Contributions Of DNA Sequence Elements, Plasmid-Encoded Proteins And Host Proteins To Maintenance Of The Yeast 2-Micron Plasmid

by

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ABSTRACT

The 2-micron plasmid is found at high copy in the nuclei of most strains of the budding yeast *Saccharomyces cerevisiae*. Despite conferring no selective advantage to its host, the plasmid is able to persist due to mechanisms that ensure equal partitioning of plasmid copies at cell division and amplification of plasmid copy number in the rare event of unequal partitioning. Partitioning requires the plasmid-encoded Rep1 and Rep2 proteins and a repeated DNA sequence at the plasmid *STB* locus. Plasmid amplification is catalyzed by plasmid-encoded Flp recombinase, and regulated by Rep protein-mediated repression of the *FLP* gene; Raf, the fourth plasmid protein, relieves this repression. The goals of this study were to identify features of the Rep proteins and plasmid sequences required for their function, and to examine the role of Raf and host proteins in ensuring plasmid maintenance.

Experimental and bioinformatics analyses were used to identify residues in Rep2 required for its association with Rep1 and Rep2. Rep2 mutants that had lost either or both associations were used to show that Rep2 plasmid partitioning function was retained provided one of these associations was intact and the Raf protein was present. This, along with additional analyses, suggests that Raf might be a Rep2 homolog.

To gain insight into sequences required for *STB* function, synthetic *STB* sequences were generated and used to determine that two identical direct repeats of a 63-bp stretch of *STB* are sufficient to confer partitioning function. Mutation of either of two TGCA sequences, a TGCA-adjacent T-tract, or a CGCG sequence in both repeat copies impaired partitioning function, while mutation of either TGCA sequence impaired Rep protein association with *STB*. Mutational analysis of plasmid gene promoters showed that TGCA sequences were required for Rep protein-mediated repression, suggesting that TGCA is a Rep protein recognition motif.

Finally, genome-wide screens identified candidate host factors that might contribute to functional chromatin arrangement at *STB*, or to the toxicity associated with Rep protein overexpression.

These findings serve to further refine the model of 2-micron plasmid maintenance, thus improving our understanding of how this extrachromosomal element persists in its eukaryotic host.

LIST OF ABBREVIATIONS USED

3-AT	3-Amino-1,2,4-triazole
ß-gal	ß-galactosidase
aa	amino acid
AD	transcriptional activation domain
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
$B42_{AD}$	B42 transcriptional activation domain
BD	DNA-binding domain
BPV1	bovine papilloma virus type I
CEN	centromere
ChIP	chromatin immunoprecipitation
CSL	*cis*-acting locus
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
FRT	Flp recombinase target
$Gal4_{AD}$	Gal4 transcriptional activation domain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IgG	immunoglobulin
IR	inverted repeat
KSHV	Kaposi's sarcoma-associated virus
$LexA_{BD}$	LexA DNA-binding domain
NLS	nuclear localization sequence
NP-40	Nonidet P-40
nt	nucleotide
ONPG	ortho-nitrophenyl-ß-D-galactopyranoside
ORF	open reading frame
ORI	origin of replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
RNA	ribonucleic acid
RNAi	RNA interference
SC	synthetic complete
SD	synthetic defined
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SGA	synthetic genetic analysis
STB-D	*STB-distal*
STB-P	*STB-proximal*

SUMO	small ubiquitin-like modifier
TE	Tris EDTA
Tris	Tris(hydroxymethyl)aminomethane
UAS	upstream activation sequence
UTR	untranslated region
WT	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
YPD	yeast extract peptone dextrose

Yeast nomenclature

XXX1	wild-type gene
xxx1	mutant gene
*xxx1*Δ	gene deletion
Xxx1	wild-type protein
$Xxx1_{XX}$	mutant protein
Xxx^{-}	absent protein

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Chapter 1 INTRODUCTION

1.1 The *Saccharomyces cerevisiae* 2µm Plasmid

The 2µm plasmid is a circular, double-stranded DNA found at high copy number in the nuclei of most strains of the budding yeast *Saccharomyces cerevisiae* (Futcher, 1988). The ubiquity of the plasmid is surprising as it confers no selective advantage to its host, and persists instead due to two efficient maintenance strategies: equal partitioning of plasmid copies at cell division, and amplification of plasmid copies in the event of unequal partitioning. The aim of this thesis is to investigate components of the 2µm plasmid partitioning system, and the regulation of plasmid amplification and gene expression.

The 2µm plasmid is organized into two unique regions containing four protein-encoding genes (*FLP*, *REP1*, *REP2* and *RAF*), separated by large inverted repeats (*IR*). The plasmid partitioning locus (*STB*), and a single origin of replication (*ORI*) are positioned adjacent to one of the inverted repeats (Figure 1). Replication of each plasmid copy is initiated once per cell cycle, during S phase, proceeding bidirectionally from the *ORI*, and mediated by the host replication machinery (Livingston and Kupfer, 1977; Zakian *et al.*, 1979).

1.1.1 2µm Plasmid Amplification

Plasmid amplification is mediated by the largest of the plasmid-encoded proteins, Flp recombinase, which belongs to a family of site-specific tyrosine recombinases that includes the Cre protein encoded by the P1 bacteriophage (Argos *et al.*, 1986). Flp catalyzes recombination between the Flp recombinase target (*FRT*) sites in the *IR* sequences (Broach *et al.*, 1982; Senecoff *et al.*, 1985), inverting one half of the plasmid relative to the other, and resulting in A and B forms of the plasmid, which are present in the host in approximately equimolar amounts (Broach and Hicks, 1980).

Flp-mediated recombination during plasmid replication has been proposed to lead to copy number amplification by inverting the direction of one replication fork relative to the other, an event made more likely by the close proximity of one of the *FRT* sites to the

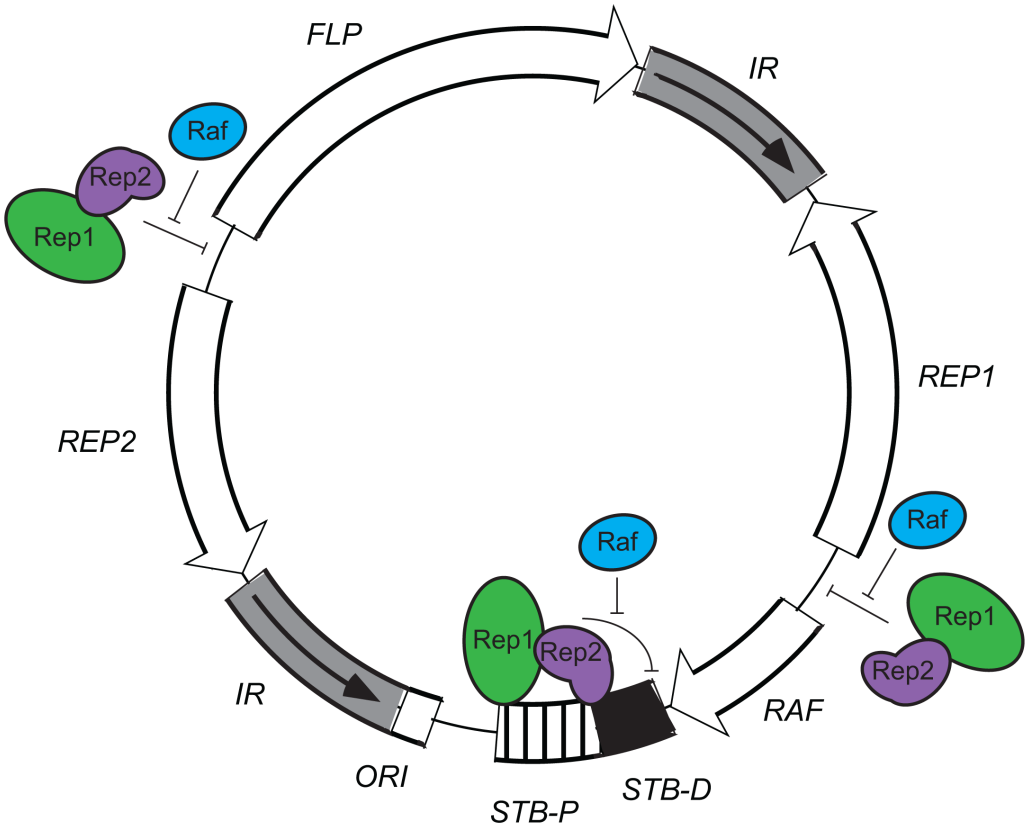

Figure 1. Map of the 2µm plasmid of *Saccharomyces cerevisiae* showing model of interaction of plasmid-encoded proteins with the plasmid. The A form of the 2µm plasmid is shown with positions of plasmid genes (white arrows), inverted repeat sequences (*IR*; grey boxes with black arrows showing orientation), origin of replication (*ORI*; white box), *STB-distal* (*STB-D*; black box) and *STB-proximal* (*STB-P*; striped box) indicated. Rep1 and Rep2 associate with *STB-P* to mediate plasmid partitioning. Rep1 and Rep2 also repress transcription driven by *STB-P* and divergent 2µm plasmid promoters; this repression is relieved by Raf. See text for references.

ORI (Futcher, 1986). This replication fork inversion results in a switch from bidirectional theta-type replication to a double rolling-circle mode of replication, in which multiple copies of the 2µm plasmid sequence are produced as a concatamer. A second Flp-mediated recombination event can restore bidirectional replication, allowing the replication forks to meet and replication to terminate. This results in one 2µm plasmid monomer and a large concatamer that can subsequently be resolved into monomers by Flp or host recombination machinery. This model is supported by the observation that plasmid amplification requires both Flp activity and mitotic growth (Volkert and Broach, 1986; Reynolds *et al.*, 1987).

1.1.2 2µm Plasmid Partitioning

Although Flp can correct downward fluctuations in 2µm plasmid copy number, it is not clear how often this is required. Analysis of DNA by density labeling *in vivo* showed that the majority of plasmid copies contain one conserved and one newly-synthesized strand after replication (Zakian *et al.*, 1979), implying that Flp-mediated amplification of plasmid copy number occurs rarely. A second, and likely more significant, contribution to 2µm plasmid maintenance is made by the active partitioning system encoded by the plasmid, which ensures equal partitioning of plasmid copies at cell division. Plasmid copy number is maintained at a similar level within cells from the same strain, but can vary between strains, with the average copy number per cell ranging from 30 to 100 (Gerbaud and Guérineau, 1980). Active partitioning is necessary to ensure faithful inheritance, despite this high copy number, as yeast undergo an asymmetrical cell division in which only 10% of nuclear material that is not actively partitioned reaches the daughter nucleus, while the rest is retained in the mother nucleus (Gehlen *et al.*, 2011). Additionally, observations made using fluorescently-tagged 2µm-based reporter plasmids suggest that 2µm plasmids aggregate into three to five clusters per nucleus, effectively reducing the number of partitioning units (Velmurugan *et al.*, 2000; Scott-Drew *et al.*, 2002); therefore, in the absence of an active partitioning mechanism, plasmid clustering would further reduce the frequency of plasmid transmission to the daughter.

1.1.3 Plasmid-Encoded Rep1 And Rep2 Proteins

Equal partitioning of the 2µm plasmid during host cell division is dependent on the plasmid-encoded Rep1 and Rep2 proteins and the *STB* locus (Jayaram *et al.*, 1983; Kikuchi, 1983); absence of any one of these three components results in a strong maternal bias in inheritance, similar to that observed for plasmids that contain an autonomously replicating sequence (*ARS*), but lack partitioning sequences (*ARS* plasmids; (Murray and Szostak, 1983)). Rep1 and Rep2 self-associate and associate with each other both *in vivo* and *in vitro* (Figure 2; (Ahn *et al.*, 1997; Velmurugan *et al.*, 1998)). The region of the Rep2 protein required for association with Rep1 does not overlap with the region required for Rep2 self-association, suggesting that these proteins can adopt higher-order arrangements (Velmurugan *et al.*, 1998; Sengupta *et al.*, 2001). We have shown that stability of the Rep1 protein is reduced in the absence of Rep2, and that Rep2 stability may be slightly reduced in the absence of Rep1 (Pinder *et al.*, 2013), suggesting that the association of Rep1 and Rep2 protects both from degradation.

In vivo, the Rep proteins are found in association with the *STB* locus throughout the cell cycle, dissociating only briefly at the G1-to-S phase transition (Velmurugan *et al.*, 1998; Yang *et al.*, 2004). The association of Rep1with *STB* does not require the presence of other 2µm proteins, while association of Rep2 with *STB* is greatly reduced in the absence of Rep1 (Pinder *et al.*, 2013). Amino acid substitutions in Rep1 that impair its ability to associate with either Rep2 or *STB* impair plasmid partitioning (Yang *et al.*, 2004). This suggests that the functional 2µm plasmid partitioning structure includes a complex of Rep1, Rep2 and *STB*.

Despite reliance on Rep1 for robust *STB* association *in vivo*, *in vitro* Rep2 has been shown to display a preference for binding *STB* DNA in a southwestern analysis, while Rep1 has not been found to bind *STB* in the absence of host proteins (Hadfield *et al.*, 1995; Sengupta *et al.*, 2001). Purified Rep1 and Rep2 were both able to associate with *STB* DNA in a plasmon resonance assay, but only in the presence of urea-solubilized yeast extracts, suggesting that host proteins may be required to mediate this interaction (Hadfield *et al.*, 1995).

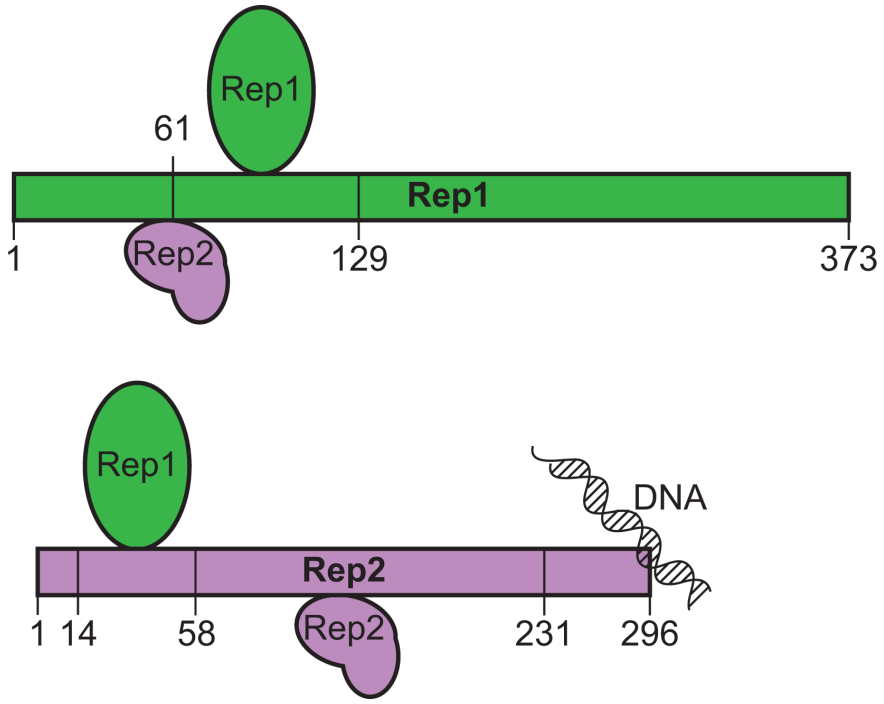

Figure 2. Cartoon representation of interacting regions of 2µm plasmid-encoded Rep1 and Rep2 proteins. Cartoon summarizes results of Sengupta and co-workers ((Sengupta *et al.*, 2001); J. Chew, unpublished results). Interacting regions are indicated by amino acid residue number either above or below the rectangular representations of Rep1 and Rep2. Amino acids 62 to 129 of Rep1 are required for Rep1 self-association, while amino acids 1 to 129 are sufficient for Rep1 self-association. Amino acids 1 to 129 of Rep1 are required and sufficient for association with Rep2. Amino acids 58 to 231 of Rep2 are required for Rep2 self-association, while residues 58 to 296 are sufficient for Rep2 self-association. Amino acids 15 to 58 of Rep2 are required for association with Rep1, while amino acids 1 to 58 of Rep2 are sufficient for association with Rep1.

1.1.4 Transcriptional Regulation Of 2µm Plasmid Promoters

In addition to their role in plasmid partitioning, Rep1 and Rep2 function to down-regulate transcription from plasmid promoters. Nine transcripts originating from the native 2µm plasmid have been detected, six of which have been mapped and shown to be polyadenylated (Sutton and Broach, 1985; Sleep *et al.*, 2001). Four are the mRNAs encoding plasmid proteins, which are transcribed from divergent promoters; *FLP* and *REP2* share a promoter (*FLP/REP2p*), as do *REP1* and *RAF* (*REP1/RAFp*). The remaining transcripts are non-coding RNAs, one of which is a 1950-nucleotide (nt) transcript that initiates in *STB* and co-terminates with the *REP1* transcript.

Transcription driven by *FLP/REP2p*, *REP1/RAFp* and *STB* is repressed upon simultaneous expression of Rep1 and Rep2 (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988). Expression of the Raf protein has been shown to relieve this Rep protein-mediated repression (Murray *et al.*, 1987), although the mechanism by which this occurs has not been determined. As expression of *RAF* is also regulated by combined expression of Rep1 and Rep2, Raf has been proposed to amplify the response to a decrease in Rep protein levels, which is a signal of a decrease in 2µm protein levels. This signal amplification would enable rapid induction of plasmid genes, including *FLP*, when plasmid copy number drops. Once expressed, Flp recombinase can mediate plasmid amplification, restoring copy number, and consequently Rep proteins, to a level sufficient to re-establish Rep-mediated repression and active partitioning.

1.1.5 Host Toxicity Associated With 2µm Plasmid Hyperamplification And Rep Protein Overexpression

In conditions where plasmid copy number is normal, repression of *FLP* is essential for 2µm plasmid maintenance. Overexpression of Flp from a heterologous promoter leads to elevated plasmid copy number and impaired host cell proliferation (Murray *et al.*, 1987; Reynolds *et al.*, 1987). This toxicity is characterized by the appearance of large, multi-budded, filamentous cells, and colonies with uneven “nibbled” borders – phenotypes suggestive of a defect in the progression of the G2/M phase of the cell cycle (Holm, 1982). Co-overexpression of Rep1 and Rep2 recapitulates this phenotype, regardless of 2µm plasmid presence (Scott-Drew and Murray, 1998),

suggesting that it is the excess of Rep proteins that is responsible for the cell-cycle defect associated with 2µm plasmid hyperamplification.

