samples from blot shown in panel A, and the copy number of pAS4 as determined from the same blot (Figure 10 panel A) were used to determine the copy number of native 2μm. The number obtained for 2μm was multiplied by 2 to account for the 2.2-kbp *Eco*RI band being only half the 2μm molecules in the AS3 [cir^+] cells.

3. *FLP* promoter assay plasmid

A 0.3-kbp *Bam*HI fragment containing the promoter region of the *FLP* gene was generated by PCR using the 2μm-based plasmid pJDB219B as the template and the following oligonucleotide primers,

FLP1: 5'GACGGATCCAAATTGTGGCATGCTTAG and FLP2: 5' GACGGATCCTGTGCAGATCACATGTC . PCR was carried out with a proof-reading DNA polymerase, ID proof (ID Laboratories), according to the manufacturer's instructions. A proof-reading thermostable DNA polymerase was used to reduce the frequency of PCR-introduced mutations normally associated with use of non-editing Taq DNA polymerase. Amplification was carried out for 30 cycles, each consisting of one minute at 94°C, two minutes at 50°C and two minutes at 72°C. *Bam*HI-restricted PCR products were cloned into the *Bam*HI site in plasmid YCpAW638 (Amy Wheeler, unpublished results), a derivative of the plasmid pJL638 originally called pBgl-lacZ (Li and Herskowitz, 1993). The result was plasmid pFLP-lacZ, a *CEN-ARS* yeast-*E. coli* shuttle vector with the *FLP* promoter immediately upstream of the *lacZ* gene (see Table A1). This plasmid can be propagated in *leu2* yeast at one copy per cell by selecting for growth on medium lacking leucine.

4. *In vitro* mutagenesis

A. Generation of *REP1* and *REP2* gene fragments by PCR

Mutated *REP1* and *REP2* gene fragments to be used in gap repair of pAS4 were generated by PCR using the 2μm-based plasmid pJDB219B (Beggs, 1978) as a template.

For the PCR amplicons to contain sufficient flanking sequences to allow homologous recombination into the gapped vector *in vivo*, the following oligonucleotide primers were used: for *REP1*, REP1-6: 5'-GCAGAGAATCGTTTACAGC and REP1-7: 5'-GGCAGCCATTGTAGAAGTG ; and for *REP2*, REP2-5: 5'-CTCGAAGTATACTCAAACG and REP2-6: 5'-GTTTAACGGAACAAGATGC . Amplification was carried out for 30 cycles, each consisting of one minute at 94°C, two minutes at 50°C and four minutes at 72° C. PCR was performed using Taq DNA polymerase (Boehringer) according to the manufacturer's instructions, except that an additional 10 mM Tris, pH 8.8, was added to the supplied buffer to improve the yields. To do mutagenic PCR, the nucleotide concentrations of dGTP, dCTP and dATP were increased to 1 mM from the standard 0.2 mM while dTTP levels were maintained at 0.2 mM (Muhlrad et al. 1992). $MgCl_2$ and $MnCl_2$ concentrations were also varied for mutagenic PCR (see Section III 5A).

B. Gap repair of pAS4

i. Generation of mutant *REP1* and *REP2* PCR amplicons

The mutated *REP1* and *REP2* gene fragments generated by PCR (see section II 4A) were used to replace *REP1* and *REP2* loci, respectively, on the test vector (pAS4) by gapped plasmid repair in yeast. The 8.5-kbp *Bam*HI/*Pvu*II fragment from pAS4 was used for gap repair with the *REP1* PCR product (Figure 6A) and the 8.2-kbp *Bam*HI/*Sph*I fragment from pAS4 was used for gap repair with the *REP2* PCR product (Figure 6B). In each case, 100 ng of the PCR product was used with 100 ng of gapped vector for co-transformation. The repair of pAS4 with non-conditional or temperature-sensitive mutant

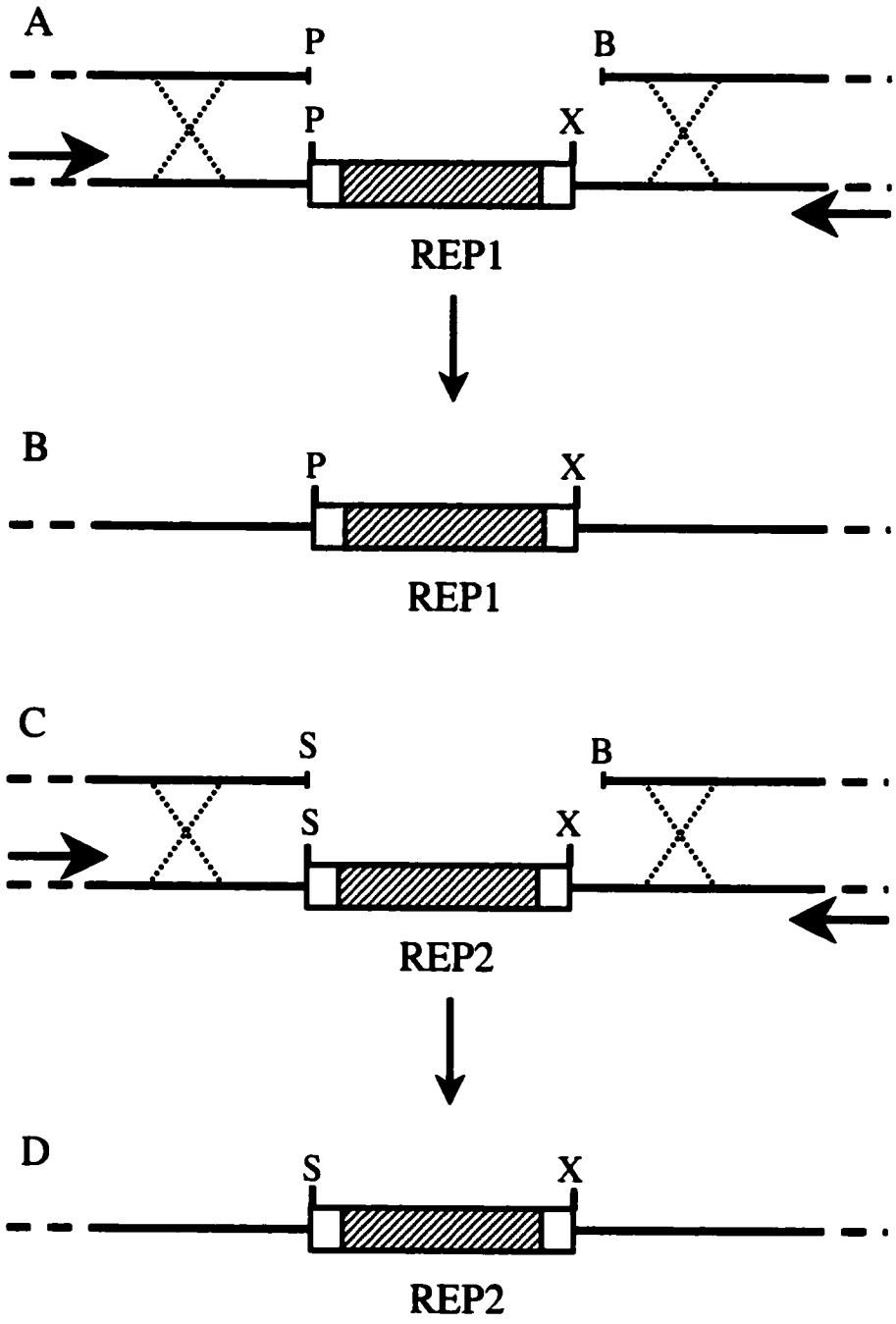

Figure 6.

Figure 6. Gap repair of pAS4. PCR-generated mutated *REP1* and *REP2* gene fragments were used to replace *REP1* (A) and *REP2* (C) loci, respectively, on the test vector pAS4 by gap repair in yeast. The 8.5-kbp *Bam*HI/*Pvu*II fragment from pAS4 (shown as the gapped top line in A) was co-transformed into the yeast with *REP1* PCR product (bottom line in A) and the 8.2-kbp *Bam*HI/*Sph*I fragment from pAS4 (shown as the gapped top line in C) was used for gap repair with *REP2* PCR product (bottom line in C). The directions of the PCR primers are indicated by arrows. Homologous recombination is indicated by the dotted crosses and the final products of the gap repair are shown in panels B and D for *REP1* and *REP2*, respectively. *REP* ORFs are indicated by the cross-hatched boxes. Relevant restriction sites are shown as follows: *Sph*I (S), *Xba*I (X), *Bam*HI (B) and *Pvu*II (P).

alleles of *REP1* or *REP2* was screened using the *ADE2/ade2* white/pink colour assay (see Section II 2B). Two different plating regimes were used dependent on the mutant alleles being sought. In screens where temperature-sensitive alleles of *REP1* and *REP2* were sought, the transformation mixes were plated on selective medium and were grown at 23°C for a week. These were then replica-plated onto two YEPD plates. One of the YEPD plates was kept at 23°C and the other one was incubated at 37°C for 24 hours and then transferred to 23°C. The plates were then incubated at 23°C for another four days. Mutants that form pink/white-sectored colonies after being heat-shocked at 37°C for 24 hours, but were white when incubated only at 23°C, were labeled as temperature-sensitive alleles. Those alleles that gave pink or pink/white sectored colonies at both temperatures were scored as non-conditional mutants.

In a second plating regime where only non-conditional *REP1* and *REP2* mutants were sought, yeast transformed with gapped pAS4 and PCR amplicons were plated on selective media and the plates were incubated at 30°C for five to seven days. Only non-conditional mutant alleles that gave either red or pink colonies were scored. Since gapped vector re-circularizing without having recombined with a PCR amplicon containing either *REP1* or *REP2* also gives pink colonies, *REP1*- and *REP2*-containing transformants were identified by colony hybridization with appropriate probes (see Section II 4C).

ii. Rescue and analysis of mutant *REP1* and *REP2* genes

The *REP1* and *REP2* genes from plasmids giving mutant phenotypes were rescued from yeast by a second PCR reaction, using standard PCR conditions (see Section II 4A) and the same primers used to generate the PCR amplicons used in the original transformation. However, instead of Taq DNA polymerase, a proof-reading thermostable DNA polymerase, ID proof (ID Laboratories), was used to minimize any further mutations being introduced. The mutated regions were then sequenced. To facilitate sequencing, the mutated *REP1* gene amplicons were digested with *Eco*RI and *Xba*I and subcloned into *Eco*RI /*Xba*I digested pBluescript (Stratagene). The mutated *REP2* gene amplicons were digested with XbaI and SphI and subcloned into *XbaI*/*Sph*I digested pTZ18R. The *REP* fragments were sequenced using a Sequenase 2.0 sequencing kit (USB) according to the manufacturer's instructions.

iii. Generation of *STB* mutants in the pAS4 stability plasmid

PCR amplicons containing mutant versions of *STB* proximal were generated using the oligonucleotide primers STB1: 5'-CTATTCATAGAGTGAATCG and STB2: 5'-CTAAGATTCTATCTTCGC . Similar to the approach used to generate mutant *REP1* and *REP2* genes in the 2μm stability vector pAS4, these mutant *STB* amplicons were used for gap repair of pAS4. The plasmid pAS4ΔX was digested with *Xho*I and co-transformed along with the PCR products into the *ade2* strain AS3 [cir^0]. Thus mutated versions of the *STB*-proximal region were introduced into the test vector pAS4. Screening for yeast cells containing mutant forms of the plasmid was carried out as described for the non-conditional *REP1* and *REP2* mutants in pAS4.

C. Colony Hybridization

The filters for colony hybridization were prepared using the zymolyase method as described by Rose et al. (1990). In brief, yeast were plated on selective medium and grown for three days at 30°C. The colonies were replica-plated onto nitrocellulose membranes placed on top of selective medium and allowed to grow on the filters for three days. The nitrocellulose membranes were then placed colony-side up on YEPD plates and incubated for about twelve hours at 30°C prior to Zymolyase treatment. The membranes were then placed on Whatman 3MM filters soaked with the following solutions: 1 M sorbitol, 20 mM EDTA, 50 mM DTT, for fifteen minutes at room temperature; then on 1 M sorbitol, 20 mM EDTA, 1mg/ml Zymolyase 100,000 (Dupont), at 37°C for 2-3 hours; then sequentially at room temperature on 0.5 N NaOH for seven minutes, twice on 0.5 M Tris-Cl pH 7.5, 10xSSC for four minutes, then on 2xSSC for two minutes, and dried for twenty minutes prior to hybridization.

A 1.1-kbp *REP1 Bam*HI fragment or a 0.9-kbp *REP2 Bam*HI fragment obtained from pAS5, and pAS6 (see Section II 5Ai), respectively, were used as probes for colony hybridizations. The probes were labelled with α-^{32}P-dCTP(Amersham) using a random priming kit from Boehringer Mannheim. The probes were purified by centrifugation through a Sephadex G-50 (Pharmacia) column. Filters were washed with 2x SSC at 65°C for 10 minutes followed by pre-hybridization in the hybridization solution, 6x SSC, 5x Denhardt's solution (0.1% Pharmacia type 400 Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 mg/ml sonicated salmon sperm DNA (Pharmacia) and 0.1%

SDS, for 1 hour at 65°C. The probes were boiled for 10 minutes, chilled on ice and added to the solution in the hybridization tubes. Hybridization was overnight at 65°C. The filters were washed with 2x SSC, 0.1% SDS for 20 minutes followed by a wash in 2xSSC for another 20 minutes. These were then exposed to Kodak X-omat film, usually for several hours to overnight at –70°C with an intensifying screen.

D. Generation of single point mutations in *REP1* and *REP2* by site-directed mutagenesis

i. Plasmids used for site-directed mutagenesis

To facilitate later subcloning in pAS4, the *Eco*RI site was removed from pTZ18R prior to insertion into pAS4. This was achieved by digesting pTZ18R with *Sma*I and *Eco*RI followed by a brief treatment with S1 nuclease and then self-ligation which resulted in the removal of the *Eco*RI, *Sst*I, *Kpn*I and *Sma*I sites from the multiple cloning site of the vector. However, at a later stage the *Sst*I site was required for the purpose of site-directed mutagenesis. For these experiments, pAS4 was digested with *Bam*HI and the pTZ18R part was replaced with *Bam*HI-digested pTZ18R containing the entire undeleted polylinker to give plasmid pAS9. The plasmid pAS10 is identical to pAS9 except with pTZ18R inserted in the opposite orientation (see Table A2).

To simplify site-directed mutagenesis, the majority of the pAS4 plasmid was deleted, leaving only either the *REP1* or the *REP2* coding regions plus the *E. coli* vector sequences. For *rep1* mutants, pAS9 was digested with *Pst*I and self ligated to give pAS11 and for *rep2* mutants, pAS10 was digested with *Sph*I and self ligated to give

pAS12 (see Table A2). Mutagenesis was carried out on plasmids pAS11 and pAS12, the plasmids were sequenced to confirm the changes, and then the deleted pAS4 sequences were returned to reconstruct the stability vector.

ii. Site-directed mutagenesis by Unique-Site Elimination

The unique-site elimination procedure (Deng and Nickoloff, 1992) uses two mutagenic primers, both of which anneal to the same strand of a denatured double-stranded plasmid DNA. One of the primers introduces a desired mutation into a known sequence of the DNA and is designated as the target mutagenic primer. The second primer eliminates a unique non-essential restriction site in the plasmid and is designated as the selection primer. Restriction digests with this restriction enzyme will differentiate between the mutated and wild-type plasmids, absence of the unique non-essential restriction site indicating presence of mutation. The newly synthesized strand of DNA will contain both mutations. The plasmids pAS11 and pAS12 were used as templates for site-specific mutagenesis of the *REP1* gene and the *REP2* gene, respectively. I designed a primer (5'-GGGAATTCGCGCGCGGTACCCGGGG) which eliminates the *Sst*I site (GAGCTC) in the multiple cloning site of pTZ18R and converts it into a *Bss*HII site (GCGCGC). This primer was used as the selection primer along with the target mutagenic primer having the desired mutation in either the *REP1* or the *REP2* gene. A primer (5'-CTGTGACTGGTGACGCGTCAACCAAGTC) from Pharmacia was used as a selection primer with a couple of target mutagenic primers (rep2E173G and

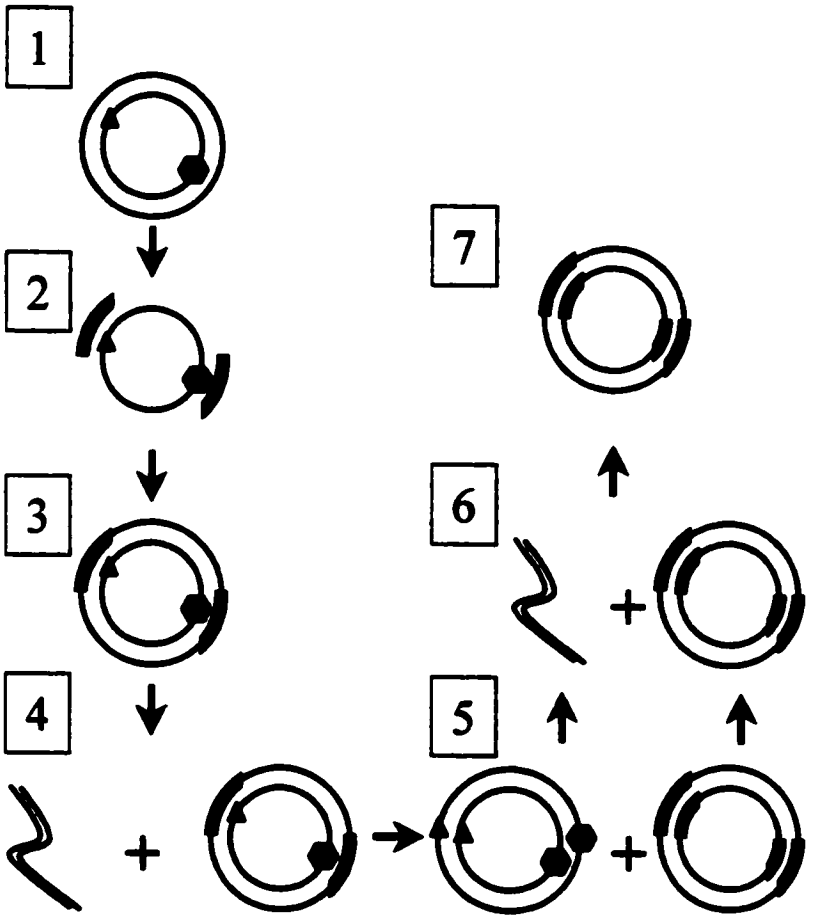

Figure 7.

Figure 7. Site-directed mutagenesis using unique-site elimination. The figure shows the steps involved in site directed mutagenesis using unique-site elimination.

1. The plasmid with unique restriction site (▲)and target site (●) was denatured.
2. The denatured plasmid was annealed to the selection primer and the target mutagenic primer. The primers are shown as thick lines.
3. The DNA was incubated with the reaction mix containing T4 DNA Polymerase, T4 DNA ligase and Gene 32 protein to obtain the mutant plasmid.
4. Primary restriction digest was performed with restriction enzymes *Sst*I or *Sca*I to give linearized parental plasmid and circular mutant plasmid.
5. The digested mix of mutated and non-mutated plasmid was used to transform a repair-defective (*mutS*) strain of *E. coli*.
6. Plasmid DNA isolated from transformed cells was subjected to a second round of restriction digests with enzymes *Sst*I or *Sca*I to increase the proportion of mutant plasmid.
7. After the second round of selection, the *E. coli* host DH5α was transformed with the digested plasmid.

TABLE 1. Target mutagenic primers used in site-directed mutagenesis

Mutation	Primer Sequence
rep1 F174S	5'-GCTTGACATGAAgACGTGAGAATG
rep1K297E/R/G	5'-CAAGACACTT(c/t)(c/t)GAACTTTGTACGACG
rep1F53S/C/Y	5'-GTACATGTCCAACA(g/c/t)ATGCAAATGC
rep1K47R	5'-GCAAATGCTAACGTTcTGTATTT
rep1V98A	5'-CTGACTAATAAATGCAgCCGATATAAACTG
rep1Q269X	5'-CCGCCTCAGTTTaATCTTCCGCTTC
rep1T339A	5'-CCCATACGGCTGcAATATGCTGCTC
rep1I338T	5'-CCATACGGCTGTAgTATGCTGCTCTTC
rep1I30T	5'-CTTGTCGTCTCGgTTACACACCTAC
rep2S108P	5'-GTATGCTCTCTcCACAAGAAGCAAG
rep2E173G	5'-CTCCAACGGAAGgAGATGTTATGAAG
rep2D174N	5'-CCAACGGAAGAAaATGTTATGAAGC
rep2H238Y	5'-GACTTCACTTGaTACGATTATG

Upper case letters represent wild-type sequences. Lower case letters represent sites with mutations

rep2S108P). The selection primer eliminates the *ScaI* site in the multiple cloning site of pTZ18R. A schematic of the procedure is given in Figure 7. The target mutagenic primers used in this study are listed in Table 1. The experiment was carried out using the U.S.E. Mutagenesis kit from Pharmacia following the manufacturer's instructions. The primers were designed to be 25-30 nucleotides in length. The mutation was introduced at the centre of each primer, with 10-15 nucleotides of uninterrupted sequence on each end of the primers. The GC content of each primer was approximately 40%, with a Tm of about 70 ^{o}C. The mutants were identified by *SstI* restriction digests (loss of the *SstI* site indicates introduction of the selection primer). This was followed by sequencing of the targeted region of *REP1* or *REP2* using a Cyclist Taq DNA sequencing kit (Stratagene) according to the manufacturer's instruction. Each of these single mutants was reconstructed into the stability vector (pAS4). The mutation was confirmed by sequencing or, if possible, by restriction digests, and transformed into the *ade2* yeast strain AS3 [cir^{0}], where the ability of the plasmid to confer either a red or white colony phenotype could be tested.

5. Rep1/Rep2 interaction

The plasmids used for *in vivo* two-hybrid assays and *in vitro* baiting assays in this study are listed in Table A3 and described in the following section.

A. Plasmids

i. Construction of pAS5 and pAS6

*Bam*HI restriction fragments containing *REP1* and *REP2* open reading frames were generated by PCR using the 2μm-based plasmid pJDB219B (Beggs, 1978) as the template and the following oligonucleotide primers: for *REP1*, REP1-1: 5'-GGATCCATATGAATGGCGAGAGACTGC and REP1-2: 5'-GGATCCATATAACCTACCCATCCAC ; and for *REP2*, REP2-1 : 5'-GGATCCAAATGGACGACATTGAAACAGCC and REP2-2 : 5'-GGATCCTCATACCCTAGAAGTATTACGTG . PCR conditions were the same as those described for standard PCR in section II 4A. The *REP1* and *REP2* PCR products so obtained were directly ligated into *Sma*I-digested $^{+}$T-tailed phagemid (Marchuk, 1991) or pTZ18R (Pharmacia), to give pAS5, and pAS6, respectively (see Table A3).

ii. Two-hybrid plasmids

The construction of the *REP1* and *REP2* two-hybrid plasmids was performed by Kristina Blomqvist, Melanie Dobson and Ying Zhang (unpublished). *Bam*HI restriction fragments containing *REP1* and *REP2* open reading frames were obtained by digesting pAS5 and pAS6, respectively, and subcloned into the *TRP1* Gal4 DNA-binding domain two-hybrid vector pGBT9 (Clontech) and the *LEU2* Gal4 activation domain two-hybrid vector, pGAD424 (Clontech), to give pGBT9-REP1, pGBT9-REP2, pGAD-REP1 and pGAD-REP2. To construct pGADrep1Δ1-129, a 0.75-kbp filled-in *StuI*/*Bam*HI fragment from pAS5, encoding the carboxy-terminal 244 amino acids of Rep1, was ligated with *Bam*HI-digested filled-in pGAD424. Similarly, a 0.9-kbp filled-in *SpeI*/*Bam*HI fragment from pAS5, encoding the carboxy-terminal 297 amino acids of Rep1, was ligated to

filled-in *Bam*HI-digested pGAD424 to give pGAD-rep1Δ1-76. pGAD-rep1Δ130-373, encoding the amino-terminal 129 amino acids of Rep1, was constructed by *StuI/BglII* digestion of pGAD-REP1 followed by fill-in and self closure. Similarly, pGAD-rep1Δ78-373, encoding the amino-terminal 77 amino acids of Rep1, was obtained by *SpeI/BglII* digestion of pGAD-REP1 followed by fill-in and self-ligation. pGADrep2Δ1-57 and pGADrep2Δ59-296 were constructed by *Eco*RI/*Nco*I or *NcoI/BglII* digestion, respectively, and fill-in of pGAD-REP2. pGADrep2Δ1-14 was a gift from J. Sherk and is a *MaeII* partial digestion product containing the truncated *REP2* ORF inserted at the *ClaI* site in pGAD424. For details of construction see Table A3 in appendix.

iii. Plasmids for two-hybrid analysis with *rep1* and *rep2* mutants

REP1 and *REP2* ORFs containing mutations were amplified by PCR and were subcloned into the two-hybrid plasmids pGBT9 and pGAD424 as described in Section II 5A. However, in this case the ORFs were generated as *Eco*RI/*Bam*HI fragments and the templates were yeast DNAs prepared from colonies identified as containing pAS4 plasmids that had either mutant *rep1* or *rep2* genes. The following oligonucleotide primers were used: for *REP1*, REP1-12: 5'- CGGAATTCATGAATGGCGAGAGACTG and REP1-13: 5'-CGGGATCCTATATAACCTACCCATC; and for *REP2*, REP2-10: 5'-CGGAATTCATGGACGACATTGAAACAG and REP2-11: 5'-CGGGATCCTCATACCCTAGAAGTATTAC. The PCR conditions were the same as those described in Section II 4A.

iv. Plasmids for baiting assays

For the expression of S-peptide-tagged histidine-tagged thioredoxin-Rep fusion proteins in *E. coli*, *Eco*RI/*SalI* fragments containing intact or truncated *REP* gene ORFs

from the appropriate pGADREP plasmids were ligated with *Eco*RI/*Sal*I-restricted pET32 LIC (Novagen).

B. Two hybrid assays

In the yeast two-hybrid assay the interaction of two proteins is tested by expressing both as fusion proteins in the same yeast cell, one of the proteins being fused in-frame with the activation domain of the yeast transcriptional activator Gal4 and the other fused with the DNA-binding domain of Gal4 (Chien et al. 1991). The yeast host strain used for transformation contains the *GAL1-10* upstream activation sequence (*UAS*) (the DNA sequence bound by Gal4) integrated at two positions, one site upstream of an *E. coli lacZ* reporter gene, and the other upstream of an otherwise promoter-less yeast *HIS3* gene. The strain is deleted for its endogenous copy of the *GAL4* gene, so the *lacZ* and *HIS3* reporter genes can only be activated if interaction of the two fusion proteins within the yeast brings the two Gal4 domains into close physical proximity. The DNA-binding domain fusion protein will bind the *UAS* and its interaction with the other fusion protein will recruit the Gal4 activation domain to the DNA and activate the *lacZ* and *HIS3* genes. Expression of the *lacZ* gene will produce β-galactosidase, which can be detected by a filter assay due to the formation of a blue colour with the substrate X-Gal (Breeden and Nasmyth, 1985). The expression of the *HIS3* gene confers resistance to 3-aminotriazole, which can be used to select for yeast containing two fusion proteins that interact. In theory, the *HIS3* reporter should also allow selection of the otherwise *his3* yeast strain on medium lacking histidine, but in my hands the host strain behaved as if it were a histidine prototroph. Addition of 50 mM 3-aminotriazole, an analogue inhibitor

of the histidine biosynthetic pathway, was required to inhibit growth of the host strain in the absence of activation of the *HIS3* reporter gene. At 50 mM aminotriazole, fusion-protein interaction resulted in large aminotriazole-resistant colonies against a background of micro-colonies.

Strain Y153 was used as the yeast host for all two-hybrid analyses (Fields and Song, 1989). Transformants and co-transformants were assayed for β-galactosidase expression by an X-gal filter assay (Breeden and Nasmyth, 1985). As a positive control, yeast were also co-transformed with the plasmids pSE1111, expressing the Gal4 DNA-binding domain fused to Snf1, and pSE1112, expressing the Gal4 activation domain fused to Snf4 (Durfee et al. 1993).

C. *In vitro* baiting assays

The experiment was carried out essentially as described by Ahn et al. (1997) using the protocol recommended by Novagen. *E. coli* transformed with pGEX-2T, pGEX2T-REP1 or pGEX2T-REP2, were grown overnight at 37°C in LB medium containing 50 μg/ml ampicillin. The overnight *E. coli* cultures for GST-Rep protein expressions were diluted one in ten in the same medium and grown at 37°C for one hour prior to being shifted to 25-28°C for induction. Induction was carried out by adding IPTG to a final concentration of 0.3 mM and the cultures were incubated with shaking at 25-28°C for 5-6 hours before the cells were harvested by centrifugation at 4000 x g for 10 minutes at 4°C. All manipulations following harvesting were carried out at 4°C. Cell pellets were resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, 1.4

mM KH_2PO_4, pH 7.3) containing 0.2% Nonidet P40 (NP-40) and a protease inhibitor cocktail, 1 x LAP (final concentration: 0.5 µg/ml leupeptin, 1 µg/ml aprotinin, 0.8 µg/ml pepstatin A and also contains 1 mM PMSF, 1mM dithiothreitol) followed by two washes in buffer A (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 2 mM methionine) and resuspension in 10 ml of buffer A. Lysozyme (100 µg/ml) was then added and the mixture was sonicated and centrifuged at 20,000 x g for 10 minutes. The supernatants were removed and made 15% in glycerol before storage at –80°C.

For pET-Rep fusions, 10 ml cultures of BL21(DE3) transformed *E. coli* were grown and induced as for GST-fusion protein-expressing *E. coli*. All manipulations following harvesting were carried out at 4°C. Cell pellets were resuspended in 1 ml cold sonication buffer (50 mM NaH_2PO_4 , pH 8.0, 10 mM Tris pH 8.0, 100 mM NaCl) containing 15% glycerol and stored overnight at –20°C, then thawed to assist subsequent lysis of cells. Cells were collected by centrifugation and resuspended in cold lysis buffer (sonication buffer + 8 M urea), sonicated and centrifuged. The supernatant containing the urea-solubilized fraction was removed, glycerol added to 15%, and stored at –20°C. Insoluble material was resuspended in 100 µl of lysis buffer containing 15% glycerol and stored at –20°C. Unfused pET and pET-Rep fusion proteins were purified from the urea-solubilized fraction using a His-tag affinity resin, Talon, according to the manufacturer's instructions (Clontech). A volume of 750 µl of the solubilized fraction was made up to 1 ml in lysis buffer and incubated with 200 µl of 50% Talon resin. The Talon-purified His6-tagged pET and pET-Rep fusion proteins were diluted to 10 ng/µl in buffer A

containing 1x LAP, 1 mM PMSF, 1 mM DTT and then dialyzed against three changes of buffer A at 15°C.