The mechanism by which high levels of Rep1 and Rep2 proteins lead to a cell cycle defect is unknown. The role of the Rep proteins in mediating plasmid partitioning requires that they act as the interface between plasmid and host factors, and this provides several avenues through which the Rep proteins could subvert host functions required for normal cell division. Additionally, co-overexpression of Rep1 and Rep2 from a heterologous promoter has been shown to decrease silencing at the *HMR* mating-type locus, suggesting that the Rep proteins may prevent the establishment of silent chromatin, which in turn could be detrimental to cell cycle progression (Papacs *et al.*, 2004). The manner in which the Rep proteins achieve this anti-silencing effect is unclear.

Another way in which Rep proteins could mediate toxicity is through inappropriate repression of the transcription of host genes. Comparisons of sequences at *STB*, where the Rep proteins have been shown to associate, with sequences at the *FLP/REP2p* and *REP1/RAFp* have identified common motifs that may be involved in recruiting Rep1 and Rep2 (Murray and Cesareni, 1986; Som *et al.*, 1988; Veit and Fangman, 1988). Most of these motifs are short, being only six to nine nucleotides in length; therefore, it seems likely that they may be found in the promoter regions of some host genes as well by chance. This possibility is further supported by the observation that overexpression of Raf, which relieves Rep-mediated transcriptional repression, also relieves Rep-mediated toxicity (Pinder, 2011).

1.2 Interface Of 2µm Plasmid With Host

Several host proteins have been shown to associate with *STB* and contribute to plasmid partitioning, including the nuclear motor protein Kip1 (Cui *et al.*, 2009), components of the RSC2 chromatin remodeling complex (Wong *et al.*, 2002; Huang *et al.*, 2004; Ma *et al.*, 2012), the centromere-specific histone H3 variant Cse4 (Hajra *et al.*, 2006), and the cohesin complex (Mehta *et al.*, 2002).

1.2.1 Host Proteins Required For 2µm Plasmid Partitioning

What roles do these host proteins play in plasmid partitioning, and how might these roles relate to their roles in chromosome segregation? A brief summary of the

functions of Kip1, RSC2, Cse4 and cohesin, and evidence for their involvement in 2µm plasmid partitioning is presented in the following sections.

1.2.1.1 Kip1

The host protein that spends the greatest portion of the cell cycle in association with *STB* is Kip1. Kip1 is a nuclear motor protein, belonging to the kinesin-5 family of ATP-dependent motor proteins, which are responsible for cross-linking and sliding apart interpolar microtubules (reviewed in (Cross and McAinsh, 2014)). Like most kinesin motor proteins, kinesin-5 proteins contain a highly conserved motor domain and an extended helical region that assists in oligomerization by participating in intermolecular coiled-coil interactions. Dimerization allows kinesins to move in a directed manner along the microtubule surface. The active configuration of kinesin-5 proteins is a homotetramer, which allows motors to bind two microtubules simultaneously, consistent with their function in microtubule cross-linking. Kinesin-5 motors have been shown to display processive movement along microtubules in both the plus-end and minus-end directions (Gerson-Gurwitz *et al.*, 2011; Roostalu *et al.*, 2011).

Kip1 is one of two kinesin-5 motor proteins in yeast, with the other being a Kip1 paralog, Cin8. Together *KIP1* and *CIN8* are a functionally redundant gene pair, as deletion of both simultaneously is lethal, while deletion of either alone is not (Hoyt *et al.*, 1992; Roof *et al.*, 1992). Of the two, Cin8 is thought to play a larger role in the cell, as the phenotypes associated with *CIN8* deletion are more severe than those associated with deletion of *KIP1* (Hoyt *et al.*, 1992; Roof *et al.*, 1992; Geiser *et al.*, 1997; de Gramont *et al.*, 2007). Most Kip1 functions, including clustering and aligning kinetochores on the metaphase spindle, overlap with those of Cin8 (Tytell and Sorger, 2006); however, Kip1 has been shown to persist at the spindle midzone in anaphase after Cin8 has dispersed (Fridman *et al.*, 2013), which is consistent with the observation that Kip1 plays the major role in mediating the second half of late anaphase spindle elongation (Straight *et al.*, 1998).

In addition to Kip1 and Cin8, yeast contain two other nuclear motor proteins, Kar3 and Kip3. Although Kip1, Cin8, Kar3 and Kip1 have all been shown to associate with *STB in vivo* in the presence of Rep1 and Rep2, deletion of the *KIP1* gene, but not the *CIN8*, *KAR3*, or *KIP3* genes, was shown to result in an increase in plasmid

missegregation (Cui *et al.*, 2009). The motor activity of Kip1 is required for its role in plasmid partitioning, suggesting that it may be required to move plasmid copies to a particular "nuclear address" to allow efficient partitioning (Cui *et al.*, 2009). Rep2 has been shown to associate with Kip1 independently of other 2µm plasmid factors (Cui *et al.*, 2009), suggesting a mechanism by which Kip1 could be recruited to *STB*. In support of this possibility, Rep1 association with both Rep2 and *STB* is required for association of Kip1 with *STB* (Cui *et al.*, 2009).

Although Kip1 would seem to be a critical host protein for 2µm plasmid maintenance, we have observed that yeast lacking Kip1 do not lose the native 2µm plasmid, and are as proficient as strains having Kip1 at partitioning a 2µm-based plasmid (Pinder, 2011); this suggests that the role of Kip1 in 2µm plasmid partitioning, if any, may be in fine-tuning aspects of this mechanism.

1.2.1.2 RSC2 Complex

RSC2 is one of two isoforms of the RSC complex, so named for its role in remodeling the structure of chromatin. The RSC complex isoforms are identical with the exception of the Rsc1 subunit, found exclusively in the RSC1 isoform, and Rsc2, which is homologous to Rsc1 and found exclusively found in RSC2. RSC is part of the SWI/SNF family of chromatin remodelers, which are highly conserved in eukaryotes from yeast to humans (reviewed in (Mohrmann and Verrijzer, 2005)). Like all chromatin remodelers, those of the SWI/SNF family have DNA-dependent ATPase activity and the ability to recognize covalent histone modifications. Chromatin remodelers mediate nucleosome remodeling, repositioning, eviction and replacement. Structural studies show that the RSC complex surrounds the nucleosome, holding it in a central cavity (Chaban *et al.*, 2008). Current models suggest that RSC binding destabilizes histone-DNA contacts, possibly due to favourable ionic conditions within the central cavity, which facilitates the ATP-dependent translocation of DNA along the nucleosome surface (Lorch *et al.*, 2010).

Yeast encode two SWI/SNF family remodelers, RSC and SWI/SNF, of which only RSC is essential (Cairns *et al.*, 1996). RSC consists of 17 subunits, two of which are shared and four of which are homologous with those of SWI/SNF. The Sth1 subunit of RSC carries a conserved ATPase domain required for nucleosome remodeling activity (Du *et al.*, 1998), and, together with Arp7, Arp9, Rsc6, Rsc8 and Sfh1, is part of the

widely-conserved core of the RSC complex. Rsc1, Rsc2, Rsc4 and Rsc9 also show some similarity to chromatin remodeler subunits found in higher eukaryotes (Xue *et al.*, 2000; Mohrmann and Verrijzer, 2005). Five RSC subunits – Rsc3, Rsc30, Lbd7, Npl6 and Htl1 – form a subdomain of RSC found only in fungal species (Wilson *et al.*, 2006). The Rsc3 and Rsc30 subunits contain zinc finger DNA-binding domains and confer sequence specificity to the association of RSC with chromatin (Badis *et al.*, 2008; Zhu *et al.*, 2009). The preferred binding sites of Rsc3 and Rsc30 are CGCGCGC and CGCGCGCGCG, respectively (Zhu *et al.*, 2009), and the motif CGCG is enriched at sites of RSC binding, as identified in genome-wide immunoprecipitation studies using several RSC subunits (Badis *et al.*, 2008). As none of the subunits of the SWI/SNF family remodelers in higher eukaryotes are known to have sequence-specific DNA-binding activity, it has been suggested that these SWI/SNF family remodelers rely more heavily on interactions with histone modifications to regulate their recruitment (Mohrmann and Verrijzer, 2005).

Chromatin remodeling by RSC is involved in a wide variety of cellular functions. Inactivation of the catalytic Sth1 subunit of RSC leads to a global decrease in mRNA, tRNA and rRNA transcription (Parnell *et al.*, 2008). RSC is recruited specifically to promoters of genes transcribed by RNA polymerases II and III (Ng *et al.*, 2002). RNA polymerase III may mediate the association of RSC with tRNA genes (Ng *et al.*, 2002; Badis *et al.*, 2008), while association of RSC with RNA polymerase II-transcribed genes is more highly regulated and can have both positive and negative effects on gene expression (Ng *et al.*, 2002). RSC has also been shown to assist in transcriptional elongation by RNA polymerase II from a nucleosomal template *in vitro*, and this activity is strongly stimulated by histone acetylation (Carey *et al.*, 2006).

The RSC complex also has several roles in genome maintenance. RSC is involved in the recruitment of cohesin to both centromeres and chromosome arms (Huang *et al.*, 2004; Lopez-Serra *et al.*, 2014), while cohesin in turn contributes to the destabilization of nucleosomes by RSC (Lopez-Serra *et al.*, 2014). RSC associates with Cse4 and influences chromatin arrangement at centromeres, although RSC is not required for localization of Cse4 or other kinetochore components to centromeres (Hsu *et al.*, 2003; Baetz *et al.*, 2004). Mutation of several RSC subunits has been shown to lead to an increase in chromosome missegregation (Tsuchiya *et al.*, 1998; Hsu *et al.*, 2003). RSC is

also involved in the repair of double-stranded DNA breaks (Chai *et al.*, 2005; Shim *et al.*, 2005; Shim *et al.*, 2007; Kent *et al.*, 2007; Chambers *et al.*, 2012) and required for the expression of both early and mid-late sporulation-specific genes (Yukawa *et al.*, 2002; Bungard *et al.*, 2004).

Although most functions of the RSC complex described above can be performed by either RSC1 or RSC2, loss of each isoform has distinct consequences. Examples of this distinction in the regulation of chromosome segregation are that RSC2 has been shown to play a larger role than RSC1 in mediating sister chromatid cohesion, and greater chromosome loss is observed upon deletion of the *RSC2* gene than the *RSC1* gene (Baetz *et al.*, 2004).

Both Rsc1 and Rsc2, along with several other RSC2 complex subunits, co-purified with the 2µm plasmid Rep1 and Rep2 proteins in tandem affinity purifications (Liu *et al.*, 2013); of the host proteins that have been shown to have roles in 2µm plasmid partitioning, RSC subunits are the only ones to do so. The fact that Rsc1 also co-purifies with Rep proteins suggests that both RSC isoforms may be involved in plasmid partitioning; however, while partitioning is impaired in the absence of Rsc2 (Wong *et al.*, 2002), or of functional Rsc8 or Rsc58 (Ma *et al.*, 2012), inheritance is not impaired in the absence of Rsc1, suggesting that only the RSC2 isoform is required for efficient partitioning (Wong *et al.*, 2002). The observation that a nuclease-sensitive site in the middle of the *STB* locus disappears upon deletion of Rsc2 suggests that the RSC2 complex may serve to remodel chromatin at *STB* (Wong *et al.*, 2002). Inactivation of the catalytic Sth1 subunit of RSC2 impairs the association of cohesin with *STB*, implying that RSC2 remodeling activity is required to recruit cohesin to this locus (Huang *et al.*, 2004). The timing of association of RSC2 subunits with *STB*, as determined by chromatin immunoprecipitation in synchronized cells (Liu *et al.*, 2013), suggests that RSC2 may also play a role in the recruitment of Kip1 and Cse4, although this has not been tested.

1.2.1.3 Cse4

Concomitant with RSC2 subunits Sth1 and Rsc2, Cse4 is recruited to *STB* in S phase. Cse4 is a centromere-specific variant of histone H3, homologs of which serve to define centromeres in all eukaroytes. Referred to collectively as CenH3 or CENP-A

proteins, these variants contain a histone fold domain similar to that of conventional histone H3, but a longer amino-terminal tail domain (reviewed in (Talbert *et al.*, 2009)).

The small genetically-defined centromere of budding yeast wraps a single Cse4-containing nucleosome (Henikoff and Henikoff, 2012), although observation of fluorescently-tagged Cse4 suggests the existence of a small pool of pericentromeric Cse4 (Haase *et al.*, 2013). Cse4 can become mis-incorporated into regions of high histone turnover if overexpressed (Hewawasam *et al.*, 2010; Ranjitkar *et al.*, 2010); however, under normal conditions, mis-localized Cse4 is rapidly degraded by ubiquitin-mediated proteolysis, among other mechanisms (Collins *et al.*, 2004). A chaperone protein, Scm3, serves to protect Cse4 from ubiquitination and guides it to the centromere through association with Ndc10, a yeast-specific protein of the inner kinetochore (Camahort *et al.*, 2007). A homolog of Scm3, HJURP, is found in mammalian cells, and performs a similar role in CenH3 chaperoning and kinetochore assembly.

While the presence of Cse4 in centromeric nucleosomes is well established, the composition of these nucleosome has been a topic of debate for nearly a decade. Nucleosomes at centromeres have been found to protect only approximately 125 base pairs (bp) of DNA from nuclease digestion, while canonical nucleosomes protect approximately 146 bp (Kurumizaka *et al.*, 2013), suggesting that centromeric nucleosome structure is atypical. Centromeric nucleosomes have also been shown to induce positive supercoiling, suggesting that DNA is wrapped around these nucleosomes in a right-handed fashion (Furuyama and Henikoff, 2009); this is in contrast to the nucleosomes of chromosome arms, which induce negative supercoils and show a left-handed DNA wrap. A variety of structures have been proposed for centromeric nucleosomes. These include hemisomes (Dalal *et al.*, 2007) and octamers (Camahort *et al.*, 2009) composed of one or two molecules each, respectively, of histones H2A, H2B, CenH3 and H4; tetrasomes composed of two molecules each of histones CenH3 and H4 (Williams *et al.*, 2009); and hexasomes composed of two molecules each of Scm3 and histones CenH3 and H4 (Mizuguchi *et al.*, 2007). Based on evidence from studies in yeast and human cells, it has also been suggested that centromeric nucleosomes transition between hemisomal and octameric arrangements in a cell cycle-dependent fashion (Bui *et al.*, 2012; Shivaraju *et*

al., 2012), a possibility that would reconcile some of the contradictory structural observations.

Association of Cse4 with *STB* is not dependent on its association with centromeres (Hajra *et al.*, 2006). Cse4 is deposited concomitantly at both *STB* and centromeres in early S phase; however, the timing of Cse4 dissociation from *STB* is distinct from that observed at centromeres, with Cse4 exiting *STB* upon spindle disassembly prior to cytokinesis, but dissociating from the centromere only briefly at the beginning of S phase (Hajra *et al.*, 2006). The observations that Cse4 association with *STB* occurs in the absence of cross-linking agents and in the presence of high salt concentrations, and that Cse4 protects *STB* DNA from restriction enzyme cleavage, suggest that Cse4 is incorporated into a nucleosome at *STB* (Hajra *et al.*, 2006; Huang *et al.*, 2011b). The nucleosomal incorporation of Cse4 is further supported by comparisons of plasmid supercoiling in the presence versus the absence of Rep proteins, which are required for association of Cse4 with *STB*. In the presence of Rep proteins, supercoiling of *STB*-containing plasmids showed a positive change of between one and two linking units, consistent with either the removal of two nucleosomes with left-handed DNA wraps, or the replacement of a nucleosome with a left-handed DNA wrap by a nucleosome with a right-handed DNA wrap, such as a Cse4-containing nucleosome (Huang *et al.*, 2011a). Positive supercoiling of *STB* in the presence of Rep proteins was also observed in experiments aimed at identifying sequence elements that prevented DNA rotation, although in this instance the authors postulated that this supercoiling was due to the tethering of *STB* to a nuclear structure by Rep1 and Rep2 (Gartenberg and Wang, 1993).

In addition to the Rep1 and Rep2 proteins, Cse4 recruitment to *STB* requires Scm3, which has been shown to associate with *STB* (Huang *et al.*, 2011b). The presence of Scm3 may explain how Cse4 escapes the proteolytic degradation encountered by most non-centromeric Cse4. Association of Cse4 with *STB* is also dependent on the Kip1 motor protein (Cui *et al.*, 2009) and spindle integrity (Hajra *et al.*, 2006). Chromatin immunoprecipitation (ChIP) experiments performed in strains harbouring a version of Cse4 shown to impair centromere function suggest that wild-type Cse4 is required for the recruitment of Rep2, RSC subunits Rsc2 and Rsc8, and cohesin subunit Mcd1 to *STB*

(Hajra *et al.*, 2006). As Rep2 and Rsc8 have been observed to associate with *STB* prior to Cse4 recruitment (Ma *et al.*, 2012), this requirement for wild-type Cse4 may be related to its role at centromeres instead of or in addition to its role at *STB*.

Quantification of the efficiency of *STB* immunoprecipitation by Cse4 indicated that association of Cse4 with *STB* was highly sub-stoichiometric, consistent with Cse4 only being present at *STB* loci of 10 to 20% of 2μm plasmid copies in the cell (Ghosh *et al.*, 2010; Huang *et al.*, 2011b). This implies that the presence of Cse4 at *STB* may not be required for their role in partitioning, or may only be required by a subset of plasmids within a cluster. In experiments where a single-copy *STB* reporter plasmid was used, association of Cse4 with *STB* was shown to be nearly stoichiometric (Huang *et al.*, 2011a), supporting the argument that this association is indeed required for partitioning function, as, were association of Cse4 with *STB* not required for partitioning function, it would likely immunoprecipitate the *STB* DNA of the single-copy plasmid in sub-stoichiometric concentrations, in a manner similar to the high-copy *STB* plasmid.

1.2.1.4 Cohesin

The final host factor reported to associate with *STB* is the cohesin complex (reviewed in (Marston, 2014)). Like the RSC complex and Cse4, cohesin is conserved throughout eukaryotes, serving to hold sister chromatids together after their replication in S phase. Cohesin is a ring-shaped structure made up of four core subunits, which in yeast are Smc1, Smc2, Mcd1 and Scc3. Smc1, Smc2 and Mcd1 make up the cohesin ring, while Scc3 interacts with the middle domain of Mcd1, and is required for establishment of the cohesin ring on chromosomes. The cohesin complex is loaded onto DNA in an ATP-dependent fashion with the help of a loader complex made up of Scc2 and Scc4 in yeast. Cohesin maintains sister chromatid cohesion by encircling the chromatids topologically. At the metaphase-to-anaphase transition, Mcd1 is cleaved, opening the cohesin ring and allowing the sister chromatids to separate.

Cohesin is not distributed evenly along the chromosome, but instead is enriched in the vicinity of the centromere and at sites of convergent transcription (Lengronne *et al.*, 2004). These regions do not correspond to sites of cohesin loading, which are enriched at transcriptional start sites and favour poly(A) tracts (Lopez-Serra *et al.*, 2014), suggesting that the cohesin ring slides along the chromatin fiber after loading. Cohesin is

also involved in cellular functions other than sister chromatid cohesion (reviewed in (Mehta *et al.*, 2013)). In addition to interchromatid cohesion, cohesin can mediate intrachromatid cohesion, generating chromatin loops that can serve to condense chromosomes during mitosis and bring regulatory regions into contact with their target genes. Cohesin is also involved in the repair of DNA double-stranded breaks and prevents non-disjunction in meiosis I.

All three subunits of the cohesin ring have been shown to associate with the *STB* locus of the 2µm plasmid, although, like that of Cse4, the association of cohesin with *STB* is highly substoichiometric (Mehta *et al.*, 2002; Ma *et al.*, 2012). Immunoprecipitation experiments suggest that a single cohesin ring embraces a pair of replicated sister plasmids (Ghosh *et al.*, 2007; Ghosh *et al.*, 2010). The Scc2-Scc4 cohesin loader complex is required for cohesin deposition at *STB*, and Scc2 has been shown to associate with *STB* in the presence of Rep1 and Rep2 (Ghosh *et al.*, 2007). Cohesin is not likely responsible for 2µm plasmid clustering, as foci of fluorescently-tagged plasmids are observed at all stages of the cell cycle, while cohesin is cleaved at the metaphase-to-anaphase transition and is not re-established until S phase (Velmurugan *et al.*, 1998; Mehta *et al.*, 2002). Temporally, cohesin is the last of the identified host partitioning proteins to associate with *STB* (Ma *et al.*, 2012), and its association is abolished in the absence of Rep1, Rep2, Kip1, Rsc2, functional Cse4 or spindle integrity (Mehta *et al.*, 2002; Hajra *et al.*, 2006; Cui *et al.*, 2009).

1.2.1.5 Timeline Of Protein Association With *STB*

ChIP experiments using synchronized cell cultures have established a timeline of *STB* association for some of the key host proteins involved in 2µm plasmid partitioning (Ma *et al.*, 2012). At the beginning of S phase, the Rsc8 and Rsc58 subunits of the RSC2 chromatin remodeling complex are recruited to the *STB* locus, where Rep1, Rep2 and Kip1 from the previous cell cycle are already bound. Rep1, Rep2 and Kip1 then dissociate briefly from *STB* before reassociating. Next, two more RSC2 subunits, Sth1 and Rsc2, and Cse4 are brought to *STB*, shortly before the cohesin subunit Mcd1 is recruited. The four RSC2 subunits, Rsc8, Rsc58, Sth1 and Rsc2, dissociate from *STB* simultaneously (Ma *et al.*, 2012). Mcd1 is released upon cohesin cleavage in late

anaphase, and Cse4 shortly thereafter (Hajra *et al.*, 2006), while Rep1, Rep2 and Kip1 remain associated through G1 and into the following S phase (Ma *et al.*, 2012).

1.2.2 Similarities And Differences Between Chromosome Segregation And 2µm Plasmid Partitioning

Although the 2µm plasmid-associated host factors are all recruited to either centromeric (in the case of Kip1 and Cse4) or pericentromeric (in the case of the RSC2 complex and cohesin) regions, proteins that form part of the kinetochore complex required for microtubule capture at centromeres, such as Ndc10 and Ncd80, do not associate with *STB* (Mehta *et al.*, 2002; Mehta *et al.*, 2005), making it unclear how *STB* mediates partitioning. Under conditions of unequal chromosome segregation, the 2µm plasmid has been shown to missegregate with chromosomes (Velmurugan *et al.*, 2000; Mehta *et al.*, 2002; Scott-Drew *et al.*, 2002; Liu *et al.*, 2013), suggesting that the plasmid may be tethered to chromosomes by the Rep proteins to ensure equal partitioning – a strategy similar to that used by many viral episomes (Frappier, 2004). Supporting this possibility is the observation that the plasmid co-localizes with chromosomes in chromosome spreads from both actively-dividing and G1-arrested cells (Mehta *et al.*, 2002). In a sucrose gradient, the 2µm plasmid was shown to co-sediment with chromosomes of actively-dividing cells, but not those from cells in G0 stationary phase, suggesting that the plasmid may recognize condensed chromosomes specifically (Taketo *et al.*, 1980). There is also evidence to suggest that the plasmid may interact with the spindle independently of chromosomes, as chromosomes segregate efficiently following spindle depolymerization and recovery, while 2µm plasmid copies are unevenly partitioned under these conditions (Mehta *et al.*, 2005). Overall, these results demonstrate that the 2µm plasmid relies on the host chromosomes and spindle apparatus for partitioning function; however, the precise mechanism by which the plasmid interacts with these components remains unclear.

1.2.3 Localization Of The 2µm Plasmid

In mitotic cells, the plasmid foci are found in the vicinity of the spindle pole body, and not with the mitotic spindle, the nuclear periphery, the nucleolus, telomeres, or sites of cohesin binding (Velmurugan *et al.*, 2000; Scott-Drew *et al.*, 2002); therefore, despite

their role in partitioning, neither the mitotic spindle nor cohesin are likely to be the direct link between 2µm plasmids and chromosomes. During meiosis, the plasmid co-localizaes with chromatin and depends on the chromosomes for equal partitioning, as it does in mitosis; however, the plasmid localizes to the nuclear periphery instead of the spindle pole bodies during meiosis I (Sau *et al.*, 2014).

1.2.4 Model Of 2µm Plasmid Partitioning

Many conditions have been identified that impair the ability of the 2µm plasmid to be evenly distributed between mother and daughter cells. However, the uneven partitioning observed when the Rep proteins are absent shows a strong maternal bias, while the uneven partitioning observed upon perturbation of cohesin function or spindle integrity is not biased toward either mother or daughter cell (Liu *et al.*, 2013). This suggests that equal plasmid partitioning has two separable requirements (Liu *et al.*, 2013). The first is Rep protein-mediated circumvention of the maternal inheritance bias, likely by tethering plasmid copies to chromosomes. The second involves a mechanism to ensure that an equal number of plasmids are tethered to chromatids destined each for the mother and daughter cell, and depends on spindle integrity and functional cohesin. Cohesin is responsible for pairing of sister plasmids after replication, and cohesin cleavage is required for the separation of foci of fluorescently-tagged 2µm plasmid-reporters at anaphase, suggesting that cohesin may participate in some sort of plasmid counting mechanism (Ghosh *et al.*, 2007). Association of the centromeric histone H3 variant Cse4 with *STB*, like that of cohesin, depends on spindle integrity (Hajra *et al.*, 2006), while association of Kip1 and components of the RSC2 complex do not (Cui *et al.*, 2009). This suggests that that the role of Cse4 in plasmid partitioning may also relate to the plasmid counting mechanism, while Kip1 and RSC2 may be involved, along with Rep1 and Rep2, in overcoming the maternal inheritance bias.

1.3 The *STB* Partitioning Locus

1.3.1 The *STB* Locus Of The 2µm Plasmid Of *S. cerevisiae*

Although significant progress toward understanding the host components that contribute to 2µm plasmid inheritance has been made in recent years, the DNA locus on which this partitioning system is established is still not well understood. The *STB* locus

was originally defined as the 606-bp sequence extending from the AvaI site near the *ORI* to the PstI site in the *RAF* gene sufficient to confer plasmid partitioning function to an *ARS*-only plasmid, provided the Rep1 and Rep2 proteins were supplied *in trans* (Kikuchi, 1983). *STB* was subsequently further divided at a HpaI site into two functional domains, *STB*-proximal (*STB-P*) and *STB-distal* (*STB-D*), so named for their positioning relative to the *ORI* (Kikuchi, 1983; Murray and Cesareni, 1986). The 294-bp *STB-P* portion of the locus is required and sufficient to confer Rep protein-mediated partitioning function in most sequence contexts (Jayaram *et al.*, 1985; Murray and Cesareni, 1986). It contains a tandem array of five 62- to 63-bp direct repeats, with another half repeat on the *ORI* side of the AvaI site. *STB-D* is 312 bp in size, and encodes transcription termination signals and a silencer that protect *STB-P* from disruptive transcription from the adjacent *RAF* gene (Murray and Cesareni, 1986).

The specific sequence elements required at *STB-P* for Rep protein recognition and partitioning are not known. Individual *STB-P* repeats have 65% to 98% sequence identity (Figure 3), suggesting that there may be some redundancy within the locus. Indeed, deletion studies have shown that removal of 125-bp from the HpaI end of *STB-P*, leaving three-and-a-half repeats, does not abolish partitioning function or Cse4 recruitment, while removal of 187 bp, leaving only two-and-a-half repeats, leads to loss of function (Jayaram *et al.*, 1985; Huang *et al.*, 2011b).

1.3.2 *STB* Loci Of 2µm Plasmid Variants

Clues about *STB-P* features that are critical for partitioning can also be gleaned from sequence analyses of 2µm plasmid variants. A survey of industrial yeast strains identified eight 2µm plasmid variants, which were classified as Type-I or Type-II based on sequence diversity observed at *STB*; all of these *STB* sequences were able to confer plasmid partitioning function in laboratory yeast (Xiao *et al.*, 1991b; Xiao and Rank, 1993). The 2µm plasmid variant found in most laboratory strains of yeast, Scp1, was classified as a Type-II plasmid, along with most of the industrial variants observed. The *STB* regions of Type-II plasmids are highly similar in sequence, but vary in the number of 62- to 63-bp repeats they encode, with number of repeats ranging from three to seven. The *STB* locus of the Type-I plasmids shows only 70% sequence identity to that of the Type-II *STB*, and the two regions that correspond to two of the 62- to 63-bp repeats of the

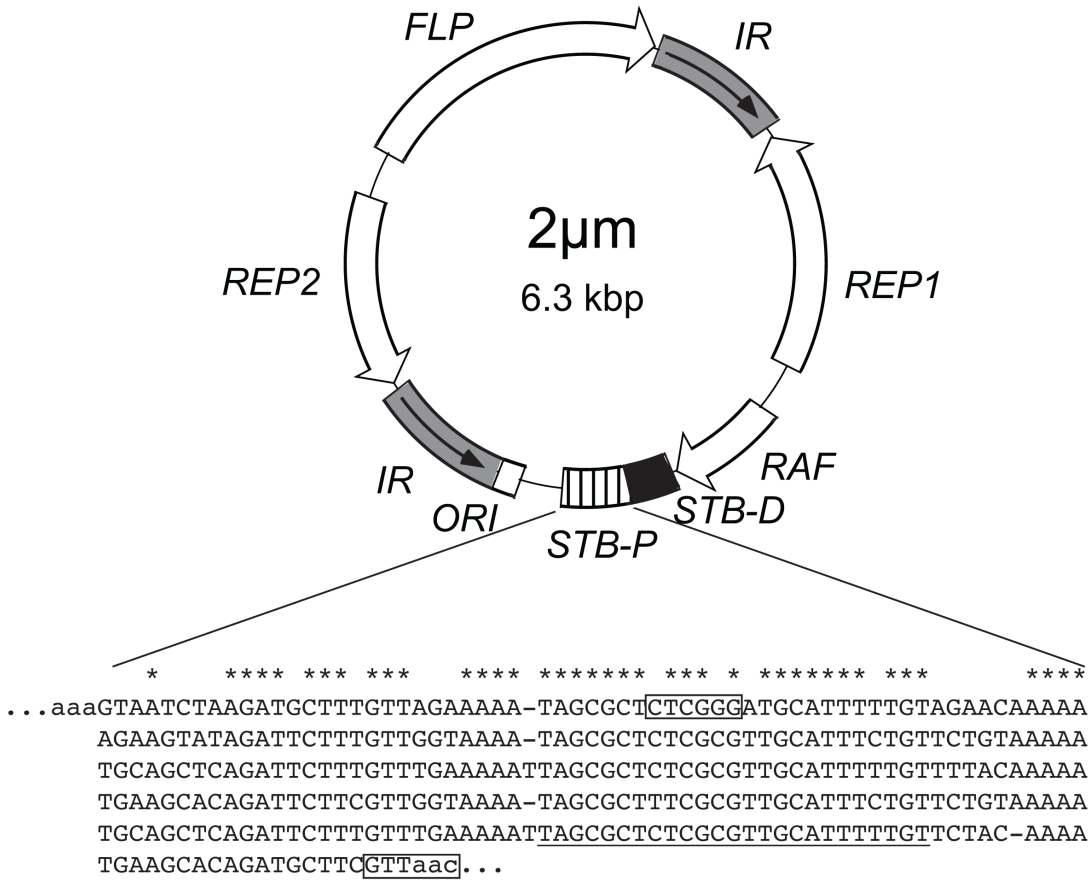

Figure 3. Map of 2µm plasmid and alignment of repeated sequences of *STB*. The repeated *STB* sequence is arranged to show the degree of similarity between the five-and-a-half tandem repeats. The AvaI and HpaI sites marking the boundaries of *STB-P* as originally defined are boxed, and the 25-bp "core" *STB* repeat consensus is underlined. Asterisks indicate positions of identical residues in the alignment.

Type-II *STB* show only ~30% sequence identity to each other (Xiao *et al.*, 1991b; Xiao and Rank, 1993). This suggests that the high degree of similarity between the *STB-P*

repeats of the more highly-studied laboratory yeast Scp1 variant is not required for function, and that the repetition of a short recognition motif may be the key to partitioning function. Candidates for this motif are likely to be found in the most highly conserved region of the repeats – a 25-bp core (Rank *et al.*, 1994b), which shows 80% identity among Type-II 2µm variants, and 60% identity among Type-I and Type-II variants, compared to only 41% identity for the entire 62- to 63-bp repeat among variants. Plasmid sequences reported by a recent study, in which the entire genomes of 93 strains of *S. cerevisiae* were sequenced, could further narrow down this conserved region to highlight sequence elements of functional importance (Strope *et al.*, 2015).

1.3.3 Partitioning Loci Of 2µm-Like Plasmids

Other budding yeast species of the Saccharomycetaceae family have also been shown to carry circular plasmids similar to the 2µm plasmid in their nuclei. In total, nine distinct plasmids have been identified in seven yeast species other than *S. cerevisiae*, ranging from 4500-bp to 6700-bp in size (Toh-E *et al.*, 1984; Araki *et al.*, 1985; Toh-e and Utatsu, 1985; Chen *et al.*, 1986; Utatsu *et al.*, 1987; Chen *et al.*, 1992; Blaisonneau *et al.*, 1997; Douradinha *et al.*, 2014). These 2µm-like plasmids show very little similarity to each other at the nucleotide level, and cannot be efficiently maintained in yeast species other than their own (Toh-e and Utatsu, 1985; Jearnpipatkul *et al.*, 1987b; Chen *et al.*, 1992; Blaisonneau *et al.*, 1997). However, the 2µm-like plasmids do show similar organization, with each having a pair of long inverted repeats that separate the plasmid into two unique regions containing three to four open reading frames (ORFs; Figure 4). On each plasmid, one of these ORFs encodes a protein with homology to Flp recombinase of the 2µm plasmid, which, where tested, has been shown to be required for intramolecular recombination (Araki *et al.*, 1985). Another encodes a homolog of the 2µm plasmid Rep1 protein, while the third and fourth ORFs, although in the positions of the *REP2* and *RAF* genes, encode proteins with no significant sequence similarity to their 2µm plasmid counterparts. Where tested, the Rep1 homolog and the product of the *REP2*-positioned ORF have been shown to be required for efficient plasmid inheritance (Bianchi, Santarelli & Frontali 1991, Toh-e, Utatsu 1985, Jearnpipatkul, Araki & Oshima

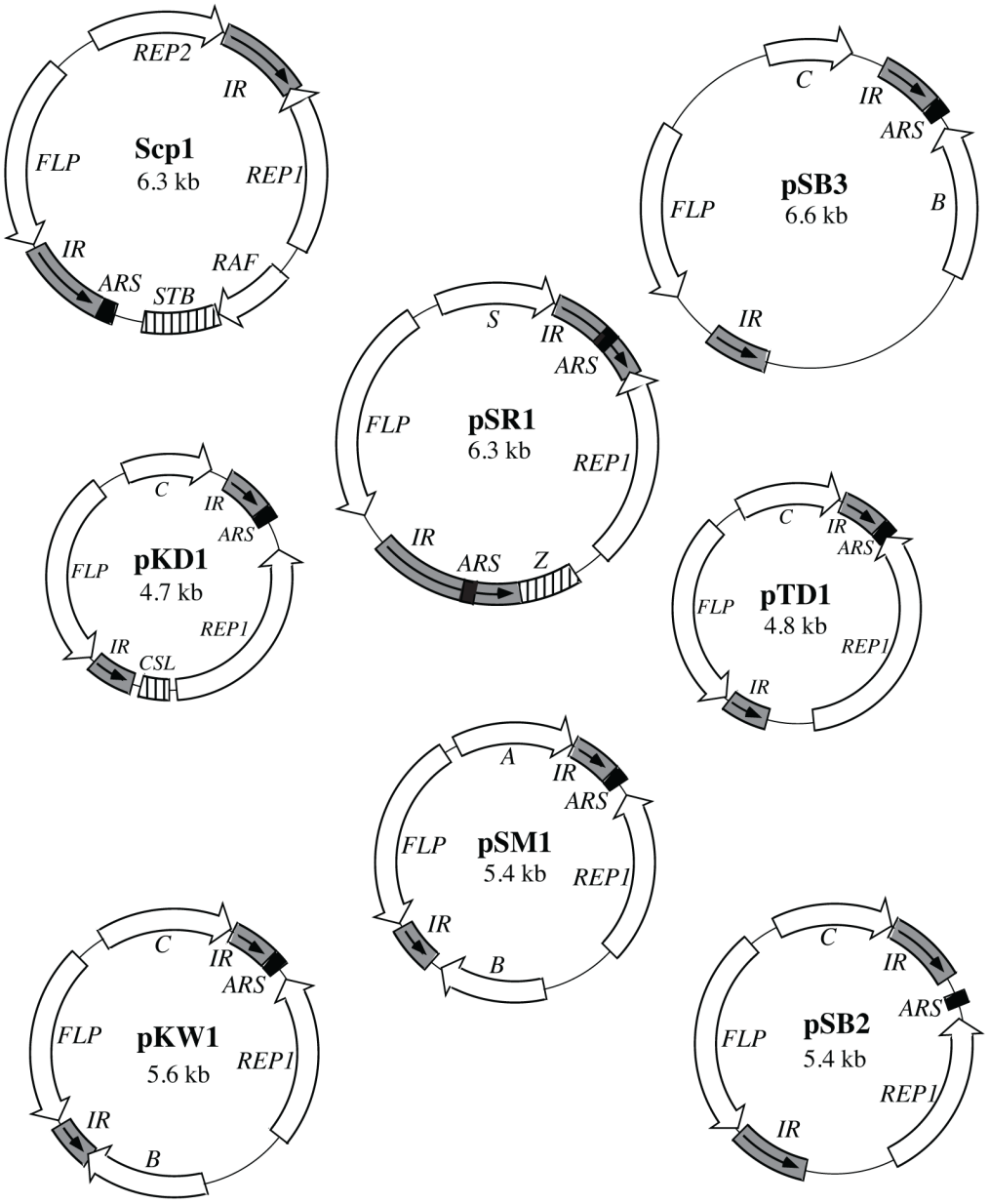

Figure 4. Maps of 2µm and 2µm-like plasmids isolated from closely-related budding yeast species. Plasmids are the 2µm plasmid from *Saccharomyces cerevisiae*, pSR1 and pSB3 from *Zygosaccharomyces rouxii*, pSB2 from *Zygosaccharomyces bailii*, pSM1 from *Zygosaccharomyces fermentati*, pKD1 from *Kluveromyces lactis*, pKW1 from *Kluveromyces waltii*, and pTD1 from *Torulaspora delbrueckii*. Autonomously replicating sequences (*ARS*) are indicated by black boxes, ORFs with putative protein products that lack significant similarity to Rep1 or Flp (*B*, *C* and *S*) are indicated by white arrows, and experimentally-identified *cis*-acting stability loci (*STB*, *Z* and *CSL*) are indicated by striped boxes; all other plasmid features are indicated as described in the legend of Figure 1.