To do the baiting assay (see Figure 8) 100 µl of the GST-Rep proteins or GST itself were pre-bound to 50 µl of a 50% suspension of glutathionine-agarose beads (Sigma). The volume was made up to 200 µl with buffer A containing 1xLAP, 1 mM DTT, 1 mM PMSF and incubated at room temperature with gentle rocking for one hour. The beads were washed with PBS, NP-40 and resuspended in 100µl of PBS, NP-40 containing 150 µg/ml of BSA. The above was mixed with 500 ng of purified pET or pET-Rep fusion proteins in a total volume of 500 µl of buffer A and incubated for one hour at room temperature with gentle mixing. Following centrifugation, the bead-bound fraction was washed with PBS, NP-40 and resuspended in 50 µl of 2x protein gel loading buffer. One-fifth of the sample was analyzed by eletrophoretic separation on a 12% SDS-PAGE and Western blotting. S-tagged thioredoxin fusion proteins expressed from the pET vectors were detected by probing with S protein-conjugated-horseradish peroxidase (see Figure 8) and the signal was detected by chemiluminescence using a Lumi-glo detection system (KPL).

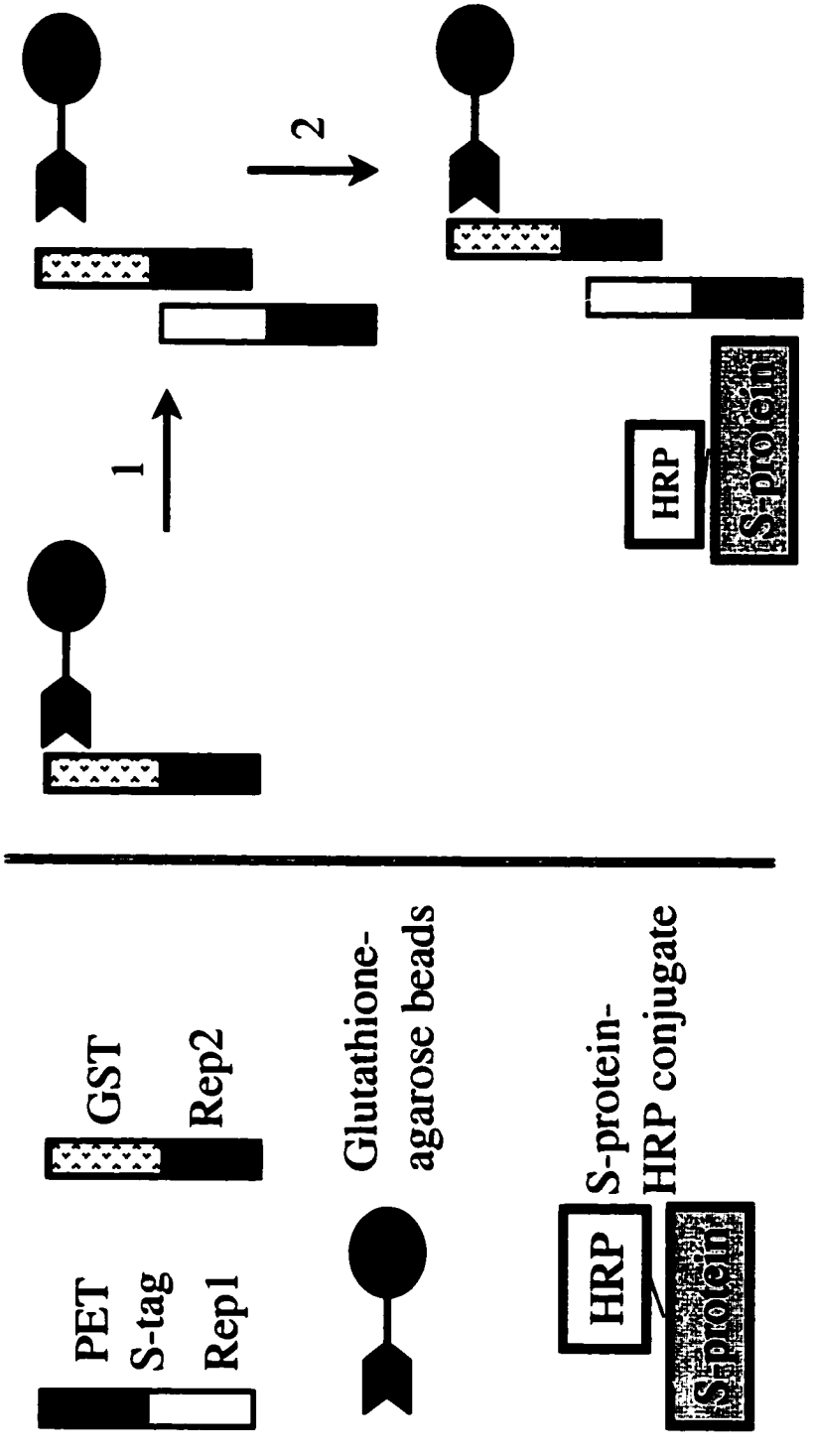
PET
S-tag
Rep1
GST
Rep2
Glutathione-
agarose beads
HRP
S-protein
S-protein-
HRP conjugate
1
2
HRP
S-protein

Figure 8.

Figure 8. *In vitro* baiting assay. The PET-Rep and GST-Rep fusions are shown on the left. The GST-Rep fusion proteins were bound to glutathione agarose beads and mixed with PET-Rep fusion protein. The sample was analyzed by electrophoretic separation on a 12% SDS-polyacrylamide gel and Western blotting. The PET fusion proteins that bound to the beads were detected by SDS-PAGE and Western blotting. Blots were probed with S protein tagged with horseradish peroxidase. S protein was detected by chemiluminescence.

D. Plasmid segregation assay

The *ADE2* plasmid pAS4Δ rep1 was co-transformed with *LEU2* plasmid pGAD-REP1, or other plasmids expressing truncated versions of Rep1 as fusions with the Gal4 activation domain, into the [cir^0] strain AS3. To select for the presence of both plasmids, co-transformants were initially grown in SD medium lacking both adenine and leucine. The cells were then grown in SD medium lacking leucine but containing adenine, where there was no selection for the *ADE2* plasmid. Both before and after the non-selective growth, cells were plated on SD medium lacking adenine and leucine as well as on SD medium lacking only leucine. The percentage of cells containing both plasmids relative to those containing only the *LEU2* plasmid was determined at both steps. The rate of plasmid loss per generation was calculated as in Section II 2C.

III. Results

1. Development of a colour assay for plasmid stability

One of the principle aims of my research was to study 2μm plasmid segregation. Since the 2μm plasmid confers no phenotype on its host yeast cell, I needed to develop an assay system that would allow me to detect the presence of a 2μm-based plasmid in the yeast and which would then allow me to monitor 2μm plasmid stability. The following were the objectives. 1. Creation of a 2μm stability vector (pAS4) which has all the 2μm sequences known to be required for efficient plasmid partitioning, the *REP1* and *REP2* genes and the *cis*-acting *STB* locus. The stability vector also has sequences that allow the plasmid to be detected. 2. To test whether pAS4 is as stable as 2μm circle itself and therefore can be used to study 2μm circle segregation. 3. To test whether absence of the *FLP* gene affects the mitotic stability of the plasmid and therefore determine whether it plays a role in 2μm circle stability. 4. To then use the assay system to study the segregation of the 2μm plasmid.

For the 2μm plasmid assay, I incorporated a yeast purine biosynthetic gene into a 2μm plasmid that would allow the presence of the plasmid in the yeast to be detected visually by a change in colony colour. The *ADE2* 2μm plasmid pAS4 was designed to mimic a 2μm plasmid in the B form but with two important differences. First, the *FLP* gene was disrupted by the insertion of the yeast *ADE2* gene. The *FLP* gene, which is a key component of the plasmid amplification system, was not expected to be required for plasmid segregation, although this has never been formally tested (Futcher, 1986; Volkert and Broach, 1986). Thus disruption of the *FLP* gene would allow analysis of partitioning of the plasmid in the absence of any contributions to stability that arise due to the copy number control system. Secondly, the plasmid pAS4 contains the *ADE2* gene so in an

ade2 yeast strain, it would complement the adenine auxotrophy and allow the strain to form white colonies. Without the plasmid the strain would otherwise form red colonies on rich medium containing adenine due to the *ade2* defect (see Section II 2B). Thus presence or absence of the plasmid can be monitored by the colour of the colony. The presence of the *ADE2*-tagged 2μm-based plasmid in the *ade2*Δ yeast also allows precise measurement of plasmid stability by a plating assay. Since only cells with plasmid are able to form colonies on medium lacking adenine, whereas on rich medium containing adenine, cells with or without plasmids will grow, the percentage of cells that have lost plasmid can be calculated. This colony colour assay system allows an easy way to test for the importance of the plasmid encoded *REP* and *STB* sequences for plasmid partitioning. If any of the components of the partitioning system, either the Rep1 or the Rep2 proteins or the *STB* region, is absent or not functioning properly due to mutation in the *REP1* or the *REP2* genes or in the *STB* region, the plasmid pAS4 should not segregate efficiently. As a result some cells should receive fewer or no plasmids and these colonies should be completely red or will show red/white sectoring depending on the kinetics of plasmid mis-segregation when plated on YEPD medium. When plated on medium lacking adenine, only those cells that have at least one copy of the plasmid will be able to form colonies. Cells with no plasmid will not grow on this medium.

The yeast host specifically created for this study was the strain AS3 [cir^0] in which the yeast *URA3* gene was used to disrupt the genomic *ADE2* locus. This disruption was designed to avoid problems of gene conversion or reversion of the mutant *ade2* allele back to *ADE2*. For the purpose of my assay this conversion or reversion would make the strain look plasmid-plus even in the absence of the plasmid pAS4. The presence of the *ADE2*-plasmid pAS4 in the strain also provides a template for correcting the *ade2* chromosomal defect by gene conversion. However, the *URA3*-disrupted *ade2*

strain that I created allows these events to be selected against by growing the strain on uracil-minus medium so that any gene convertants, which are now uracil auxotrophs, cannot grow.

A. Testing the colour assay system

To study this assay system, the *ade2* strain AS3 [cir^0] was separately transformed with pAS4 as well as with its derivatives in which the *REP1* gene, the *REP2* gene, or the *STB* proximal region was deleted, namely pAS4Δrep1, pAS4Δrep2 and pAS4ΔX, respectively, and the transformants selected by growth on medium lacking adenine. Transformants were obtained at high efficiency (>10^4 transformants/μg) with each plasmid, which indicates the presence of a functional *ADE2* gene on the plasmids. The ability of even the *REP*-gene-deleted plasmids to efficiently transform was not surprising, since even a non-2μm-based plasmid containing only an *ARS* and a selectable marker can transform yeast with high frequency (Murray and Szostak, 1983). The results are shown in Table 2. When AS3 [cir^0] was transformed with the 2μm stability plasmid pAS4, the colonies were all white. It can be concluded from this that the plasmid pAS4, which has all the components of the 2μm plasmid partitioning system, segregates efficiently in this [cir^0] host strain. When the *REP1* or *REP2* gene was deleted from the plasmid, such as in plasmids pAS4Δrep1 or pAS4Δrep2, adenine prototrophic colonies were obtained at high frequency. The colonies that grew on the SD medium lacking adenine were all pink in colour (not shown) rather than the white colonies observed for pAS4 transformants. This shows that the cells have received plasmid otherwise they would not be able to grow on SD medium lacking adenine. The pink colour may arise due to the mixture of cells within each colony, some containing and others lacking the plasmid. The overall pink colour of the colonies would be consistent with the mis-segregation of the *ADE2* plasmid

such that at any given cell division within the colony, only a proportion of the daughter cells receive plasmid. As has been reported in previous studies, the present study also shows that absence of either *REP1* or *REP2* conferred an identical phenotype, in this case pink colony colour, suggesting that loss of either *REP* gene affects plasmid stability to the same degree. In contrast, yeast transformed with pAS4ΔX lacking *STB*-proximal formed colonies almost as white as those of the pAS4 transformants, suggesting that the absence of *STB*-proximal is not as deleterious as loss of the *REP* genes. The results obtained from pAS4ΔX thus differ from those reported previously, where absence of *STB*-proximal was shown to affect stability of 2μm circle to the same extent as deletions of either the *REP1* or *REP2* genes (Kikuchi, 1983; Jayaram, 1985).

B. Mitotic stability of 2μm-based plasmids

Unlike *ARS* plasmids, the 2μm plasmid is equally distributed between the mother and the daughter cells during cell division (Murray and Szostak, 1983). If the 2μm stability vector pAS4 contains all the components required for efficient partitioning it should be as stable as the native 2μm plasmid. To determine how efficiently the plasmid pAS4 segregates in a [cir^0] host, a plasmid stability assay was performed using the AS3 [cir^0] transformants described in the previous section and the results are shown in Table 2. Less than 0.01% of cells lose the pAS4 plasmid at each cell division, a value very close to that reported for native 2μm plasmid (Futcher and Cox, 1983). The stability of pAS4 is significantly greater than most other reported 2μm-based plasmids (Kikuchi, 1983; Jayaram et al. 1983). The plasmid pAS4B (pAS4 lacking *E. coli* vector sequences) was lost at about 2.1±1.5% and is therefore slightly less stable than pAS4. Thus the *E. coli* vector sequences do not affect its mitotic stability and, indeed, their absence from pAS4B seems to have a minor destabilizing effect. Therefore these two plasmids are

TABLE 2. Mitotic stability of pAS4 and its derivatives in the yeast strain AS3 [cir^0]

Plasmid	Colony Colour	[a]Initial Plasmid$^+$ Cells (%)	[a]Final Plasmid$^+$ Cells (%)	No. of gen. in YEPD	Plasmid Loss Per Generation (%)
pAS4	White	95.7 (±3.7)	95.1 (±6.5)	11 (±2.8)	0.003 (±0.002)
pAS4B	White	96.8 (±2.8)	80.2 (±15.3)	12.2 (±0.7)	2.1 (±1.5)
pAS4 Δ rep1	Pink	19.0 (±2.6)	3.3 (±1.3)	11.8 (±0.1)	15.3 (±3.1)
pAS4 Δ rep2	Pink	11.1 (±3.3)	1.6 (±0.7)	12 (±0.4)	16.5 (±2.8)
pAS4ΔX	[b]White/pale pink	61.1 (±9.4)	30.8 (±6.1)	14.9 (±1.3)	4.7 (±1.3)

[a]The initial and final percentages of cells containing plasmid (plasmid$^+$) were obtained from the ratio of the number of colonies growing on medium lacking adenine to those growing on medium containing adenine (see Section II 2C for details). Data represent an average obtained from the results of four independent transformants for each plasmid. Standard deviations are shown in parentheses.
[b]Colonies had to be plated on YEPD medium for the pale pink colour to be observed.

good model systems for studying endogenous 2μm circle function. However, when either *REP1* or *REP2* was deleted from the pAS4 plasmid, the rate of plasmid loss per generation rose to 15.3% and 16.5% for pAS4Δrep1 and pAS4Δrep2, respectively. Thus the presence of wild-type *REP1* and *REP2* genes in the plasmid is required for the plasmid to segregate in a [cir^0] host. The results show that loss of *REP1* or *REP2* makes the plasmid pAS4 behave like *ARS*-only plasmids, which have loss rates of approximately 18% per generation (Kingsman et al. 1979). Since the pAS4 stability vector was able to segregate as efficiently as native 2μm, it was of interest to determine whether similar levels of Rep proteins were expressed from the two plasmids.

C. Steady state levels of Rep1 and Rep2 proteins

In an effort to confirm that the pAS4 2μm test plasmid was indeed a good model for studying 2μm plasmid segregation, I examined expression of the Rep1 and Rep2 proteins from the plasmid pAS4 and compared these to that of native 2μm plasmid. Western blot analysis was performed to determine the steady-state levels of the Rep1 and Rep2 proteins in AS3 [cir^0] yeast transformed with pAS4 and its derivatives as well as for AS3 [cir^+] yeast containing native 2μm. Whole-cell protein extracts from the transformants were isolated and analyzed by SDS-PAGE and Western blotting using affinity-purified polyclonal antibodies specific for Rep1 and Rep2. The results are shown in Figure 9. The anti-Rep1 antibody detects a protein of apparent molecular weight of 66 kDa in all extracts examined. Since one extract is from [cir^0] yeast (lane 1), the protein cannot be encoded by the 2μm plasmid and must therefore be a non-specific interaction between the anti-Rep1 serum and this unknown host protein. Comparing [cir^+] and [cir^0] lanes, it can be seen that there are two bands detected in [cir^+] extract in lane 2 which are

not present in the [cir^0] extract in lane 1. The higher band has a mobility consistent with a 50-kDa protein, which is only slightly larger than the predicted molecular weight of 43 kDa for native Rep1 protein (Hartley and Donelson, 1980). The absence of this band in [cir^0] extracts, its mobility, and its recognition by anti-serum raised and affinity-purified against recombinant Rep1 protein support the identification of the 50-kDa species as native Rep1. The lower-molecular-weight species of 36 kDa recognized by the Rep1 anti-serum in [cir$^+$] but not in [cir^0] extracts may represent a degradation product of the full-length Rep1 protein, or a product of premature termination of translation of the Rep1 mRNA. Comparing lanes 2 and 3 in Figure 9A, it can be seen the Rep1 protein levels expressed by native 2μm plasmid in the strain AS3 [cir$^+$] are similar to those of the 2μm test plasmid pAS4 in strain AS3 [cir^0].

The anti-Rep2 antibody detects a protein of apparent molecular weight of 55 kDa in all extracts examined. Since one extract is from a [cir^0] yeast (Figure 9B, lane 1), the protein cannot be encoded by 2μm plasmid and must therefore be a non-specific interaction between the anti-Rep2 serum and this unknown host protein. Comparing [cir$^+$] and [cir^0] yeast, it can be seen that there is a major band migrating with an apparent molecular weight of 35 kDa in [cir$^+$] extract in lane 2 which is not present in the [cir^0] extract in lane 1. The size of the band is consistent with the 33 kDa predicted molecular weight of native Rep2 (Hartley and Donelson, 1980). The absence of this band in [cir^0] extracts, its mobility, and its recognition by anti-serum raised and affinity-purified against recombinant Rep2 protein support the identification of the 35-kDa species as native Rep2. In addition, there are two faint bands detected by the anti-Rep2 antibody

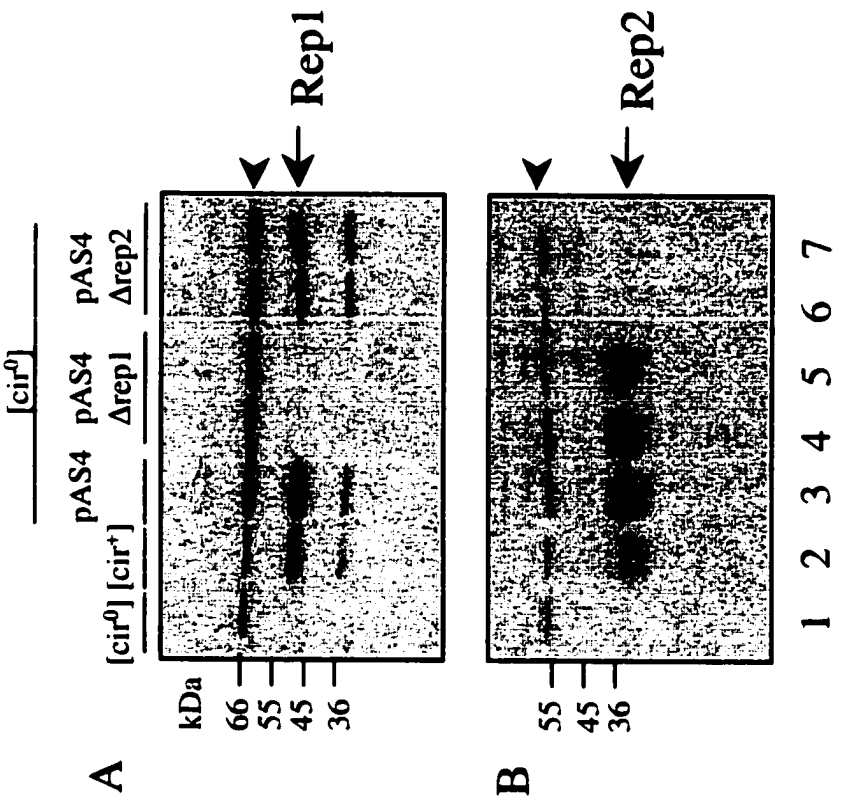
[cir0]
pAS4
pAS4 Δrep2
[cir0] [cir+]
pAS4 Δrep1
A
kDa
66
55
45
36
Rep1
B
55
45
36
Rep2
1 2 3 4 5 6 7

Figure 9.

Figure 9. Western blot analysis of steady-state Rep1 and Rep2 proteins levels. Duplicate Western blots of total protein extracted from the isogenic strains AS3 [cir^0] (lane 1), AS3 [cir^+] (lane 2) and AS3 [cir^0][pAS4] (lane 3) were incubated with anti-Rep1 (A) and anti-Rep2 (B) antibodies. Data from two independent transformants of AS3 [cir^0][pAS4Δrep1] (lanes 4 and 5) and AS3 [cir^0][pAS4Δrep2] (lanes 6 and 7) are shown. The Rep1p and the Rep2p bands are indicated by arrows. The non-specific bands detected by the antibodies are shown by black arrowheads and reflect the relative loading of protein in each lane.

only in [cir$^+$] extracts, one immediately below and the other immediately above the major band of 35 kDa. The apparent molecular weights of these minor species are 33 kDa and 38 kDa respectively. The lower band may be a degradation product or a premature termination of translation product of Rep2. The upper faint band (38 kDa) could be Rep2 with the major band being a breakdown product, but this seems less likely since even the major species has a higher apparent molecular weight than is predicted. Also, an *in vitro* translation product of the *REP2* ORF runs with the mobility of the major band (Dobson, unpublished results). The upper band could be a modified form of Rep2 protein. The level of the Rep2 protein in the AS3 [cir$^+$] strain is comparable to that observed in the AS3 [cir^0] strain transformed with the 2μm-based plasmid pAS4 (data not shown). These results show that as well as segregating efficiently as a 2μm plasmid, the pAS4 stability vector expresses steady-state levels of Rep1 and Rep2 proteins that are comparable to those observed in [cir$^+$] cells containing native 2μm circle.

2. Effect of *REP* gene deletions on Rep protein expression

Having established that the pAS4 stability vector mimics the 2μm circle with respect to both its efficient segregation and the level of expression of the two Rep proteins, I wanted to determine whether Rep1 and Rep2 protein levels were dependent on one another. Steady-state levels of Rep1 and Rep2 proteins in AS3 [cir^0] transformed with plasmids pAS4Δrep1 and pAS4Δrep2 were determined by Western blotting. The results are shown in Figure 9, lanes 4 to 7. It is clear from the Western blot that the level of the Rep2 protein is unaffected by the absence of the Rep1 protein (lanes 4 and 5 in figure 9B). In contrast, the Rep1 protein level goes down in the pAS4Δrep2-transformed yeast, as shown in lanes 6 and 7 in figure 9A. Thus, absence of the Rep2 protein affects Rep1 steady-state protein levels but not *vice versa*. This result was unexpected since

previous studies of transcript levels suggested that loss of Rep2 might induce *REP1* gene expression (Reynolds et al. 1987; Som et al. 1988).

3. Copy numbers of 2μm-based plasmids

The 2μm-based pAS4 behaves like 2μm with respect to its segregation and expression of the Rep proteins. This stability is observed despite the disruption of the *FLP* gene in pAS4. Since pAS4 is stable but cannot amplify, it was of interest to determine whether the copy number of the test vector was similar to that of native 2μm. It has been suggested that the Flp system only comes into play when copy number drops to a very low level but may not play a significant role during normal propagation of the yeast. I therefore set out certain objectives. I wanted to determine whether the 2μm-based pAS4 behaves like 2μm circle with respect to the number of copies maintained in yeast and also assess the effect of loss of either *REP1* or *REP2* on the copy number of pAS4. To compare the copy numbers of pAS4 (the 2μm stability vector) and pAS4B (lacks the *E. coli* vector sequences but otherwise is identical to pAS4) with native 2μm plasmid, DNA was prepared from strains AS3 [cir^0][pAS4] and AS3 [cir^0][pAS4B] which were grown in medium lacking adenine to select for the presence of plasmid. DNA was also prepared from strain AS3 [cir^+], which was grown in YEPD medium. These growth conditions were identical to those used for the cultures for Rep protein extracts analyzed in Figure 9. Each of these DNAs was digested with *Eco*RI and *Bam*HI and analyzed by gel electrophoresis and Southern blotting (Figure 10). Two identical blots were prepared by running half of each restricted DNA sample on each of two

separate gels that were run under identical conditions. The copy numbers were determined by phosphorimaging and using Molecular Analyst software from Bio-Rad (see Section II 2E).

The average copy numbers per cell for native 2μm, pAS4, pAS4B, pAS4Δrep1 and pAS4Δrep2 are given in Table 3. As the data shows, native 2μm had a higher average copy number (130 per cell) than pAS4 (20 per cell). Despite this lower copy number, the steady-state levels of Rep1 and Rep2 expressed from pAS4 in a [cir^0] host are high, and comparable to those produced by native 2μm circle (see Figure 9 in Section III1C). The copy numbers of plasmids pAS4B, pAS4Δrep1 and pAS4Δrep2 are comparable to that of pAS4. However, once the percentage of cells that contain plasmid is taken into consideration, the data suggest that at least some of the cells containing pAS4Δrep1 and pAS4Δrep2 have copy numbers similar to those of native 2μm, while many others contain no plasmid. In this regard pAS4 does differ from 2μm circle and this probably reflects the contribution that *FLP* makes to overall copy number for native 2μm circle.

4. Effect of 2μm encoded Rep proteins on *FLP* promoter

It had previously been suggested that the *FLP* promoter activity was regulated by the level of Rep proteins in the yeast cell, thereby forming the basis of a feedback loop that could negatively regulate 2μm circle copy number (Reynolds et al. 1987). I decided to test this hypothesis by examining the level of expression directed by the *FLP* promoter either in the presence or absence of the 2μm circle or the 2μm stability vector pAS4. A single-copy *CEN ARS* plasmid containing the *E. coli lacZ* gene under the control of the

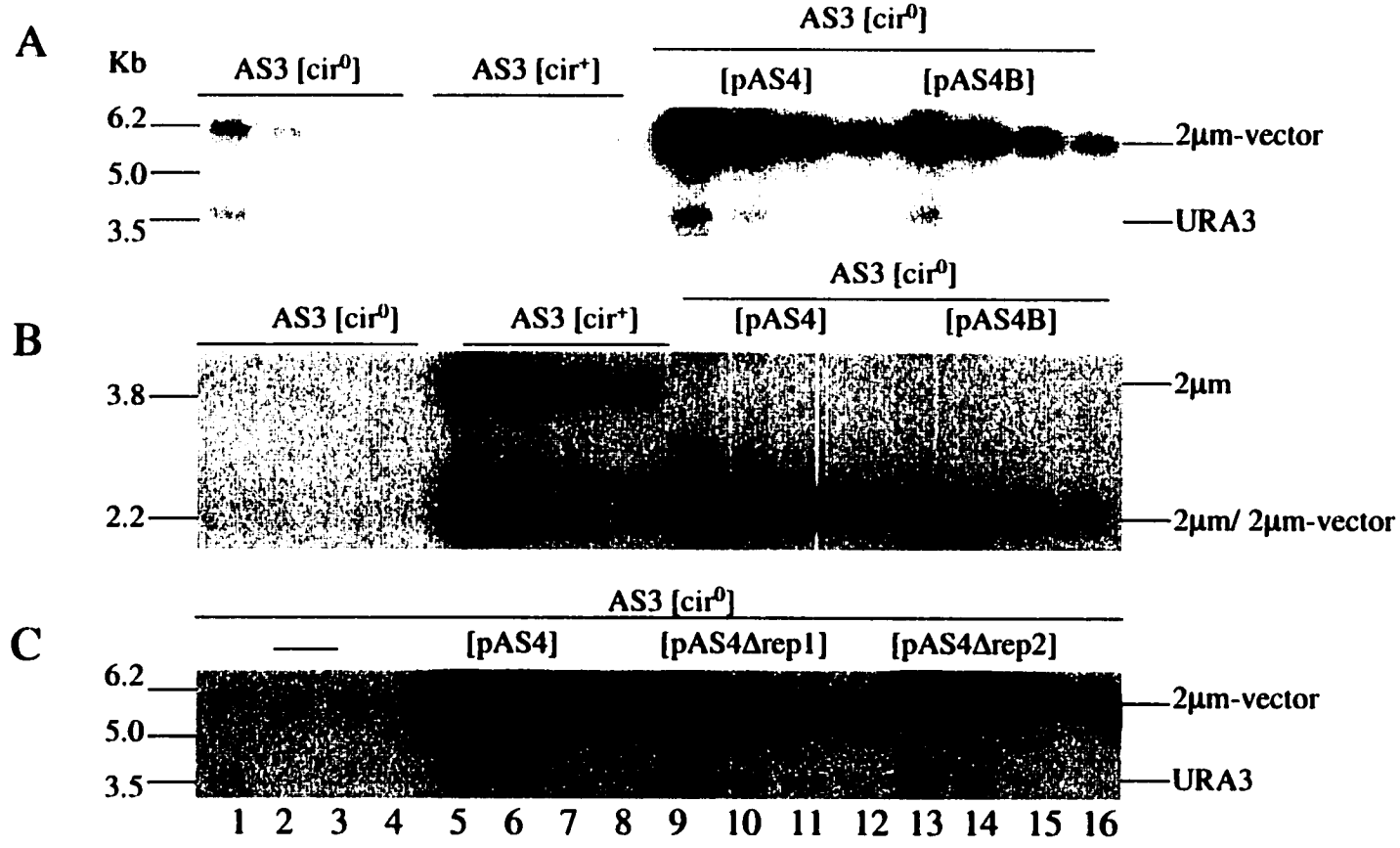
A
Kb
AS3 [cir0]
AS3 [cir+]
AS3 [cir0]
[pAS4]
[pAS4B]
6.2
5.0
3.5
2µm-vector
URA3
B
AS3 [cir0]
AS3 [cir+]
AS3 [cir0]
[pAS4]
[pAS4B]
3.8
2.2
2µm
2µm/ 2µm-vector
C
AS3 [cir0]
[pAS4]
[pAS4Δrep1]
[pAS4Δrep2]
6.2
5.0
3.5
2µm-vector
URA3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 10.

Figure 10. Southern blots for determination of copy number of 2μm circle in AS3 [cir$^+$] or other 2μm-based plasmids in AS3 [cir^0]. Total yeast DNA preparations were digested with restriction enzymes *Eco*RI and *Bam*HI, resolved in 1% agarose gels, and transferred to nylon membranes. Four two-fold serial dilutions of the restriction digests were loaded in adjacent lanes. **Panels A and B:** lanes 1 to 4: strain AS3 [cir^0]; lanes 5 to 8: AS3 [cir$^+$]; lanes 9 to 12: strain AS3 [cir^0][pAS4]; lanes 13 to 16: strain AS3 [cir^0][pAS4B]. The Southern blot shown in panel A was probed with a 2.4-kbp *Eco*RI/*Bam*HI fragment, purified from the plasmid pAS3, containing the yeast *URA3* gene flanked by the 5' and 3' non-coding regions of the yeast *ADE2* gene. This probe recognizes a 3.5-kbp fragment at the genomic *URA3* locus in the strain and also a 5.8-kbp band from plasmids pAS4 and pAS4B. It also recognizes a 6.2-kbp *Eco*RI/*Bam*HI fragment indicating the *ADE2* locus disrupted with the *URA3* gene. The Southern blot shown in panel B was probed with a 1.4-kbp *Xba*I/*Stu*I fragment, purified from plasmid pAS4, containing 2μm origin of replication, part of one of the *IRS* and *STB* region. This probe detects a 2.2-kbp *Eco*RI fragment that is common to all pAS4-based plasmids used in this study and also present in the B form of native 2μm circle. It also recognizes a 3.8-kbp fragment and a 2.5-kbp fragment from the A form of the 2μm circle. **Panel C:** lanes 1 to 4: strain AS3 [cir^0]; lanes 5 to 8: AS3 [cir^0][pAS4]; lanes 9 to 12: AS3 [cir^0][pAS4Δrep1]; lanes 13 to 16: AS3 [cir^0][pAS4Δrep2]. This blot was probed with the same probe as the blot shown in panel A. This probe recognizes a 5.8-kbp *Eco*RI/*Bam*HI fragment from plasmid pAS4Δrep1 and 7.1-kbp *Eco*RI/*Bam*HI fragment from plasmid pAS4Δrep2, in addition to the bands mentioned above.

TABLE 3. Copy number of 2μm circle in AS3 [cir^+] or other 2μm-based plasmids in the yeast strain AS3 [cir^0].

Plasmid	Average Copy number/cell	[a]Percentage of plasmid containing cells (%)	Copy number/plasmid-containing cell
Native 2μm	130	N/A	-
pAS4	20	96	20
pAS4B	10	97	10
pAS4Δrep1	10	19	70
pAS4Δrep2	20	11	200

See Section II 2E for details.
[a]Values taken from Table 2

FLP promoter, pFLP-lacZ, was transformed separately into each of the strains AS3[cir^0], AS3[cir$^+$] and AS3[cir^0][pAS4]. The expression of the *lacZ* gene was measured by a β-galactosidase filter assay (Rose et al. 1990). Transformants from all three strains showed equal levels of expression of β-galactosidase (blue colour) (data not shown), suggesting that the *FLP* promoter was not repressed in the presence of the 2μm Rep proteins expressed either from native 2μm or the 2μm stability vector. This was a surprising finding given that current models for 2μm circle copy number control postulate that the *FLP* promoter is regulated by the combined action of Rep1 and Rep2 proteins (Reynolds et al. 1987). Since no repression was observed, I was not able to use this as an assay system to measure Rep protein function.

5. *In vitro* mutagenesis of *REP1* and *REP2*

One approach to determine the function of a protein is to look for mutations that inactivate the protein. I therefore undertook mutational analysis of the *REP1* and *REP2* genes in an attempt to identify residues that were important for the segregation functions of the encoded proteins. Having determined that colony colour of AS3 [cir^0] yeast transformed with the 2μm stability vector pAS4 or its *REP1* and *REP2*-deleted versions does indeed reflect the mitotic stability of the transforming plasmids, I used this system to identify mutations in *REP1* and *REP2* that abolished function. Mutated *REP1* or *REP2* gene fragments were generated by PCR and were used to replace the *REP1* or the *REP2* gene in the test vector pAS4 by gap repair in the *ade2* [cir^0] yeast strain AS3 (see sections II 4A and II 4B for details). If a single mutation or a combination of mutations introduced into either of these genes by PCR results in loss of function, the gap-repaired pAS4 should mis-segregate, as was demonstrated for pAS4Δrep1 and pAS4Δrep2. This

will result in the formation of pink or red coloured colonies instead of white colonies. Sequencing of the mutant *REP* genes from the mis-segregating plasmid would then identify the mutation that was leading to loss of mitotic stability.

A. Gap repair of pAS4 with mutagenic PCR amplicons

Initially conditions for mutagenic PCR were used to generate the *REP1* and the *REP2* gene fragments for *in vivo* repair of the gapped pAS4. The results are given in Table 4. Scoring of the red and white colonies gives a minimum estimate of the percentage of mutants obtained. If any unrestricted pAS4 contaminated the gel-purified DNA fragment excised from pAS4, it would also give white colonies when the DNA was transformed into the *ade2* yeast host. A few white colonies were observed when gapped vector only was used for transformation and probably arise from contamination with intact pAS4 plasmid. The percentage of *REP1* and *REP2* mutants increased when Mg^{+2} concentration was kept constant but increasing concentrations of Mn^{+2} were used. Sequencing of these mutant *REP1* and *REP2* genes revealed that there was at least one mutation per 150 bases for most of the mutants sequenced. As mentioned, (see Section II 4B) temperature-sensitive mutants were obtained for both *REP1* and *REP2* using the mutagenic PCR approach. Out of the five temperature-sensitive mutants that I completely sequenced, only one *rep2* mutant had a single point mutation, while the others contained multiple changes, as was found for the non-conditional *REP1* and *REP2* mutants that were sequenced. The temperature sensitive (t.s.) mutant *rep2H10*, which has a single mutation, is currently being analyzed in the Dobson laboratory, while no further experiments were carried out with the other multiply-mutated-*REP* mutants.

TABLE 4. Gap repair of pAS4 with *REP1* or *REP2* amplicons generated using different mutagenic PCR conditions

	Mg^{+2} (mM)	Mn^{+2} (mM)	% of transformants giving red colonies at 23°C and 37°C	% of transformants giving red/white sectored colonies at 23°C and 37°C	% of transformants giving red/white sectored colonies at 37°C but white at 23°C	% of transformants giving white colonies at 23°C and 37°C	Total number of traansformants
	3.5	0.25	24.6	2.3	2.9	70.2	524
REP1	3.5	0.3	51.2	4.2	11	33.6	238
	3.5	0.4	52.5	3.5	8.6	35.4	429
	3.5	0.5	72.3	2.5	5.0	20.2	119
	3.5	0.25	43	27	0	30	100
REP2	3.5	0.3	40	22.7	8.7	28.6	150
	3.5	0.4	58	2.7	27.3	12	150
	3.5	0.5	12	70	14	4	50

The results of four independent transformations for both *REP1* and *REP2* with the appropriately gapped pAS4 (see Sections II 4A and B) are shown.

B. Results from "standard" PCR

Since the aim was to obtain a single mutation per gene, the mutation rate from initial conditions for mutagenic PCR was too high. Mutated *REP1* and *REP2* genes were therefore generated from standard PCR reaction conditions. I found that standard PCR conditions provided an adequate error rate to prepare a bank of random *REP1* and *REP2* mutants.

The results obtained from gap repair of pAS4 with *REP1* and *REP2* amplicons generated using standard PCR conditions instead of mutagenic conditions are shown in Table 5. As expected, a higher percentage of white colonies were obtained when amplicons generated by standard PCR, as compared to mutagenic PCR, were used for gap repair. Out of the total number of 713 colonies scored for the *REP1* gene only 20 were pink, and for *REP2*, 38 out of the 434 colonies scored were pink. A Southern blot was carried out to demonstrate proper integration of the PCR fragments into the gapped vector (data not shown). The *REP1* and *REP2* genes were PCR-amplified from yeast that had formed pink colonies, cloned and sequenced. Most of these recovered *REP* genes were found to have more than one mutation; typically two to three mutations per gene were observed (see Tables 6 and 7 respectively). Most of the mutants, such as *rep1 A5-1*, *rep1 A9-1*, *rep1 B9-1*, *rep1 B10-1*, *rep2 B1-1* and *rep2 A1-2*, had premature stop codons that were likely to be the causative mutation. The mutant *rep1 C10-1* has a thymidine nucleotide missing at position 469, which generates a stop codon which would result in expression of a truncated Rep1 protein of only 189 amino acids. The mutants *rep2 A2-1* and *rep2 A5-2* have mutations in the upstream promoter region that might affect expression of the downstream *REP2* gene.

TABLE 5. Results of gap repair of pAS4 in yeast with *REP1* or *REP2* DNA fragments generated using standard PCR conditions

Transforming DNA	% (number) of Ade$^+$ transformants with red colonies	% (number) of Ade$^+$ transformants with white colonies	Total number of transformants
8.5-kbp *Bam*HI/*Pvu*II pAS4	88.2 (45)	11.8 (6)	51
8.2-kbp *Bam*HI/*Sph*I pAS4	82.2 (74)	17.8 (16)	90
8.5-kbp *Bam*HI/*Pvu*II pAS4 + *REP1* PCR amplicon	2.8 (20)	97.2 (693)	713
8.2-kbp *Bam*HI/*Sph*I pAS4 + *REP2* PCR amplicon	8.0 (38)	92 (434)	472

See Section II 4B for details.

TABLE 6. PCR-generated *rep1* mutants

Mutant	Sequence alteration(s)	Amino acid Change(s)	Expected size of protein	Mobility observed	Interaction with Rep2p
rep1 A9-1	c307t	Q103Stop	12 kDa	Nd	Not done
rep1 A11-1	a133g	K45E	43.2 kDa	>WT	Yes
rep1C10-1	t469del	L189Stop	20 kDa	Nd	Not done
rep1 D3-1	t605c	I202T	43.2 kDa	WT	Yes
rep1 D4-1	t583c	C195R	43.2 kDa	WT	Yes
rep1A5-1	g706a g786a	E236K W262Stop	30 kDa	Nd	Not done
rep1 B10-1	t158c c805t	F53S Q269Stop	31 kDa	31kDa	No
rep1 C11-1	t89c a140g	I30T K47R	43.2 kDa	WT	No
rep1 B6-1	t293c t520c a889g	V98A F174S K297E	43.2 kDa	WT	Yes
rep1 B9-1	a125g t424c g539a	N42S F142L W180Stop	20 kDa	Nd	Not done

Table 6. PCR-generated *rep1* mutants. The numbering used for showing sequence alterations in the *REP1* gene (column 2) starts at 1 for the first nucleotide in the translation initiation codon. The mobility observed for the mutant Rep1 proteins are shown in column 5, where WT refers to the same mobility as unmutated Rep1, Nd represents not detected and >WT indicates a mobility higher than that of unmutated Rep1. The interactions between mutant Rep1 proteins and wild-type Rep2 (column 6) were detected by two-hybrid assay. Activation of the *lacZ* reporter gene in the two-hybrid host was measured by a β-galactosidase filter assay (Breeden and Nasmyth, 1985). Detection of blue colonies within nine hours or less indicated positive interaction.

TABLE 7. PCR-generated *rep2* mutants

Mutant	Sequence Alteration (s)	Amino acid change(s)	Expected size of protein	Mobility observed	Interaction with Rep1	Interaction with Rep2
rep2B1-1	a22t	K8Stop	<1kDa	Nd	Not done	Not done
rep2B8-1	a518g	E173G	33.2kDa	WT	Not done	Not done
rep2C1-1	c712t	H238Y	33.2kDa	WT	Not done	Not done
rep2E12-1	c653t	A218V	33.2kDa	WT	Yes	Yes
rep2A8-2	a529 g	K177E	33.2 kDa	>WT low level	No	No
rep2A2-1	a11g a661t	A-7G N221Y	33.2 kDa	Nd	Yes	No
rep2B9-1	t322c g520a	S108P D174N	33.2kDa	<WT	Yes	Yes
rep2A4-2	t29a a283g	L10Q K95E	33.2kDa	Nd	No	No
rep2A5-2	a13g t527c	A13G M176T	33.2kDa	Nd	No	No
rep2A1-2	a26g g340a g694t	N9D A114T G232Stop	25kDa	25 kDa	Yes	Yes

Table 7. PCR-generated *rep2* mutants. The numbering used for showing sequence alterations in the *REP2* gene (column 2) starts at 1 with the first nucleotide in the *REP2* translation initiation codon. The mobility observed for the mutant Rep2 proteins are shown in column 5, where WT refers to the same mobility as unmutated Rep2, Nd represents not detected, >WT indicates a mobility higher than, and <WT a mobility less than that of unmutated Rep2. The interactions between mutant Rep2 proteins and wild-type Rep1 (column 6) or wild-type Rep2 (column 7) were detected by two-hybrid assay. Activation of the *lacZ* reporter gene in the two-hybrid host was measured by a β-galactosidase filter assay (Breeden and Nasmyth, 1985). Detection of blue colonies within nine hours or less indicated positive interaction.

C. Generation of single point mutations in *REP1* and *REP2* by site-directed mutagenesis

Since one of the primers used to generate the *REP1* PCR fragment annealed to the 3' region of the *REP2* gene, there was a possibility that gap repair with this amplicon might have introduced mutations in the 235 bases of the 3' end of the *REP2* gene. No mutations was found in the 3' end of the *REP2* gene in these *rep1* mutants. Similarly, the 3' primer used to generate the *REP2* PCR fragment annealed to the 3' end of the *REP1* gene. However, in this case some presumptive *REP2* mutants were associated with mutations in the 3' region of the *REP1* gene. For these cases it was necessary to find out whether it was the mutation in *REP2*, in *REP1*, or in both that was responsible for the segregation defect. Similarly, for multiple mutations in either *REP1* or *REP2*, it was necessary to determine which of the mutations was responsible for the loss of function. Since most *rep1* and *rep2* mutants identified by gap repair had more than one nucleotide change, I made an attempt to generate these as single mutations which could then be independently assayed in the stability vector for effects on the segregation of the 2μm-based plasmid. I used the procedure of site-directed mutagenesis by unique-site elimination (Pharmacia). The mutagenesis reaction was carried out separately for each mutation using a combination of the selection primer and the particular target mutagenic primer (primers are listed in Table 1) to introduce that mutation (see Section II 4D). After mutations were confirmed by sequencing, each one was introduced into the 2μm-based *ADE2* test vector pAS4. The yeast strain AS3 [cir^0] was transformed separately with each of these plasmids that now carry a single mutation in either the *REP1* or the *REP2* gene. Visual inspection of the colour of the colonies was used to determine whether the mutation was causing defective segregation of the plasmid. The results are shown in Table 8.

TABLE 8. Colony-colour stability assay for 2μm-based pAS4 carrying a single mutation in either *REP1* or *REP2* generated by site-directed mutagenesis

Parent *rep1* mutant	*rep1/rep2* mutants by site-directed mutagenesis	Colony colour
rep1B6-1	rep1V98A	White
V98A		
F174S	rep1F174S	White
K297E		
	rep1K297R	White
rep1B10-1	rep1F53C	White
F53S		
Q269X	rep1F53Y	White
	rep1Q269X	Pink
rep1C11-1	rep1I30T	White
I30T		
K47R	rep1K47R	White
Parent *rep2* mutant		
rep2B9-1	rep2S108P	White
S108P		
D174N	rep2D174N	White
rep2C1-1	rep2H238Y	White
H238Y		
rep1I338T	rep1I338T	Pink
rep2B8-1		
E173G	rep2E173G	White
rep1W262STOP		
rep2A4-2		
L10Q	rep1T339A	White
K95E		
rep1T339A		

Table 8. Colony-colour stability assay for 2μm-based pAS4 carrying a single mutation in either *REP1* or *REP2* gene generated by site-directed mutagenesis:
Column 1 shows the original allele containing the *rep1* or *rep2* mutants. The multiple point mutations each separated by site-directed mutagenesis are shown in column 2. These single mutants (column 2) with nucleotide changes in the *REP1* or *REP2* gene are named by the gene in which the mutation is present, followed by the particular amino acid change for convenience in identification.

[a]In the *REP1* mutant *rep1 B6-1*, two out of the three mutations (V98A and F174S) were generated in the 2μm stability vector and were introduced separately into the strain AS3 [cir^0]. The mutation K297E was not obtained despite using a degenerate primer that could give both K297E and K297R. Each of the *rep2* mutants *rep2B8-1*, *rep2C1-1* and *rep2A4-2* had a mutation in the *REP1* gene and are shown in column 1 along with the *rep2* mutation. Results of the colony colour assay for the single mutants (in column 2) are shown in column 3.

The results show that each of the three mutants *rep1V98A*, *rep1F174S* or *rep1K297R* separately does not cause any defect in segregation of the 2μm plasmid. The K297E mutation was not obtained despite screening numerous candidates. The K297R mutation does change this same residue and did not abolish function, but a change from lysine to arginine is a conservative change, so it cannot be concluded whether the single non-conservative substitution of lysine to glutamate at this site would result in a partitioning defect. Thus we do not know whether it is the single mutation K297E or a combination of the three mutations V98A, F174S and K297E that is responsible for loss of function of *rep1B6-1*. In the original *rep1 B10-1* mutant there were two mutations, F53S and Q269X. These were separated and the mutation Q269X, which creates a premature stop codon, was found to independently give rise to pink colonies and is therefore unable to mediate proper plasmid segregation. A degenerate primer was used to introduce a mutation at position 53 which could change the phenylalanine to a cysteine, tyrosine, or a serine. I sequenced twenty transformants and obtained the two mutations F53C and F53Y. Since the mutation F53S creates an *Sfa*NI restriction site, I did PCR amplification and *Sfa*NI restriction digests on an additional twenty transformants, none of which had the mutation. Thus it is not possible to conclude whether changing phenylalanine to serine at position 53 is by itself able to cause defective segregation. However, it is clear that changing this phenylalanine to either cysteine or to tyrosine does not affect segregation, as pAS4 carrying these single mutations gave white colonies when transformed into the AS3 [cir^0] yeast. The mutant *rep1 C11-1* has two mutations, I30T and K47R. When separately analyzed, neither of the mutations results in plasmid mis-segregation. It seems that it is the combined effect of the two mutations that is affecting function.

Of the *REP2* mutants, each of the two mutations S108P and D174N in *rep2 B9-1*, when separately introduced into the *REP2* gene by site-directed mutagenesis, gave white colonies in the *ade2* strain AS3 [cir^0], indicating that the individual mutations were not affecting function. Apparently the combined effect of the two mutations is required to impair plasmid segregation. As previously mentioned, some *REP2* PCR fragments, when used for gap repair of *REP2*-deleted pAS4, introduced mutations within the 3' region of the *REP1* gene. As a result, three of the *REP2* mutants had a mutation in the *REP1* gene. In addition to the mutation E173G in the *REP2* gene in the mutant *rep2 B8-1*, there was the mutation W262STOP in the *REP1* gene. When separately analyzed, the mutant *rep2E173G* formed white colonies, indicating that the mutation E173G in the *REP2* gene was not responsible for the segregation defect. Instead, it is most likely the premature termination of the Rep1 protein due to the mutation W262STOP that gave the pink phenotype in the original mutant. The mutant *rep2 C1-1* had the mutation H238Y in the *REP2* gene as well as a mutation I338T in the *REP1* gene (see Table 8). When separately introduced into the strain AS3 [cir^0], the mutant *rep2H238Y* formed white colonies and the mutant *rep1I338T* formed pink colonies, proving that it was the mutation in the *REP1* gene that affected segregation in the double mutant. In addition to the two mutations L10Q and K95E in the *REP2* gene in the mutant *rep2 A4-2*, there was the mutation T339A in the *REP1* gene (see Table 8). When separately introduced into the *ade2* strain, the mutation T339A formed white colonies. Thus in this case the mutation T339A in the *REP1* gene is not the one affecting function. It is either one of the mutations in the *REP2* gene, L10Q or K95E, or the combined effect of the two, that is affecting segregation.

D. Analysis of Rep1 and Rep2 proteins in the mutants

The *rep1* and *rep2* mutants in pAS4 were originally isolated based on the pink colony phenotype that arises due to mis-segregation of this *ADE2* 2μm-like plasmid.

This defect in segregation could be due to lack of expression, or a lower abundance, of the Rep1p or Rep2p proteins in the *rep1* or the *rep2* mutants, respectively. To determine the steady-state levels of the proteins, whole-cell protein extracts from the transformants were isolated and resolved on SDS-PAGE. Proteins were analyzed by Western blotting using affinity-purified polyclonal anti-Rep1 and anti-Rep2 antibodies. The results are shown in Figures 11 and 12.

As with the previous Western blot analysis of Rep protein levels (see Section III 1C), the anti-Rep1 antibody detects a non-specific band of approximately 66 kDa in all protein extracts derived from [cir^0] and [cir$^+$] yeast (Figure 11A). As expected, native Rep1 is observed as a band with a mobility of approximately 50 kDa, and is only present in [cir$^+$] yeast but not in [cir^0] yeast. In protein extracts from AS3 [cir^0] yeast transformed with the pAS4 stability vector having *rep1 A5-1*, *rep1 A9-1* or *rep1 B9-1* mutations, all of which create premature stop codons, no Rep1 protein was detected (Figure 11A, lanes 3, 4 and 7). Although *rep1 A11-1* and *rep1 B6-1* have levels of Rep1 protein that are not too dissimilar to those expressed by unmutated pAS4, the protein expressed from *rep1 A11-1* has a slightly higher mobility than native Rep1 protein (Figure 11A, lanes 5 and 6). The Rep2 protein level for all these mutants is comparable to that expressed from the unmutated pAS4 plasmid (Figure 11B, lanes 3 to 7).

The mobility of the anti-Rep1 immuno-reactive species for *rep1 B10-1* was calculated as around 31kDa, which is close to the size expected given that this mutation creates a premature stop codon at amino acid 269 (Figure 11A, lane 10). No anti-Rep1 immuno-reactive species were detected for *rep1 C10-1*, which has a frameshift mutation at nucleotide 469, with a missing thymidine nucleotide at that position thus introducing a

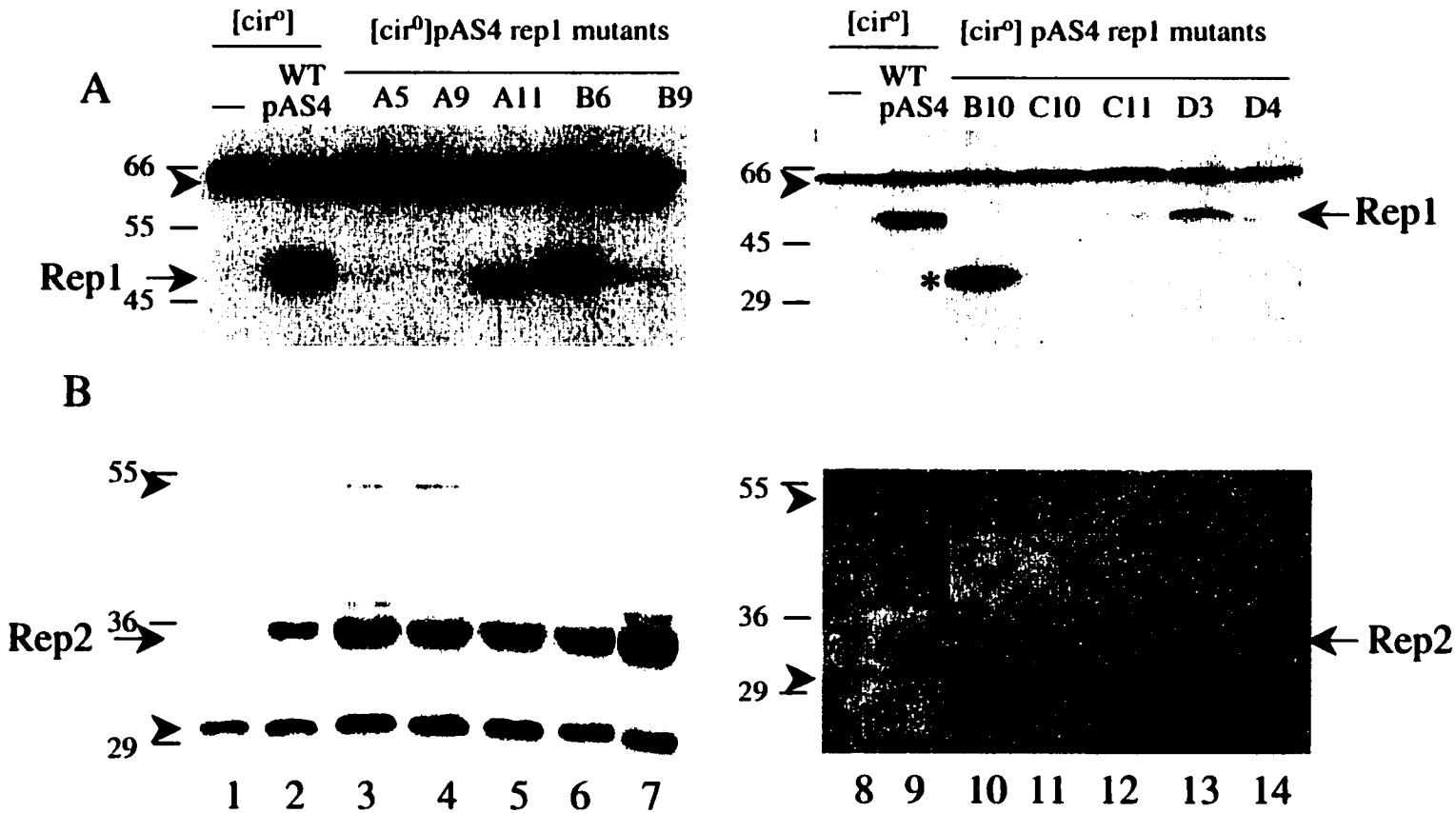
A
[cir°]
[cir°]pAS4 rep1 mutants
WT
pAS4
A5
A9
A11
B6
B9
66
55
Rep1
45
[cir°]
[cir°] pAS4 rep1 mutants
WT
pAS4
B10
C10
C11
D3
D4
66
45
29
Rep1
B
55
Rep2
36
29
1 2 3 4 5 6 7
55
36
29
Rep2
8 9 10 11 12 13 14

Figure11.

Figure 11. Western blot analysis of Rep1 mutants. Total protein extracted from the AS3 [cir^0][pAS4] transformants containing mutations in the *REP1* gene, resolved by a 10% SDS-PAGE and immunoblotted against affinity-purified anti-Rep1 (Panel A) and anti-Rep2 (Panel B) antibodies.

Protein extracts from AS3 [cir^0] (lanes 1 and 8) and AS3 [cir^0][pAS4] (lanes 2 and 9). Lanes 3 to 7: extracts from mutants *rep1 A5-1* (A5), *rep1 A9-1* (A9), *rep1 A11-1* (A11), *rep1 B6-1* (B6) and *rep1 B9-1* (B9), respectively; lanes 10-14: extracts from mutants *rep1 B10-1* (B10), *rep1 C10-1* (C10), *rep1 C11-1* (C11), *rep1 D3-1* (D3) and *rep1 D4-1* (D4), respectively. The positions of the non-specific bands detected by the antibodies are indicated by arrowheads. The bands representing native Rep1 (Panel A) and Rep2 (Panel B) proteins are shown with black arrows. The mutant protein expressed from the mutant *rep1B10-1* which has a higher mobility than wild-type Rep1 is indicated by a star. Protein molecular-weight markers (Sigma) are shown on the left. Sizes are in kDa.

premature stop at codon 189. There was significantly less anti-Rep1 immuno-reactive protein expressed from mutants *rep1 C11-1*, *rep1 D3-1* and *rep1 D4-1* (Figure 11A, lanes 12-14). Rep2 protein was detected for all these mutants (Figure 11B,). The mobility and amount of the Rep2 antigenic band for the *rep1* mutants was similar to the wild-type (Figures 11B, lanes 10 to 14).