1987), suggesting that the product of the *REP2*-positioned ORF may indeed be related to Rep2.

These 2µm-like partitioning proteins are presumed to interact with DNA loci on their respective plasmids; however, of the seven sequenced 2µm-like plasmids, none encodes an array of direct tandem repeats as striking as those found at the 2µm plasmid *STB-P* locus. The *Z* locus of pSR1 and the *cis*-acting locus (*CSL*) locus of pKD1 have been shown to be sufficient to confer partitioning function *in cis* when both of their respective partitioning proteins are provided *in trans* (Jearnpipatkul *et al.*, 1987b; Bianchi *et al.*, 1991). These loci are 383-bp and approximately 200-bp in length, respectively, and both contain three to four copies of short direct repeat sequences, separated by stretches of non-repeated sequence. These observations support the inference made based on studies of 2µm plasmid variants, that a series of long tandemly-arrayed repeats are not required for partitioning of the 2µm family of plasmids.

1.3.4 Theory Of Common Evolutionary Origin For *STB* And Budding Yeast Centromere

In addition to possessing 2µm-like plasmids, species of the Saccharomycetaceae lineage of budding yeast are nearly unique among eukaryotes in that they also have genetically-defined point centromeres. Point centromeres are short (~125 bp), genetically-defined loci, having sequence elements for recruitment of components of the inner kinetochore, three of which (Cep3, Ctf13 and Ndc10) are specific to the Saccharomycetaceae family (Meraldi *et al.*, 2006). This is in contrast to most other eukaryotes, including closely-related fungal species such as *Candida albicans* and the fission yeast *Schizocaccharomyces pombe*, which have epigenetically-defined regional centromeres. Most epigenetically-defined centromeres are embedded in regions of heterochromatin maintained by RNA interference (RNAi) mechanisms, while yeast of the Saccharomycetaceae family do not possess the machinery for generation of centromeric heterochromatin or for RNAi (reviewed in (Malik and Henikoff, 2009)).

The coincidence of 2µm-like plasmids and point centromeres has led to the proposal that yeast point centromeres evolved from the partitioning loci ancestral 2µm-like plasmids (Malik and Henikoff, 2009). According to this theory, when 2µm-like plasmids first appeared in budding yeast, they may have survived by co-opting

components of the host centromere, such as Cse4. In strains in which the plasmid partitioning locus became integrated into the chromosome, selection for components required for the maintenance of epigenetically-defined centromeres, such as the RNAi machinery, may have been relaxed, allowing the loss of epigenetically-defined centromeres in favour of partitioning locus-based centromeres. The host would also have required the 2µm plasmid proteins responsible for the recruitment of centromeric proteins to these new, genetic centromeres, namely Rep1 and Rep2. Rep1 and Rep2 are not recruited to the budding yeast centromere in their current form (Mehta *et al.*, 2002). However, the authors of this theory propose that at least two of the Saccharomycetaceae-specific subunits of the inner kinetochore may have evolved from Rep1 and Rep2, with the rapid evolution of 2µm-like plasmids obscuring this relationship, as it has the relationship between Rep2 of the 2µm plasmid and products of *REP2*-positioned ORFs from 2µm-like plasmids.

1.4 Other Circular DNA Plasmids

Many other plasmids found in a wide range of host species are also actively partitioned in order to avoid the generation of plasmid-free cells that would out-compete plasmid-bearing members of the population under conditions in which the plasmid confers no advantage or a disadvantage to the host cell. A general strategy for plasmid partitioning in both prokaryotic and eukaryotic cells involves one or two plasmid-encoded proteins that associate with a repetitive *cis*-acting plasmid sequence to localize the plasmid in a manner that favours its equal distribution between daughter cells at cell division (reviewed in (Funnell and Phillips, 2004)).

1.4.1 Bacterial Plasmids

The organization of the *par* system of partitioning used by the majority of bacterial plasmids (reviewed in (Funnell and Slavcev, 2004)), including the F plasmid and the plasmid prophage of the P1 bacteriophage, is similar to that of the 2µm plasmid in many ways. Both consist of two partitioning proteins that help to cluster plasmid copies and regulate their own expression, and a *cis*-acting locus with which these proteins interact. However, instead of tethering the plasmid to the host chromosome, most bacterial partitioning systems use the formation and destabilization of protein filaments to

localize themselves within the cell independently of the chromosome. These protein filaments are made up of subunits of one of the two partitioning proteins, which is an ATPase.

The bacterial plasmids that use *par* systems to ensure their maintenance are low copy plasmids. High copy bacterial plasmids had been thought not to use an active partitioning mechanism, but instead to be maintained due to the low probability of plasmid-free cell generation. However, high copy bacterial plasmids have been observed in clusters held at specific locations within the cell, suggesting that their inheritance is more regulated than originally thought (Million-Weaver and Camps, 2014). These plasmids may be tethered to a host structure, perhaps even chromosomal DNA, to ensure their equal partitioning, although further study is required to validate this possibility.

It is possible that the 2µm plasmid partitioning system originally evolved from that of a bacterial plasmid. The most striking evidence for this is the similarity of the Cre recombinase enzyme encoded by the P1 bacteriophage and the Flp recombinase of the 2µm plasmid, which both belong to the same family of tyrosine recombinases. Cre is responsible for circularizing the P1 genome and resolving multimeric forms of the P1 plasmid, and has been shown to recognize the *FRT* site, although this is with 20-fold lower affinity than recognition by Flp (Argos *et al.*, 1986).

1.4.2 Mammalian Viral Plasmids

The 2µm plasmid partitioning system also bears some resemblance to the partitioning systems of mammalian viral plasmids, including gammaherpesviruses, such as Epstein-Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV); and papillomaviruses (reviewed in (Van Doorslaer *et al.*, 2012)). These viruses encode one partitioning protein (EBNA1 in the case of EBV, LANA1 in the case of KSHV and E2 in the case of bovine papillomavirus 1 (BPV1)). This protein associates with a DNA locus on the viral plasmid to mediate equal partitioning of plasmid copies at cell division by tethering the plasmid to mitotic host chromosomes. A number of host proteins involved in this tethering have been elucidated, and include a kinesin-like motor (Yu *et al.*, 2007) and proteins associated with the centromere and mitotic spindle (Si *et al.*, 2008; Xiao *et al.*, 2010), which suggests that aspects of viral plasmid partitioning in mammalian cells may be analogous to 2µm plasmid partitioning in yeast. Overall, the picture of plasmid

tethering in eukaryotic cells that emerges from the study of 2µm and viral plasmids is one in which chromosomal tethering is not mediated by a single host protein, but requires different proteins for establishment and maintenance of tethering, and may be mediated differently at different points in the cell cycle (Van Doorslaer *et al.*, 2012).

In addition to the similarities in their modes of tethering, viral plasmids may also employ a plasmid counting mechanism, similar to the cohesin-dependent counting mechanism of the 2µm plasmid, to ensure equal partitioning of plasmid copies instead of relying on stochastic tethering. Observation of equal distribution of plasmid copies between daughter cells and of the pairing of plasmids in G2 provide support for this hypothesis (Frappier, 2013).

1.5 Overview

Although the findings described above present a general picture of how 2µm plasmid partitioning proceeds, the nature of the association between Rep1, Rep2 and the *STB* locus is unclear. Are any host proteins required to mediate association of Rep1 with *STB*? Is association of Rep2 with itself or with *STB* required for plasmid partitioning? What plasmid sequences are required for partitioning and for Rep protein-mediated transcriptional repression? What host proteins are required for this transcriptional repression? And how is Raf involved?

To answer these questions, I used *in vivo* assays, as well as bioinformatic and genetic approaches. I have identified functional redundancy in the interactions of the Rep2 protein with itself and Rep1 that reveal a role for Raf in 2µm plasmid partitioning, and provide support for our theory that Raf may be a highly divergent Rep2 paralog (Section 3.1). I created synthetic versions of the *STB* locus, and used these to show that two copies of a single 63-bp *STB* sequence are sufficient to mediate plasmid partitioning (Section 3.2). I showed that two TGCA sequences, a TGCA-adjacent T-tract and a CGCG sequence found in the *STB* repeat contribute to partitioning function. Analyses of both synthetic *STB* sequences (Section 3.2) and the *FLP/REP2* promoter (Section 3.3) suggest that CGCG sequences mediate transcription and TGCA sequences mediate Rep protein association at both loci. Finally, I used genome-wide screens to identify candidate genes involved in the regulation of either the promoter activity of *STB* or Rep protein-mediated toxicity (Section 3.4).

The sum of my results improves our understanding of the protein interactions and DNA sequences required for maintenance of the 2μm plasmid, which in turn contributes to our understanding of extra-chromosomal plasmids maintenance.

Chapter 2 METHODS

2.1 Strains And Media

Yeast strains used in this study are listed in Table 1. Yeast were cultured in YPAD (1% yeast extract, 2% Bacto Peptone, 0.003% adenine, 2% glucose), synthetic defined (SD; 0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 0.003% adenine, 0.002% uracil, and all required amino acids), or synthetic complete (SC; 0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 0.003% adenine, 0.002% uracil, 1% Difco casamino acids, 0.002% tryptophan) medium at 28˚C (Burke *et al.*, 1997). For induction of galactose-inducible promoters, 2% glucose was replaced with 2% galactose. For selection of plasmids or gene replacements tagged with nutritional genes, SC or SD medum lacking the appropriate nucleotide or amino acid was used. For selection of *kanMX4*- or *natMX4*-tagged plasmids and gene replacements, YPAD supplemented with 200 mg/L geneticin (G418, Sigma) or 100 mg/L nourseothricin (clonNAT, Werner BioAgents), respectively, was used. Yeast were transformed using the Li/SSS-DNA/PEG method (Gietz *et al.*, 1995).

Strains lacking the 2µm circle, designated cir^0, were derived from strains containing the 2µm circle, cir^+, by expression of a defective Flp recombinase from the plasmid pBIS-GALkFLP-(TRP1) (Tsalik and Gartenberg, 1998). Yeast gene-deletion strains were created by targeted replacement of wild-type alleles with *kanMX4* gene-deletion alleles amplified by polymerase chain reaction (PCR) from appropriate strains in the EUROSCARF yeast gene-deletion strain collection using recommended primers and conditions (Winzeler *et al.*, 1999). Transformed yeast were selected for G418-resistance, and gene deletions confirmed by PCR.

Escherichia coli strain DH5α was used for propagation of plasmids. *E. coli* were cultured and manipulated according to standard protocols (Sambrook *et al.*, 1989).

2.2 Plasmids

Plasmids used in this study are listed in Table 2. Oligonucleotides used as primers for PCR or as linkers for addition of restriction sites are listed in Table 3. Phusion

Table 1. Strains used in this study.

Strain	Genotype	Parent Strain/Source
W303a/α [cir$^+$]	*MAT**a**/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1* [*cir*$^+$]	(Pinder *et al.*, 2013)
W303a/α [cir^0]	*MAT**a**/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1* [*cir*^{0}]	(Pinder *et al.*, 2013)
MD83/7a	*MATα ade2-1 his3-11, 15 trp1-1 leu2-3,112 ura3-1 [cir$^+$]*	(Dobson *et al.*, 2005)
MD83/1c	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 [cir^0]*	(Dobson *et al.*, 2005)
MD83/1b	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1* [*cir*^{0}]	(Dobson *et al.*, 2005)
JP48/2b	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1* [*cir*$^+$]	W303 a/α [cir$^+$]
JP49/6b	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1* [*cir*^{0}]	W303 a/α [cir^0]
AG8/5	*MAT**a** ade2Δ::URA3 his3-11,15 leu2-3, 112 trp1-1 ura3-1 [cir^0]*	W303 a/α [cir^0]
JP98/2	*MAT**a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 rsc2Δ::natMX4* [*cir*^{0}]	(Pinder *et al.*, 2013)
WSWI4dk/1	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 swi4Δ::kanMX4* [*cir*$^+$]	MD83/7a
WSWI4dk/2	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 swi4Δ::kanMX4* [*cir*^{0}]	MD83/1c
WSWI6dk/1	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 swi6Δ::kanMX4* [*cir*$^+$]	MD83/7a
WSWI6dk/2	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 swi6Δ::kanMX4* [*cir*^{0}]	MD83/1c
WSWI6dn/2	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 swi6Δ::natMX4* [*cir*^{0}]	WSWI6dk/2
JSK17/2b	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 SWI4-6HA::kanMX4* [*cir*$^+$]	JP48/2b
CTY10/5d	*MAT**a** gal4Δ gal80Δ ade2 his3-200 trp1-901 leu2-3,112 met thr URA3::*(lexAop)$_8$-*lacZ* [*cir*$^+$]	(Bartel *et al.*, 1993)
CTMD/3a	*MAT**a** ade2-1 his3 trp1 leu2-3,112 met URA3::*(lexAop)$_8$-*lacZ* [*cir*^{0}]	(Pinder *et al.*, 2013)
EP7/2	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3:: lacZ [cir^o]*	MD83/1c
JP98/2-EP7	*MAT **a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 rsc2Δ::natMX4 URA3:: lacZ* [*cir*^{0}]	JP98/2
WSWI6dk/2-EP7	*MAT **a** ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 swi6Δ::katMX4 URA3:: lacZ* [*cir*^{0}]	WSWI6dk/2
EP8/1-*STB*(O)	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::STB-P(O)-lacZ* [*cir*$^+$]	MD83/7a
EP8/2-*STB*(O)	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::STB-P(D)-lacZ* [*cir*^{0}]	MD83/1c
EP8/1-*STB*(D)	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::STB-P(O)-lacZ* [*cir*$^+$]	MD83/7a
EP8/2-*STB*(D)	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::STB-P(O)-lacZ* [*cir*^{0}]	MD83/1c
MM26/1c	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 swi4Δ::kanMX4 URA3::STB-P(O)-lacZ* [*cir*$^+$]	MD83/1c

Strain	Genotype	Parent Strain/Source
MM27/4d	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 swi4Δ::kanMX4 URA3::STB-P(O)-lacZ* [cir^0]	MD83/1c
EP9/1	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 swi4Δ::kanMX4 URA3::STB-P(D)-lacZ* [cir^+]	MD83/1c
EP9/2	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 swi4Δ::kanMX4 URA3::STB-P(D)-lacZ* [cir^0]	MD83/1c
MM28/2b	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 swi6Δ::kanMX4 URA3::STB-P(O)-lacZ* [cir^+]	MD83/1c
MM29/1b	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 swi6Δ::kanMX4 URA3::STB-P(O)-lacZ* [cir^0]	MD83/1c
MM41/2	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 swi6Δ::kanMX4 URA3::STB-P(D)-lacZ* [cir^+]	MD83/1c
MM42/2	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 swi6Δ::kanMX4 URA3::STB-P(D)-lacZ* [cir^0]	MD83/1c
EP8/2-1O-WT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::6/7-1O-lacZ* [cir^o]	MD83/1c
JP98/2-1O-WT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::6/7-1O-lacZ rsc2Δ::natM4* [cir^o]	JP98/2
EP8/2-2O-WT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::6/7-2O-lacZ* [cir^o]	MD83/1c
JP98/2-2O-WT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::6/7-2O-lacZ rsc2Δ::natMX4* [cir^o]	JP98/2
EP8/2-3D-WT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::6/7-3D-lacZ* [cir^o]	MD83/1c
JP98/2-3D-WT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::6/7-3D-lacZ rsc2Δ::natMX4* [cir^o]	JP98/2
WSWI6dk/2-3D-WT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::6/7-3D-lacZ swi6Δ::katMX4* [cir^o]	WSWI6dk/2
EP8/2-3D-AAA	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::8/9-3D-lacZ* [cir^o]	MD83/1c
JP89/2-3D-AAA	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::8/9-3D-lacZ rsc2Δ::natMX4* [cir^o]	JP98/2
WSWI6dk/2-3D-AAA	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::8/9-3D-lacZ swi6Δ::katMX4* [cir^o]	WSWI6dk/2
EP8/2-1O-TGTG	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::10/11-1O-lacZ* [cir^o]	MD83/1c
JP98/2-1O-TGTG	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::10/11-1O-lacZ rsc2Δ::natMX6* [cir^o]	JP98/2
EP8/2-2O-TGTG	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::10/11-2O-lacZ* [cir^o]	MD83/1c
JP98/2-2O-TGTG	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::10/11-2O-lacZ rsc2Δ::natMX6* [cir^o]	JP98/2
EP8/2-3D-TGTG	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::10/11-3D-lacZ* [cir^o]	MD83/1c
JP98/2-3D-TGTG	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::10/11-3D-lacZ rsc2Δ::natMX4* [cir^o]	MD83/1c
EP8/2-1O-TGAA	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::32/33-1O-lacZ* [cir^o]	MD83/1c
EP8/2-1O-CTAGm	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::16/17-1O-lacZ* [cir^o]	MD83/1c