Figure 12 shows the Western blot analysis of the *rep2* mutants using anti-Rep2 (Figure 12A) and anti-Rep1 (Figure 12B) antibodies. As with the previous Western blot analysis, the results here show that steady-state Rep1 and Rep2 protein levels in AS3 [cir^+] are similar to that observed in AS3 [cir^0][pAS4]. No Rep2 protein was detected for *rep2 B1-1* (Figure 12A, lane 4), in which the mutation introduces a premature stop codon near the 5' end of the open reading frame. As mentioned in the previous section, *rep2 B8-1* and *rep2 C1-1* have mutations in the 3' region of the *REP1* gene, and it is the *rep1* mutation in each of these mutants that is responsible for loss of segregation. As can be seen from Figure 12A (lanes 5 and 7), the Rep2 protein level is similar to wild-type for both *rep2 B8-1* and *rep2 C1-1*. The Rep1 protein is also present at a level similar to those expressed from pAS4 in both these mutants, although the anti-Rep1 immuno-reactive band has an apparent mobility of 35 kDa in the mutant *rep2 B8-1* (Figure 12B, lanes 5 and 7). This 35-kDa species is expected to be the mutant form of Rep1 based on its recognition by the antibody, and because the mutation introduces a stop codon in the *REP1* gene at codon 262 that would generate a truncated protein. The anti-Rep2 antibody detects a band with an apparent mobility of 45 kDa in the mutant *rep2 B9-1* (Figure 12A, lane 6). The reduced mobility of this mutant form of Rep2 protein may be due to the loss of the negatively charged aspartic acid residue (D174N). The size and

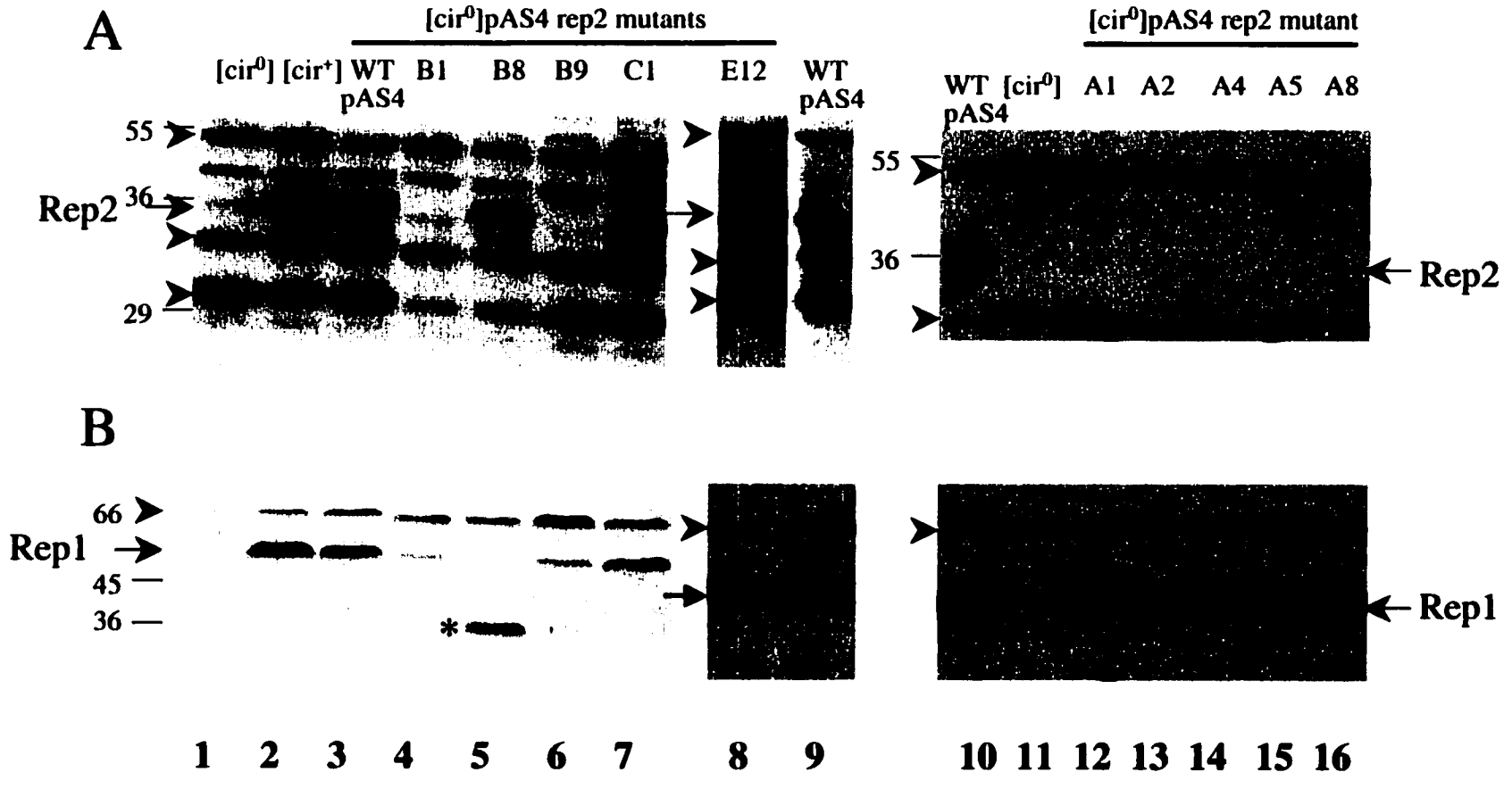
A
[cir0]pAS4 rep2 mutants
[cir0]pAS4 rep2 mutant
[cir0] [cir+] WT pAS4 B1 B8 B9 C1 E12 WT pAS4
WT pAS4 [cir0] A1 A2 A4 A5 A8
55
36
29
Rep2
Rep2
B
66
Rep1
45
36
*
Rep1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 12.

Figure 12. Western blot analysis of Rep2 mutants. Total protein extracted from the AS3 [cir^0] pAS4 transformants containing mutations in the *REP2* gene, resolved by a 10% SDS-PAGE and immunoblotted against affinity-purified anti-Rep2 (Panel A) and anti-Rep1 (Panel B) antibodies.

Protein extracts are from AS3 [cir^0] (lanes 1 and 11), AS3 [cir^+] (lane 2), AS3 [cir^0][pAS4] (lanes 3, 9 and 10); lanes 4 to 8: extracts from mutants *rep2 B1-1* (B1), *rep2 B8-1* (B8), *rep2 B9-1* (B9), *rep2 C1-1* (C1) and *rep2 E12-1* (E12), respectively, and lanes 12 to 16 from mutants *rep2 A1-2* (A1), *rep2 A2-1* (A2), rep2 *A4-2* (A4), *rep2 A5-2* (A5) and *rep2 A8-2* (A8), respectively. The positions of the non-specific bands detected by the antibodies are indicated by arrowheads. The bands representing Rep2 (Panel A) and Rep1 (Panel B) proteins are shown by arrows. The Rep2 protein expressed from mutant *rep2 B9-1* (Panel A, lane 6) that has mobility less than native Rep2, the mutant Rep2 proteins expressed from *rep2A1-2* and *rep2 A8-2* (Panel A, lanes 12 and 16) with mobilities higher than native Rep2 and the mutant Rep1 protein expressed from *rep2 B8-1* (Panel B, lane 5) with mobility higher than native Rep1, are all indicated by stars. Protein molecular-weight markers (Sigma) are shown on the left. Sizes are in kDa.

intensity of the band detected by the anti-Rep2 antibody in the mutant *rep2 E12-1* are comparable to wild-type (Figure 12A, lane 8). The size of the Rep1 protein is similar to wild-type for the mutants *rep2 B1-1, B9-1* and *E12-1*, but the amount is significantly less than that expressed from unmutated pAS4 (Figure 12B, compare lanes 4, 6 and 8 to lane 9). No anti-Rep2 immuno-reactive species could be detected for the mutants *rep2 A2-1, A4-2* and *A5-2* (Figure 12A, lanes 13, 14 and 15). The anti-Rep2 antibody detected a faint band with an apparent mobility of 30 kDa in the mutant *rep2 A8-2*, which may be the mutant form of Rep2 (Figure 12A, lane 16). A higher mobility band, with a molecular weight of approximately 25 kDa, is detected by the Rep2 antibody in the mutant *rep2 A1-2* (Figure 12A, lane 12). This mutation introduces a stop codon in the *REP2* gene at the position that would specify amino acid 232, and is therefore predicted to give a smaller truncated Rep2 protein of about 25 kDa. The mobility of the Rep1 protein expressed from all five of these *rep2* mutants is similar to wild-type Rep2 protein but the levels are lower than wild-type (Figure 12B, compare lanes 12-16 to lane 10).

E. Plasmid stability assay

The analysis of the Rep1 and Rep2 steady-state protein levels expressed by the PCR-generated *REP1* and *REP2* mutants in pAS4 indicated some mutations which were likely to have disrupted segregation of the plasmid due to low abundance of either Rep1 and Rep2 protein. However, for some mutants the Rep protein levels were close to wild-type so it was essential to prove that these proteins were indeed incapable of promoting proper segregation of the plasmid. The pink colour of the colonies in the *ade2* strain AS3 [cir^0] had been used to determine this segregation defect phenotypically, but it was

possible that the inability to fully complement the Ade$^-$ phenotype might be due to altered expression of the *ADE2* gene on the plasmid rather than mis-segregation. It was also possible that some of the mutants might confer a less severe segregation defect than the complete deletion of *REP1* or *REP2*. To determine the effect of the *REP1* and *REP2* mutations on the segregation of the plasmid, four independent colonies from each mutant were assessed for mitotic stability by calculating plasmid loss after ten to fouteen generations in non-selective medium (described in Section II 2C).

The results of the plasmid segregation assay for the *rep1* and the *rep2* mutants in the strain AS3 [cir^0] are given in Tables 9 and 10, respectively. The results show that the rate of loss of plasmid with mutations in the *REP1* gene varies from 14.2% for the mutant *rep1 I338T*, which has a single mutation in the carboxy terminal region, to 27.7% for *rep1 C10-1*, which has a frame-shift mutation leading to a premature stop codon. The difference between these loss rates is not statistically significant. The values for the remaining mutants lie between these two. The loss rate of pAS4Δrep1 is 15.3±3.1% and is similar to some of these values. These loss rates are similar to those reported for *ARS*-only plasmids (Kingsman et al. 1979). Thus in the presence of any of these mutations in the *REP1* gene, the 2μm test plasmid is behaving as if the 2μm segregation function is completely dysfunctional just like *ARS*-only plasmids.

The rates of loss of plasmid per generation for the *rep2* mutants are also similar to *ARS*-only plasmids. The values range between 13.3% for *rep2 A8-2* to 18.4% for *rep2 E12-1*, but the differences are not statistically significant. The loss rate of pAS4Δrep2 (16.5±2.8%) is comparable to these values.

The results from this plasmid segregation assay indicate that all mutations identified in either *REP1* or *REP2* on the basis of the colony colour assay, do affect the segregation of the 2μm plasmid.

TABLE 9. Plasmid segregation assay for *rep1* mutants

Plasmid	Colony colour	Initial plasmid+ cells (%)	Final plasmid + cells (%)	No. of generations in YEPD	Plasmid loss per generation (%)
pAS4 rep1A5-1	Pink	16.5 (±7.9)	1.5 (±0.6)	12.0(±0.5)	19.5 (±3.5)
pAS4 rep1A9-1	Pink	23.6 (±3.4)	3.8 (±1.4)	12.0(±0.5)	15.8 (±2.2)
pAS4 rep1A11-1	Pink	22.2 (±2.0)	3.2 (±1.3)	12.4(0.3)	15.6 (±2.5)
pAS4 rep1B6-1	Pink	21.8 (±2.5)	2.0 (±0.6)	10.7(±1.8)	21.8 (±3.0)
pAS4 rep1B9-1	Pink	17.9 (±3.0)	1.8 (±1.1)	12.8(±0.2)	19.4 (±4.0)
pAS4 rep1B10-1	Pink	20.6 (±2.8)	1.9 (±0.4)	12.3(±0.1)	19.1 (±2.1)
pAS4rep1C10-1	Pink	20.8 (±1.0)	0.8 (±0.4)	12.2(±0.2)	27.7 (±3.9)
pAS4rep1C11-1	Pink	18.1 (±3.5)	2.3 (±0.5)	12.1(±0.2)	17.0 (±0.4)
pAS4rep1D3-1	Pink	32.9 (±6.5)	5.3 (±0.4)	12.2(±0.1)	14.9 (±1.8
pAS4rep1D4-1	Pink	17.8(±2.0)	1.9(±0.3)	12.7(±0.3)	18.0(±1.5)
pAS4rep1I338T	Pink	40.1(±8.1)	5.8(±1.8)	14.1(±0.5)	14.0(±0.8)
pAS4rep1W262X	Pink	18.7(±1.3)	13.1(±0.2)	13.1(±0.2)	21.5(±1.6)

TABLE 10. Plasmid segregation assay for *rep2* mutants

Plasmid	Colony colour	Initial plasmid+ cells (%)	Final plasmid + cells (%)	No. of generations in YEPD	Plasmid loss per generation (%)
pAS4 rep2A2-1	Pink	28.0(±3.9)	2.9(±0.2)	14.5(±0.4)	15.6(±0.9)
pAS4 rep2B9-1	Pink	29.2(±7.8)	4.7(±0.6)	13.4(±0.2)	13.4(±1.3)
pAS4 rep2E12-1	Pink	26.1(±4.6)	3.7(±2.3)	11.4(±0.2)	18.4(±5.9)
pAS4 rep2A1-2	Pink	17.8(±2.8)	2.0(±0.8)	12.4(±0.2)	17.9(±2.7)
pAS4 rep2A4-2	Pink	21.3(±2.2)	2.9(±0.5)	12.3(±0.03)	16.3(±1.1)
pAS4 rep2A5-2	Pink	20.9(±5.7)	2.6(±0.9)	12.7(±0.5)	16.5(±1.6)
pAS4rep2A8-2	Pink	31.9(±6.1)	7.8(±2.5)	10.7(±0.2)	13.3(±2.0)

F. Mutagenic experiment with the *STB* locus

In addition to generating mutations in the *REP1* and *REP2* genes, I was also interested in the *STB* locus which is required in *cis* for mitotic stability of the 2μm circle. Therefore I created mutations in the *STB* locus and studied the effects of these mutations on plasmid segregation. Mutated fragments of the *STB* proximal region were generated by using both mutagenic and standard PCR conditions as described for the *REP1* and *REP2* genes (see Sections III 5A and 5B and Section II 4B). These mutated fragments were used to replace the proximal part of the *STB* region by gap repair of XhoI digested plasmid pAS4ΔX in the *ade2* strain AS3 [cir^0]. As with the *REP1* and *REP2* mutants, most transformants produced white colonies, consistent with the plasmid not having acquired mutations that affect plasmid stability. Pink colonies were observed, but their colour was much paler than previously observed for mutations in either *REP1* or *REP2*, and was best observed by replica-plating onto YEPD medium. Less than 1% of the colonies looked light pink in colour for experiments where the transforming fragment was generated by "standard" PCR. When the fragment used for gap repair was generated by mutagenic PCR, the number of pink colonies on YEPD medium was approximately 25%. Yeast plasmid DNA was prepared from the pink transformants and transformed into *E. coli* to facilitate sequencing. Sequencing of the *STB* proximal region from nine mutants obtained by standard PCR revealed no mutations in the *STB* repeats in any of these. It is possible that mutations in the *STB*-distal region, which is included in the PCR-mutagenized fragment used for gap repair, are responsible for the mutant phenotype. Further work on those mutants is being undertaken in the laboratory.

6. A two-hybrid assay to detect proteins interacting with Rep proteins

A two-hybrid genetic screen was carried out to identify cloned yeast genes encoding proteins that interact with Rep1 protein *in vivo*. Three yeast genomic libraries, YL1, 2 and 3 (Chien et al. 1991), each containing *Sau*3A partial digests of yeast genomic DNA inserted in one of the three possible reading frames downstream of the portion of the *GAL4* gene encoding the Gal4 activation domain, were used to identify interacting proteins. Approximately $2x10^5$ independent co-transformants of yeast strain Y153 were obtained from each of the three genomic libraries representing the three possible reading frames in the *GAL4* activation-domain fusion vector in combination with the Gal4 DNA-binding domain ($Gal4_{DB}$)-Rep1 fusion-protein expressing plasmid pGBT9-REP1. These primary transformants were selected on 50 mM aminotriazole (AT) and then screened by X-gal colony filter assay. Initially a total of 1398 AT-resistant transformants were obtained from the three libraries, out of which 253 were positive in the X-gal assay. The library plasmids were isolated from each of these and co-transformed back into yeast to retest the ability to activate the reporter genes in the presence or absence of the $Gal4_{DB}$-Rep1 fusion protein and a different Gal4 activation-domain fusion protein (see Materials and Methods for details). The two-hybrid experiment had a high false-positive rate, since all 253 positive clones were able to activate the *lacZ* reporter gene even when co-transformed with a plasmid expressing a different $Gal4_{AD}$-fusion protein. Therefore, no proteins that interacted specifically with Rep1 were detected using this approach. A similar two-hybrid screen was carried out by Ying Zhang to attempt to identify proteins interacting with Rep2. In this case, as with Rep1, the two-hybrid approach gave a high

level of false positives and did not identify any specific Rep2-interacting proteins (Ying Zhang, unpublished results).

7. Rep1 and Rep2 interact *in vivo*

Both Rep1 and Rep2 proteins are required for 2μm plasmid segregation as well as for repression of the *FLP* gene (Reynolds et al. 1987). Loss of either one leads to the same degree of loss of repression. These observations, and results of a study showing that specific levels of the Rep1 and Rep2 proteins are required to reconstitute the segregation activity of a 2μm-based plasmid (Dobson et al. 1988), have been used to suggest that the Rep proteins may function together as part of a complex. A two-hybrid genetic assay was used to determine that the Rep proteins interact *in vivo* (Kristina Blomqvist, unpublished results). Using Gal4 activation and DNA-binding domain fusions, it was established that Rep1 and Rep2 interacted and that Rep2 could self associate (Kristina Blomqvist, unpublished results). To extend these results, I looked at the interactions of various deletion derivatives of both *REP1* and *REP2* using the two-hybrid system. The details of plasmid constructions are given in Materials and Methods (Section 5A). The results of the two-hybrid assay are shown in Figure 13. None of the Rep protein fusions in the expression vectors, when singly transformed into the yeast host, could activate the *lacZ* reporter gene indicating that they had no intrinsic transcriptional activation function. We could not detect any interaction of Rep1 with itself using this assay. Full-length Rep1 showed interaction with full-length Rep2, as previously demonstrated by Blomqvist (unpublished results). The carboxy-terminal

$Gal4_{AD}$	$Gal4_{BD}$	β-galactosidase filter assay
REP1	–	–
REP2	–	–
–	REP1	–
–	REP2	–
REP1	REP1	–
REP1	REP2	++
rep1Δ130-373	REP2	+++
rep1Δ78-373	REP2	–
rep1Δ1-76	REP2	–
rep1Δ1-129	REP2	–
SNF4	SNF1	++
REP2	REP1	++
rep2Δ1-14	REP1	++
rep2Δ1-57	REP1	–
rep2Δ59-296	REP1	+
REP2	REP2	++
rep2Δ1-14	REP2	++
rep2Δ1-57	REP2	–
rep2Δ59-296	REP2	–

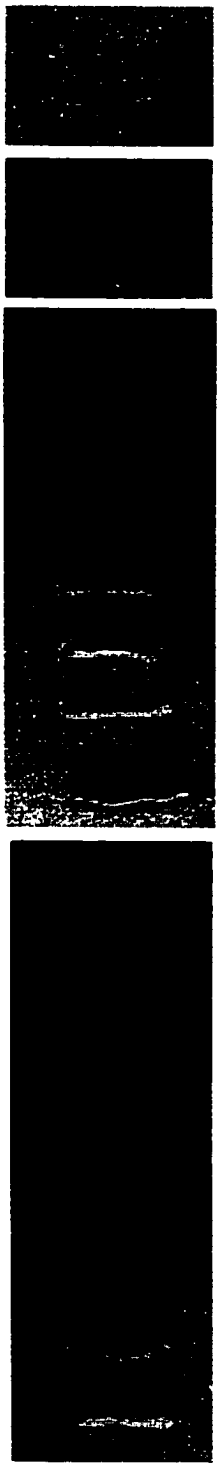

Figure 13

Figure 13. Two-hybrid assay for Rep protein interaction in yeast. Yeast strain Y153 was transformed with the plasmids shown at the left and the results of the β-galactosidase filter assay are shown at the right. Time for development of blue colour, < 1 hour (+++), 2-4 hour (++), 9 hour (+), white after 24 hour (-). Details of plasmid construction are given in Table A3 in appendix.

truncation of Rep1, rep1Δ130-373, gave a stronger activation of the *lacZ* reporter gene when co-expressed with Rep2 as compared to the level when full-length Rep1 was co-expressed with the same Rep2 fusions. None of the other deleted forms of Rep1, such as rep1Δ78-373, rep1Δ1-76 or rep1Δ1-129, interacted with full-length Rep2, as indicated by lack of β-galactosidase activity above background levels. β-galactosidase activity was detected when pGAD-REP2 and pGBT9-REP2 were co-transformed into the yeast host, suggesting self-association of Rep2. Of the three deleted forms of Rep2, rep2Δ1-14 and rep2Δ59-296 both gave β-galactosidase activity when co-expressed with full-length Rep1 fusions, although the rep2Δ59-296 gave a much lower level of activity. The rep2Δ1-14 fusion also interacted with full-length Rep2, whereas rep2Δ1-57 could not activate the *lacZ* reporter gene when co-transformed with either pGBT9-REP1 or pGBT9-REP2.

These two-hybrid results suggest that the Rep1 protein interacts with the Rep2 protein *in vivo* with the first 129 amino acids of Rep1 being sufficient for interaction, while the first 76 amino acids of Rep1 are required but not sufficient for interaction with Rep2. The first 58 amino acids of Rep2 are sufficient for its interaction with Rep1 but not for self-association, and the first 14 amino acids of Rep2 are not required for its interaction with either Rep1 or Rep2. It can be concluded that the amino-termini of the Rep proteins are required for interactions with each other.

A. Rep fusion protein expression

The two-hybrid assay provided evidence for interaction between the Rep proteins. β-galactosidase expression for yeast co-transformed with full-length Rep2 and rep1Δ130-373 or with full-length Rep1 and rep2Δ59-296 indicated that the domains of

the proteins required for their interaction were localized to the amino-terminal regions of the two proteins. No β-galactosidase expression could be detected when yeast was co-transformed with full-length Rep2 and any of the other three truncated versions of Rep1. Similarly, β-galactosidase activity above background levels was also not detected for yeast co-transformed with full-length Replor Rep2 and rep2Δ1-57 or with full-length Rep2 and rep2Δ59-296. The negative two-hybrid results could be due to lack of interaction between the various Rep fusion proteins or could be due to lack of expression of the Rep-fusion proteins from the two-hybrid vectors. Others have shown that some two-hybrid fusion proteins are expressed at very low levels inside yeast cells. To examine the steady-state levels of the fusion proteins, a Western blot analysis of protein extracts from yeast cells expressing the fusions was performed using affinity-purified anti-Rep1, anti-Rep2 and anti-Gal4 $_{AD}$ antibodies. The results are shown in Figures 14 and 15. Figures 14A and 14B show the western blots using anti-Rep1 and anti-Rep2 antibodies, respectively. The mobility of Rep proteins present in [cir$^+$] strains are indicated (Figure 14A and B, lanes 2-5). Their identification is confirmed by their absence from an isogenic strain that lacks 2μm circle (Figures 14A and B, lane 1). In addition to native Rep1, a new Rep1-antigenic band with a mobility of approximately 70 kDa was observed in protein extracts form yeast transformed with pGBT9-REP1, consistent with the 70-kDa size expected for the Gal4$_{DB}$-Rep1 fusion protein (Figure 14A, lane 4). The relative abundance of this protein was similar to that of native Rep1. Another new Rep1-antigenic band with a mobility of about 71 kDa was observed in protein extracts from yeast transformed with pGAD-REP1 and the size is as expected for the Gal4 $_{AD}$-Rep1 fusion protein (Figure 14A, lane 5). The level of this fusion protein was also comparable to that of native Rep1 levels. The anti-Rep2 antibody detects a band with a mobility of approximately of 56 kDa in protein extracts from yeast transformed

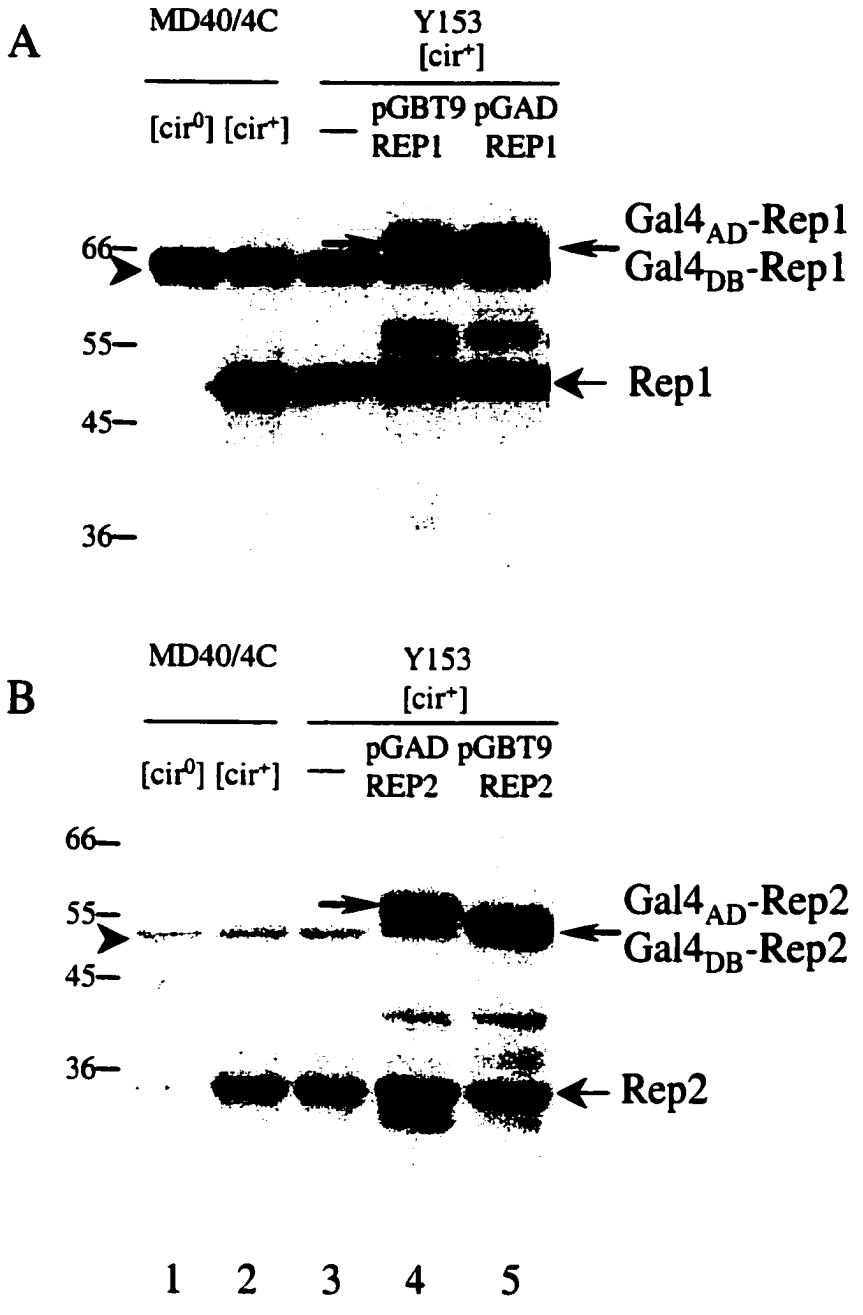
A
MD40/4C
Y153
[cir+]
[cir0] [cir+]
—
pGBT9 pGAD
REP1 REP1
66
55
45
36
Gal4AD-Rep1
Gal4DB-Rep1
Rep1
B
MD40/4C
Y153
[cir+]
[cir0] [cir+]
—
pGAD pGBT9
REP2 REP2
66
55
45
36
Gal4AD-Rep2
Gal4DB-Rep2
Rep2
1 2 3 4 5

Figure 14.

Figure 14. Rep fusion protein expression in yeast two-hybrid co-transformants. Total protein was extracted, resolved by SDS-PAGE and immunoblotted against affinity-purified anti-Rep1 (Panel A) or anti-Rep2 (Panel B) antibodies. Extracts were made from isogenic strains MD40/4c [cir^0] (lane 1) and MD40/4c [cir^+] (lane 2), Y153 [cir^+] (lane 3), Y153 [cir^+][pGBT9-REP1][pGAD-REP2] (lane 4), and Y153 [cir^+][pGBT9-REP2][pGAD-REP1] (lane 5). The native Rep1 (Panel A, lanes 2-5) and Rep2 (Panel B, lanes 2-5) proteins are shown. The positions of native Rep and Rep-fusion proteins are indicated by arrows. Protein molecular-weight markers (Sigma) are shown on the left. Sizes are given in kDa. The non-specific bands detected by the anti-Rep1 and anti-Rep2 antibodies are shown by arrowheads.

with pGAD-REP2 and is consistent with the size expected for the Gal4 $_{AD}$-Rep2 fusion protein (Figure 14B, lane 4). A different band migrating at approximately 55 kDa was observed for yeast extracts transformed with pGBT9-REP2 and the size is as expected for the fusion protein Gal4$_{DB}$-Rep2 (Figure 14B, lane 5). The molecular weights indicate that these Gal4 domains have been fused to the full-length Rep2 protein. The relative abundance of both these Gal4-Rep2 fusion proteins are comparable to that of native Rep2 protein.

Figure 15 shows the Western blots for the various truncated Rep1 and Rep2 proteins fused to Gal4$_{AD}$, using anti-Rep1 (Figure 15A), anti-Rep2 (Figure 15C) and anti-Gal4$_{AD}$ (Figure 15B and 15D) antibodies. The truncated versions of the two Rep proteins are likely to have lost anti-Rep antibody epitopes so the intensity of the bands detected by Western blotting is expected to be less, even if the truncations are expressed at the same level as the non-truncated form. In the case of Gal4$_{AD}$ fusions the use of anti-Gal4$_{AD}$ antibodies circumvents this difficulty and comparison of the results with both antibodies allows both the identity of the bands as Rep protein fusions and their steady-state levels to be confirmed. In Figure 15A (lane 3), anti-Rep1 antibody detects a band with a mobility of approximately 61 kDa, consistent with the size of 61 kDa expected for the fusion protein Gal4$_{AD}$-Rep1Δ1-76. The steady-state level of this fusion protein is, however, much less than that of the full-length Rep1 fusion. A novel band migrating at approximately 53 kDa and positioned immediately above the 50 kDa Rep1 band expressed from native 2μm was observed, and its size is consistent with the 53 kDa expected for the Gal4$_{AD}$-Rep1Δ1-129 fusion (Figure 15A, lane 4). In Figure 15A (lane

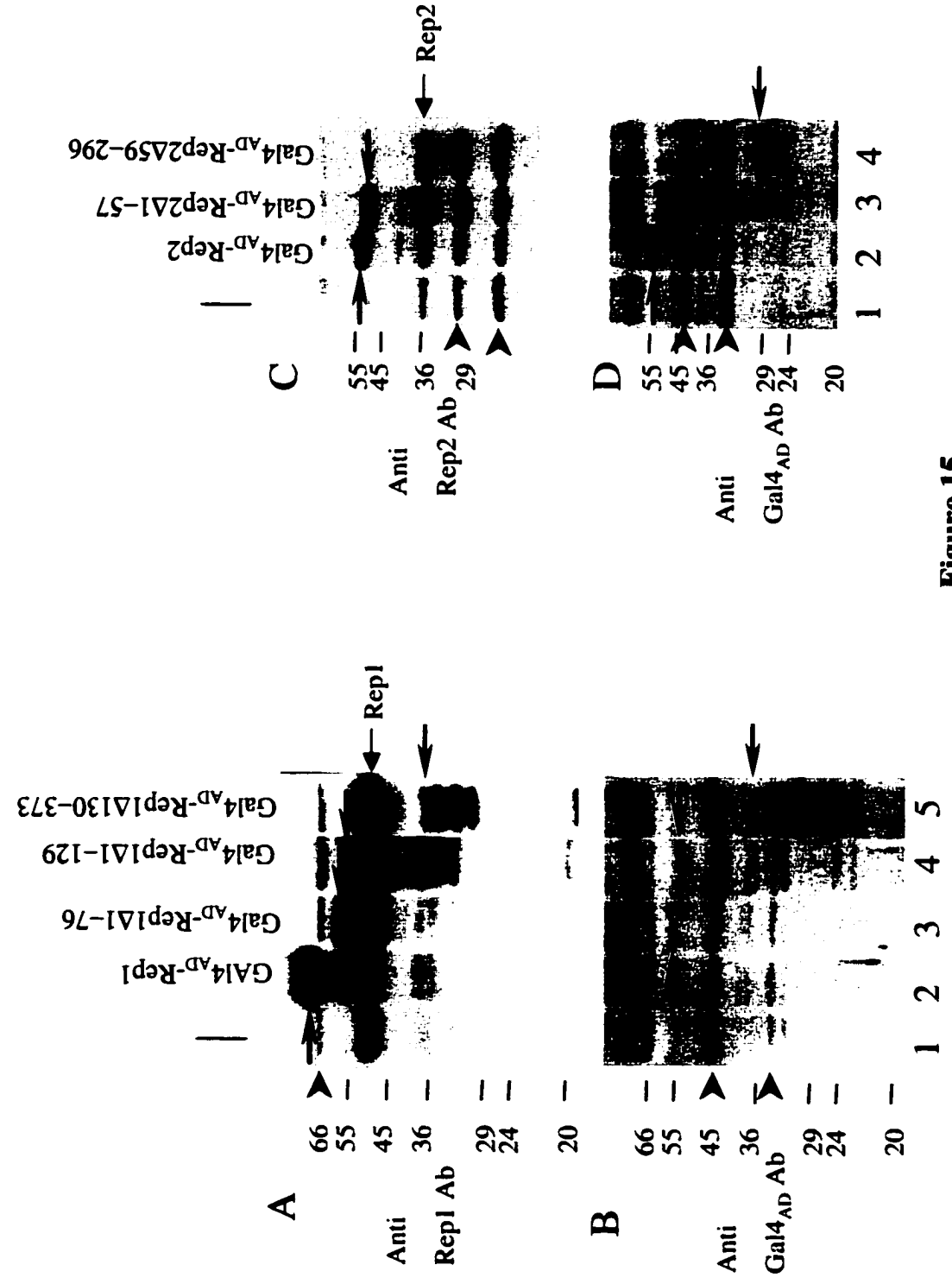
A
Anti
Rep1 Ab
66
55
45
36
29
24
20
GAl4AD-Rep1
Gal4AD-Rep1A1-76
Gal4AD-Rep1A1-129
Gal4AD-Rep1A130-373
Rep1
B
Anti
Gal4AD Ab
1 2 3 4 5
C
Anti
Rep2 Ab
Gal4AD-Rep2
Gal4AD-Rep2A1-57
Gal4AD-Rep2A59-296
Rep2
D
Anti
Gal4AD Ab
1 2 3 4

Figure 15.

Figure 15. Rep fusion protein expression in yeast two-hybrid co-transformants. Total protein was extracted from yeast, resolved by SDS-PAGE and immunoblotted against affinity-purified anti-Rep1 (Panel A), anti-Rep2 (Panel C) or anti-$Gal4_{AD}$ (Panel B and D) antibodies.

Panels A and B: The Western blot shown in panel B is the same blot as panel A, stripped and re-probed with anti-$Gal4_{AD}$ antibody. Extracts were made from strains Y153 [cir^+] (lane 1), Y153 [cir^+][pGBT9-REP2][pGAD-REP1] (lane 2), and Y153 [cir^+] [pGBT9-REP2] co-transformed with [pGADrep1Δ1-76] (lane 3), [pGADrep1Δ1-129] (lane 4) or [pGADrep1Δ130-373] (lane 5).

Panels C and D: The Western blot shown in panel D is the same as in panel C, stripped and re-probed with anti-$Gal4_{AD}$ antibody. Extracts were made from strains Y153 [cir^+] (lane 1), Y153 [cir^+][pGBT9-REP1][pGAD-REP2] (lane 2), and Y153 [cir^+] [pGBT9-REP1] co-transformed with [pGADrep2Δ1-57] (lane 3) or [pGADrep2Δ59-296] (lane 4). The positions of native Rep and Rep-fusion proteins are indicated by arrows. Protein molecular-weight markers (Sigma) are shown on the left. Sizes are given in kDa. Non-specific bands recognized by the antibodies are indicated by arrowheads.

5), four new bands are detected with anti-Rep1 antibody with apparent mobilities ranging from 37 to 30 kDa. The expected size for the $Gal4_{AD}$-Rep1Δ130-373 fusion is 38 kDa, consistent with the slowest migrating form being this fusion protein, while the other bands presumably represent breakdown products of the fusion. New species smaller in size and having a mobility of approximately 20 kDa are also recognized by this antibody for protein extracted from yeast containing either the $pGAD_{AD}$-Rep1Δ1-129 or $pGAD_{AD}$-rep1Δ130-373. These are probably further degradation products of these fusion proteins. The proteins recognized by the anti-$Gal4_{AD}$ antibody in these same extracts are shown in Figure 15B. Both the anti-$Gal4_{AD}$ and the anti-Rep1 antibodies detect bands of similar mobilities in each transformant tested. However, in some cases the intensities of the bands differ between the two antibodies. Since the $Gal4_{AD}$ and Rep1 antibodies recognize different epitopes in these fusion proteins, the differences in intensity probably reflect the number of epitopes available for recognition by the antibodies. For $Gal4_{AD}$-Rep1Δ130-373, the lower molecular-weight species are more abundant, with the most prominent species having an apparent mobility of 30 kDa. The greater intensity of the smaller species detected with the anti-$Gal4_{AD}$ antibody relative to the anti-Rep1 antibody thus indicates that these species are premature truncations of the full-length fusion and retain very little of the Rep1 protein sequence. There are, however, enough epitopes of the $Gal4_{AD}$ protein present in this smaller species to be recognized by the anti-$Gal4_{AD}$ antibody. Considering the results obtained with the two antibodies, all the truncated Rep1 fusion proteins have much lower levels of expression than full-length Rep1 fusion (Figures 15A and 15B). This is most obvious from the Western blot analysis

performed with anti-Gal4$_{AD}$ antibody (Figure 15B), which shows that all three truncations of Rep1 (lanes 3-5) have much lower levels of the fusion proteins than full-length Rep1 (lane 2). However, when the intensity of the 37-kDa band obtained from Gal4$_{AD}$- Rep1Δ130-373 fusion is compared to the intensities of the highest-molecular-weight specific bands obtained for the other two truncations of Rep1 (Figure 15B, lanes 3 and 4) as detected by the anti-Gal4$_{AD}$ antibody, they are of similar intensity, suggesting their steady-state levels are similar. Although the steady-state levels of all three fusion proteins, namely Gal4$_{AD}$-Rep1Δ1-76, Gal4$_{AD}$- Rep1Δ1-129 and Gal4$_{AD}$- Rep1 Δ130-373, are very low compared to that of full-length Rep1 fusion, Gal4$_{AD}$-Rep1Δ130-373 when co-transformed with Gal4$_{DB}$-Rep2 protein results in strong activation of the *lacZ* reporter gene in the two-hybrid assay. This suggests interaction between Rep2 and this truncated Rep1 protein. In contrast, the other two fusion proteins, Gal4$_{AD}$-Rep1 Δ1-76 and Gal4$_{AD}$-Rep1Δ1-129, when co-transformed with Rep2 are unable to activate the reporter gene. Thus low levels of fusion protein does not correlate with loss of activation of reporter gene in all cases. The very strong activation of the *lacZ* reporter gene in the two-hybrid host yeast expressing both Gal4$_{AD}$-Rep1Δ130-373 and Gal4$_{DB}$-Rep2 may also reflect the abundance of the lower-molecular-weight truncated Rep1 fusion proteins in this transformant. If this is the case, the easiest interpretation is that this truncated Rep1 fusion protein is still capable of interacting with full-length Rep2 expressed from the pGBT9-REP2 vector. Since the most abundant species has an apparent molecular weight of 30 kDa and is recognized by both anti-Gal4$_{AD}$ and anti-Rep1 antibodies, it would represent a product of a premature translational termination event. This 30 kDa species

would retain all the amimo-terminal Gal4 activation domain and approximately the first 100 amino acids of Rep1. No fusion protein could be detected in yeast transformed with the $GAL4_{AD}$-Rep1Δ78-373 plasmid with either anti-Rep1 antibody or anti-$Gal4_{AD}$ antibody (not shown). It is possible that for this deleted allele the protein is not expressed or the protein has a reduced half-life.

Western blot analysis with anti-Rep2 (Figure 15C) and anti $Gal4_{AD}$ (Figure 15D) antibodies was used to determine the relative abundances of the truncated Rep2 proteins fused to $Gal4_{AD}$. The Rep2 antibody detects a band migrating at approximately 50 kDa for $Gal4_{AD}$-Rep2Δ1-57 fusion protein (lane 3 in Figure 15C), which is close to the expected size for this fusion protein. The intensity of this fusion protein band detected by the Rep2 antibody is similar to that of the 56-kDa full-length Rep2 fusion (Figure 15C, lane 2). This 50-kDa band is not detected by the anti-$Gal4_{AD}$ antibody (lane 3 in Figure 15D). A different band with a mobility of approximately 43 kDa is detected by the anti-$Gal4_{AD}$ antibody (lane 3 in figure 15D) and presumably represents a breakdown product or truncated version of this fusion protein. The presence of this smaller species detected with the anti-$Gal4_{AD}$ antibody and not with the anti-Rep2 antibody suggests that this species is a premature truncation of the fusion and retains very little of the Rep2 epitopes recognized by the anti-Rep2 antibody. It is possible that the $Gal4_{AD}$ antibody is recognizing a degraded product instead of the full fusion protein due to the retention of enough epitopes of the $Gal4_{AD}$ protein in this smaller breakdown species. The anti-Rep2 antibody did not detect any novel Rep2-antigenic bands in extracts from yeast expected to be expressing $Gal4_{AD}$-Rep2Δ59-296 fusion protein (lane 4 in Figure 15C). The

expected size of this fusion is 28 kDa, and it is therefore possible that it might be obscured by the non-specific band detected by the anti-Rep2 antibody even in protein extracts from [cir^0] yeast. Since this fusion protein contains only the first 58 amino acids of the Rep2 protein, it is also possible that it may not be recognized by the anti-Rep2 antibody. The anti-Gal4$_{AD}$ antibody does detect a new band with an apparent mobility of 28 kDa in these extracts, consistent with the expected molecular weight of the Gal4$_{AD}$-Rep2Δ59-296 fusion protein (lane 4 in Figure 15D). The protein is less abundant than full-length Rep2 fusion but is present at levels similar to those of the truncated Rep1 fusion proteins (see Figure 15B).

It can be concluded from the molecular weights of the fusion proteins observed in the Western blotting analysis that the fusion proteins from the truncated versions of Rep1 and Rep2 were present at levels much lower than full-length Rep1 or Rep2 fusions. Although the steady-state protein levels are low, the Gal4$_{AD}$-Rep1Δ130-373 fusion protein is able to activate the two-hybrid reporter gene when co-expressed with Gal4$_{DB}$-Rep2, as is Gal4$_{AD}$-Rep2Δ59-296 co-expressed with Gal4$_{DB}$-Rep1. Therefore the absence of β-galactosidase activity for the other co-transformants can not necessarily be attributed to a lack or low-level of expression of the truncated fusion protein. Some truncations still lead to reporter-gene activation even when expressed at low steady-state levels, suggesting that in the other truncations, the loss of activation is due to the loss of interaction between the Rep domains that remain in the two-hybrid fusion proteins.

B. Plasmid segregation assay

The results from the two-hybrid assay suggest interaction between the Rep proteins. Plasmid segregation assays were carried out to test whether there is a correlation between truncations that affect Rep protein interactions *in vivo* and the plasmid segregation mediated by the Rep proteins. The *rep1*-deleted derivative of the 2μm stability plasmid pAS4, pAS4Δrep1, was used for this assay. As mentioned above, pAS4 with wild-type *REP1* and *REP2* segregates in a [cir^0] host almost as efficiently as does the 2μm circle, but in the absence of either of the *REP* genes the plasmid has a high loss rate at mitosis, behaving like an *ARS*-only plasmid. To test whether the truncated versions of the Rep proteins could restore the wild-type Rep1 function, the *ade2 leu2* yeast strain AS3 [cir^0] was co-transformed with the *ADE2* plasmid pAS4Δrep1 and the *LEU2* two-hybrid plasmids expressing full-length or truncated versions of the Gal4 $_{AD}$-Rep1 fusion proteins. A stability assay was carried out with these co-transformants. The Gal4 activation-domain plasmid pGAD424 is itself a 2μm-based plasmid containing only the *ARS* and *cis*-acting *STB* locus from 2μm. Its stability is dependent on the Rep1 and Rep2 proteins that are usually supplied in *trans* from the native 2μm plasmids in the two-hybrid host strain. In the assay performed here, the host has no native 2μm plasmid, Rep2 is provided by the *rep1*-deleted stability plasmid pAS4Δrep1, and Rep1 is supplied by the Gal4 $_{AD}$-Rep1 fusion expressed from the two-hybrid plasmid. Cells grown under conditions selecting for both plasmids will form colonies on medium lacking adenine and leucine only if they have both plasmids. To receive both plasmids, partitioning needs to function efficiently during cell division and this will only occur if the Gal4 $_{AD}$-Rep1 fusion protein is able to supply Rep1 function. If the Gal4 $_{AD}$-Rep1 fusion protein is unable to supply Rep1 function, most cells grown in the absence of selection for the *ADE2* plasmid will not contain both plasmids due to inefficient partitioning of the

plasmids between the mother and the daughter cells. In this assay, the rate of loss of the *ADE2* plasmid, pAS4Δrep1, from cells containing the *LEU2* plasmid expressing the Gal4 $_{AD}$-Rep1 fusion protein was calculated. This value will be high if the Rep1 function cannot be supplied by the fusion protein and low if the Gal4 $_{AD}$-Rep1 fusion protein provides functional Rep1. The results of this stability assay are shown in Table 11. When pAS4Δrep1 was present as the only plasmid in strain AS3 [cir^0], it was lost at a high rate of 15.3% per generation, as expected for an *ARS*-only plasmid (see Table 2). When co-transformed with the *LEU2* vector pGAD424, the loss rate was 13.3%, a value comparable to pAS4Δrep1 alone in the [cir^0] host indicating that there was no segregation function supplied by the *ADE2*-tagged pAS4Δrep1 plasmid. The rate of loss of the *rep1*-deleted pAS4 was decreased to only 0.4% in the presence of the pGAD-REP1 plasmid expressing the full-length Rep1 fusion protein. This indicates that full-length Rep1 fused to Gal4 $_{AD}$ is able to restore mitotic stability to the *rep1*-deleted 2μm stability plasmid pAS4Δrep1; more importantly it shows that the Gal4 sequences do not interfere with this Rep1 function. However, none of the amino-terminal or the carboxy-terminal truncations of Rep1 fused to Gal4$_{AD}$ and expressed from the plasmids pGAD-rep1Δ1-76, pGAD-rep1Δ1-129, pGAD-rep1Δ130-373 or pGAD-rep1Δ78-373 could restore the mitotic stability of pAS4Δrep1. Thus deletion of either the amino-terminal 76 or 129 amino acids, or the carboxy-terminal 244 or 298 amino acids, of Rep1 abolishes its ability to mediate plasmid segregation. Therefore, although the first 129 amino acids of Rep1 are sufficient for interaction with Rep2, this truncated Rep1 could not increase the stability of pAS4-Δrep1, which indicates that the carboxy-terminal region of Rep1 must play some role required for plasmid segregation other than mediating interaction with Rep2. For the other truncated Rep1 proteins, their inability to restore plasmid segregation might be due

TABLE 11. Plasmid segregation assay

LEU2 Plasmid	*ADE2* Plasmid	Ratio of Leu$^+$ Ade$^+$/Leu$^+$ cells during selective growth (%)	Rate of loss of *ADE2* plasmid from Leu$^+$ cells/generation [a] during non-selective growth
pGAD-REP1	pAS4-Δrep1	96.0(±7.7)	0.4(±1.4)
pGAD424	PAS4-Δrep1	66.7(±14.7)	13.3(±8.0)
pGAD-rep1Δ1-76	PAS4-Δrep1	61.7(±12.3)	13.8(±6.2)
pGAD-rep1Δ1-129	pAS4-Δrep1	72.1(±5.6)	13.3(±4.8)
pGAD-rep1Δ130-373	pAS4-Δrep1	44.9(±12.2)	15.9(±5.1)
pGAD-rep1Δ78-373	pAS4-Δrep1	52.7(±4.8)	23.8(±3.0)

[a] Generations in non-selective medium ranged from ten to twelve for all cultures. Data represent an average obtained from the results of four independent co-transformants. Standard deviations are shown in parentheses.

to their low steady-state levels relative to native Rep1 but it most likely reflects the loss of residues critical for Rep1 protein function.

A similar analysis was attempted with the Rep2 fusion proteins, but in this case, expression of full-length $Gal4_{AD}$-Rep2 from the pGAD-REP2 vector did not give stable co-transformants with the *rep2*-deleted pAS4. Over-expression of the Rep2 fusion protein from the two-hybrid vector may have been responsible, since over-expression of native Rep2 has also been shown to block the propagation of 2μm-based plasmids (Dobson et al. 1988).

C. Two-hybrid assay for the *rep1* and the *rep2* point mutants

To further define the domains in Rep1 and Rep2 required for interaction with each other, with other proteins, and for self-association, a two-hybrid assay was carried out with the *rep1* and *rep2* point mutants generated by PCR mutagenesis and gap repair (see Section III 5) and which had been selected on the basis of the ability to cause loss of plasmid segregation. Point mutations which had been shown to give reduced steady-state levels of Rep1 or Rep2 and where loss of function was most likely due to loss of the appropriate level of the respective Rep protein were not further analyzed. For the point mutations where steady-state levels of the mutants were not significantly reduced, suggesting that loss of plasmid stability arose due to some other cause (see Section III 5D), the two-hybrid assay was used to test the ability of the mutant proteins to interact with Rep1 and Rep2. The results of the assay for the *rep1* mutants are shown in Table 6. The yeast host was transformed with pGBT9-REP2 and each of the *rep1* mutants cloned in pGAD424 to be expressed as a $Gal4_{AD}$-fusion. The only *rep1* mutants which did not interact with Rep2 were *rep1B10-1* and *rep1C11-1*. The mutant *rep1C11-1* had two mutations, I30T and K47R. Each of these mutations, when separately introduced into

pAS4 and transformed into the *ade2* host AS3 [cir^0], did not produce a segregation defect (Table 8). Since the colonies were white for the mutants *rep1I30T* and *rep1K47R*, two-hybrid analysis was not carried out with the single mutants. It seems that for the mutant *rep1 C11-1*, the double mutation is required to abolish both interaction and segregation function. For the mutant *rep1B10-1*, since it was not possible to separately analyze the mutation F53S, it cannot be determined whether this mutation alone issufficient to abolish both Rep1/Rep2 interaction and plasmid segregation. The other two mutations F53C and F53Y created at the same position by site-directed mutagenesis could not affect plasmid segregation when separately introduced into pAS4 and transformed into the *ade2* [cir^0] host. The stop codon at position 269 should not interfere with interaction since the amino-terminal interacting domain of Rep1 is still present and steady-state protein levels were high enough to be detected by Western blotting. Thus it is possible that the change from phenylalanine to serine at position 53 in Rep1 disrupts interaction with Rep2.

Similarly, the two-hybrid host was transformed with either pGBT9-REP1 or with pGBT9-REP2 and each of the *rep2* mutants inserted in pGAD424 to be expressed as Gal4 $_{AD}$-Rep2 fusions. The results are shown in Table 7. According to the results of the two-hybrid experiment, the first 58 amino acids of Rep2 are sufficient for interaction with Rep1 but not for self-association. The rep2 mutants *rep2A5-2* and *rep2A8-2*, which did not activate the reporter gene when co-expressed with either the Gal4$_{DB}$-Rep1 or Gal4$_{DB}$-Rep2 fusions, have mutations in the carboxy-terminal region at amino acids 176 and 177, respectively. The double mutant *rep2 A4-2*, which has mutations in the amino-terminal region at amino acids 10 and 95, also did not activate the reporter gene in either two-hybrid assay. The lack of β-galactosidase activity in these cases could be due to the lack or lower abundance of the fusion proteins carrying these mutations, rather than change in the amino acid residue necessarily altering a Rep protein interacting domain. However,

the *rep2* mutant *rep2 A2-1*, which had two mutations, one in the upstream promoter region and the other in the carboxy-terminal region, interacts with Rep1 but not with Rep2. This mutant protein, however, could not be detected by Western blot analysis when expressed in an unfused form from pAS4 (see Figure 12). So it is possible that it is the promoter mutation which leads to reduced expression from pAS4, whereas in the two-hybrid vector, this part of the sequence is removed and the only mutation would then be the N221Y substitution that could impair Rep2-Rep2 interaction. For the mutant *rep2 B9-1*, which had two mutations, S108P and D174N, each of these mutations, when separately introduced into pAS4 and transformed into the *ade2* host AS3 [cir^0], did not produce a segregation defect (Table 8). Since the colonies were white for the mutants *rep2 S108P* and *rep2 D174N*, no two-hybrid experiment was carried out for the single mutants. The double mutant was able to activate the reporter gene when co-expressed with either the Rep1 or the Rep2 $Gal4_{DB}$ fusions. So these two altered residues must affect some other aspect of Rep2 function that is critical for 2μm circle segregation.

IV. Discussion

1. Segregation of the 2μm circle and 2μm circle-based plasmids

One of the aims of my research was to develop a model system that would allow the study of 2μm circle partitioning. The test plasmid pAS4 was designed to include not only the sequences required for partitioning of 2μm circle, namely the *REP1* and *REP2* genes, the *cis*-acting *STB* locus (Kikuchi, 1983), and an *ARS*, but a marker, the yeast *ADE2* gene, that would allow the presence of the plasmid in the cell to be detected visually. Previous studies have shown that 2μm circle is lost at a frequency of about 10^{-4} per generation (Futcher and Cox, 1983). The pAS4 plasmid was found to be comparable to native 2μm in terms of stability, with a loss rate of 0.003±0.002% per generation. None of the hybrid plasmids used previously for studying the partitioning of the 2μm circle were as stable as pAS4. The plasmids reported by Kikuchi (1983) contained various parts of 2μm DNA and when used singly were propagated in a [cir^+] host to provide the *trans*-acting factors. When a [cir^0] host was used, two plasmids, each of them containing the *STB* region and one or the other of the *REP* genes were co-transformed. The plasmids were *flp*$^-$ and even when the transformed yeast were grown in medium selecting for the presence of plasmid, only 80% of cells were plasmid-containing. Deletions made these plasmids even less stable. Similarly, Jayaram et al. (1983, 1985) used hybrid 2μm-based plasmids which were less stable than pAS4. Plasmid CV20, which has the entire 2μm circle genome with pBR322 and the yeast *LEU2* gene inserted

at the *Eco*RI site within the *FLP* gene, has been reported to be quite stable, with 90% of the cells containing plasmid when grown in medium selecting for the presence of plasmid. The most stable of these is the plasmid pJDB219 (Beggs, 1978), with a loss rate of 0.26+0.03% in a [cir^0] host (Futcher and Cox, 1984), a value higher than for pAS4. Like pAS4, pJDB219 contains all the sequences required for 2μm segregation and has a disrupted *FLP* gene. It also has a marker gene, the *LEU2-d* allele, a version of the *LEU2* gene which lacks most of the promoter region resulting in a very low level of expression of the gene. The relative inefficiency of the gene means that many copies of the plasmid are required for the cells to be viable on media lacking leucine. Growth of pJDB219-transformed yeast on medium lacking leucine selects for cells with extremely elevated plasmid levels, and this high copy number may account for the low instability of this plasmid (Futcher and Cox, 1984). The loss rates per generation for other 2μm-based plasmids vary and can be as high as 5% (Futcher and Cox, 1984). Like pAS4 and pJDB219, these hybrid plasmids have insertions of *E. coli* vector and yeast chromosomal sequences into the 2μm circle that allow propagation and selection in *E. coli* and yeast. One of the insertions, usually bacterial sequences, is at the internal *Eco*RI site of the *FLP* gene, which is known to result in loss of Flp site-specific recombinase activity (Dobson et al. 1980b). In pAS4 the yeast *ADE2* gene is inserted at this *Eco*RI site and the *E. coli* vector sequences are inserted in the intergenic region between the *REP1* and *REP2* genes. None of the other hybrid plasmids have the second insertion at this position; instead, the yeast *LEU2* gene, either a fully functional *LEU2* gene or the impaired *Leu2-d* allele (Beggs, 1978), is inserted at the *Pst*I site of the *STB*-distal in most cases. It seems that

STB-distal might contribute towards the stability of the 2μm plasmid (see section IV 2B) and it is possible that any insertion around this region can reduce the stability of the plasmid. However, this does not explain the instabilities observed for other 2μm-based plasmids. YpR141, which has only one insertion, the plasmid pMB9 at the internal *Eco*RI site of *FLP*, has an instability of 4% (Futcher and Cox, 1984), a value much higher than that of pAS4. The use of a different yeast gene for selection, *ADE2* for example instead of the *LEU2* as is the case in most of these hybrid plasmids, might also change the stability of the plasmid. Another consideration is that the stability assays for pAS4 were performed in a [cir^0] host. pAS4 cannot undergo Flp-mediated site-specific recombination even if Flp is provided in *trans* or if pAS4 is propagated in a [cir^+] host, since one of the *FRT* (Flp recognition target) sites is non-functional. However, in a [cir^+] host, the native 2μm might interfere with the stability of pAS4 because homologous recombination between the two plasmids can still occur and the two plasmids may now compete for the segregation apparatus. Since pAS4 is almost as stable as native 2μm in a [cir^0] host, it is unlikely that its stability will be greatly affected even in a [cir^+] background. However, stability assays of pAS4 in a [cir^+] host would have to be performed to test the hypothesis. Thus this flp^- *ADE2* test plasmid, pAS4, is more stable than any 2μm-based hybrid plasmid reported so far and can be used as a model system for studying the partitioning system of 2μm circle.

Removal of the *E. coli* sequences from pAS4 slightly increased the loss rate of the plasmid pAS4B to 2.07± 1.5% per generation. Murray and Cesareni (1986) have shown that transcription through the *STB* locus can destabilize 2μm-derived plasmids. In their

experiment, galactose-inducible transcription from a *GAL* promoter was directed into *STB* from the distal side. The decrease in stability was even more pronounced for plasmids from which the transcription terminator in *STB*-distal had been removed. It has been suggested that transcription arising in the bacterial sequences inserted in hybrid 2μm-based plasmids can encounter *STB* similar to the *GAL*-induced transcription, and cause instability of these plasmids (Murray and Cesareni, 1986). However, the low loss rates per generation for plasmids pAS4 and pAS4B suggest that any destabilization due to bacterial associated transcripts is absent in this case. In fact, the bacterial sequences in this case seem to have a stabilizing effect, perhaps by blocking *REP1* transcription from running antisense through the *REP2* gene and *vice versa*.

2. Analysis of 2μm partitioning using the pAS4 stability vector

The stability of pAS4 has enabled its use in determining the contribution of the *REP* genes and *STB* to plasmid partitioning.

A. Role of *REP1* and *REP2* in partitioning of the 2μm circle

The plasmids pAS4Δrep1 and pAS4Δrep2, with deletions of *REP1* or *REP2*, were as unstable as *ARS* plasmids, with loss rates of 15.3±3.1 % and 16.5±2.8 %, respectively. This agrees with previous results that both *REP1* and *REP2* are important for partitioning of 2μm plasmid (Kikuchi, 1983; Jayaram et al. 1985 and Cashmore et al. 1986).

Analysis of the copy number of pAS4 and its *rep*-deleted variants also provides further support for the role of the Rep proteins in mediating plasmid segregation. For

wild-type pAS4, about 95% (see Table 2) of the cells contain plasmid when growing under selective conditions. In this case the average copy number in plasmid-containing cells can be calculated to be about 17, and is similar to the average plasmid copy number per cell (see Table 3). This reflects equal distribution of plasmids among the cells. In the case of the *rep1*- and *rep2*-deleted versions of pAS4, the average copy numbers per cell are similar to that of wild type pAS4, but since only a small percentage of the cells contain plasmid, the average copy numbers per plasmid-containing cell are much higher. This reflects an uneven distribution of plasmids, a pattern identical to that reported for *ARS* plasmids (Murray and Szostak, 1983). This has been observed by others in calculating copy numbers of 2μm-based plasmids (Futcher and Cox, 1984; Kikuchi, 1983; Cashmore et al. 1986). The DNA for the copy-number measurements of the *ADE2* plasmids was extracted from cells that had been grown in medium lacking adenine to select for the presence of the plasmid. Since partitioning is poor for pAS4Δrep1 and pAS4Δrep2, all the plasmids accumulate in a small population of cells and, because of selection, only these cells can grow. This combination of poor partitioning and strong selection is the most likely explanation for the high copy number per plasmid-containing cell observed for these *rep* plasmids. Since the *FLP* gene is disrupted on pAS4Δrep1 and pAS4Δrep2 and cannot increase the copy number of the plasmids in those cells with low copy number, average number of plasmids per cell does not increase and remains the same as that observed for the segregation-competent pAS4.