Strain	Genotype	Parent Strain/Source
EP8/2-2O-CTAGm	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::16/17-2O-lacZ [cir^o]*	MD83/1c
EP8/2-1O-TCAGj	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::22/23-1O-lacZ [cir^o]*	MD83/1c
EP8/2-2O-TCAGj	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::22/23-2O-lacZ [cir^o]*	MD83/1c
EP8/2-1O-CTAG2	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::18/19-1O-lacZ [cir^o]*	MD83/1c
EP8/2-2O-CTAG2	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::18/19-2O-lacZ [cir^o]*	MD83/1c
EP8/2-1O-TTAAT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::20/21-1O-lacZ [cir^o]*	MD83/1c
EP8/2-2O-TTAAT	*MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::20/21-2O-lacZ [cir^o]*	MD83/1c
EP8/2-FLPp	*MAT ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::FLPp-lacZ [cir^o]*	MD83/1b
EP4MD/2	*MAT**a**/MATα GAL4/gal4 GAL80/gal80 ADE2/ade2-1 his3-11,15/his3-200 leu2-3,112/leu2-3,112 trp1-1/trp1-901 MET/met THR/thr URA3::STB-P(O)-HIS3* [*cir*^{0}]	MD83/1c, CTY10/5d
MM37/1	*MAT ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::FLPp-HIS3 [cir$^+$]*	JP48/2b
MM38/2	*MAT ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::FLPp-HIS3 [cir^o]*	JP49/6b
MM39/1	*MAT ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::REP1p-HIS3 [cir$^+$]*	JP48/2b
MM40/2	*MAT ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 URA3::REP1p-HIS3 [cir^o]*	JP49/2b
BY4741 [cir$^+$]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 [cir$^+$]*	(Brachmann *et al.*, 1998)
BY4741 [cir^0]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 [cir^0]*	(Brachmann *et al.*, 1998)
Y2454 [cir$^+$]	*MATα his3Δ1 leu2Δ0 lys2Δ0 lys2Δ0 ura3Δ0 [cir$^+$]*	(Tong *et al.*, 2001)
Y2454 [cir^0]	*MATα his3Δ1 leu2Δ0 lys2Δ0 lys2Δ0 ura3Δ0 [cir^0]*	This study
Y2454/2-STB(O)	*MATα his3Δ1 leu2Δ0 lys2Δ0 lys2Δ0 ura3Δ0 URA3::STB-P(O)-lacZ [cir^0]*	Y2454

Table 2. Plasmids used in this study.

Plasmid name	Relevant Features	Source
pGAD424	*LEU2 2µm ADH1p-GAL4$_{AD}$*	Clonetech
pGAD-REP1	*LEU2 2µm ADH1p-GAL4$_{AD}$-REP1*	(Sengupta *et al.*, 2001)
pGAD-REP2	*LEU2 2µm ADH1p-GAL4$_{AD}$-REP2*	(Sengupta *et al.*, 2001)
pGAD-RAF	*LEU2 2µm ADH1p-GAL4$_{AD}$-RAF*	This study
pSH2-1	*HIS3 2µm ADH1p-LexA$_{BD}$*	(Lech *et al.*, 1988)
pSH-REP1	*HIS3 2µm ADH1p-LexA$_{BD}$-REP1*	(Sengupta *et al.*, 2001)
pSH-rep1$_{1-129}$	*HIS3 2µm ADH1p-LexA$_{BD}$-rep1$_{1-129}$*	(Sengupta *et al.*, 2001)
pSH-rep1$_{62-373}$	*HIS3 2µm ADH1p-LexA$_{BD}$-rep1$_{62-373}$*	This study
pSH-rep1$_{130-373}$	*HIS3 2µm ADH1p-LexA$_{BD}$-rep1$_{130-373}$*	(Sengupta *et al.*, 2001)
pSH-REP2	*HIS3 2µm ADH1p-LexA$_{BD}$-REP2*	(Sengupta *et al.*, 2001)
pSH-rep2$_{D22N}$	*HIS3 2µm ADH1p-LexA$_{BD}$-rep2$_{D22N}$*	This study
pSH-rep2$_{AA}$	*HIS3 2µm ADH1p-LexA$_{BD}$-rep2$_{L185A,L186A}$*	This study
pSH-rep2$_{NAA}$	*HIS3 2µm ADH1p-LexA$_{BD}$-rep2$_{D22N, L185A,L186A}$*	This study
pSH-rep2$_{1-58}$	*HIS3 2µm ADH1p-LexA$_{BD}$-rep2$_{1-58}$*	(Sengupta *et al.*, 2001)
pSH-rep2 A1	*HIS3 2µm ADH1p-LexA$_{BD}$-rep2$_{1-231}$*	A. Sengupta*
pSH-rep2$_{58-296}$	*HIS3 2µm ADH1p-LexA$_{BD}$-rep2$_{58-296}$*	(Sengupta *et al.*, 2001)
pSH-RAF	*HIS3 2µm ADH1p-LexA$_{BD}$-RAF*	This study
pSH-raf$_{1-73}$	*HIS3 2µm ADH1p-LexA$_{BD}$-raf$_{1-73}$*	This study
pSH-raf$_{47-181}$	*HIS3 2µm ADH1p-LexA$_{BD}$-raf$_{47-181}$*	This study
pMM2	*TRP1 2µm GAL1p-B42$_{AD}$-HA*	(Pinder *et al.*, 2013)
pMM2-REP1	*TRP1 2µm GAL1p-B42$_{AD}$-HA-REP1*	(Pinder *et al.*, 2013)
pMM2-RAF	*TRP1 2µm GAL1p-B42$_{AD}$-HA-RAF*	This study
pMM3	*TRP1 CEN GAL1p-B42$_{AD}$-HA*	(Pinder *et al.*, 2013)
pMM3-REP1	*TRP1 CEN GAL1p-B42$_{AD}$-HA-REP1*	(Pinder *et al.*, 2013)
pMM3-rep1$_{S330A}$	*TRP1 CEN GAL1p-B42$_{AD}$-HA-rep1$_{S330A}$*	This study
pMM3-REP2	*TRP1 CEN GAL1p-B42$_{AD}$-HA-REP2*	(Pinder *et al.*, 2013)
pGAL-TRP	*TRP1 CEN GAL1p*	(Pinder *et al.*, 2013)
pGAL-TRP-REP1	*TRP1 CEN GAL1p-REP1*	(Pinder *et al.*, 2013)
pGAL-TRP-REP2	*TRP1 CEN GAL1p-REP2*	(Pinder *et al.*, 2013)
pGAL-TRP-R1R2	*TRP1 CEN GAL1/10p-REP1/REP2*	This study
pGAL-TRP-RAF	*TRP1 CEN GAL1p-RAF*	(Pinder, 2011)
pGAL-LEU	*LEU2 CEN GAL1p*	(Pinder *et al.*, 2013)
pGAL-LEU-REP1	*LEU2 CEN GAL1p-REP1*	(Pinder *et al.*, 2013)
pGAL-LEU-REP1(ES)	*LEU2 CEN GAL1p-REP1* (high-level expression)	(Pinder *et al.*, 2013)
pGAL-LEU-REP2(ES)	*LEU2 CEN GAL1p-REP2* (high-level expression)	(Pinder *et al.*, 2013)
pGAL-LEU-R1R2	*LEU2 CEN GAL1/10p-REP1/REP2*	This study
pGAL-LEU-RAF	*LEU2 CEN GAL1p-RAF*	(Pinder, 2011)
pGAL-FLAG-RAF	*LEU2 CEN GAL10p-FLAG-RAF*	This study
pBM272	*URA3 CEN GAL1/10p*	Yang *et al.*, 2004)
pBM272-R1R2	*URA3 CEN GAL1/10p-REP1/REP2*	(Yang *et al.*, 2004)
pBM272-R1R2 $_{D22N}$	*URA3 CEN pBM272-R1R2 rep2$_{D22N}$*	This study
pBM272-R1R2$_{AA}$	*URA3 CEN GAL1/10p-REP1/rep2$_{AA}$*	This study
pNAT	*natMX4 CEN GAL1/10p*	This study
pNAT-R1R2	*natMX4 CEN GAL1/10p-REP1/REP2*	This study
pAS4	*ADE2 2µm flp- REP1 REP2 RAF*	(Sengupta *et al.*, 2001)
pKan4	*kanMX4* 2µm *flp- REP1 REP2 RAF*	(Pinder *et al.*, 2013)
pKanΔREP1	*kanMX4* 2µm *flp- rep1Δ REP2 RAF*	(Pinder *et al.*, 2013)
pKan19	*kanMX4* 2µm *flp- rep1$_{S330A}$ REP2 RAF*	J. Pinder*
pKan147	*kanMX4* 2µm *flp- REP1 rep2$_{AA}$ RAF*	This study
pKan148	*kanMX4* 2µm *flp- REP1 rep2$_{NAA}$ RAF*	This study

Plasmid name	Relevant Features	Source
pKan149	*kanMX4* 2µm *flp- REP1 REP2 raf-*	This study
pKan150	*kanMX4* 2µm *flp- REP1 rep2$_{D22N}$ raf-*	This study
pKan151	*kanMX4* 2µm *flp- REP1 rep2$_{AA}$ raf-*	This study
pKanΔSTB	*kanMX4* 2µm *flp- STB-P*Δ	This study
pKan-STB-P	*kanMX4 flp- STB-P*Δ XhoI *STB-P*	This study
pKan-1-WT	*kanMX4 2µm STB-P*Δ *1xWT*	This study
pKan-2-WT	*kanMX4 2µm STB-P*Δ *2xWT*	This study
pKan-3-WT	*kanMX4 2µm STB-P*Δ *3xWT*	This study
pKan-4-WT	*kanMX4 2µm STB-P*Δ *4xWT*	This study
pKan-5-WT	*kanMX4 2µm STB-P*Δ *5xWT*	This study
pKan-2-TTT	*kanMX4 2µm STB-P*Δ *2xTTT*	This study
pKan-2-TGTG	*kanMX4 2µm STB-P*Δ *2xTGTG*	This study
pKan-3-TGTG	*kanMX4 2µm STB-P*Δ *3xTGTG*	This study
pKan-4-TGTG	*kanMX4 2µm STB-P*Δ *4xTGTG*	This study
pKan-5-TGTG	*kanMX4 2µm STB-P*Δ *5xTGTG*	This study
pKan-2-TGTA	*kanMX4 2µm STB-P*Δ *2xTGTA*	This study
pKan-2-CTAGm	*kanMX4 2µm STB-P*Δ *2xCTAGm*	This study
pKan-3-CTAGm	*kanMX4 2µm STB-P*Δ *3xCTAGm*	This study
pKan-4-CTAGm	*kanMX4 2µm STB-P*Δ *4xCTAGm*	This study
pKan-5-CTAGm	*kanMX4 2µm STB-P*Δ *5xCTAGm*	This study
pKan-2-CTAG2	*kanMX4 2µm STB-P*Δ *2xCTAG2*	This study
pKan-2-TTAAT	*kanMX4 2µm STB-P*Δ *2xTTAAT*	This study
pKan-3-TTAAT	*kanMX4 2µm STB-P*Δ *3xTTAAT*	This study
pKan-4-TTAAT	*kanMX4 2µm STB-P*Δ *4xTTAAT*	This study
pKan-2-TCAGj	*kanMX4 2µm STB-P*Δ *2xTCAGj*	This study
pKan-3-TCAGj	*kanMX4 2µm STB-P*Δ *3xTCAGj*	This study
pKan-4-TCAGj	*kanMX4 2µm STB-P*Δ *4xTCAGj*	This study
pKan-5-TCAGj	*kanMX4 2µm STB-P*Δ *5xTCAGj*	This study
pKan-2-CG3	*kanMX4 2µm STB-P*Δ *2xCG3*	This study
pKan-2-TGAA	*kanMX4 2µm STB-P*Δ *2xTGAA*	This study
pCD1	*TRP1*	C. Desroches Altamirano*
pCD1-STB-P	*TRP1 STB-P*	C. Desroches Altamirano*
pCD1-2-WT	*TRP1 2xWT*	C. Desroches Altamirano*
pCD1-5-WT	*TRP1 5xWT*	M. Dobson*
pCD1-FLPp	*TRP1 FLPp*	J. Lacoste*
pBS-STBX	*STB-P*	This study
pEP1	*URA3 (no UASp)-HIS3*	(Pinder *et al.*, 2013)
pEP1-STB-P(O)	*URA3 STB-P(O)-HIS3*	(Pinder *et al.*, 2013)
pEPM-FLPp	*URA3 FLPp-HIS3*	This study
pEPM-REP1p	*URA3 REP1p-HIS3*	This study
pJL638	*URA3 (no UASp)-lacZ*	(Li and Herskowitz, 1993)
pJL638-STB-P(O)	*URA3 STB-P(O)-lacZ*	This study
pJL638-STB-P(D)	*URA3 STB-P(D)-lacZ*	This study
pSTB-1O-WT	*URA3 1xWT(O)-lacZ*	This study
pSTB-2O-WT	*URA3 2xWT(O)-lacZ*	This study
pSTB-3D-WT	*URA3 3xWT(D)-lacZ*	This study
pSTB-3D-TTT	*URA3 3xTTT(D)-lacZ*	This study
pSTB-1O-TGTG	*URA3 1xTGTG(O)-lacZ*	This study
pSTB-2O-TGTG	*URA3 2xTGTG(O)-lacZ*	This study
pSTB-3D-TGTG	*URA3 3xTGTG(D)-lacZ*	This study

Plasmid name	Relevant Features	Source
pSTB-1O-TGAA	*URA3 1xTGAA(O)-lacZ*	This study
pSTB-2O-TGAA	*URA3 2xTGAA(O)-lacZ*	This study
pSTB-1O-CTAGm	*URA3 1xCTAGm(O)-lacZ*	This study
pSTB-2O-CTAGm	*URA3 2xCTAGm(O)-lacZ*	This study
pSTB-1O-2CTAG	*URA3 1x2CTAG(O)-lacZ*	This study
pSTB-2O-2CTAG	*URA3 2x2CTAG(O)-lacZ*	This study
pSTB-1O-TTAAT	*URA3 1xTTAAT(O)-lacZ*	This study
pSTB-2O-TTAAT	*URA3 2xTTAAT(O)-lacZ*	This study
pSTB-1O-TCAGj	*URA3 1xTCAGj(O)-lacZ*	This study
pSTB-2O-TCAGj	*URA3 2xTCAGj(O)-lacZ*	This study
pSTB-1O-CG3	*URA3 1xCG3(O)-lacZ*	This study
pSTB-2O-CG3	*URA3 2xCG3(O)-lacZ*	This study
pSTB-1O-TGAA	*URA3 1xTGAA(O)-lacZ*	This study
pSTB-2O-TGAA	*URA3 2xTGAA(O)-lacZ*	This study
pFLPpr-lacZ	*URA3 FLPp-lacZ*	J. Pinder*
pREP1pr-lacZ	*URA3 REP1p-lacZ*	J. Pinder*
pREP2pr-lacZ	*URA3 REP2p-lacZ*	J. Pinder*
pRS315	*LEU2 CEN*	(Sikorski and Hieter, 1989)
pRS-FLPp-lacZ	*LEU2 CEN FLPp-lacZ*	(Sengupta, 2000)
pRS-STB-P(O)-lacZ	*LEU2 CEN STB-P(O)-lacZ*	This study
pRS-CYC1p-lacZ	*LEU2 CEN CYC1p-lacZ*	This study
pBIS-GALk-FLP(TRP1)	*TRP1 CEN GAL-flp$_{H305L}$*	(Tsalik and Gartenberg, 1998)

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Table 3. Oligonucleoties used as PCR primers or linkers for the addition of restriction sites.