B. Role of *STB*-proximal in partitioning of the 2μm circle

A surprising finding when yeast were transformed with the *ADE2* stability plasmid lacking the repeat region of *STB*-proximal, plasmid pAS4ΔX, was that transformants were almost as white as those formed by pAS4 transformants. This is in contrast to the transformants containing plasmids pAS4Δrep1 or pAS4Δrep2 in which the *REP1* or *REP2* genes were deleted and the yeast, although able to grow on medium lacking adenine, formed colonies that were a uniform pink colour. Similarly, replacement of the *STB* locus on pAS4 by gap repair with PCR-mutagenized versions of *STB* gave pink colonies much paler than those observed when mutations were introduced into either *REP1* or *REP2*. This colour difference between colonies transformed with *STB*-mutated *versus REP1*- or *REP2*-mutated pAS4 suggests that the absence of *STB*-proximal is not as deleterious for segregation of 2μm circle as is the deletion of either *REP1* or *REP2*. Further support for this conclusion comes from a stability assay which measured the rate of loss of a pAS4 plasmid which lacked this *STB*-proximal repeat region. This plasmid, pAS4ΔX, was lost from a [cir^0] yeast at a rate of approximately 5% per generation (Table 2), whereas pAS4Δrep1 and pAS4Δrep2 were both lost at rates of approximately 16% per generation, a value close to that expected for *ARS*-only plasmids. The colour of the colonies as well as the stability data suggest that absence of *STB*-proximal reduces stability of the plasmid, but does not disrupt segregation to the same extent as deletions or mutations in the *REP* genes. Taken together, these data suggest that sequences other than those between the *Ava*I and *Hpa*I sites of *STB*-proximal

are able to confer a significant degree of mitotic stability on the 2μm circle. This is an unexpected result, since previous studies assessing the stability of 2μm-based plasmids concluded that the tandem repeats of *STB*-proximal are solely responsible for the segregation activity of *STB*, while the flanking distal region merely plays a protective role (Kikuchi, 1983, Jayaram et al. 1985; Murray and Cesareni, 1986). The identity of the sequences that confer this residual mitotic stability on pAS4ΔX are not known. Half of one of the *STB*-proximal repeats is retained in pAS4ΔX on the *AvaI* side of the deletion and may still confer some segregation. Alternatively, recent experiments suggest that sequences in *STB*-distal may contribute more significantly to 2μm stability than has been previously reported. A point mutation that abolishes binding of a yeast host protein to *STB*-distal DNA *in vitro* was introduced into *STB*-distal region of pAS4ΔX. The rate of loss per generation in a [cir^0] host was measured for this plasmid, pAS4M-ΔX, and was found to be about 8%, a value higher than that for pAS4ΔX but still less than that for *ARS*-only plasmids (Dobson, unpublished results). This point mutation does not affect the transcription termination sequences at the end of the *RAF* gene. The ability of a point mutation in *STB*-distal to further reduce the stability of the plasmid pAS4ΔX suggests that *STB*-distal may also contribute to the efficient partitioning of 2μm circle. The stability of pAS4M-ΔX is higher than for the *rep*-deleted plasmids, suggesting that other sequences in *STB*-distal are contributing to plasmid segregation. Again, the results differ from those reported earlier where *STB*-proximal was found to be absolutely necessary for 2μm plasmid partitioning (Kikuchi, 1983) and was suggested to be the only part of *STB* required for plasmid partitioning (Jayaram et al. 1985). The results reported here reflect

the first time that the involvement of *STB* in partitioning has been studied using a test plasmid such as pAS4 that segregates as efficiently in a [cir^0] host as native 2μm. The previous studies used hybrid plasmids containing various 2μm DNA segments. None of these plasmids were as stable as native 2μm and deletions around the *STB* region further decreased their stability (Kikuchi, 1983; Jayaram et al. 1985; Murray and Cesareni, 1986). The reduced stability of these plasmids even prior to the introduction of deletions may have precluded the ability to measure the contribution of sequences other than those in *STB*-proximal. Murray and Cesareni (1986) have shown the presence of a silencer-like element in the *STB*-distal region which represses transcription from upstream promoters. It has also been reported that silencer sequences from the yeast silent mating type locus *HMR* have the ability to confer efficient segregation to a non-2μm plasmid (Brand et al. 1987). This ability of a silencer to perform centromere-like segregation function and the fact that *STB*-distal has silencer sequences further suggest that *STB*-distal might be playing a role in the segregation of the 2μm circle. It would be interesting to see how the segregation function is affected by deletions in *STB*-distal in this test plasmid pAS4.

C. Does Flp protein play any role in partitioning of the 2μm circle?

Since pAS4 is efficiently segregated but has a disrupted *FLP* gene, there was no contribution from this plasmid-encoded protein towards the stability of the plasmid. It further establishes that Flp is not required for partitioning and confirms the results from previous studies that demonstrated that the 2μm circle-encoded *REP1* and *REP2* genes

and the *cis*-acting *STB* locus form the partitioning system (Kikuchi, 1983; Jayaram et al. 1985 and Cashmore et al. 1986).

D. Comparison of copy number of 2μm-based plasmids

The average copy number per cell of pAS4 in a [cir^0] yeast was significantly less than that of native 2μm circle in the isogenic [cir^+] strain. Different values for the average copy number of 2μm have been reported, with some of the differences being due to strain background (Gerbaud and Guerineau, 1980). The difference in copy number observed for pAS4 compared to native 2μm should not be due to strain differences since an isogenic pair was used. There are several other reasons why differences in copy number might have been observed in my experiment. Firstly, copy number was determined with DNA prepared from only one transformant for each plasmid and there may be clone-to-clone variation in copy number (Futcher and Cox, 1984). It is possible that determination of copy number from several transformants would have shown variation in copy number. Secondly, the plasmid pAS4 carries the *ADE2* selectable marker and the initial selection for the transformant required growth in medium lacking adenine. This selection probably sets a value for the copy number of this plasmid which may relate to the requirement for the product of the *ADE2* gene, with higher copy number facilitating growth in a medium lacking adenine. Although the *ADE2* gene should be required at one copy per cell for viability, it must be considered that the expression of the *ADE2* gene from a plasmid may be different from that expressed from the chromosome. Higher copies of the *ADE2* gene may be necessary when expressed from a plasmid to

produce an amount of the product equivalent to that when expressed from a single copy in the genome. This setting of the copy number due to selective pressure imposed by growth in medium lacking adenine is quite different to the Flp-mediated amplification of the 2μm plasmid, which apparently operates in the absence of any selective pressure on the host cell to retain higher copy number. A third possibility to explain the observed plasmid copy numbers is that the presence of the *ADE2* gene on the plasmid pAS4 may not be the selective pressure, but rather that intrinsic factors of the 2μm plasmid, such as the levels of the Rep proteins, may dictate the copy number of pAS4.

3. Analysis of Rep proteins

A. Steady-state levels of Rep1 and Rep2 proteins expressed from native 2μm circle

Another aim of my research has been to determine the function of the 2μm circle-encoded Rep1 and Rep2 proteins. The anti-Rep1 antibody developed and used for the first time in this study detects one major band with an apparent mobility of 50 kDa in protein extracts from [cir$^+$] yeast that is absent in extracts from [cir^0] yeast (Figure 9A). The anti-Rep2 antibody shows a band migrating with a molecular weight of 35 kDa in all protein extracts from [cir$^+$] cells that is not present in the extract from [cir^0] yeast (Figure 9B). This shows that there are proteins whose presence is dependent on the presence of 2μm circle, and also shows the apparent mobility of the Rep1 and Rep2 proteins. The antibodies, however, are not specific for Rep1 and Rep2, since there are other bands detected by Western blotting. The observed size of 50 kDa for the larger of the two

bands detected by the anti-Rep1 antibody in [cir$^+$] yeast is slightly larger than the 43 kDa predicted molecular weight for native Rep1 protein (Hartley and Donelson, 1980). The size is, however, similar to that observed by Wu et al. (1987), where they confirmed that this 50 kDa band was Rep1 by microsequencing. The higher mobility may be due to post-translational modification such as phosphorylation or acetylation, making the protein appear larger than the expected size. It is also possible that the amino acid constitution of Rep1 (high proportion of charged residues) or the structure of the Rep1 protein (it is predicted to have a large rigid coiled-coil domain in its carboxy-terminal region) may contribute to its reduced mobility in SDS-PAGE gels. The smaller [cir$^+$]-specific anti-Rep1 antigenic band is less abundant than the 50-kDa species and is most likely a degradation product of Rep1.

The size of the band recognized by the anti-Rep2 antibodies in [cir$^+$] but not [cir^0] protein extracts is consistent with the 33-kDa predicted molecular weight of native Rep2 protein (Hartley and Donelson, 1980). As with Rep1, a smaller, less abundant species was also detected by the antibody and probably represent Rep2 degradation products. Interestingly, low amounts of an approximately 38-kDa Rep2 antigenic band were also observed in protein extracted from [cir$^+$] cells, suggesting this might be a post-translationally modified form of Rep2.

B. Steady-state levels of Rep proteins expressed from pAS4 and its deleted derivatives

Having ascertained that the 2μm-like plasmid pAS4 segregates as efficiently as 2μm circle, I used Western blot analysis to determine whether Rep1 and Rep2 protein levels expressed from this plasmid were similar to those expressed from 2μm. No significant differences were observed between Rep1 and Rep2 steady-state protein levels expressed from native 2μm circle in the strain AS3 [cir^+] or from the 2μm test plasmid, pAS4, in strain AS3 [cir^0] (Figure 9), despite the approximately eight-fold difference in copy number between native 2μm circle and pAS4 in these yeast (Table 3). First, it suggests that the amount of the Rep proteins produced per plasmid must differ between the two plasmids, although this difference could be at a transcriptional or post-transcriptional level. Secondly, the data also shows that the copy number is not dictating the steady-state level of the Rep proteins. Since both 2μm and pAS4 are able to segregate efficiently, the similarity in levels of the two Rep proteins between the two plasmids may be a reflection of their functions. The amount of the two proteins may be the only significant factor in determining whether the plasmid will be efficiently segregated irrespective of its copy number. The similar level of Rep proteins expressed from quite different numbers of plasmids indicates the existence of feed back mechanisms at the level of transcription or translation for the *REP* genes. Two studies have shown that the *REP1* gene is negatively regulated by both Rep1 and Rep2 proteins (Som et al. 1988; Veit and Fangman, 1988). Veit and Fangman (1988) have also reported repression of the *REP2* gene by the presence of both Rep proteins, as shown by an increase in transcript

level of *REP2* when either *REP1*, *REP2*, or both genes are disrupted. Both studies suggest that this Rep1- and Rep2-mediated regulation of the *REP* genes is probably at the level of transcription. My observation that the levels of the Rep proteins are the same for native 2μm and the stability plasmid pAS4, in spite of the differences in copy number of the plasmids in strain AS3, is consistent with this model of autoregulation of the *REP1* and *REP2* genes. With increasing copy number, the Rep1 and Rep2 proteins would increase in concentration to a steady-state level when they would repress further expression of the *REP1* and *REP2* genes. Above this copy number, there would be no further increase in Rep1 or Rep2 protein levels.

Western blot analysis of Rep protein levels expressed from pAS4 and its *rep1* or *rep2* deletion derivatives showed that in the absence of the Rep1 protein, the Rep2 protein level was comparable to that from wild-type pAS4. In contrast, in the absence of Rep2 protein, the Rep1 protein level was less than that from wild-type pAS4. If the *REP1* gene is repressed by the presence of both Rep1 and Rep2, the absence of *REP2* should derepress *REP1* and lead to an increase in the level of the Rep1 protein. Thus the result showing a decrease in the level of the Rep1 protein in the absence of *REP2* might look contradictory to the previous studies by Som et al. (1988) and Veit and Fangman (1988). Som et al. (1988) have shown that expression of *REP1* directed by its own promoter is repressed by 2μm-encoded products. They have shown this repression by measuring the transcript level of *REP1* integrated as a single copy in the chromosome in a [cir^0] strain and comparing this level to that of transcripts obtained in an isogenic [cir^+] strain. Measuring the β-galactosidase activity produced by the *lacZ* coding region

controlled by *REP1* promoter in strains harbouring different 2μm circle coding regions fused to inducible *GAL* promoters and integrated into the chromosome, the authors concluded that expression of both *REP* genes is necessary for this repression. Based on their results, these authors concluded that the repression of the *REP1* gene by the combined activity of the Rep1 and Rep2 proteins is mostly at the level of transcription. However, they did not look at the steady-state levels of the Rep proteins. The possibility of regulation at the level of translation or the existence of post-translational regulation cannot be ruled out. Thus the reduced level of the Rep1 protein in the absence *REP2* in my study could be due to degradation of the Rep1 protein as a consequence of post-translational regulation. An important point to be noted is the requirement for stoichiometric amounts of Rep1 and Rep2 for efficient partitioning. Insufficient levels of Rep1 or overexpression of Rep2 were both found to impair 2μm plasmid segregation (Cashmore et al. 1986; Dobson et al. 1988). Recently, Scott-Drew and Murray (1998) also showed that the ratios of the Rep proteins affect the localization of the proteins to sub-nuclear foci, suggesting the requirement for a proper regulation of Rep protein ratios. Overexpression of either of the Rep proteins caused reduced localization of the over-expressed protein and at the same time enhanced the localization of the other Rep protein at the foci suggesting that the foci, contain quantitative ratios of Rep1 and Rep2 proteins (Scott-Drew and Murray, 1998). Although both Rep1 and Rep2 proteins have been shown to be nuclear, Rep1 is nuclear only in the presence of Rep2 (Scott-Drew and Murray, 1998). Balanced amounts of the two proteins may be necessary to form a complex and the presence of a stoichiometric amount of Rep2 may be required for the

stability of the Rep1 protein. If this were the case, in the absence of Rep2, Rep1 might be more readily degraded. In contrast, the steady-state level of Rep2 is not affected by the absence of Rep1, suggesting that Rep2 is stable even if unable to complex with Rep1.

C. Regulation of *FLP* by Rep1 and Rep2

It has been suggested previously that the combined activity of the Rep proteins negatively regulates 2μm copy number through the repression of the *FLP* gene (Som et al. 1988; Veit and Fangman, 1988; Reynolds et al. 1987). To test this hypothesis, the plasmid pFLP-lacZ, containing the *E. coli lacZ* gene under the control of the *FLP* promoter, was transformed separately into the strains AS3 [cir^0], AS3 [cir^+] and AS3 [cir^0] [pAS4]. Transformants from all three strains showed equal levels of expression of β-galactosidase as measured by a filter assay. The results are contradictory to those from a similar study by Reynolds et al. (1987), where they showed repression of the *FLP* gene by 2μm-encoded proteins. There are, however, differences in the way the experiments were performed in the two laboratories. Reynolds et al. (1987) used a [cir^0] strain containing multiple copies of the *FLP-lacZ* fusion integrated into the chromosome at the *LEU2* gene. The strains were mated with [cir^0] and [cir^+] strains and β-galactosidase activities expressed by the diploid cells were measured. The expression of a gene integrated at a locus in the chromosome may not be the same as from a plasmid. In our study we have used a *CEN-ARS* plasmid which is maintained as a single copy per cell and a β-galactosidase filter assay was performed. In the Reynolds et al. (1987) study the presence of multiple tandem arrays of the *FLP-lacZ* gene fusion would have provided a

substrate for variable removal of some of the reporter cassettes by intra-chromosomal recombination. In the [cir$^+$] yeast, this recombination might have been enhanced by the presence of multiple copies of the 2μm plasmid with homology to the *FLP* promoter region on the integrated reporter cassettes. This plasmid/chromosome recombination would not occur in the [cir^0] diploids used in their study. In the same study, the [cir^0] haploid containing the integrated *FLP-lacZ* fusions was also mated to a [cir^0] strain where both the *REP1* and *REP2* genes had been integrated into the chromosome at one or three copies per cell. In this experiment, no repression of the *FLP* promoter was observed. However, in that case the *REP* ORFs were controlled by their own promoters and even at three copies/cell would be at a lower copy number than on the 2μm plasmid. In my experiment, haploid [cir$^+$] strains were used which had normal levels of 2μm plasmid and one copy of the plasmid containing a single *FLP-lacZ* fusion gene, and thus should be more representative of the normal levels of expression of the *REP1* and *REP2* genes. No repression of *FLP* was observed. Reynolds et al. (1987) also reported repression of the *FLP* gene when they used strains overexpressing both *REP1* and *REP2* from *GAL* promoters, but again the Rep protein levels were unlikely to be physiologically relevant. The *FLP-lacZ* fusion used in the Reynolds et al. (1987) study had 350 codons of the *FLP* gene and it is therefore possible that the lower level of β-galactosidase activity observed in their [cir$^+$] diploids as compared to their [cir^0] diploids may have been due to post-transcriptional effects. In contrast, we have used a precise fusion of the *FLP* promoter to the *lacZ* gene, which would only allow us to see transcriptional effects. To summarize: 1. *REP1* and *REP2* expressed from their own promoters at one to three

copies per cell (Reynolds et al. 1987), or at 130 copies per cell as in the strain AS3 used in our studies, does not produce transcriptional repression of the *FLP* promoter. 2. Overexpression of both *REP1* and *REP2* using a *GAL* promoter produces transcriptional or post-transcriptional repression of *FLP* expression (Reynolds et al. 1987). 3. The normal 2μm level of *REP1* and *REP2* may give post-transcriptional repression of *FLP* (Reynolds et al. 1987). The available data therefore do not provide clear-cut support for the simple model by which Rep1 and Rep2 repress transcription of the *FLP* promoter to limit copy number amplification. Clarification will depend on identifying proteins that do interact directly with the *FLP* promoter.

4. Functional domains of Rep1 and Rep2

Mutational analyses and two-hybrid genetic assays were performed to study the roles of the Rep1 and Rep2 proteins in partitioning of the 2μm circle. To date little is known about which portions of the two Rep proteins are required for their function and how these proteins are involved in the actual mechanism of partitioning. Both proteins are localized to the nucleus (Ahn et al. 1997; Scott-Drew and Murray, 1998). The nuclear targeting signal for both proteins seems to be near the carboxy-terminus, within the terminal 25 amino acids for Rep1 and terminal 20 amino acids for Rep2 (Velmurugan et al. 1998). The interaction between Rep1 and Rep2 has also been shown both by two-hybrid analysis (Ahn et al. 1997; Blomqvist unpublished results) and by *in vitro* baiting assays (Ahn et al. 1997; Scott-Drew and Murray, 1998; Velmurugan et al. 1998; Joyce Chew, unpublished results). The Rep1 and Rep2 proteins have also been shown to self-

associate (Ahn et al. 1997; Velmurugan et al. 1998; Scott-Drew and Murray, 1998; Blomqvist, unpublished results, Joyce Chew, unpublished results).

The results of the two-hybrid analysis presented in this thesis (see Results section 7), suggest that the two Rep proteins interact with each other. The two-hybrid assay also suggested that Rep2 had homotypic interactions (Kristina Blomqvist, unpublished results). Interaction of Rep1 with itself could not be detected by this assay, although it has been shown by Ahn et al. (1997). The levels of expression of the $Gal4_{DB}$-Rep1 and $Gal4_{AD}$-Rep1 fusion proteins were similar to the level of the Gal4-Rep2 fusion proteins as judged by Western blotting. Also, the two Gal4-Rep1 fusions were capable of giving a two-hybrid interaction when co-expressed with the appropriate Gal4-Rep2 fusion proteins, indicating that there was no problem with expression of fusion proteins from these plasmids. It is possible that the $Gal4_{DB}$ portion of the $Gal4_{DB}$-Rep1 fusion used in our study interferes with the Rep1-Rep1 interaction. Ahn et al. (1997) used LexA-Rep fusions with LexA as the DNA-binding component in their two-hybrid assays. Using a different DNA-binding domain may have facilitated the interaction of Rep1 with itself. It is also possible that native Rep1 protein present in the two-hybrid host Y153, used in our studies, interferes with the $Gal4_{DB}$-Rep1 fusion proteins.

To determine the domains of interaction of the two Rep proteins, I looked at the interactions of various deletion derivatives of both Rep1 and Rep2 using the two-hybrid assay. The results suggest that Rep1 and Rep2 interact with each other through the amino-terminal regions of the proteins. The carboxy-terminal truncation of Rep1, $Gal4_{AD}$-rep1Δ130-373, gave a better activation of the reporter gene *lacZ* when co-

expressed with $Gal4_{DB}$-Rep2 as compared to the level when full-length Rep1 was co-expressed with the same Rep2 fusions. It is possible that removal of the carboxy-terminal coiled-coil domain makes the Rep1 protein more soluble or changes the tertiary structure of the Rep1 protein thereby improving the interaction with Rep2 in the two-hybrid experiment. None of the other deleted forms of Rep1 expressed as $Gal4_{AD}$ fusions could activate the *lacZ* reporter gene when co-expressed with full-length Rep2 expressed as a $Gal4_{DB}$ fusion. Of the different deleted forms of Rep2, rep2Δ1-14 and rep2Δ59-296 both gave β-galactosidase activity when co-expressed with full-length Rep1 fusions. The rep2Δ1-14 fusion also showed interaction with full-length Rep2. Summarizing the results of the two-hybrid assay, it can be concluded that the first 129 amino acids of Rep1 are sufficient for its interaction with Rep2. The first 76 amino acids of Rep1 are required but not sufficient for its interaction with Rep2. The first 58 amino acids of Rep2 are sufficient for its interaction with Rep1 but not with itself, and the first 14 amino acids of Rep2 are dispensable for its interaction with either Rep1 or Rep2.

The results of the two-hybrid assay were followed by *in vitro* baiting assays (see Materials and Methods section 5C for details of experiments). The *in vitro* assay shows direct interaction between the Rep proteins as well as the self-association of Rep2. Self-association of Rep1 was also detected. Since the *REP1* ORFs were subcloned directly from the two-hybrid vectors into the *E. coli* expression vectors used for the baiting assay and the proteins produced were shown to interact *in vitro*, the lack of interaction of the $Gal4_{DB}$-Rep1 fusion with the $Gal4_{AD}$-Rep1 fusion may have been due to the Gal4 moiety fused to the amino terminus of Rep1. This lack of interaction could also

be due to competition with native Rep1 *in vivo*. The *in vitro* results corroborate the two-hybrid data reported here that show the first 129 amino acids of Rep1 are sufficient for association with Rep2 while the first 76 amino acids of Rep1 are required for this interaction. It also confirms the interaction between Rep1 and first 58 amino acids of Rep2. The *in vitro* baiting assays also showed that a truncated Rep2 protein containing only the carboxy-terminal 238 amino acids was capable of interacting with both Rep1 and Rep2, while the first 58 amino acids of Rep2 could interact weakly with Rep2 (Joyce Chew, unpublished results). Although the results of the baiting assay indicate direct interactions between the Rep proteins and provide additional information about the domains of the proteins required for interaction, it cannot be concluded from the data whether these interactions are modulated by any host factors under *in vivo* conditions.

The data from the plasmid segregation assay suggest that although the amino-terminal 129 amino acids of Rep1 are sufficient for its interaction with Rep2, this region alone is not capable of mediating proper plasmid segregation. The carboxy-terminal 25 amino acids of Rep1 contains a nuclear targeting signal (Velmurugan et al. 1998), so loss of these sequences may account for the inability of the $Gal4_{AD}$-Rep1Δ130-373 truncated protein to provide segregation function. However, it is equally likely that the deleted region, which bears similarity to intermediate filament proteins (Wu et al. 1987), may play some other role required for plasmid segregation.

A. Mutant Rep proteins

As an alternative approach to analyzing Rep proteins, mutated *REP1* and *REP2* genes that could not support 2μm plasmid segregation were generated by PCR and identified by their inability to function when they replaced wild-type *REP1* and *REP2* in the stability plasmid pAS4 in a [cir^0] yeast strain. This approach allowed me to identify a number of *rep1* and *rep2* mutants. The results of the plasmid segregation assay for the *rep1* and *rep2* point mutants (Tables 9 and 10) show that all mutations identified in each *REP* gene on the basis of the colony colour assay do affect the segregation of the 2μm stability vector. It was important to demonstrate that the pink colour of the colonies in the *ade2* strain AS3 [cir^0], which was used to determine this segregation defect of the plasmid phenotypically, was not due to altered expression of the *ADE2* gene in the plasmid pAS4, since this was another possible explanation for the colony colour. The data from the segregation assay confirmed that it was indeed plasmid mis-segregation that resulted in the pink phenotype.

The mutations in these genes that might be expected to abolish function can be grouped into several categories.

Mutations that lead to the introduction of a nonsense codon give rise to proteins that were variably carboxy-terminally truncated depending on where the STOP codon was introduced. In all cases, for both Rep1 and Rep2 these proteins would now lack nuclear targeting. It has been shown by Velmurugan et al. (1998) that carboxy-terminal deletions as small as 25 and 20 amino acids in Rep1 and Rep2, respectively, abolished the nuclear localization of the Rep proteins as indicated by Green fluorescent protein

(GFP)-Rep fusions. The authors also demonstrated that different carboxy-terminal deletion derivatives of Rep1 fused to GFP protein could not restore stability of a 2μm-based test plasmid containing *STB* and *REP2* in a [cir^0] host, while full-length Rep1 fused to GFP could. Similarly, full-length Rep2 fused to GFP could stabilize a 2μm-based plasmid containing *STB* and *REP1* in a [cir^0] host. However, various carboxy-terminal deletions of Rep2 fused to GFP protein could not restore stability of this test plasmid. Addition of an SV40 NLS (nuclear localization signal) to the shortest carboxy-terminal deletions of Rep proteins, in which 25 and 20 amino acids were removed from Rep1 and Rep2, respectively, could localize the proteins to the nucleus as well as restore the stability of the test plasmids. Based on the data, Velmurugan et al. (1998) have suggested that the extreme carboxy-terminal regions of the Rep proteins are not directly involved in mediating plasmid segregation. Instead, they conclude that these portions of the Rep proteins affect function indirectly by playing a role in targeting these proteins to the site of action, which is the nucleus.

Premature STOP codons, those that are near the 5' end of the ORF, can also cause low steady-state levels of proteins due to nonsense-mediated decay of RNA (Ruiz-Echevarria et al. 1996). In other words, the stop codon in the RNA targets the RNA for degradation, reducing the amount of RNA and thereby decreasing the protein level. Even if the RNA is stable, the truncated protein may be unstable and have a shorter half-life than the normal Rep protein.

Missense mutations might also reduce protein levels if they change amino acids that affect folding, subcellular localization of the mutant protein, or its ability to interact with other proteins.

Both nonsense and missense mutations were obtained in my *REP1* and *REP2* mutagenesis experiment.

B. Rep1 mutants

Results of the Western blot analysis for the *rep1* mutants show that for most mutations which create premature stop codons, such as A9-1, A5-1, B9-1 and C10-1, no protein could be detected. For these point mutants, the defect in segregation is most likely due to lack of expression of the protein, just as is seen for plasmid pAS4Δrep1, where the *REP1* gene is deleted. The low abundance of the Rep1 protein from the mutants *rep1 C11-1*, *rep1 D3-1* and *rep1 D4-1* might be responsible for the lack of segregation of the plasmid for these cases. In contrast, the level of the Rep1 protein expressed from the mutants *rep1 A11-1*, *rep1 B6-1* and *rep1 B10-1* is similar to that expressed from unmutated pAS4. The mobility of the protein expressed from *rep1 A11-1* is, however, slightly greater than that observed for native Rep1. This is not surprising, as the mutation in *rep1 A11-1* created an amino acid change from lysine to glutamate at position 45 and probably accounts for the observed gel mobility shift. This is a change from a positively charged residue to a negatively charged one and can affect the ionic environment of the protein, leading to a change in the secondary or tertiary structure altering their migration on SDS-PAGE gels. The Rep1 protein expressed from

rep1 B10-1 is missing about 104 amino acids from the carboxy terminus. Since carboxy-terminal deletions reduce the capacity of the Rep proteins to be localized to the nucleus (Velmurugan et al. 1998), the absence of the nuclear localization signal might be sufficient to cause the reduced function for the mutant *rep1B10-1*. Similarly, the *rep1* mutation W262STOP is missing 111 amino acids from the carboxy end and is probably incapable of being targeted to the nucleus. Thus for these four mutations it is not the level of the expressed protein, but the changes in the amino acid residue within the protein, that is affecting the function. The steady-state level of the mutant protein expressed from *rep1I338T* is also similar to wild-type. It is interesting to note that the *rep1* mutant *rep1I338T* is a single point mutant, in which a threonine is substituted for an isoleucine at position 338. This change in amino acid is close to the carboxy end of the protein but not within the terminal 25 amino acids reported to be required for nuclear targeting (Velmurugan et al. 1998). Therefore it is unlikely that its loss of function is due to defect in NLS. Instead, this substitution probably affects some other role of this domain.

The steady-state level of the Rep2 protein is similar to wild-type in all these *rep1* mutants. This result is similar to that observed for pAS4Δrep1, where complete absence of the Rep1 protein did not seem to affect the level of the Rep2 protein. Thus the steady-state level of the Rep2 protein is not affected by any changes in abundance of the Rep1 protein.

C. Rep1 proteins of *S. cerevisiae* 2μm circle and other 2μm-like yeast plasmids

Proteins with functions similar to *S. cerevisiae* 2μm circle Rep1 and Rep2 are encoded by other 2μm-like plasmids in several closely related yeast species (Utatsu et al. 1987). A limited degree of amino acid sequence similarity is shared between the 2μm circle Rep1 and the Rep1-equivalent proteins encoded by the 2μm-like plasmids, while the Rep2 equivalent proteins are too dissimilar to be aligned (Murray et al. 1988). An alignment of the amino acid sequences for three of these Rep1-like sequences, namely the products of the B ORF of plasmid pKD1 from *K. drosophilarum* (Chen et al. 1986), the P ORF of plasmid pSR1 from *Z. rouxii* (Toh-e et al. 1982), and the ORF B of pSB3 from *Z. bisporus* (Toh-e et al. 1984), with that of *S. cerevisiae* 2μm Rep1 was used to identify three conserved Rep1 domains (Murray et al. 1988). Domain 1, in the amino-terminus, shows about 50% amino acid similarity across a 60 amino acid stretch, while the other two domains show about 30% similarity (Murray et al. 1988).