Oligo	Sequence (5'->3')	Relevant Details
RAF2	GCGGATCCGAATGCCTTATAAAACAGC	generate *RAF* ORF with *Bam*HI overhangs
RAF3	CGGGGATCCTTCCGTAAAGCGCTAGAC	
RAF9	GTTTTTTAAGGCATTCAATGGACAG	generate *RAF* Stop at codon 3
RAF10	TTGAATGCCTTAAAAAACAGCTATAGATTGCATAG	
REP2-10	CGGAATTCATGGACGACATTGAAACAG	generate *REP2* ORF with *Eco*RI/*Bam*HI overhangs
REP2-11	CGGGATCCTCATACCCTAGAAGTATTAC	
REP2-26	GAATGTATGTCGGCTGTTTATTG	genearte *REP2 D22N* mutant allele
REP2-27	CAGCCGACATACATTCCAGACGCTATAAGCTG	
REP2-28	GTTACCCAAGCTGCTACTTTAGTTCCACCAG	generate *REP2 L185A*, *L186A* mutant allele
REP2-29	GTAGCAGCTTGGGTAACGACGCTTAC	
REP1-7	GGCAGCCATTGTAGAAGTG	used with RAF9 and RAF10 for *RAF* mutagenesis
REP1-9	CTGTCGGCTATTATCTC	
REP1-12	CGGAATTCATGAATGGCGAGAGACTG	generate *REP1* ORF with *Eco*RI/*Bam*HI overhangs
REP1-13	CGGGATCCTATATAACCTACCCATC	
REP1-14	CCGGAATTCGACACACCGGTAATTG	generate $REP1_{62\text{-}373}$ truncated allele
BglII linker	CCCTCGAGATCTCCTCGAGGG	linker for pKanΔSTB
UF243	TTGGTTAAAAAATGAGCTG	universal primer -243 forward
HIS3-BamHI-F	CTGGATCCTATCCAAAGATGACAGAG	generate *HIS3* ORF to clone into pEPM one-hybrid reporter
HIS3-Pvu-R	CACAGCTGACACGTATAGAATGATG	
pSHR1-f	GGAAGAAGGGTTGCCGCTGGTAGGTCGTGTGGCTGCCGG	generate *REP1* ORF from any plasmid for gap repair of pSH plasmids
	TGAACCTCGACCGGAATTCATGAATGGCGAGAGACTG	
pSHR1-r	ATAAAAATCATAAATCATAAGAAATTCGCCCGGAATTAG	
	CTTGGCTGCAGGTCGACTACCCATCCACCTTTC	
pSHR2-f	GGAAGAAGGGTTGCCGCTGGTAGGTCGTGTGGCTGCCGG	generate *REP2* ORF from any plasmid for gap repair of pSH plasmids
	TGAACCTCGACCGGAATTCATGGACGACATTGAAACAG	
pSHR2-r	ATAAAAATCATAAATCATAAGAAATTCGCCCGGAATTAG	
	CTTGGCTGCAGGTCGATCATACCCTAGAAGTATTAC	
pBMREP2-f	AAACTTCTTTGCGTCCATCCAAAAAAAAAGTAAGAATTT	generate *REP2* ORF from any plasmid for gap repair of pBM272 plasmids
	TTGAAAATTCGAATTCATGGACGACATTGAAACAG	
pBMREP2-r	GACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTT	
	TCGTCTTCAAGAATTCTCATACCCTAGAAGTATTAC	
Lac i	GAGGCGGTTTGCGTATTG	amplify promoters in pRS-promoter-lacZ plasmids
REP1p-1	CAGGATCCATTCATATTTCAGTTATT	generate *REP1* promoter with *Bam*HI overhangs
REP1p-2	CAGGATCCAGTAGACGGAGTATAC	
REP2p-3	TCCGGATCCGTCCATTTTGGTTTTCTT	generate *REP2* promoter with *Bam*HI overhangs
REP2p-4	CAGGATCCTAACTTTAATTGTCCTGTT	

Oligo	Sequence (5'->3')	Relevant Details
FLP1	GACGGATCCAAATTGTGGCATGCTTAG	generate *FLP* promoter with *Bam*HI overhangs
FLP2	GACGGATCCTGTGCAGATCACATGTC	
FLP8	CACAAGACAAACAGGACAATTAAAG	mutation of $CGCG_{5448}$ (CGCG1) to CACA in *FLP/REP2p*
FLP9	GTCCTGTTTGTCTTGTGTTCTTTCGAAAAATGCAC	
FLP19	GGCTAGTTTTTCGAAAGAACGCGAG	mutation of $TGCA_{5430}$ (TGCA1) to CTAG in *FLP/REP2p*
FLP20	CTTTCGAAAAACTAGCCGGCCGCGCATTATT	
FLP21	TAATGTGTGGCCGGTGCATTTTTC	mutation of $CGCG_{5421}$ (CGCG2) to CACA in *FLP/REP2p*
FLP22	CACCGGCCACACATTATTTGTACTGCGAAA	
FLP23	CAGCAAAGGCTAGTTTTTAAAATATGAAATGAAG	mutation of $TGCA_{5358}$ (TGCA2) to CTAG in *FLP/REP2p*
FLP24	TAAAAACTAGCCTTTGCTGCTTTTCC	
REP1-10	CGCAGCTGGGGTGATTG	amplification of *REP1/RAFp* for ChIP
REP1-ChR	GTATTCAGTACATGTCCAAC	
FLP6	CAAGATAGTACCGCAAAAC	amplification of *FLP/REP2p* for ChIP
FLP7	CAATTGATATCTAGTTTCAAC	
CEN3-F	CATAAACATGGCATGGCGATCA	amplification of *CEN3* for ChIP
CEN3-R	CACCAGTAAACGTTTCATATATCC	
CLN2-ChF	GTTTCCTTCTGTAAGTTATCTCA	amplification of *CLN2* promoter for ChIP
CLN2-ChR	CAACAGTTGTTTCAGTTGGTGT	
TRP1QTFwd	CTGCATGGAGATGAGTCGTG	amplification of *TRP1* for ChIP
TRP1QTRev	CCATTTGTCTCCACACCTCC	
STBP-ChF	ATTATAGAGCGCACAAAGGAGA	amplification of *STB-P* for ChIP
STBP-ChR	TGCACTTCAATAGCATATCTTTG	

Polymerase (Thermo Scientific) was used for all PCR-based cloning, as recommended by supplier. All oligonucleotides and plasmid sequences generated by PCR were confirmed by sequencing after introduction into plasmids used to assess their function.

2.2.1 Plasmids For Expression Of 2µm-Encoded Proteins In Yeast For One- And Two-Hybrid Assays

Plasmids used for expressing Rep1 or Rep2 fused to the transcriptional activation domain (amino acids 768 to 881) of Gal4 ($Gal4_{AD}$; pGAD-REP1 and pGAD-REP2), to the DNA-binding domain (amino acids 1 to 87) of the bacterial repressor protein LexA ($LexA_{BD}$; pSH-REP1 and pSH-REP2) (Dobson *et al.*, 2005), or to an HA epitope-tagged B42 activation domain ($B42_{AD}$-HA; pMM2-REP1 and pMM2-REP2 (multi-copy) or pMM3-REP1 and pMM3-REP2 (single copy)) (Pinder *et al.*, 2013), in yeast, have been previously described. Plasmids used for expressing Raf fused to $Gal4_{AD}$ (pGAD-RAF) to $LexA_{BD}$ (pSH-RAF), and to $B42_{AD}$-HA (pMM2-RAF), in yeast, were created by PCR amplification of the *RAF* open reading frame (ORF) with flanking *Bam*HI sites, and cloning of the resulting *Bam*HI fragment at the *Bam*HI site in the vectors pGAD424, pSH2-1, pMM2 and pMM3.

Plasmids expressing truncated versions of Rep1 and Rep2 fusion proteins used in this study have been previously described (Sengupta *et al.*, 2001) with the exception of the $Rep1_{62\text{-}373}$ fusion. The sequence encoding codons 62-373 of Rep1 was amplified by PCR using primers REP1-13 and REP1-14, which introduced flanking *Eco*RI and *Bam*HI sites, allowing it to be cloned in *Eco*RI /*Bam*HI-digested pSH-2-1, creating pSH-$REP1_{62\text{-}373}$. Plasmids expressing truncated versions of Raf fused to $LexA_{BD}$ were created by digesting pSH-RAF with *Sal*I and *Stu*I followed by fill-in and self-ligation to give pSH-$RAF_{1\text{-}73}$, and by ligating the 5.0-kbp *Sma*I/*Eco*RI-filled pSH-RAF fragment to the 4.0-kbp *Sma*I pSH2-1 fragment to give pSH-$RAF_{47\text{-}181}$.

pSH plasmids expressing mutant versions of Rep2 fused to $LexA_{BD}$ were generated either by amplification of the *REP2* ORF from the appropriate pKan plasmid with pSHR2 primers, and replacement the wild-type *REP2* coding region in pSH-REP2 by the mutant *REP2* coding region using homologous recombination-mediated gap repair in yeast (see below; pSH-$REP2_{D22N}$, pSH-$REP2_{AA}$); or by ligation of the *Nco*I/*Sal*I

fragment of the *rep2$_{AA}$* ORF from pSH-REP2$_{AA}$ into *Nco*I/*Sal*I-digested pSH-REP2$_{D22N}$ (pSH-REP2$_{NAA}$).

2.2.2 Plasmids For Galactose-Inducible Overexpression Of 2µm-Encoded Proteins

Plasmids expressing untagged Rep1, Rep2 or Raf under the control of a galactose-inducible *GAL1/10* promoter, individually at high (pGAL-LEU-REP1(ES), pGAL-TRP-REP2(ES)) or low (pGAL-LEU-REP1, pGAL-LEU-RAF, pGAL-TRP-REP1, pGAL-TRP-REP2, pGAL-TRP-RAF) levels, or together at high level (pBM272-R1R2) have been described previously (Yang *et al.*, 2004; Pinder, 2011). Plasmids expressing untagged Rep1 and Rep2 simultaneously under the control of the *GAL1/10* promoter at low level (pGAL-LEU-R1R2, pGAL-TRP-R1R2) were generated by ligation of the 0.9-kbp *Sma*I/*Bgl*II *REP2* ORF fragment from pGAD-REP2 into *Eag*I-filled/*Bgl*II digested pGAL-LEU-REP1 or pGAL-TRP-REP1. To switch the selectable marker in the pBM272 (Johnston and Davis, 1984) and pBM272-R1R2 (Yang *et al.*, 2004) plasmids from *URA3* to *natMX4*, the 1.6-kbp *Eag*I/*Sma*I *URA3*-encoding fragment on these plasmids was replaced with the 1.1-kbp *Eag*I/*Eco*RV fragment from plasmid p4339 encoding the *natMX4* cassette (Tong *et al.*, 2001), generating plasmids pNAT and pNAT-R1R2. A single-copy *ARS/CEN* plasmid expressing Raf under the control of the *GAL1* promoter with an amino-terminal FLAG tag (pGAL-FLAG-RAF) was created by cloning the *RAF* ORF *Bam*HI fragment at the *Bgl*II site in pGAL-LEU.

2.2.3 2µm-Based Plasmids

The plasmid pKan4 (referred to hereafter as pKan) was derived from the *flp*$^{-}$ *ADE2*-tagged 2µm plasmid, pAS4 (Sengupta *et al.*, 2001). pKan consists of a B-orientation 2µm plasmid in which the *FLP* gene is disrupted by insertion of the *kanMX4* gene cassette, and the *FRT* site in the inverted repeat downstream of the *REP1* and *REP2* genes has been replaced with the *E. coli* vector pTZ18R. A derivative of the pKan plasmid that lacks the *REP1* gene (pKanΔREP1) has previously been described (Pinder *et al.*, 2013).

Site-directed mutagenesis of *REP2* and *RAF* genes in pKan was carried out by gap repair. PCR amplicons containing either the *REP2* ORF flanked by ~900 bp upstream and ~600 bp downstream, or ~400 bp of the 5' terminus of the *RAF* ORF flanked by

~1100 bp upstream, and containing the designated point mutation(s), were created by assembly PCR, and co-transformed into yeast with *Sph*I-digested pAS4 or *Stu*I-digested pKan-based plasmids, respectively. pKan versions of pAS4 plasmids with point mutations in *REP2* were generated from these by replacement of the *ADE2* marker gene with the *kanMX4* gene cassette. Plasmids were created by gap repair in yeast and isolated in *E. coli*. Mutations were confirmed by sequencing, and plasmids re-transformed into yeast for subsequent experiments. Oligonucleotides used are listed in Table 3.

To create a version of pKan lacking *STB-P* (pKanΔSTB), a *Bgl*II site upstream of the kan^R gene in the *kanMX4* cassette was removed by digestion with *Bgl*II and *Sal*I, followed by fill-in of the overhangs and self-closure. The 0.3-kbp *Hpa*I/*Ava*I fragment encoding *STB-P* was then replaced with a linker containing a *Bgl*II site flanked by *Xho*I sites (Table 3). To create an *Xho*I fragment containing *STB-P* suitable for re-insertion in pKanΔSTB, the 0.3kbp *Hpa*I/*Ava*I *STB-P* fragment was flush-ended and cloned at the *Eco*RV site of the vector pBluescript (Stratagene). An *Xho*I linker was introduced at the *Sma*I site in the vector to produce plasmid pBS-STBX, from which *STB-P* could be excised as a 346-bp *Xho*I fragment. This *Xho*I fragment was cloned into *Xho*I-digested pKanΔSTB to create pKan-STB-P, which has the 296-bp native *STB-P* sequence re-introduced in the original orientation and position, but separated from the normal flanking sequences on either side by 25-bp of linker sequence.

A 63-bp stretch of *STB-P* (nucleotides 2986 to 3048 in the Scp1 2µm A form, NCBI GenBank J01347.1 GI: 172190) was selected as the basis of the synthetic *STB* repeats used in this study. To facilitate creation of *STB* loci with varying numbers of synthetic repeats or altered sequence composition, overhangs matching *Bam*HI and *Bgl*II restriction sites were included on the ends of each synthetic repeat.

Pairs of complementary oligonucleotides (Table 4) were annealed to form synthetic *STB* single repeat duplexes. To create tandem arrays of synthetic *STB* repeats, duplexes were phosphorylated and then incubated with T4 ligase. Head-to-head and tail-to-tail ligation products were eliminated by digestion with *Bam*HI and *Bgl*II. The resulting *Bam*HI/*Bgl*II fragments encoding different numbers of *STB* repeats were cloned at the unique *Bgl*II site in pKanΔSTB, creating the pKan-STB series of plasmids. Plasmids with inserts in the orientation of the native *STB-P* repeats were assessed for

Table 4. Oligonucleotides used for generation of synthetic *STB* duplexes.

Oligo	Sequence (5'->3')	Relevant Details
STB6	GATCCATTTTGTAGAACAAAAATGCAACGCGAGAGCGCTAATTTTT CAAACAAAGAATCTGAGCTGCA	WT duplex
STB7	GATCTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT TGCATTTTTGTTCTACAAAATG	
STB8	GATCCATTTTGTAGAACAAAAATGCAACGCGAAAGCGCTAATTTTT CAAACAAAGAATCTGAGCTGCA	TTT duplex
STB9	GATCTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTTTCGCGT TGCATTTTTGTTCTACAAAATG	
STB10	GATCCATTTTGTAGAACAAAAATGCAACACAAGAGCGCTAATTTTT CAAACAAAGAATCTGAGCTGCA	TGTG duplex
STB11	GATCTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTTGTGT TGCATTTTTGTTCTACAAAATG	
STB14	GATCCATTTTGTAGAACAAAAATACAACGCGAGAGCGCTAATTTTT CAAACAAAGAATCTGAGCTGCA	TGTA duplex
STB15	GATCTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT TGTATTTTTGTTCTACAAAATG	
STB16	GATCCATTTTGTAGAACAAAAACTAGACGCGAGAGCGCTAATTTTT CAAACAAAGAATCTGAGCTGCA	CTAGm duplex
STB17	GATCTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT CTAGTTTTTGTTCTACAAAATG	
STB18	GATCCATTTTGTAGAACAAAAACTAGACGCGAGAGCGCTAATTTTT CAAACAAAGAATCTGAGCCTAG	2CTAG duplex
STB19	GATCCTAGGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT CTAGTTTTTGTTCTACAAAATG	
STB20	GATCCATTTTGTAGAACAATTATGCAACGCGAGAGCGCTAATTTTT CAAACAAAGAATCTGAGCTGCA	TAATT duplex
STB21	GATCTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT TGCATAATTGTTCTACAAAATG	
STB22	GATCCATTTTGTAGAACAAAAATGCAACGCGAGAGCGCTAATTTTT CAAACAAAGAATCTGAGCCTGA	TCAGj duplex
STB23	GATCTCAGGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT TGCATTTTTGTTCTACAAAATG	
STB24	GATCCATTTTGTAGAACAAAAATGCAACGCGCGAGCGCTAATTTTT CAAACAAAGAATCTGAGCTGCA	CG3 duplex
STB25	GATCTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCGCGCGT TGCATTTTTGTTCTACAAAATG	
STB32	GATCCATTTTGTAGAACAAAAATTCAACGCGAGAGCGCTAATTTTT CAAACAAAGAATCTGAGCTGCA	TGAA duplex
STB33	GATCTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT TGAATTTTTGTTCTACAAAATG	

partitioning function.

2.2.4 Non-2µm-Based Plasmids For Assaying Partitioning Function

The pCD1 series of plasmids was generated to assess partitioning function conferred by synthetic *STB* repeats and by specific native 2µm plasmid sequences in a non-2µm context. *Bam*HI/*Sca*I fragments from pSTB plasmids and pFLPp-lacZ (ranging from 0.5 to 0.8 kbp depending on the source plasmid) were introduced into *Bam*HI/*Nru*I digested pYR7, a plasmid that consists of the 1.45-kbp yeast genomic *Eco*RI fragment encoding *TRP1* and the adjacent *ARS* cloned into the *Eco*RI site of *E. coli* vector pBR322 (Stinchcomb *et al.*, 1979).

2.2.5 Plasmids Encoding Reporters For One-Hybrid And Transcriptional Assays

Plasmids encoding a DNA sequence of interest upstream of a reporter gene were used to assess both transcription and protein-DNA associations *in vivo* in a one-hybrid assay system (described below). The *Xho*I fragment from pBS-STBX was inserted upstream of a *lacZ* gene lacking an upstream activation sequence (UAS) at the *Xho*I site in the integrating *URA3*-tagged pJL638 plasmid, to generate pJL638-STB-P(O) and pJL638-STB-P(D), in which the *ORI*-proximal or *STB-D*-proximal end of *STB-P* was placed closest to the *lacZ* gene, respectively. To insert synthetic *STB* sequences in the place of the native *STB-P*, synthetic sequences with *Bgl*II/*Bam*HI overhangs were used to replace the *Bgl*II *STB-P* fragment, generating the pSTB plasmid series (Table 2). In the case of pEP1-STB-P(O), the *lacZ* gene was replaced with an *Msc*I/*Sst*I fragment encoding the *HIS3* ORF with its transcriptional termination sequences (Pinder *et al.*, 2013).

Integrating plasmids encoding promoters as transcriptional fusions with the *lacZ* gene (pFLPpr-lacZ, pREP1pr-lacZ, pREP2pr-lacZ and pCYC1p-lacZ) were generated by amplification of promoter sequences with primers that introduced flanking *Bam*HI sites. These amplicons could then be used to replace the *Bam*HI fragment encoding the basal promoter upstream of the *lacZ* gene on pJL638. To generate a *CEN*-based *lacZ* reporter plasmid (pRS-STB-P(O)-lacZ), the 4.1-kbp *Sma*I/*Dra*I-filled *STB-P(O)-lacZ* reporter fragment from pJL638-STB-P(O) was ligated to 5.6-kbp *Xho*I/*Dra*I-filled fragment (which lacks the multiple cloning site) of pRS315, a *CEN*-based *LEU2*-tagged vector

(Sikorski and Hieter, 1989). To create promoter-*lacZ* reporters, the PCR-generated *Bam*HI promoter fragments were cloned into the *Bam*HI sites upstrearm of *lacZ* on pJL638-STB-P(O), replacing *STB(O)* and the basal promoter. pEP1-FLPp and pEP1-REP1p were generated by replacement of the *lacZ* gene on pJL638-based promoter plasmids with a PCR-generated *HIS3* ORF flanked by *Bam*HI and *Pst*I sites that positioned *HIS3* in-frame with the first codons of the *FLP* and *REP1* genes, respectively.

2.3 Multiple Sequence Alignments

Sequences of conceptual translations of non-*FLP*, non-*REP1* ORFs encoded by 2µm-like plasmids were obtained from the NCBI GenBank database: Rep2 (AAB59343.1) and Raf (AAB59342.1) of the Scp1 2µm plasmid variant from *Saccharomyces cerevisiae*, ORF S (YP_355330.1) of pSR1 and ORF C (XP_001728568.1) of pSB3 from *Zygosaccharomyces rouxii*, ORF C (NP_040497.1) of pSB2 from *Zygosaccharomyces bailii*, ORF A (NP_040492.1) and ORF B (NP_040493.1) of pSM1 from *Zygosaccharomyces fermentati*, ORF C (CAA27592.1) of pKD1 from *Kluveromyces lactis*, ORF C (CAA39901.1) and ORF B (CAA39903.1) of pKW1 from *Kluveromyces waltii*; and ORF C (CAA71933.1) of pTD1 from *Torulaspora delbrueckii*. Protein sequences were aligned using ClustalW2 with default settings for all parameters, except 'iteration type,' which was set to 'tree,' to perform iterations at each step of the alignment; and 'maximum number of iterations to perform,' which was set to five. Alignments generated by ClustalW2 were shaded using Boxshade 3.21 with default settings.

2.4 Assays For Transcriptional Activity

The transcriptional activity of DNA sequences of interest was assayed by monitoring the ability of these sequences to drive expression of a *lacZ* reporter gene. This was done using *cir*^{0} strains containing either a *Stu*I-digested pJL638-based plasmid integrated at the *URA3* locus, or a pRS315-based *CEN* plasmid. Rep1, Rep2 or mutant versions thereof were expressed from pGAL-LEU- or pGAL-TRP-based plasmids. Expression of *lacZ* was monitored using assays for the enzymatic activity of the *lacZ* gene product, ß-galactosidase (ß-gal) as previously described (Burke *et al.*, 1997). In qualitative assays, patches of yeast were grown on nitrocellulose filters overlaid on solid

medium, lysed by freeze-thaw and incubated with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol) containing 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal), which is cleaved by ß-gal to give a blue precipitate. In quantitative assays, activity of ß-gal was measured by a permeabilized cell assay with ß-gal substrate ortho-nitrophenyl-ß-D-galactopyranoside (ONPG), which is cleaved to give a yellow product, the development of which was monitored spectrophotometrically. For all ß-gal assays, a minimum of 3 independent transformants were assayed.

2.5 One- And Two-Hybrid Assays

To monitor protein-protein associations *in vivo*, plasmids expressing either $Gal4_{AD}$ (pGAD) or $B42_{AD}$-HA (pMM2 and pMM3) fusions of Rep1, Rep2 or Raf; and $LexA_{BD}$ fusions of Rep1, Rep2, Raf or their truncated or mutated derivatives (pSH) were used to co-transform CTMD/3a (Pinder *et al.*, 2013), a cir^0 reporter strain containing 8 copies of the LexA operator sequence integrated upstream of a *lacZ* reporter gene at the *URA3* locus. Activity of ß-gal was monitored by a filter assay, as described (Section 2.4).

To monitor protein-DNA associations *in vivo*, one-hybrid assays were performed, in which a protein of interest fused to an activation domain is expressed in a strain containing a DNA sequence of interest upstream of a reporter gene. To test for association of Rep1, Rep2 and Raf with *STB-P*, pMM3-REP1, pMM3-REP2 or pMM2-RAF were used to transform EP4MD cir^0 (Pinder *et al.*, 2013), which contains an *STB-P(O)-HIS3* reporter; Rep1 and Rep2 were expressed from pGAL-LEU plasmids, as indicated. To monitor association of Rep1, Rep2 and Raf with *FLP/REP2p* and *REP1/RAFp*, StuI-digested pEPM-FLPp and pEPM-REP1p were used to transform JP48/2b cir^+ and JP49/6b cir^0 to uracil prototrophy; these strains were subsequently transformed with either pGAD- or pMM3-based plasmids expressing activation domain fusions of Rep1, Rep2, Raf or mutant derivatives thereof. Activity of the *HIS3* gene product, an enzyme of the histidine biosynthetic pathway, was used as a measure of association between the activation domain fusion protein and DNA sequence of interest. Activity of the *HIS3* gene product was determined by monitoring the growth of a series of five five-fold dilutions (ranging from 1.25×10^6 to 2×10^3 cells/mL) of strains of interest on media lacking histidine and containing concentrations of 3-aminotriazole (3-

AT, a competitive inhibitor of the *HIS3* gene product) ranging from 2.5 mM to 10.0 mM. Serial dilutions were also grown on medium containing histidine and lacking 3-AT, as a growth control. All dilutions were applied to media in 5 µL volumes, so that the most concentrated spots contained ~6250 cells, while the least concentrated contained ~10 cells.

To test for association of Rep proteins with synthetic *STB* sequences, StuI-digested pSTB plasmids were integrated at the *URA3* locus of cir^0 yeast strain MD83/1c, generating a series of yeast strains (EP8) with native or synthetic *STB* sequences upstream of a *lacZ* reporter gene (Table 2). EP8 strains were co-transformed with either pGAD-REP1 and pGAL-TRP-REP2 or pGAD-REP2 and pGAL-TRP-REP1, and *lacZ* activity was monitored as described (Section 2.4).

2.6 Plasmid Inheritance Assays

Plasmid inheritance, as a measure of partitioning function, was monitored by determining either the fraction of plasmid-bearing cells or the rate of generation of plasmid-free cells, as previously described (Pinder, 2011; Pinder *et al.*, 2013). These assays are summarized below.

Inheritance of pKan plasmids with synthetic *STB* repeats in place of the native repeats, or with mutant *REP1*, *REP2* or *RAF* alleles, were assayed in yeast strain AG8/5, a cir^0 strain in which the *URA3* gene has been inserted into the mutant *ade2-1* allele at the *ADE2* locus, to enable selection against recombination between the *kanMX4*-flanking *ADE2* sequences on pKan and the chromosomal *ADE2* locus. Inheritance of the wild-type pKan plasmid was assayed in JP98/2 and WSWI6dn/2, cir^0 strains in which the *RSC2* or *SWI6* gene was deleted, respectively. Inheritance of the non-2µm-based pCD1 plasmids carrying selected synthetic or native 2µm plasmid sequences was assayed in JP48/2b, which is cir^+ and thus provided Rep proteins *in trans*; or, to ensure that no recombination between pCD1 and the native 2µm plasmid could occur, JP49/6b, which is cir^0, with Rep1 and Rep2 expressed from the pGAL-LEU-R1R2 plasmid.

In most cases, the fraction of plasmid-bearing cells was used as a measure of plasmid inheritance. To determine the fraction of plasmid-bearing cells, 4 to 8 independent isolates of each strain were cultured for 16 to 24 hours (6 to 8 generations) in selective medium (YPAD+G418 for pKan transformants, SD-trp for pCD1 transformants,

or SD-leu-trp for pCD1 and pGAL-LEU-R1R2 co-transformants). The fraction of cells containing the pKan or pCD1 plasmid was determined by calculating the ratio of colonies on selective (YPAD+G418, SD-trp or SD-leu-trp) versus non-selective (YPAD, SD or SD-leu) medium. Yeast were cultured in medium containing glucose for all assays except those performed with *cir*^{0} yeast containing the pGAL-LEU-R1R2 plasmid, in which galactose was substituted for glucose to induce expression of Rep proteins from the *GAL1/10* promoter.

For comparisons of strains with different growth rates (i.e., *SWI6* and *swi6*Δ), the rate of generation of plasmid-free cells per cell doubling was used as a measure of plasmid inheritance. To determine the rate of generation of plasmid-free cells, the fraction of plasmid-bearing cells was determined as described above. Strains were then cultured for 10 to 12 generations in non-selective medium, and the fraction of plasmid-bearing cells determined following this period of non-selective growth. The percentage of plasmid-free cells generated per doubling was calculated as previously described from the fraction of plasmid-bearing cells both before and after non-selective growth, and the number of doublings undergone (Dobson *et al.*, 1988).

2.7 Western Blotting Analysis

Protein was extracted from yeast cultures using alkali lysis as previously described (Yaffe and Schatz, 1984; Chen *et al.*, 2005). Briefly, ~1x10^{8} yeast cells were pelleted and resuspended in 0.2 mL lysis solution (1.85 M NaOH, 7.4% ß-mercaptoethanol). After 10 minutes on ice, 0.2 mL 50% TCA was added, and protein cell lysates were kept on ice for a further 10 minutes before centrifugation at 16000 X g for 1 minute. Protein pellet were washed twice with acetone, dried, and resuspended in equal volumes of urea extraction buffer (8 M urea, 100 mM NaH_2PO_4/Na_2HPO_4, 50 mM Tris) and 2 X protein gel loading buffer (125 mM Tris pH6.8, 4.0% sodium dodecyl sulfide (SDS), 20% glycerol, 4.0% ß-mercaptoethanol, 1 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol). Protein suspensions were heated at 37˚C for 5 minutes and centrifuged at 16000 X g for 1 minute, and the soluble fraction resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins of interest were detected by western blotting as previously described (Sengupta *et al.*, 2001). Primary antibodies used were rabbit polyclonal anti-Rep1 or anti-Rep2 (Sengupta *et al.*, 2001), rabbit monoclonal

anti-LexA (Pierce), mouse monoclonal anti-Pgk1 (Molecular Probes), and mouse monoclonal anti-HA (Sigma). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG (KPL), along with Clarity Western ECL Substrate kit (BioRad), were used for chemiluminescent detection of anti-Rep1, anti-Rep2, anti-LexA and anti-HA antibodies; Dylight 549 or 649-conjugated goat anti-mouse IgG (Rockland) was used for fluorescent detection of anti-Pgk1 antibodies. Images were captured digitally using a charge-coupled device (CCD) camera in a VersaDoc 4000 MP imaging system, equipped with the appropriate LED/filter combination for fluorescence detection, and Quantity One software (BioRad). Quantification of band densities was performed using ImageLab software (BioRad).

For detection of Rep1, Rep2 and Pgk1 in the same sample, western transfers from duplicate gels were probed with either anti-Rep1 and anti-Pgk1, or anti-Rep2 and anti-Pgk1. In some cases, blots were stripped and re-probed. To strip, blots were washed 5 times in phosphate-buffered saline (PBS)-Tween, incubated 1 hour in 50 mL stripping solution (50 mM Tris pH 6.8, 1% SDS,10 mM 2-mercaptoethanol) at 60˚C, and washing 5 times in PBS-Tween; each was was 5 minutes. After stripping, western blotting was performed as previously described (Sengupta *et al.*, 2001).

2.8 Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) was performed essentially as previously described (Pinder *et al.*, 2013). Briefly, yeast were cultured in 50 mL YPAD or YPA + galactose to a density of ~1.5 x 10^7 cells/mL, and fixed for 15 minutes in 1% formaldehyde to cross-link DNA and proteins. Cross-linking was quenched by addition of 125 mM glycine. Cells were harvested and washed 3 times with water before being resuspended in 400 µL lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% TritonX-100 (Sigma), 0.1% SDS, 1 x complete protease inhibitor cocktail (Roche)) and 1 volume of 450 to 600 µm acid-washed silanized glass beads. Cells were lysed by 12 rounds of vortexing for 2 minutes, with each round being followed by 1 minute on ice, to lyse. Lysates were recovered by centrifugation, and DNA was sheared by 8 rounds of 12 second sonication on a Branson 250 Sonifier at power setting 3, 50% cycle duty (Branson), giving DNA fragments 0.1 to 1.0-kbp in length. To remove insoluble material, lysates were centrifuged for 15 minutes at 16000 X g before the

supernatant was transferred to a new tube and centrifuged 10 minutes at 16000 X g. A 50 µL volume of supernatant was set aside as input, and the remaining volume was divided into aliquots in fresh tubes. Lysis buffer was added to each aliquot to bring the total volume to 500 µL.

Antibodies were added to ChIP aliquots, and samples were incubated for 4 to 16 hours at 4˚C with gentle agitation. Antibodies used were rabbit polyclonal anti-Rep1 and anti-Rep2, and mouse monoclonal anti-HA and anti-FLAG (Sigma). Protein A Sepharose CL-4B (in the case of rabbit-derived antibodies; Amersham Biosciences) or Protein G-Agarose (in the case of mouse-derived antibodies; Roche Diagnostics) beads were washed 3 times with water, then equilibrated in lysis buffer with 0.1 mg/mL sonicated salmon sperm DNA (Sigma) and 0.1 mg/ml bovine serum albumin (New England Biolabs) for 1 hour at 4˚C. Following equilibration, beads were resuspended in lysis buffer as a 50% slurry, and 40 µL added to each ChIP aliquot taken. ChIP aliquots were incubated a further 1 hour at 4˚C with gentle agitation. Beads were washed twice with 1 mL ChIP Wash Buffer I (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% sodium deoxycholate), once with 1 mL ChIP Wash Buffer II (45 mM HEPES-KOH pH 7.5, 435 mM NaCl, 0.9 mM EDTA, 0.9% TritonX-100, 0.09% sodium deoxycholate), once with ChIP Wash Buffer III (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% nonidet-P40 (Sigma-Aldrich), 0.5% sodium deoxycholate) and once with Tris-EDTA buffer (TE; 50 mM Tris pH 8.0, 10 mM EDTA). Immunoprecipiated material was eluted using SDS, crosslinks were reversed by heating, proteins were degraded by proteinase K digestion (Invitrogen), and DNA was recovered by phenol:chloroform extraction and ethanol precipitation.

DNA isolated by ChIP was resuspended in 40 µL TE, and the enrichment of DNA loci of interest in eluates was evaluated by semi-quantitative PCR using Platinum Taq (Invitrogen), as recommended by supplier. DNA templates were diluted to give products within the linear range of PCR with the following conditions: 4 minute hot start at 94˚C, followed by 30 cycles of 1 minute denaturation at 94˚C, 1 minute annealing at 48˚C and 1 minute extension at 72˚C, and final 6 minute extension at 72˚C. Primers used are shown in Table 3. A 329-bp region of the *TRP1* gene was amplified to ensure that no antibodies were immunoprecipitating DNA non-specifically (data not shown). PCR products were

separated by agarose gel electrophoresis, stained with ethidium bromide, and digitally imaged using a charge-coupled device (CCD) camera in a VersaDoc 4000 MP imaging system and Quantity One software (BioRad). Yield of products obtained from immunoprecipitated DNA was quantified by densitometry using ImageLab software (BioRad) and compared to that of input DNA to calculate percent of input DNA immunoprecipitated.

2.9 Synthetic Genetic Analysis

For all synthetic genetic analyses (SGA) performed in this study, query strains were mated to the complete collection of viable haploid yeast gene deletion strains (EUROSCARF; (Winzeler *et al.*, 1999)), which consists of approximately 5000 haploid yeast strains, each with a different single gene replaced by the *kanMX4* cassette. The resultant diploid strains were sporulated, and *MAT* ***a*** haploid segregants bearing the gene deletion and reporter of interest were selected. Matings, sporulation and haploid selection were performed by the Dalhousie Enhanced Gene Analysis and Discovery Facility using a robotic pinning platform, as described by Tong and Boone (Tong and Boone, 2006; Baryshnikova *et al.*, 2013).

2.9.1 Construction Of A Strain To Assay *STB-P* Transcriptional Activity By SGA

A yeast strain suitable for synthetic genetic array (SGA) analysis with *STB-P* integrated in the genome upstream of a *lacZ* reporter gene (Y2454/2-STB(O)) was generated in several steps. First, a wild-type copy of the *URA3* gene had to be introduced in the robot query strain (Y2454 cir^0), which has a complete deletion of the *URA3* coding region. To do this, a 1.1 kbp *Sma*I/*Sph*I fragment from pHR81 (Nehlin, 1989) carrying the *URA3* gene was used to transform yeast strain Y2454 to uracil prototrophy to generate the strain Y2454-URA3. Next, the wild-type *URA3* gene was replaced by a mutant *URA3* gene, *ura3*Δ*17*, which has a 17-bp deletion in the middle of the *URA3* gene. The *ura3*Δ*17* mutant was created by the digestion of YDp-U (Hilger, 1991) with EcoRV and NcoI, fill-in with the large fragment of DNA polymerase I to give blunt ends and self-ligation to generate the plasmid YDp-U-ura3Δ17. YDp-U-ura3Δ17 was digested with *Bam*HI and used to transform strain Y2454-URA3 to 5-fluoroorotic acid (5-FOA) resistance. This new strain, Y2454-ura3Δ17, could then be transformed to uracil

prototrophy using the plasmid pJL638-STBX linearized by digestion with StuI, which cuts the *URA3* gene on this plasmid to allow integration at the *URA3* locus.

2.10 Statistical analysis

Statistical significance of results was assessed using unpaired, two-tailed Student's t-tests, performed using Microsoft Excel for Mac 2011, version 14.5.3 (Microsoft).

Chapter 3 RESULTS

3.1 Functional Analysis Of The Rep2 And Raf Proteins

Saccharomyces cerevisiae is not the only budding yeast species that encodes a small circular plasmid. 2µm-like plasmids have been found in seven other species belonging to the Saccharomycetaceae family (Blaisonneau *et al.*, 1997; Douradinha *et al.*, 2014). Despite not showing any similarity to each other at the nucleotide level, these plasmids all have a similar organization (Figure 4), which suggests that proteins encoded by genes in equivalent positions might be homologs. Sequence homology has been observed for conceptual translations of ORFs in the positions of *FLP* and *REP1* (Chen *et al.*, 1986; Murray *et al.*, 1988); however, conceptual translations of ORFs in the positions of *REP2* and *RAF* do not show sufficient similarity to conceptual translations of ORFs in equivalent positions to be classified as homologs on this basis (C. Stairs, personal communication). Where tested, the *REP2*-positioned ORF has been shown to be required, along with the *REP1*-positioned ORF, for efficient plasmid inheritance (Toh-e and Utatsu, 1985; Jearnpipatkul *et al.*, 1987a; Bianchi *et al.*, 1991); this suggests that Rep2 and proteins encoded by the Rep2-positioned ORF perform similar functions and may therefore be related, despite their lack of significant sequence similarity.