Sequences for several other 2μm-like plasmids have now been reported. A CLUSTAL W (1.7) multiple alignment of six Rep1-like proteins was performed (Figure 16). These include products of the P ORF of plasmid pSR1 from *Z. rouxii* (Toh-e et al. 1982), B ORF of plasmid pSB2 from *Z. bailii* (Utatsu et al. 1987), D ORF of plasmid pKW1 from *K. waltii* (Chen et al. 1992), B ORF of plasmid pTD1 from *T. delbrueckii* (Blaisonneau et al. 1997), B ORF of plasmid pKD1 from *K. marxianus* (Chen et al. 1986) and 2μm Rep1 from *S. cerevisiae*. It is possible to define domains that are conserved

```
ZrouxiiP        ----------MFTSQDAR-DSRPDRE------------LRMMNDVLMTYPYTVIHLPAQN
ZbailiiB        ----------MFSREEVR-ASRPTKE------------MKMIFDVLMTFPYFAVHVPSKN
KwaltiiD        ----------MSEAQKIPKITDHDGY------------HNCLKECCLVGTVDVLYVPQLK
Torula.B        MTDNLHLLPPLGLNRTTPSWADPVVQDAIETPPINKEVRAYLSECFFLLPHRVIYCPEHQ
Scp1REP1        --------------------MNGERL------------LACIKQCIMQHFQPMVYDESRC
KmarxiaB        -----MSAPSESSETAAPLLSDLSPEEQ-KGVQQVREIYRALLDSFKFKNEYNAYDPLNN
                                                      : :          :

ZrouxiiP        MLSTAKGMVNIAENYRDYPILAIFYVKYLMKKL--PYGVIPVNLEWPEPYVVLNTILKRL
ZbailiiB        ILITPKGTVEIPENYQNYPILAIFYVKYLMKKN--PYDLLPSTVNWPEPYVVVNTITKRF
KwaltiiD        ELVSPYGSTKLPKEYSEHPQLALFYYSYMINKNTASAARSELTFEWPKPYVVLNTIVKRL
Torula.B        CVVGMRGYMNVPDPYAEYPMLAIYFAKYRLRKY--PFDILRNDITWPEPYVVLNTIMRRL
Scp1REP1        VIETTRGTFPVPDNYKKYKTLAFAFVGHVLNTD--DTPVIEKELDWPDPALVYNTIVDRI
KmarxiaB        TIITQHGSYEVPEAFAQCTYLAQIYVSYKINSL--PYQTYIKDLEWISPAEVYKLIMERL
                 :    *   :.. : .   **  :  : :..            . * .*  * : *  *:

ZrouxiiP        KEHKFFANKDKED-FAERLHKLIAPDVSIPESRKDEILGQQKKERV--VTKTINENFLDP
ZbailiiB        QDHKLFANKNADV-YVERLQNAIASGIKIPESKKNERLGQPKKTKN--VTKEIEETFIDA
KwaltiiD        ESHIFVVRNKYTQ-LPSIVKKHIGEGIECPTNDSIEELTPFLDPRK--RHPIIIR--LGP
Torula.B        RTHRFLKGNADAASIPDDIRKVIAPNLDIPSGMEGELLEVPYSERK--LGANRVMKVFEG
Scp1REP1        INHPELSQFISVA-FISQLKATIGEGLDI--NVKGTLNRRGKGIR---RPKGVFFRYMES
KmarxiaB        QKNKYFLRKQVQA--MEKIKVLLCPSPELLENNYVDDNLKISSHKVTQRHPEKVYDLMTY
                  :  .           . ::  :  . .   .             :            :

ZrouxiiP        VNARPRLQRFFEKLHNGTLVENLEVGLCKVEILVSSKAMLG-QSFKLQIMAANVRELWVG
ZbailiiB        TNARKELDEYFRKLQDGTLTGDLEGGLCKVKTLISCKALFGGHTQELQFMATNVRKVWIG
KwaltiiD        K--REALD-FFTELTDLEAAQSKYENLSKLEVRISCRQWFG-SSLDVERSARTIRRLWLL
Torula.B        QDARLEMERFFDELLGSSHSELYPD--SSVIRVVSDYPLRS--QADVIVFSEYLRKLWYI
Scp1REP1        PFVNTKVTAFFSYLRDYNKIASEYHNNTKFILTFSCQAYWA-SGPNFSALKNVIRCSIIH
KmarxiaB        QEIADGIDDFFSLLEMPNLSLAFVN-KTSIKLNISCSGANNHIGSFIGRTARTIRHYWIK
                      :  :*  *              ..   .*          .      :*
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Figure 16

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ZrouxiiP        EMVCNMITN-----ETDYGFDE--GGGDDDEGSSVEVQNSQSASPGQDQEAQRAPEAPET
ZbailiiB        EIVCGMVSNKNAIDDNDLEEEERNASGEQTTTAREESEALDTTSNGLD-ALNTQINAIET
KwaltiiD        EMVMSRAES-----------------IQQAAAAEDQEPTVDNESQGES--SGSGPPIDH-
Torula.B        EKILVLMNMVP-----DSVRDR--PDLEDPSIVWDLLEDLQRQPVYEP-SQNERMTQVAA
Scp1REP1        EYISKFVER---------EQDK--GHIGDQELPPEEDPS-RELNNVQHEVNSLTEQDAEA
KmarxiaB        NAILEFADY--------KKKTVPYPFVGPPRDPGLFEHIVDDCSTGAP--INWDDFSIDS
                : :

ZrouxiiP        SSQLFDKIVSALQDDPDSAKAQLGQCRKLASYLLSHKREQEDFFMQTKDTRARLYLDLKG
ZbailiiB        EESFWEAIR-ALHNELRTSPTQLEECRKAAVFLLGHKKILQTFTKQKDTARALFYINLKE
KwaltiiD        -AAFWDELS------------QLERNQLLLMSMVTKRTLSQDYLIALGEPKSF-------
Torula.B        LLKLWPDALEEFQ-------QQRKDCRTFIYSKIRAWLPKKNKVKIYRGTSVLEDGSVKV
Scp1REP1        DEGLWGEIDSLCEKWQSEAEDQTEAE-IIADRIIGNSQRMANLKIRRTKFKSVLYHILKE
KmarxiaB        -YASYVTVMEHLEEVVELTDRHRRVIEYLGMYIASDLHEEGKLTRARKLDRTTLFHTVRD
                    :                   :

ZrouxiiP        CLGSSWKFSIYRGVRCKQNGQLKVSLKPSNSGHLSGFWVNIKMTSQGNTL-DDIRLQVRC
ZbailiiB        CLGTSWNLEYTEASDAR-----KMAIK----GELQN-----------------------
KwaltiiD        ------------------------------------------------------------
Torula.B        SLNFTEYSYIKSAYISFR----PVSSRE-APHHPEKVLATFECRHVTREG-SESHEEDAG
Scp1REP1        LIQSQGTVKVYRGSSFSHDS-IKISLHY-EEQHITAVWVYLTVKFEEHWKPVDVEVEFRC
KmarxiaB        VLLHDGSYTRHKGVRCLDNGSVRVSIRL-KHRQLTATWIELEPIIENGEV-KDVMFKLST

ZrouxiiP        DILGKDTNRRGGSEKDPEDQSETESSG
ZbailiiB        ---------------------------
KwaltiiD        ---------------------------
Torula.B        DAGSSE---------------------
Scp1REP1        KFKERKVDG------------------
KmarxiaB        RVQYTEENE---NETRPESSSE-----
```

Figure 16 (contd.)

Figure 16. Alignment of the amino acid sequences of *S.cerevisiae* Rep1 and Rep1-like proteins from closely related yeast species using the CLUSTAL W (1.7) multiple sequence alignment programme. Sequences were as follows: the P ORF from *Z. rouxii* plasmid pSR1 (Toh-e et al. 1982), the B ORF from *Z. bailii* plasmid pSB2 (Utatsu et al. 1987), the D ORF from *K. waltii* plasmid pKW1 (Chen et al. 1992), the B ORF from *T. delbrueckii* plasmid pTD1 (Blaisonneau et al. 1997), the B ORF of *K. marxianus* plasmid pKD1(Chen et al. 1986), and the *REP1* ORF from *S. cervisiae* 2μm Scp1.

between 2μm Rep1 and the other Rep1-like proteins from other yeast plasmids. Two domains, one between amino acids 30 and 120 and the other between amino acids 147 and 206 are shown in Figure 17. The presence of the conserved domains might indicate some similarities in structure and function of the various Rep1 proteins. Although the Rep1 proteins are unable to substitute functionally for one another (Murray et al. 1988), substitution of individual domains has not been attempted. In the case of *S. cerevisiae* 2μm circle, domain 1 at the amino terminus of the Rep1 protein includes the region that we have shown to interact with Rep1 and Rep2. The results of the two-hybrid assays and *in vitro* baiting assays taken together show that the first 129 amino acids of Rep1 are required for association with both Rep1 and Rep2. Deletion of the first 76 amino acids abolishes association with Rep2 but not with Rep1, only impairing the Rep1 interaction slightly. The presence of a conserved domain might suggest a common mode of interaction between Rep1 proteins and their species-specific Rep2 analogues. However, the Rep2 analogues do not show any sequence conservation and they cannot complement the segregation defect of *rep2* derivatives of any 2μm-like plasmids apart from their own. The conserved domains may perform other functions such as self-association or maintaining a particular secondary structure. Since *in vitro* experiments have shown Rep1 to self-associate (Joyce Chew, unpublished data; Velmurugan et al. 1998), and the Rep1-Rep1 interacting domain is also within the first conserved block of amino acids, therefore this amino-terminal domain might be expected to be conserved since the Rep1 proteins are themselves conserved.

Some of the point mutations in Rep1 generated by mutagenesis are present in these conserved domains (Figure 17). The mutations that alter amino acids in domain 1 are K45E (*rep1 A11-1*), I30T, K47R (*rep1 C11-1*) and F53S (*rep1 B10-1*). The mutant *rep1 B6-1* has three mutations, V98A, F174S and K297E, of which the V98A substitution does fall into conserved domain 1. However, this mutation, V98A, alone did not affect segregation function of the 2μm plasmid. Although the substitution F174S is within conserved domain 2 and a phenylalanine is found at this position in the various Rep1-like proteins in other yeasts, the mutation F174S by itself also did not affect segregation of the plasmid. The third mutation in *rep1 B6-1*, K297E, is not within a conserved domain and a lysine is not found at this position in various Rep1-like proteins from other yeasts. This mutation was not analyzed separately, but it seems likely that this substitution might be responsible for the loss of function. Two other *rep1* mutants that have amino acid changes within conserved domain 2 are *rep1 D3-1*, with the mutation I202T, and *rep1 D4-1*, that has the mutation C195R. While the cysteine at position 195 seems to be unique to *S. cerevisiae* Rep1 and is not conserved among the other species, an isoleucine is found at the position equivalent to 202 in Rep1 proteins from other yeast species. Any changes in this conserved domain may affect the function of protein.

The lysine at position 45 in 2μm circle Rep1 is within a conserved domain, but none of the other five Rep1-like proteins have a positively charged residue at a similar position. However, this residue is important for Rep1 function, as substituting it with glutamate abolishes the ability of Rep1 to mediate efficient 2μm plasmid segregation. The lysine at position 47, also within this conserved domain 1, when substituted with

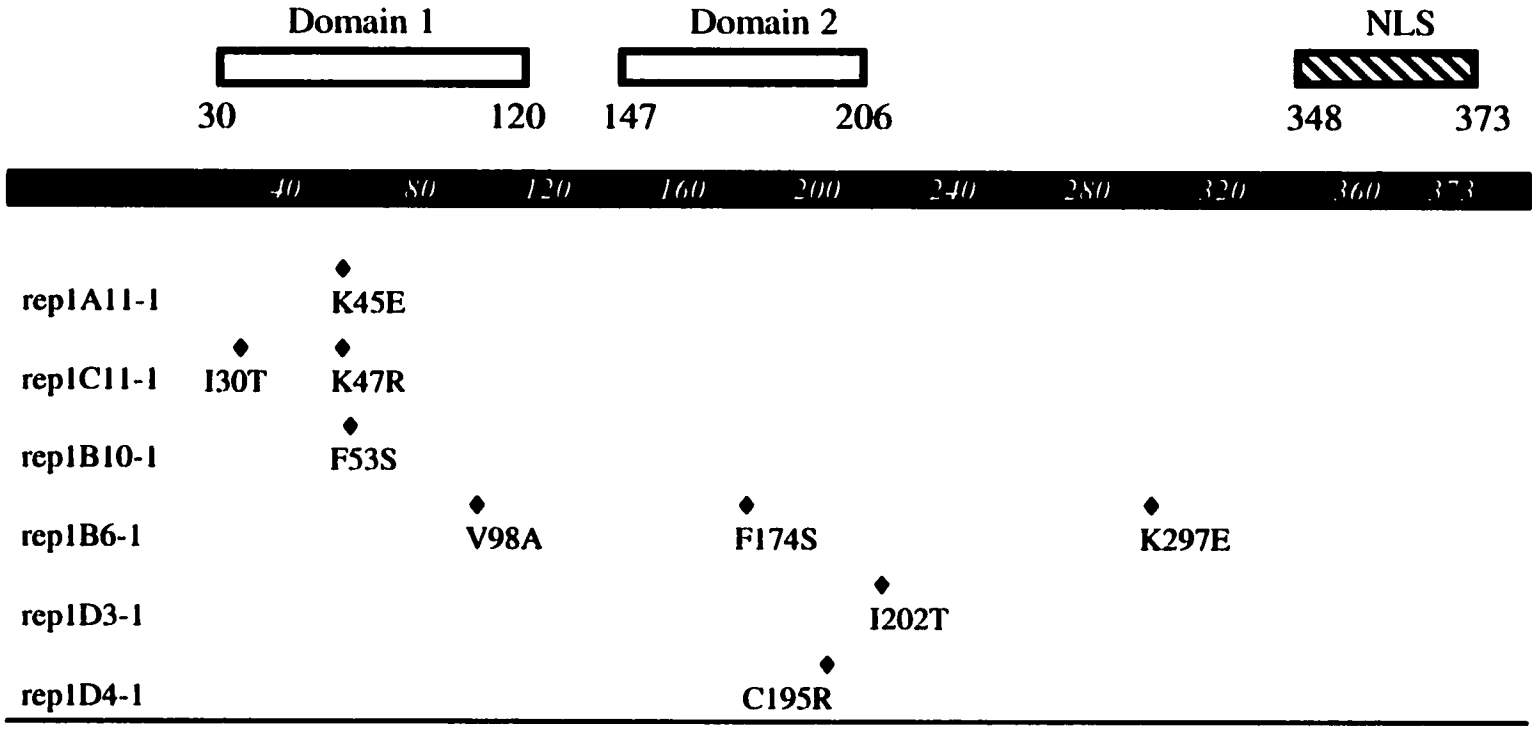
Domain 1
Domain 2
NLS
30
120
147
206
348
373
40
80
120
160
200
240
280
320
360
373
rep1A11-1
K45E
rep1C11-1
I30T
K47R
rep1B10-1
F53S
rep1B6-1
V98A
F174S
K297E
rep1D3-1
I202T
rep1D4-1
C195R

Figure 17.

Figure 17. Distribution of deleterious amino acid changes in Rep1 relative to Rep1 putative conserved domains. Two conserved domains (domain 1 between amino acids 30 and 120, domain 2 between amino acids 147 and 206) have been predicted based on CLUSTAL W (1.7) alignment. The amino acids included in each domain are shown using numbering of amino acids in *S. cerevisiae* Rep1. The *rep1* mutants isolated in my mutational screen are shown on the left, with their position and corresponding changes in amino acids included in each domain as indicated. Nuclear localization signal (NLS) is indicated by a striped box (Velmurugan et al. 1998).

another positively charged residue, arginine, did not affect segregation function. The substitution of the isoleucine at position 30 with threonine could not alone disrupt segregation of the plasmid. It is the combined effect of two mutations, I30T and K47R, that affects function. The phenylalanine at position 53, while not conserved among all Rep1 proteins, does lie within one of the more conserved domains of Rep1. However, when this phenylalanine was substituted for tyrosine or cysteine in the 2μm Rep1, the plasmid segregated properly. Even if not conserved among themselves, these residues are all found within a conserved region of Rep1 protein. Since this domain 1 includes the region of the Rep1 protein that the two-hybrid analysis and *in vitro* baiting assay show is required for its interaction with Rep1 and/or Rep2, this segregation deficiency could be due to lack of interaction of the mutant Rep1 with itself or with Rep2.

To further define the domains in Rep1 and Rep2 required for interaction, a two-hybrid assay was carried out with *rep1* mutants. In brief, all but two *rep1* mutants with amino acids altered in the conserved domain 1, namely *rep1B10-1* and *rep1 C11-1*, showed interaction with full-length Rep2. The *in vitro* assays, however, showed weak interaction of these mutants with Rep2. The Rep1-Rep1 interaction was not affected by these mutations, as indicated by the *in vitro* baiting assays (Joyce Chew, unpublished results). However, it must be considered that the two-hybrid experiment is an *in vivo* assay and the proteins are in their normal physiological state. In contrast, the baiting assay is an *in vitro* experiment where the proteins may not be properly folded. Also, in the case of two-hybrid assays, native Rep1 and Rep2 are present *in vivo* and may compete

or assist with interactions. The *in vitro* results are consistent with the two-hybrid data for *repIA11-1* where this mutant Rep1 protein has been shown to interact with wild-type Rep2. The K45E mutation therefore does not abolish interaction between Rep1 and Rep2 but may affect the secondary or tertiary structure of the Rep1 protein. The lack of interaction between the *rep1* mutant *C11-1* and Rep2 in the two-hybrid assay could be due to low level of expression of this mutant protein (see Figure 11), therefore affecting segregation function.

As described above (see Section III 7C), the mutant Rep1 protein expressed from *rep1 B10-1* (F53S and Q269X) did not interact with full-length Rep2 in the two-hybrid assays. Since the Rep1 protein level is similar to wild-type, the lack of interaction of this mutant Rep1 and Rep2 in the two-hybrid assay can not therefore be due merely to low steady-state levels of the mutant protein making the interaction impossible to detect. Instead, the result suggests that either the F53S mutation or the loss of the carboxy-terminal portion of Rep1 impairs its association with Rep2. Since it was not possible to separately analyze the mutation F53S, it could not be determined whether this mutation alone was sufficient to abolish both interaction with Rep2 and plasmid segregation. Although two other mutations, F53C and F53Y, did not affect plasmid segregation, it is possible that a change from phenylalanine to serine at the same position disrupts interaction with Rep2. However, it could not be concluded from this particular point mutation whether this phenylalanine residue at position 53 is indeed important for interaction with Rep2.

To summarize, it can be concluded that conserved domain 1 is important for Rep1 protein to function, as illustrated by the detrimental effects of amino acid substitutions at

positions 30, 45 and 47. Similarly, there is a second conserved domain (amino acids 147 to 206) that is important for Rep1 protein function, whose role is demonstrated by the effect of substitutions at positions 195 and 202 within this region.

D. Rep2 mutants

Figure 18 shows the distribution of the rep2 mutants relative to the domains required for Rep protein interaction as observed in two-hybrid and *in vitro* assays. No Rep2 protein was detected for mutant *rep2 B1-1*, which is not surprising considering that the mutation (K8STOP) created a premature stop codon at the extreme 5' end of the coding region. The absence of any anti-Rep2 immuno-reactive protein species for the mutants *rep2 A2-1*, *rep2 A4-2* and *rep2 A5-2* seems most likely due to the mutations affecting half-life of the protein. For the mutant *rep2 A4-2*, it is at present unknown whether it is the change from leucine to glutamine at position 10 or from lysine to glutamate at position 95, or a combination of both mutations, that is affecting the abundance of the protein. Although the change from leucine to glutamine is a non-conservative change, a change from positively charged lysine to negatively charged glutamate would seem more likely to affect the protein structure. The significantly lower level of Rep2 protein expressed from *rep2 A8-2*, rather than the amino acid change *per se*, may be responsible for its inability to supply partitioning function. The slightly greater mobility for the protein could again be explained based on a substitution of a positively charged lysine residue with a negatively charged glutamate. The levels of the Rep2 protein expressed from *rep2* mutants *A1-2*, *B9-1*, *C1-1* and, *E12-1* are comparable to wild-type. The mobility of the Rep2-antigenic band for *rep2 A1-2* is higher than the

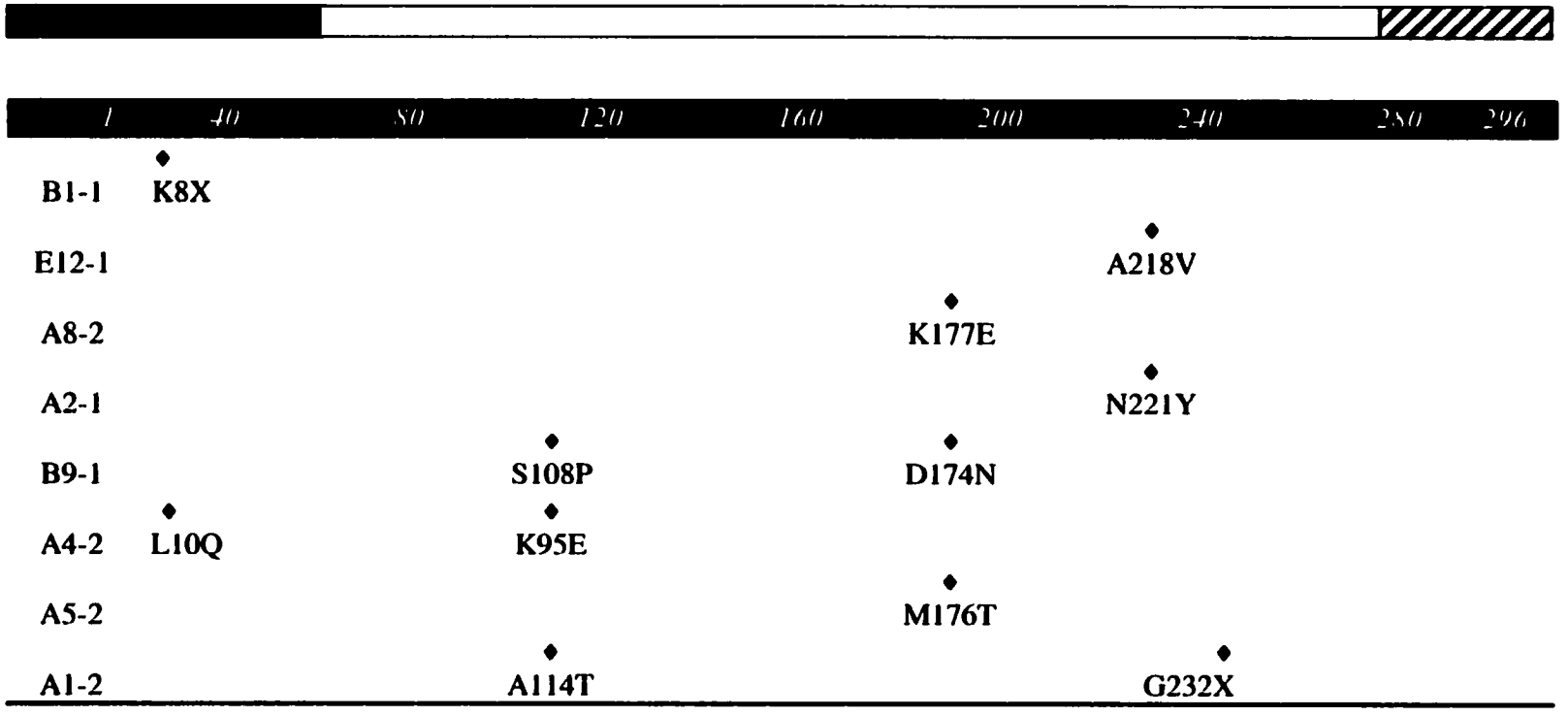
1
40
80
120
160
200
240
280
296
B1-1
K8X
E12-1
A218V
A8-2
K177E
A2-1
N221Y
B9-1
S108P
D174N
A4-2
L10Q
K95E
A5-2
M176T
A1-2
A114T
G232X

Figure 18.

Figure 18. Distribution of Rep2 mutants. The two domains of Rep2 used in this study are shown at the top, the black box indicating amino acids 1 to 58 and the white box amino acids 59 to 296, with the nuclear localization signal at the extreme carboxy-terminal shown in black and white stripe. Domain (1-58) includes the region interacting with Rep1. Domain (59-296) is the region interacting with Rep1 and Rep2. DNA-binding activity has also been shown for the latter domain. The mutants are shown on the left with the corresponding alterations in amino acids indicated.

native Rep2 and the size is consistent with that predicted, based on the presence of a premature stop codon. In this case it is possible that the deletion of the nuclear targeting signal (Velmurugan et al. 1998) due to the creation of the premature stop codon is responsible for loss of function. In the case of the mutant *rep2 E12-1*, although the change from alanine to valine is a conservative substitution and steady-state level of Rep2 protein is similar to wild-type, it is possible that this alanine is a critical residue and therefore this change is abolishing function of the protein. Data from *in vitro* baiting assays show that amino acids 58-296 of Rep2 are capable of mediating interaction with both Rep1 and Rep2 (Joyce Chew, unpublished results). Any mutation in this portion of Rep2, such as A218V, might abolish either or both these interactions. A Southwestern assay showed that this same portion of Rep2 has DNA-binding activity and can bind equally well to a radiolabeled DNA fragment containing the repeat region of *STB* or to another unrelated repeated DNA sequence (Dobson, unpublished data). If Rep2 is responsible for binding to *STB*, any mutation in that region that can interfere with this binding may also abolish segregation function. The Rep2 protein expressed from *rep2 B9-1* has a mobility lower than native Rep2 with an apparent molecular weight of about 45 kDa. In this case it is the combined effect of two mutations, S108P and D174N, that is impairing Rep2 function (see Section III 5D). While a substitution of aspartate with asparagine is a non-conservative change and might be expected to be deleterious, the change from serine to proline could also affect the secondary structure of the protein.

The two-hybrid results show that most *rep2* mutants that gave levels of proteins detectable by Western blotting were capable of interacting with both Rep1 and Rep2. For the mutant A8-2, the very low level of expression of Rep2 protein may be the reason for

lack of interaction. The *rep2* mutant *A2-1* interacted with wild-type Rep1 but not with Rep2. Although no protein could be detected for *rep2 A2-1* (see Results section 5D), it is possible that fusion protein is expressed when this mutant Rep2 protein is fused to $Gal4_{AD}$ protein. In the two-hybrid fusion only the *REP2* ORF was used, so the upstream promoter mutation in *rep2 A2-1* was not included. The only mutation that would be present in the two-hybrid fusion is N221Y in the carboxy terminus of the Rep2 protein. The interaction with Rep1 is consistent with the two-hybrid result that the carboxy-terminal portion of Rep2 that contains this altered residue is not required for interaction with Rep1. Since no interaction with wild-type Rep2 could be detected, it seems that this altered residue at position 221 abolishes Rep2/Rep2 interaction. However, the level of mutant two-hybrid fusion protein for *rep2 A2-1* would need to be determined since it is formally possible that the Rep1/Rep2 association does not require the same abundance of the Rep2 fusion protein as does Rep2 self association.

The level of the Rep1 protein expressed from most *rep2* mutants (except for *B8-1* and *C1-1*) is lower than that from wild-type pAS4. This lower level of Rep1 in the *rep2* mutants is apparent when the intensity of the non-specific 66-kDa band detected by the anti-Rep1 antibody, which reflects the amount of protein loaded in the lane, is compared between pAS4 and the *rep2* mutants (see Figure 12B). The Western blot shows that more protein was loaded for extracts from cells containing *rep2* mutants compared to the "wild-type" pAS4. In spite of this loading difference, more Rep1 protein was detected in extracts from cells containing wild-type pAS4. The results are consistent with those derived from studies of pAS4Δrep2 and support the assumption that absence or lower

abundance of the Rep2 protein affects the level of the Rep1 protein. In two of the *rep2* mutants, *B8-1* and *C1-1*, the amount of Rep1 protein was comparable to that expressed from wild-type pAS4. As mentioned above (see Section III 5C), these two mutant plasmids also have mutations in the *REP1* gene, and it is the *rep1* mutation in each case that affects function, the mutations in the *REP2* gene being silent.

Thus for both Rep1 and Rep2, mutations were obtained that resulted in lower steady-state levels of the proteins, and in these cases it is probably the absence or lower abundance of the proteins that is affecting segregation function. However, some mutant Rep proteins were expressed at levels comparable to wild-type, suggesting that in these mutants the changes in the amino acid residues are significant to the structure of the protein or alter its interaction with other proteins.

5. Partitioning of 2μm circle

Three different models have been proposed to explain how the Rep system promotes efficient partitioning of the 2μm circle. In the "passive diffusion" model the Rep proteins act to free plasmids from their sites of attachment, which would allow the molecules to diffuse freely and get into the daughter nuclei by random diffusion (Broach and Volkert, 1991; Wu et al. 1987). The "attachment" model proposes that the Rep proteins form an extended intra-nuclear structure that would provide a dispersed set of attachment sites for plasmid molecules. The assembly of this structure would be the driving force to distribute newly synthesized molecules throughout the growing nucleus and thus ensure that both mother and daughter cells receive plasmids during cell division

(Wu et al. 1987). The "active partitioning" model suggests a more active process where the Rep proteins may form a connector to a nuclear component, such as the mitotic spindle, that is partitioned equally between mother and daughter cells (Wu et al. 1987). Scott-Drew and Murray (1998) extended the third model based on their recent studies showing that Rep1 and Rep2 localize to sub-nuclear foci that double in number during mitosis and are segregated equally between mother and daughter nuclei. They predicted that 2μm plasmids are also associated with such foci and as a result get segregated. Scott-Drew and Murray (1998) also suggest that these Rep foci also correspond to sites of some important cellular function. Association of the plasmids with such foci would ensure an efficient segregation of these plasmid molecules mediated by the Rep proteins. Recent studies have shown that the Rep proteins accumulate near the spindle pole bodies, and that the Rep1 and Rep2 proteins colocalize with a 2μm-based plasmid inside the yeast nucleus. These two observations further suggest that the 2μm circle may attach to the mitotic spindle through the Rep/*STB* system to ensure its efficient partitioning (Velmurugan et al. 2000).

A. Model

On the basis of the data presented here and in previous studies, I would like to propose the following model for partitioning of the 2μm plasmid.

Step I – Nuclear localization of Rep proteins

The Rep1 and Rep2 proteins are targeted to the nucleus. This nuclear targeting may occur by virtue of each having its own NLS (Velmurugan et al. 1998).

Alternatively, Scott-Drew and Murray (1998) suggest that nuclear localization of Rep1 is dependent on the presence of Rep2 protein. It is unclear at present whether the formation of the Rep1/Rep2 complex is necessary for targeting Rep1 to the nucleus. Since both proteins have NLS (Velmurugan et al. 1998), it is possible that the formation of Rep1/Rep2 complex is required for the retention of the Rep1 protein within the nucleus.

Step II-Formation of Rep1/Rep2 complex

Once targeted to the nucleus, the Rep1 and Rep2 proteins interact with each other to form a complex that is the functional unit mediating plasmid segregation. The same or a different Rep complex may be responsible for regulation of the *REP* genes and *FLP*. Evidence for a complex is discussed below (see Section IV 5B).

Step III-Interaction of Rep proteins with *STB*

In step III the two Rep proteins interact with *STB* either directly or indirectly through a host factor(s). Hadfield et al. (1995) reported *in vitro* binding of an uncharacterized yeast host protein to *STB*. This binding could be detected only in urea-solubilized yeast extracts isolated from cells expressing both Rep proteins, a fraction which contains most of the soluble Rep1 and Rep2 (Dobson, unpublished results). The requirement for urea solubilization of the proteins suggests that these proteins may be associated with a sub-cellular structure (Hadfield et al. 1995). One candidate for the host protein that may be required to mediate the interaction of the Rep1 and Rep2 proteins with *STB* is the Shf1 protein, recently isolated on the basis of its ability to interact with the *STB* in a one-hybrid assay (Velmurugan et al. 1998).

Step IV-Interaction of 2μm circle with the host's segregation apparatus

Rep1 or Rep2, or the Rep1/Rep2 complex, attaches to some nuclear structure such as the mitotic spindle or some other nuclear component that is segregated equally between the mother and the daughter cells during cell division. Scott-Drew and Murray (1998) and Velmurugan et al. (2000) have shown that Rep1 and Rep2 are localized within the nucleus as discrete patches or foci that double during cell cycle and are segregated during mitosis. The Rep proteins could therefore form a bivalent connector attached to the plasmid molecules through the *STB* locus on one hand and some cellular component such as the mitotic spindle on the other hand. In the process the plasmid molecules would be equally partitioned (Step V) as the nucleus divides.

B. Evidence for a Rep protein complex

Segregation of the 2μm plasmid requires the Rep1 and Rep2 proteins to be present in certain stoichiometric amounts, which has been cited as evidence that the proteins form a complex *in vivo* (Dobson et al. 1988; Cashmore et al. 1986). Further support for such a balance between Rep1 and Rep2 comes from the observation that the levels and the ratio of the two Rep proteins expressed from the plasmid pAS4, which displays efficient segregation in a [cir^0] strain, did not differ significantly from those characteristic of the native 2μm circle in an isogenic strain. This was observed even though there was an eight-fold difference in plasmid copy numbers between pAS4 and native 2μm in the strain. The present study also shows that in the absence of Rep2 the Rep1 protein levels were decreased relative to wild-type. This lower level of Rep1

protein could be due to lower expression of the *REP1* gene, or due to reduced stability of either Rep1 mRNA or Rep1 protein in the absence of Rep2. Rep protein-mediated regulation of the *REP* genes has been proposed (Som et al. 1988; Veit and Fangman, 1988). The *REP1* gene has been shown to be downregulated by the products of both *REP* genes, as indicated by measurements of transcript levels (Som et al. 1988; Veit and Fangman, 1988). Those transcriptional studies would suggest that the *REP1* mRNA expression should increase in the absence of Rep2, since both Rep1 and Rep2 are required for repression of the *REP1* gene. One would then expect the level of the Rep1 protein expressed from the plasmid pAS4Δrep2 to be higher than what is found for wild-type pAS4. In contrast, the results from the Western blot analysis used in this study indicate lower steady-state levels of Rep1 in the absence of Rep2. It is possible that in the presence of the Rep2 protein, the Rep1 protein has a longer half-life due to its participation in the Rep1/Rep2 complex. If this is the case, then the results from the present study can be reconciled with the previously reported models that are based on transcriptional regulation. In all previous studies any regulation of the *REP* genes at the level of translation or post-translation would not have been observed, since *REP* promoter-*lacZ* fusion genes were used to assess transcriptional regulation and Rep proteins themselves were not analyzed. The current data, however, are not sufficient to conclude that in the absence of the Rep2 protein, Rep1 is less stable. Further experimental data are necessary to support the hypothesis. An analysis of *REP* mRNA levels and Rep protein half-lives is needed. Experiments such as S1 nuclease and RNAse protection assays will indicate whether in the absence of Rep2 the *REP1* mRNA levels

are increased, as suggested by the increased activity of β-galactosidase expressed from a *REP1* promoter fused to the *lacZ* gene in the earlier studies (Som et al. 1988; Reynolds et al. 1987). If native *REP1* mRNA is less stable in the absence of Rep2, this would be consistent with the observed decrease in Rep1 protein levels and would suggest that the *REP1* mRNA itself contains determinants that affect its stability in the absence of Rep2. To determine whether the Rep1 protein has a shorter half-life in the absence of Rep2, pulse-chase experiments can be performed to look at the stability of the Rep1 protein. The data, however, suggest that Rep proteins are maintained in certain stoichiometric amounts, since the levels of the proteins expressed from pAS4 are similar to that of native 2μm circle even though the copy numbers are different. Future experiments as mentioned will elucidate how the Rep1 and Rep2 proteins are maintained at defined levels, and whether this reflects their ability to form a stable Rep1/Rep2 complex.

C. How does 2μm circle compare to bacterial plasmids in terms of segregation?

Like the yeast 2μm plasmid, partitioning of bacterial plasmids requires the actions of *trans*-acting proteins on a *cis*-acting site on the plasmid. For example, the *par* locus (Meacock and Cohen, 1980) of the intermediate copy number plasmid pSC101 binds the host-encoded DNA gyrase and may be considered similar to *STB* in this respect, since *STB* also seems to interact directly or indirectly with host proteins (Hadfield et al. 1995; Velmurugan et al. 1998). The stability system of 2μm circle seems most similar to the F-plasmid of *E. coli,* which also includes two *trans*-acting proteins encoded by the plasmid genes *sopA* and *sopB* and a *cis*-acting locus, *sopC*. Studies have predicted that the SopB

protein might be necessary for positioning of plasmids through its interaction with the *sopC* site. This positioning of plasmid molecules is probably one of tethering the replicated molecules in areas away from each other until the mid-cell septum is formed (Kim and Wang, 1998; Jensen and Gerdes, 1999). In analogy with the bacterial system, either or both Rep proteins may also be involved in attaching the replicated plasmid molecules to some structure within the nucleus, such as the mitotic spindle (Velmurugan et al. 2000) and the plasmids are thus segregated during cell division. For the bacterial plasmid R1, which has a partition system similar to the F plasmid in terms of its organization, it has been shown *in vitro* that two DNA molecules containing the *cis*-acting *parC* locus are paired by the partitioning proteins (Jensen and Gerdes, 1998). If such pairing of 2μm plasmid takes place through the *STB* locus and is mediated by the Rep proteins, and the Rep proteins are also able to attach to a cellular component such as the mitotic spindle that is able to direct the attached pair of plasmids towards opposite poles in a dividing nucleus, this may be the molecular basis for 2μm plasmid segregation in yeast.

D. Polymerization of plasmid-encoded protein facilitates partitioning and silencing of bacterial plasmid

The distal portion of the 2μm *STB* locus contains a silencer-like element which represses transcription from upstream promoters (Murray and Cesareni, 1986). The contribution of this silencer to 2μm plasmid partitioning is still unknown, but recent studies of the bacterial plasmid P1 suggest that silencing and partitioning may be linked. The P1 protein ParB binds to the partitioning locus *parS*, and has been shown to silence genes

flanking *parS* (Rodionov et al. 1999). This silencing requires polymerization of the ParB protein along the DNA from a nucleation site at *parS* and extending along the length of the DNA, thus forming a nucleoprotein filament. ParB mutants that were silencing-defective were unable to extend along the DNA beyond *parS* and were also partition-defective, suggesting that ParB polymerization may be important for partitioning of P1 plasmid (Rodionov et al. 1999). By analogy with these studies of bacterial plasmids, the studies reported here and by others show that the Rep1 and Rep2 proteins of the 2μm plasmid can self-associate and that Rep2 seems to have a DNA-binding capacity (Dobson, unpublished results). Although it has not yet been demonstrated, *STB* might be a nucleation site for polymerization of Rep proteins that is primarily mediated through Rep2. The formation of these extended filamentous structures may explain the reduced accessibility of the transcriptional apparatus to the plasmid DNA sequences, hence the "silencing". This filamentous structures may facilitate tethering of the plasmids to a larger structure in the host cell that is partitioned during cell division. However, the correlation between partitioning and the capacity for silencing as found for P1, or the presence of a silencer in the 2μm plasmid, is still unclear.

E. Does 2μm circle partitioning involve attachment to centromere-kinetochore complex?

The centromere-kinetochore complex (CKC) mediates the attachment of chromosomes to the mitotic spindle and ensures proper chromosome segregation during cell division. The *STB* locus of 2μm circle is functionally equivalent to the centromere.

Both *STB* and centromere are *cis*-acting sites which, when present on *ARS* plasmids, can stabilize those plasmids. Both these sequences seem to be the sites for the assembly of protein complexes and mediate protein-protein interaction. Considering the high mitotic stability of 2μm circle, it is reasonable to predict that this plasmid uses the same machinery as chromosomes for its efficient partitioning. The data presented here show that Rep1 and Rep2 self-associate and interact, and may therefore form a multimeric complex *in vivo*. Interaction between *STB* and the Rep proteins has also been suggested (Hadfield et al. 1995; Velmurugan et al. 1998). The kinetics of chromosome and 2μm segregation are identical (Velmurugan et al. 2000). Thus, in the absence of a *CEN* sequence on 2μm, *STB* might be able to act like *CEN*, associating with proteins that allow it to be attached to the spindle apparatus in a way that is similar to kinetochore attachment. *STB* is most likely connected to the protein complex through Rep1 and Rep2 which may be mediated through some host factor. For centromeres the CDE III element seems to be the nucleation site for kinetochore assembly and the Cbf3 protein complex is the key component. Similarly, the *STB* locus may be the site for protein assembly in 2μm circle and the Rep proteins may form the main component of this complex. The interaction of the kinetochore with the microtubules of the spindle is critical for the segregation of the chromosomes. That the 2μm plasmids may be segregated using the same machinery, being attached to the spindle apparatus through *STB* and the Rep protein, is supported by data from two studies. Scott-Drew and Murray (1998) have shown that the Rep proteins localize to discrete nuclear foci that duplicate during the cell cycle and become segregated to both mother and daughter cells. The other study shows

that the Rep proteins accumulate near the poles of the mitotic spindle (Velmurugan et al. 2000). The associations of the Rep proteins with plasmid through *STB* and with the spindle mediated by host protein further suggest that 2μm circle may be utilizing the host's segregation machinery and segregate with a kinetics that is identical to that of the chromosome (Velmurugan et al. 2000).

Although a large number of proteins involved in kinetochore assembly and stability of the spindle have been identified, the available data have not established how kinetochores interact with the spindle. Isolation of such proteins and description of their modes of interaction with each other will help elucidate the process of chromosome segregation and 2μm circle partitioning.

F. Is there any evidence for nuclear control of plasmid segregation?

One way of finding nuclear components that are also involved in partitioning of 2μm circle is to look for host mutations that affect plasmid functioning. One such mutation is *nib1*, a mutation in the yeast chromosomal gene *NIB1*, which confers a nibbled colony morphology only in the presence of 2μm plasmids (Holm, 1982). The nibbled colony morphology arises due to the death of lineages within the colony where the cells have acquired elevated 2μm circle copy number (Holm, 1982). This lethal sectoring phenotype in *nib1* yeast is thus 2μm circle-dependent. The nib1 mutation causes a lag in the G2/M stage in the cell cycle in [cir$^+$] yeast (Holm, 1982) implying that the Nib1 protein may be required for progression through mitosis, perhaps playing a role in both chromosome and plasmid segregation. The *NIB1* gene has been cloned in our

laboratory and has been shown to be identical to the yeast *ULP1* gene (Picket, 1998). The *ULP1* gene (Li and Hochstrasser, 1999) encodes a protease that cleaves a ubiquitin-like protein, Smt3 (Johnson et al. 1997) (SUMO-1 in mammalian cells [Matunis et al. 1996; Mahajan et al. 1997]) from protein targets to which it has been added as a post-translational modification. Depending on the protein substrate, Smt3/SUMO-1 modification can be involved in different cellular functions, including targeting of protein to a particular subcellular location. There is evidence that Smt3 modification itself may be sufficient to target a protein to the mitotic spindle. For example, SUMO-1 modification has been shown to target Ran GTPase-activating protein (RanGAP1) from the cytosol to the nuclear pore complex during interphase and to the spindle apparatus during mitosis (Matunis et al. 1996). The increase in copy number of 2μm plasmid in the presence of the *nib1* mutation suggests that a critical component of the plasmid's copy number control or segregation systems may be regulated by Smt3 modification. The Western blot analysis presented in this study shows a higher form of the 2μm Rep2 protein that has been found to be more abundant in a *nib1* mutant (Dobson, unpublished results). This implies that Rep2 may be Smt3-modified, and this higher-molecular-weight anti-Rep2 immuno-reactive band may be this modified form of Rep2.

Both Rep1 and Rep2 proteins together are required for repression of the *FLP* gene (Som et al. 1988; Veit and Fangman, 1988; Reynolds et al. 1987). If Rep2 is Smt3-modified, deconjugation of Smt3 may be required for maintaining proper stoichiometry of the Rep proteins, which together form the repressor complex. In the presence of the mutant form of Nib1 the modified form of Rep2 accumulates (Dobson, unpublished

results), which may affect the formation of the Rep1/Rep2 repressor complex resulting in Flp-mediated amplification of plasmid. An elevated 2μm circle copy number might be a burden on the host's replication and/or segregation machinery, resulting in cell death. At present, however, it is not known whether Rep2 or any of the other 2μm circle-encoded proteins is Smt3-modified. Recent data suggest that the mammalian kinetochore protein CenpC, which is a homologue of the yeast kinetochore protein Mif2, is Smt3-modified, so Smt3 modification may be directly required for chromosome segregation. If Rep2 protein accumulates due to Flp-mediated over-amplification of the 2μm, there may be a competition between Rep2 and Mif2 for a limited number of Smt3 molecules, hence the lag at G2/M observed in [cir$^+$] *nib1* mutants. The yeast *SMT3* gene itself was cloned as a high-copy-number suppressor of a mutation in the kinetochore protein Mif2 (Meluh and Koshland, 1995, 1997). Interestingly, not only deconjugation but conjugation of Smt3 to target proteins, the latter mediated by Ubc9, is required for progression through the G2/M phase of the cell cycle (Seufert et al. 1995). Identification of the protein targets for Smt3 modification is necessary to reveal the mechanisms by which 2μm circle utilizes the host's segregation machinery for its stable maintenance.

That the 2μm circle uses components of the chromosome segregation machinery to ensure its stable maintenance is further supported by the finding that the yeast chromosomal mutation *Ipl1-2* causes missegregation of both the chromosomes and 2μm plasmid (Velmurugan et al. 2000). The Rep1 and Rep2 proteins have also been shown to interact with the yeast protein Brn1 (Velmurugan et al. 2000), which shares homology with the barren gene product of *Drosophila* and a condensin subunit of *Xenopus*, both

proteins being involved in chromosome segregation (Bhat et al. 1996; Hirano et al. 1997). These data, together with the visual evidence that the Rep proteins are present near the spindle poles (Velmurugan et al. 2000), suggest that the partitioning of the 2μm plasmids involves the plasmid-encoded Rep proteins interacting with host proteins to attach the plasmids to the spindle apparatus.

G. Multiple interactions of the Rep proteins required for 2μm circle functioning

The data presented here suggest that Rep1 and Rep2 proteins interact with each other and also self-associate. Deletions or point mutations in either the amino- or the carboxy-terminal regions of the two proteins affect segregation of the plasmid. Based on the observations presented here and work from other laboratories, it is obvious that no single sub-domain in either of the Rep proteins is solely responsible for the segregation function. Some functions of the Rep proteins have been delineated by the studies reported here or carried out by others. How these Rep protein interactions or their interaction with *STB* overcome the maternal bias in inheritance and cause equal distribution of plasmids between the mother and daughter cells is still unclear. On the basis of the available data, it is possible to predict a model for 2μm circle partitioning (Figure 19). When partitioning is not required, Rep1 monomers self-associate through their amino-terminal regions to form complex A, which may be mediated through host factors F. As mentioned, the stability of Rep1 may depend on the presence of Rep2. Thus this Rep1/Rep1 complex may be stabilized through association with Rep2, although

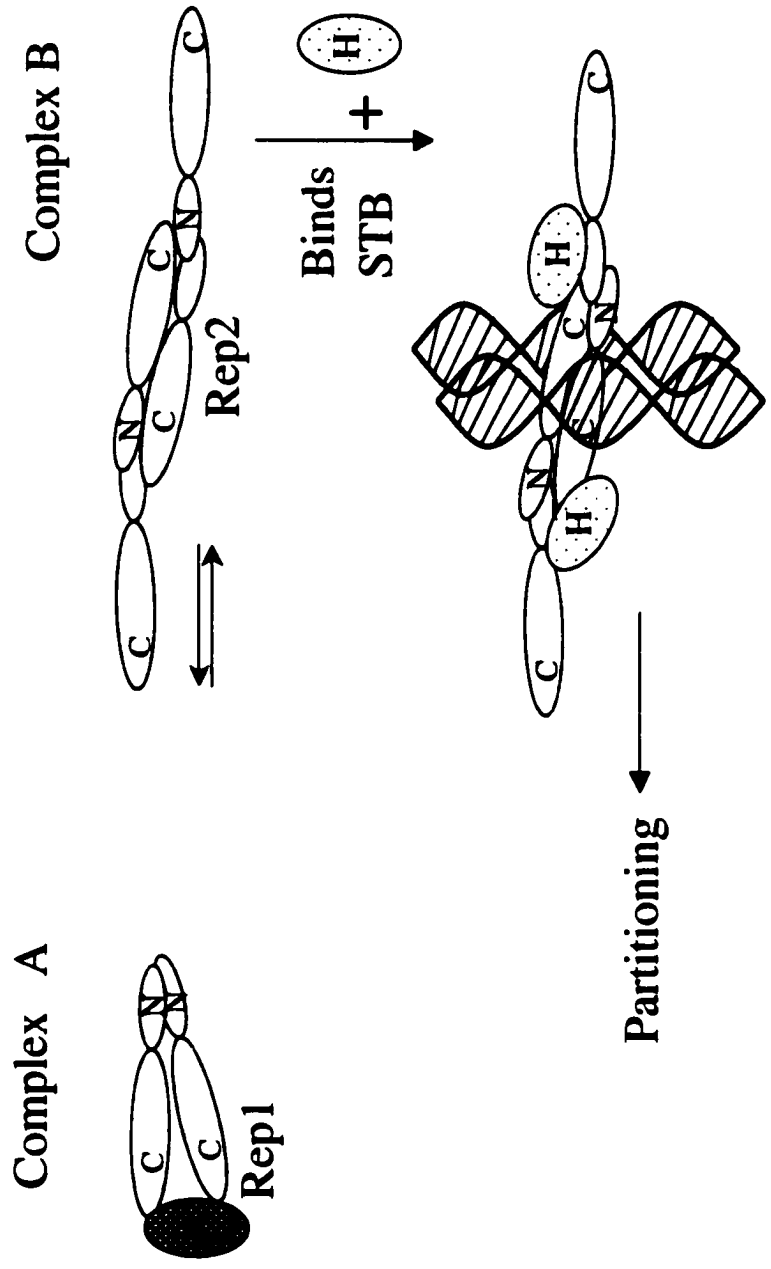

Figure 19.

Figure 19. Model for Rep protein interactions and their association with the 2μm *STB* locus. The Rep1 monomers are shown as hatched and the Rep2 monomers are indicated as shaded molecules. Rep1 monomers self-associate through their amino-terminal regions to form complex A, which may be mediated by host factors, indicated as F. The amino terminus of Rep1 protein can also interact with both amino and carboxy termini of the Rep2 protein. Rep2 is also capable of self-association. The interactions between the Rep proteins and their self-association result in the formation of a multimeric Rep protein complex B. When partitioning is required complex B is formed. The *STB* locus, acting as a nucleation site for the formation of DNA-protein complex, recruits the Rep1/Rep2 complex B as well as host factors (H). Plasmids may pair at the *STB* loci through this complex. The host factors may interact with either or both Rep proteins and/or with *STB*. The self-association of Rep2 together with its DNA-binding ability might facilitate polymerization of this protein complex along the tandem repeats within *STB*. The resulting DNA-protein complex interacts with the host's segregation machinery and promotes partitioning of the plasmids.

not necessarily through direct interaction of Rep1 and Rep2, for this stabilization may be through the interaction of Rep2 and host factors. The amino terminus of Rep1 protein can also interact with both amino and carboxy termini of Rep2 protein. Rep2 is also capable of self-association. When partitioning is required, the interactions between the Rep proteins, and their self-association, result in the formation of a multimeric Rep complex B. Within the yeast cell, the distribution between the two complexes A and B may be determined by cell-cycle-mediated alterations to the Rep proteins themselves or to other host proteins with which they interact. When partitioning is required, complex B is formed. These interactions of Rep1 and Rep2 with each other and self-association of Rep2 probably give rise to a nucleoprotein filamentous structure, as described for the bacterial plasmid P1. The *cis*-acting *STB* locus, acting as a nucleation site for the formation of DNA-protein complex, recruits complex B as well as host factors (H). The host factors may interact with either or both Rep proteins and/or *STB*. The self-association of Rep2 protein together with its DNA binding ability might facilitate polymerization of this protein complex along *STB*. For the sake of clarity, only two Rep1/Rep2 molecules are shown on the DNA in Figure 19, but further complexes would associate with these to spread along the *STB*-proximal repeats. As shown in the model, this complex contains two *STB* loci and could pair plasmids in preparation for segregation. This DNA-protein complex along *STB* probably interacts with the mitotic spindle, the interaction being mediated through proteins that are involved in chromosome segregation. The detection of the Rep proteins near the spindle poles (Velmurugan et al. 2000) further supports the interaction between 2μm plasmids and the spindle apparatus.

It is possible that Smt3 modification of Rep2 may itself be sufficient to attach the paired *STB*/protein complex to the spindle, and as the spindle bodies separate during cell division the paired plasmids are pulled towards opposite directions along with the chromosomes.

The overlap in Rep1 of the regions required for its interaction with Rep2 and for self-association suggests that there may be a competition between homo- and heterodimerization of Rep1. Further support for this competition comes from *in vitro* assays where Rep1 binding to GST-Rep1 was reduced when both Rep1 and Rep2 fusion proteins were added simultaneously, compared to when either Rep protein was added alone (Scott-Drew and Murray, 1998). The findings suggest that Rep1 and Rep2 may be competing for the same site on Rep1. Removal of the carboxy-terminal portion of Rep1 enhanced its self-association, as shown by *in vitro* baiting assays (Joyce Chew, unpublished results), suggesting this region of Rep1 may inhibit its self-association thereby promoting interaction with Rep2. It is possible that the Rep1 self-association *in vivo* involves some host factor that interacts with the carboxy-terminal region of Rep1 and promotes formation of the Rep1/Rep1 complex A. During partitioning, formation of the Rep1/Rep2 complex B would be required, but once the cells have divided, this complex could dissociate, perhaps promoted by Rep1 homodimerization. The assembly and disassembly of the Rep1/Rep2 complex B may allow attachment and detachment of the plasmid from the spindle apparatus. Although the Rep1/Rep2 complex may be required for plasmid segregation, the presence of an excess amount of this complex may not be ideal for maintenance of the plasmid, especially if it has secondary role in

regulating expression of plasmid genes. Thus self-association of Rep1 may be a requirement to maintain the proper balance of the Rep proteins complexes.

Stoichiometric amounts of the Rep1 and Rep2 proteins are required for efficient partitioning (Cashmore et al. 1986; Dobson et al. 1988). When sufficient Rep1 is present within the cell, enough of the Rep1/Rep2 complex B will be formed to bind *STB* and promote efficient partitioning. If the Rep1 protein is more stable when present as a complex with Rep2 (see sections IV), then under normal physiological conditions of the cell, formation of the Rep1/Rep2 complex B (see Figure 19), as opposed to the formation of the Rep1 homodimer, may be favored, but its level will be dependent on the level of Rep2 protein. The same or a different Rep1/Rep2 complex may be responsible for regulating the *FLP* gene and probably the *REP* genes as well, as has been previously reported (Murray et al. 1987; Som et al. 1988; Veit and Fangman, 1988), although my own data throw some questions on the relevance of this repression under normal levels of expression of the Rep proteins. If the Rep1/Rep2 complex is the key component required for 2μm circle function, it is important that the complex is present at an optimum level. In that case, overexpressing Rep1 should not affect function, provided a normal level of Rep2 is maintained within the cell. Dobson et al. (1988) had shown that over-expressing *REP1* from a heterologous promoter on a 2μm-based vector did not affect the stability of a 2μm-based plasmid in a [cir$^+$] host. In contrast, over-expression of *REP2*, while not toxic to the cell, led to an inability to propagate a 2μm-based plasmid in the over-expressing strains (Dobson et al. 1988). The presence of excess Rep2 may prevent formation of sufficient Rep1 homodimer. Instead, Rep2 might titrate the Rep1 molecules

and lead to an excess of the Rep1/Rep2 heterodimer B. Simultaneous over-expression of both Rep proteins might also lead to an increase in the amount of the Rep1/Rep2 complex B, which in turn might titrate host proteins and affect normal cellular functions. Once partitioning has occurred, disassembly of the Rep1/Rep2 complex B and formation of the Rep1/Rep1 complex A may be critical for the release of any bound host proteins which may be involved in functions other than segregation within the cell. In this respect, mutations affecting Rep1/Rep1 interaction may affect the balance between homo- and heterodimerization of Rep1.

At present our laboratory is continuing work with the *rep* mutants. *In vitro* baiting assays will be used to determine whether these mutations affect self-association of Rep1 and whether the mutants affect post-translational modification of Rep2. Identification of host proteins that interact with 2μm-encoded proteins, and determination of the nature of the Rep1/Rep2 complex as well as the complexes formed with host factors, may elucidate the mechanisms used by this eukaryotic plasmid to ensure its stable persistence within the host cell.

Appendix

Table A1. Plasmids to create AS3 strains, *FLP* promoter assay and pAS4.

Plasmid name	Parental vector	Fragment inserted/deleted	Relevant details
pAS1	pBR322	3.5-kbp fragment from pL909 containing yeast *ADE2* gene (Gottschling et al. 1990) inserted	4.0-kbp *Eco*RI/*Bam*HI fragment from pBR322 ligated to 3.5-kbp *Eco*RI/*Bam*HI fragment from pL909
pAS3	pAS1	1.1-kbp yeast genomic *Hind*III fragment containing *URA3* gene from plasmid pRB58 (Carlson and Botstein, 1982)	5.3-kbp *Hind*III fragment from pAS1 ligated to 1.1-kbp *Hind*III fragment from pRB58.
pFLP-lacZ	pJL638 (Li and Herskowitz, 1993)	1. *FLP* promoter as a *Bam*HI PCR amplicon 2. *ARS-CEN* fragment	1. *FLP* promoter replaced *CYC1* promoter 2. Replaced 2μm sequences
pAS4	2μm circle B form	1. *E. coli* vector pTZ18R inserted (*Eco*RI, *Sst*I, *Kpn*I, and *Sma*I sites removed from polylinker) 2. Genomic *ADE2* gene inserted	1. Inserted at the *REP1/REP2* intergenic region at a unique *Bam*HI site created by brief BAL31 digestion at the *Xba*I site of *IRS* followed by closure on *Bam*HI oligonucleotide 2. *Eco*RI of *FLP* flush-ended and ligated with a filled-in 3.5-kbp fragment containing genomic *ADE2* gene from pAS1

Table A2. Derivatives of plasmid pAS4.

Plasmid name	Parental vector	Fragment inserted/deleted	Relevant details
pAS4ΔX	pAS4	Deletion of *STB*-proximal	9.3-kbp *BglII/HpaI* fragment from pAS4 ligated with 3.1-kbp *BglII/AvaI* fragment from pAS4 in the presence of *XhoI* linkers
pAS4Δrep1	pAS4	Deletion of *REP1* ORF	*PvuII* digestion and self-ligation of pAS4
pAS4Δrep2	pAS4	Deletion of *REP2* ORF	*SphI* digestion and self-ligation of pAS4
pAS4B	pAS4	Deletion of *E. coli* vector	*BamHI* digestion and self-ligation of pAS4
pAS9	pAS4	pTZ18R containing entire undeleted polylinker replaced original *E. coli* sequences in pAS4	*BamHI* digested pAS4 ligated with *BamHI* digested pTZ18R
pAS10	pAS4	pTZ18R containing entire undeleted polylinker replaced original *E. coli* sequences in pAS4	*BamHI* digested pAS4 ligated to *BamHI* digested pTZ18R in opposite orientation to that of pAS9
pAS11	pAS9	Deletion of all 2μm sequences except *REP1* ORF	*PstI* digestion and self-ligation of pAS9
pAS12	pAS10	Deletion of all 2μm sequences except *REP2* ORF	*SphI* digestion and self-ligation of pAS10

Table A3. Plasmids used in two-hybrid experiments

Plasmid name	Parental vector	[a]Fragment inserted/deleted	Relevant details
pAS5	pTZ18R	*REP1* ORF inserted	*REP1* ORF (nucleotides 1 to 1122) as a *Bam*HI PCR amplicon using primers REP1-1and REP1-2 ligated to *Bam*HI digested pTZ18R
pAS6	pTZ18R	*REP2* ORF inserted	*REP2* ORF (nucleotides 1 to 895) as a *Bam*HI PCR amplicon using primers REP2-1and REP2-2 ligated to *Bam*HI digested pTZ18R
pGADREP1	pGAD424 (Clontech)	*REP1* ORF inserted in frame with $Gal4_{AD}$	*Bam*HI fragment containing *REP1* ORF from pAS5 ligated to *Bam*HI digested pGAD424
pGADrep1Δ1-76	pGAD424 (Clontech)	*REP1* ORF (nucleotides 1 to 228 deleted)	A 0.9-kbp filled-in *SpeI/Bam*HI fragment from pAS5 ligated to filled-in *Bam*HI digested pGAD424
pGADrep1Δ1-129	pGAD424 (Clontech)	*REP1* ORF (nucleotides 1 to 387 deleted)	A 0.75-kbp filled-in *StuI*/BamHI fragment from pAS5 ligated to filled-in *Bam*HI digested pGAD424
pGADrep1Δ78-373	pGADREP1	*REP1* ORF (nucleotides 229 to 1122 deleted)	*SpeI/BglII* digested pGADREP1 filled-in and self-ligated
pGADrep1Δ130-373	pGADREP1	*REP1* ORF (nucleotides 388 to 1122 deleted)	*StuI/BglII* digested pGADREP1 filled-in and self-ligated

[a]The numbering used for nucleotides in *REP* genes starts at 1 for the first nucleotide in the translation initiation codon.

Table A3 (contd.).

Plasmid name	Parental vector	[a]Fragment inserted/deleted	Relevant details
pGADREP2	pGAD424 (Clontech)	*REP2* ORF inserted in frame with $Gal4_{AD}$	*Bam*HI fragment containing *REP2* ORF from pAS6 ligated to *Bam*HI digested pGAD424
pGADrep2Δ1-14 (a gift from J. Sherk)	pGAD424 (Clontech)	*REP2* ORF (nucleotides 1 to 42 deleted)	. *Mae*II partial digestion of *REP2* inserted at *Cla*I site of pGAD424
pGADrep2Δ1-57	pGADREP2	*REP2* ORF (nucleotides 1 to 171deleted)	*Eco*RI/*Nco*I digested, filled-in and self-ligated pGADREP2
pGADrep2Δ59-296	pGADREP2	*REP2* ORF (nucleotides 175 to 891deleted)	*NcoI*/*Bgl*II digested, filled-in and self-ligated pGADREP2
pGBT9REP1	pGBT9 (Clontech)	*REP1* ORF inserted in frame with $Gal4_{DB}$	*Bam*HI fragment containing *REP1* ORF from pAS5 ligated to *Bam*HI digested pGBT9
pGBT9REP2	pGBT9 (Clontech)	*REP2* ORF inserted in frame with $Gal4_{DB}$	*Bam*HI fragment containing *REP2* ORF from pAS6 ligated to *Bam*HI digested pGBT9

[a]The numbering used for nucleotides in *REP* genes starts at 1 for the first nucleotide in the translation initiation codon.

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