3.1.1 Alignment Of Rep2 With Rep2-Equivalent Proteins Identifies Potentially Conserved Sequence Features

Acting under the assumption that ORFs of 2µm-like plasmids found in the position of the *REP2* gene on the 2µm plasmid were related to *REP2*, an alignment of Rep2 with conceptual translations of *REP2*-positioned ORFs was generated (Figure 5). The purpose of this alignment was to identify sequences in the Rep2 protein that are similar to those in the hypothetical Rep2-equivalent proteins, and may therefore be of functional relevance.

Sequences of conceptual translations of *REP2*-positioned ORFs were initially retrieved from the NCBI GenBank database (Section 2.3). The sequence of the *REP2*-positioned ORF *C* of pSB3 was shorter than the rest, encoding a protein only 157 amino acids in length; however, examination of the nucleotide sequence of the pSB3 plasmid revealed that addition of an adenine nucleotide to the run of adenines from 2439 to 2442

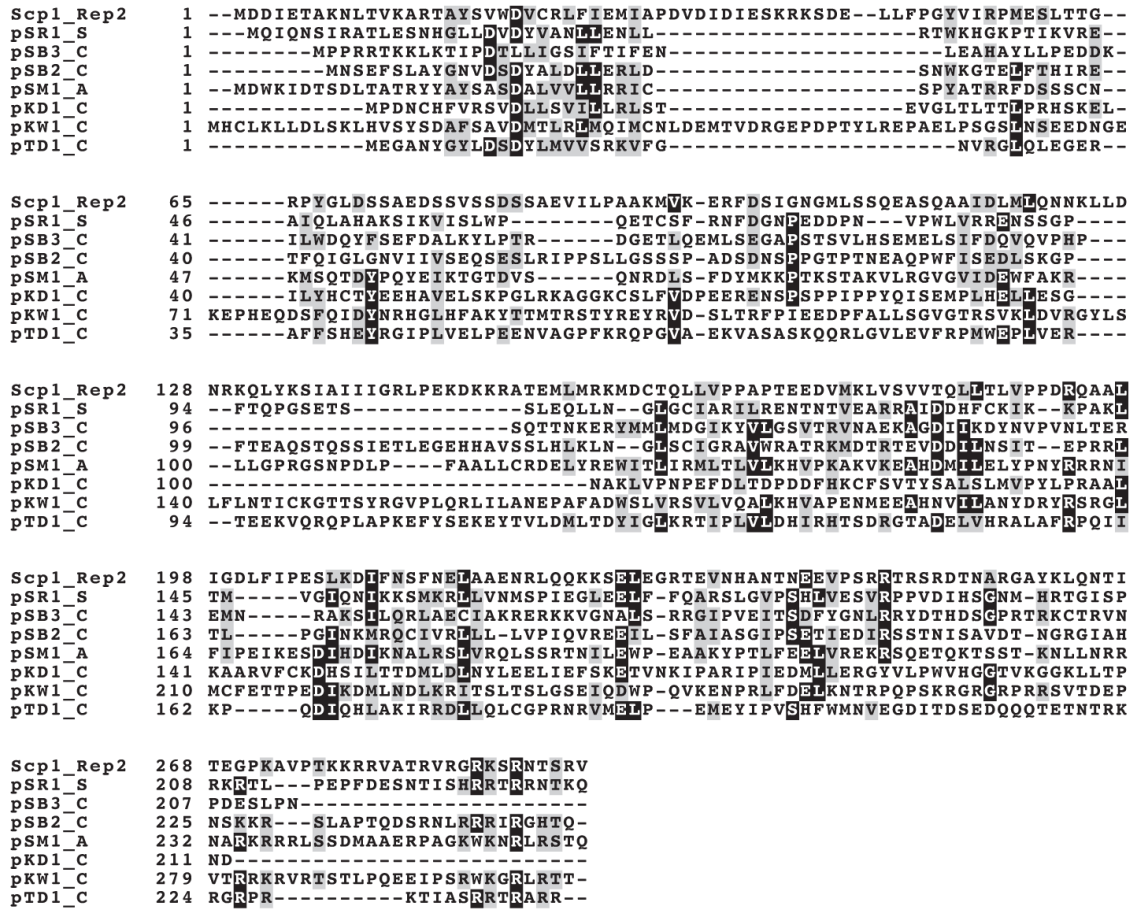


Figure 5. Alignment of Rep2 with conceptual translations of ORFs in the position of *REP2* on 2µm-like plasmids. Protein sequences were aligned using ClustalW2 and shaded using Boxshade 3.21 (Section 2.3); a black background indicates a residue that is conserved in that position in 50% or more of the aligned sequences, while a grey background indicates a residue that is similar to others at that position in 50% or more of the aligned sequences. Plasmid name and ORF name or letter are shown at left.

would shift the frame of this ORF so as to extend the protein encoded to 212 amino acids in length. The hypothetical translation of this frameshifted ORF was included in the alignment instead of the original predicted pSB3 *C* ORF translation.

The alignment of Rep2 and Rep2-equivalent proteins showed a few regions in which the density of chemically-conserved residues was greater than that of the majority of the alignment (Figure 5). *In vivo* and *in vitro* interaction assays have demonstrated that residues 15 to 58 of Rep2 are required for association with Rep1, (Figure 2; (Sengupta *et al.*, 2001); J. Chew, unpublished results). As all 2µm-like plasmids encode a conserved Rep1-like protein, the region of the alignment encompassing residues 15 to 58 of Rep2 was examined specifically for conserved features that might be required for association with Rep1. This region contained a cluster of hydrophobic residues preceded by an aspartic acid residue; it was therefore predicted that the conserved aspartic acid residue, found at position 22 in Rep2, might be involved in mediating association of Rep2 with Rep1.

Truncations of Rep2 consisting of residues 1 to 231 or residues 58 to 296 are capable of mediating association of Rep2 with itself (Sengupta, 2000; Sengupta *et al.*, 2001), suggesting that residues 58 to 231 of Rep2 encompass features required for Rep2 self-association. There is some conservation of hydrophobic residues spaced at regular intervals in the region of the Rep2 and Rep2-equivalent protein alignment corresponding to Rep2 residues 155 to 230. Secondary structure algorithms (Drozdetskiy *et al.*, 2015) predict mainly alpha helical conformations for this region of Rep2 and Rep2-equivalent proteins (data not shown). When projected on to a helical wheel plot, the hydrophobic residues in these regions of predicted helical structure cluster on one side of the plot (data not shown), suggesting that this region contains one or more amphipathic helices. On the basis of this analysis, I hypothesized that the hydrophobic faces of the alpha helices in this central portion of Rep2 might interact to facilitate Rep2 self-association.

Truncation of the 20 amino acids at the carboxy terminus of Rep2 leads to the loss of Rep2 nuclear localization, suggesting that this region, which contains many basic residues, encompasses the Rep2 nuclear localization signal (NLS; (Velmurugan *et al.*, 1998)). A similar patch of basic residues is found at the carboxy termini of most Rep2-equivalent proteins, suggesting that the NLS for these proteins might also be located at

the extreme carboxy-terminus. For the conceptual translation of the *C* ORF of pSB3, this basic patch does not show the same degree of similarity as those of the other Rep2-equivalent proteins, but is nevertheless present.

3.1.2 Amino Acid Substitutions At Residues In Rep2 Predicted To Be Functionally Relevant Based On Sequence Alignment Impair Association With Rep1 And Rep2 *In Vivo*

To test whether the regions of conservation indicated by the sequence alignment contributed to Rep2 function, mutant versions of the Rep2 protein were generated, in which features deemed to be characteristic of these regions were altered. The highly conserved aspartic acid 22 in Rep2 was replaced with asparagine to create a mutant Rep2 protein, $Rep2_{D22N}$, in which any role of the negative charge of the aspartic acid would be disrupted, but the size of the residue at position 22 would remain relatively unchanged. In a separate Rep2 mutant, a pair of leucines at positions 185 and 186, located within one of the predicted amphipathic helices, were replaced with alanines to create the mutant Rep2 protein $Rep2_{AA}$; because alanine is less hydrophobic than leucine, this substitution would weaken associations mediated by hydrophobicity. A mutant was also generated in which all three of these substitutions (aspartic acid 22 to asparagine, and leucines 185 and 186 to alanines) were present, $Rep2_{NAA}$.

The ability of the $Rep2_{D22N}$, $Rep2_{AA}$ and $Rep2_{NAA}$ mutants to associate with wild-type Rep1 and Rep2 was tested *in vivo* using a two-hybrid assay (Section 2.5). A strain that did not contain native 2µm plasmid (cir^0) was used for these assays, as wild-type Rep2 is able to associate with both Rep1 and Rep2 simultaneously (Sengupta *et al.*, 2001), and could therefore potentially bridge an association between wild-type Rep1 and mutant Rep2 proteins. The $Rep2_{D22N}$ mutant was unable to associate with Rep1, but not impaired for association with Rep2, while $Rep2_{AA}$ was unable to associate with Rep2, but not impaired for association with Rep1 (Figure 6 A). The $Rep2_{NAA}$ mutant, in which the substitutions from both $Rep2_{D22N}$ and $Rep2_{AA}$ were present, was unable to associate with either Rep1 or Rep2. Western blotting analysis with antibodies recognizing the LexA DNA-binding domain ($LexA_{BD}$), to which Rep2 proteins had been fused to perform the two-hybrid assay, showed that these substitutions did not alter Rep2 protein levels (Figure 6 B). Consistent with the hypotheses presented above (Section 3.1.1), these

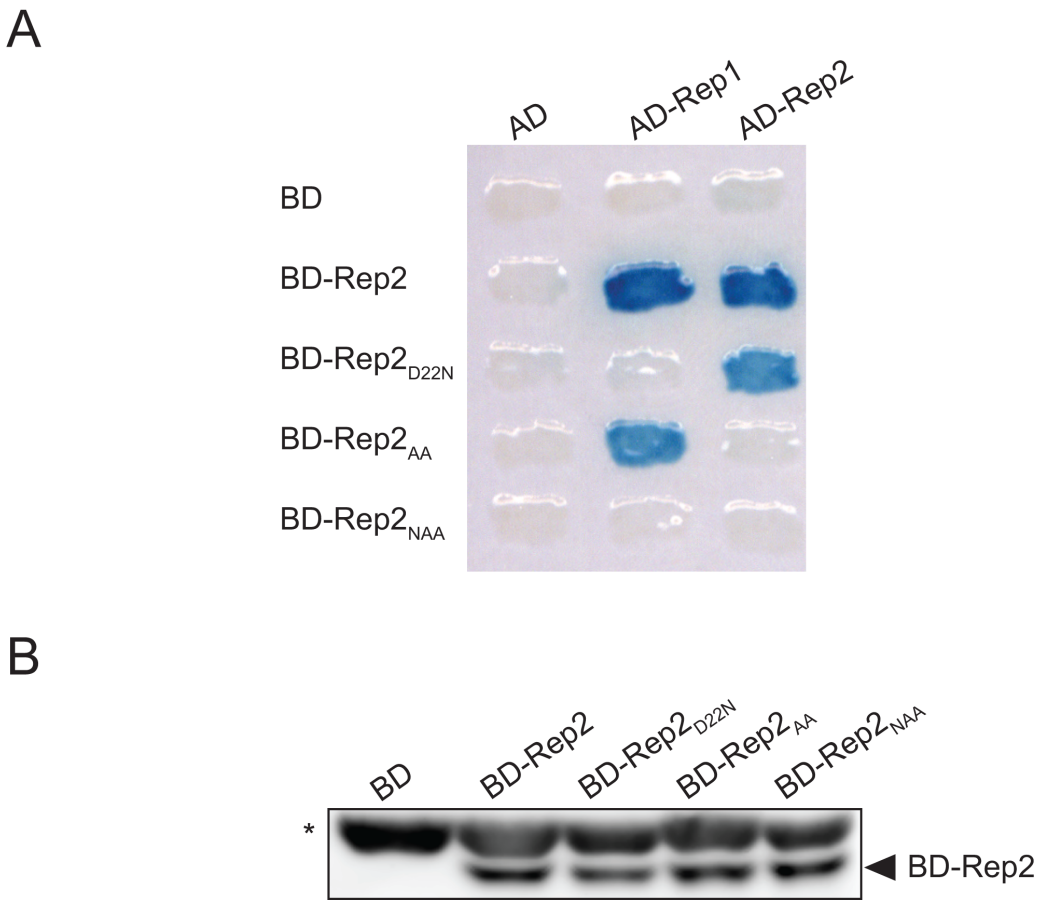


Figure 6. D22N substitution in Rep2 impairs association with Rep1; L185A and L186A substitutions in Rep2 impair self-association. A *cir*^{0} two-hybrid reporter strain was co-transformed with two plasmids, one expressing $Gal4_{AD}$ alone or $Gal4_{AD}$ fused to Rep1 or Rep2, and the other $LexA_{BD}$ alone or fused to wild-type Rep2, $Rep2_{D22N}$, $Rep2_{AA}$ or $Rep2_{NAA}$. "AD" denotes $Gal4_{AD}$; "BD" denotes $LexA_{BD}$. (A) Co-transformants were grown in patches on a nitrocellulose filter overnight (16 to 24 hours) and activation of the *lacZ* reporter gene, an indication of association between the two fusion proteins, was monitored by a filter assay using the substrate X-gal, which is cleaved to yield a blue precipitate in the presence of ß-gal activity. (B) Total protein from the co-transformants shown in (A) was extracted and analyzed by western blotting with an anti-LexA antibody, which detects the BD ($LexA_{BD}$). An asterisk (*) denotes a non-LexA host protein detected by the antibody.

results suggest that the highly conserved aspartic acid residue at position 22 in Rep2 is required for association of Rep2 with Rep1, and that the regularly-spaced hydrophobic residues in the central portion of Rep2 are required for Rep2 self-association. These results also imply that domains encoded by Rep2 and Rep2-equivalent proteins may be functionally conserved, despite their lack of significant sequence similarity, and that it may be possible to predict other residues required for Rep2 function based on the alignment of Rep2 and Rep2-equivalent proteins.

3.1.3 Association Of $Rep2_{D22N}$ Mutant With *STB-P* Is Impaired *In Vivo*

The accepted model of 2μm plasmid partitioning predicts that both Rep1 and Rep2 must associate with the *cis*-acting partitioning locus *STB-P* for the establishment of a functional partitioning complex (Yang *et al.*, 2004). This is based mainly on results obtained with mutant Rep1 proteins, showing that Rep1 mutants that are unable to associate with Rep2 or *STB-P*, or both, are unable to function in plasmid partitioning (Yang *et al.*, 2004), and is supported by the observation that Rep1, Rep2 and *STB-P* co-localize in nuclear foci (Ahn *et al.*, 1997; Velmurugan *et al.*, 2000). To determine whether Rep2 self-association or association of Rep2 with Rep1 is required for Rep2 association with *STB-P*, mutant Rep2 proteins were tested for *STB-P* association using a one-hybrid assay. Wild-type or mutant Rep2 proteins were expressed fused to the B42 transcriptional activation domain with an HA epitope tag ($B42_{AD}$-HA) in a cir^0 strain in which the *STB-P* sequence was integrated in the chromosome upstream of a *HIS3* reporter gene. Association of $B42_{AD}$-HA-Rep2 with *STB-P* would activate the *HIS3* reporter gene, allowing cells to form colonies on medium lacking histidine and containing a competitive inhibitor of the *HIS3* gene product, 3-AT. As robust association of Rep2 with *STB-P* has previously been shown to be dependent on the presence of Rep1 (Pinder *et al.*, 2013), Rep1 was expressed in these cells from a galactose-inducible promoter.

Expression of wild-type $B42_{AD}$-HA-Rep2 resulted in growth on medium lacking histidine and containing 3-AT, indicating that wild-type Rep2 associates with *STB-P* in the one-hybrid assay as previously reported (Figure 7 A). Expression of $B42_{AD}$-HA-$Rep2_{D22N}$ resulted in no growth, while expression of $B42_{AD}$-HA-$Rep2_{AA}$ resulted in growth similar to that observed for wild-type $B42_{AD}$-HA-Rep2 on medium lacking

histidine and containing 3-AT. These results with mutant Rep2 fusion proteins indicate that $Rep2_{AA}$, but not $Rep2_{D22N}$, is able to associate with *STB-P* in this one-hybrid assay system. Western blotting analysis with anti-HA antibodies showed that, similar to the $LexA_{BD}$-fused Rep2 mutants used in the two-hybrid assay, the steady-state levels of the $B42_{AD}$-HA-Rep2 were not impaired by the D22N or AA substitutions in Rep2 (Figure 7 B).

The observation that $Rep2_{D22N}$, which is impaired in its ability to associate with Rep1 (Figure 6 A), is also impaired in its association with *STB-P* (Figure 7 A) suggests that the association of Rep2 with *STB-P* is dependent not only the presence of Rep1, but also the ability of Rep2 to associate with Rep1. The observation that $Rep2_{AA}$, which is impaired for self-association (Figure 6 A), associates with *STB-P* in a one-hybrid assay in a manner similar to wild-type Rep2 (Figure 7 A) implies that Rep2 self-association is not required for Rep2 association with *STB-P*.

3.1.4 Plasmid Partitioning Function Is Impaired For $Rep2_{NAA}$, But Not $Rep2_{D22N}$ Or $Rep2_{AA}$, In A Wild-Type Strain

Based on the model of a tripartite Rep1-Rep2-*STB-P* partitioning complex, the results of the one-hybrid assay described above suggest that $Rep2_{D22N}$, which is impaired for association with Rep1 (Figure 6 A) and *STB-P* (Figure 7 A), might be unable to form a functional partitioning complex and mediate plasmid inheritance. $Rep2_{AA}$ was able to associate with *STB-P* in the one-hybrid assay (Figure 7 A), but was impaired for self-association in the two-hybrid assay (Figure 6 A), and so might also be unable to form a functional partitioning complex if this complex requires Rep2 self-association.

To determine whether association of Rep2 with *STB-P* and Rep1, or with Rep2, is required for plasmid partitioning, inheritance of a 2µm-based plasmid, pKan, encoding either wild-type or mutant Rep2 was assayed in a cir^0 yeast strain. The pKan plasmid was used because the 2µm plasmid confers no obvious phenotype to its host. pKan is a 2µm-based plasmid in which the *kanMX4* cassette was introduced in the *FLP* ORF, which serves to confer resistance to the aminoglycoside antibiotic G418, and to served to disrupt the *FLP* gene, preventing Flp expression and plasmid copy number amplification, thus ensuring that plasmid inheritance was entirely dependent on the presence of an intact partitioning system. We have previously demonstrated that the pKan plasmid is

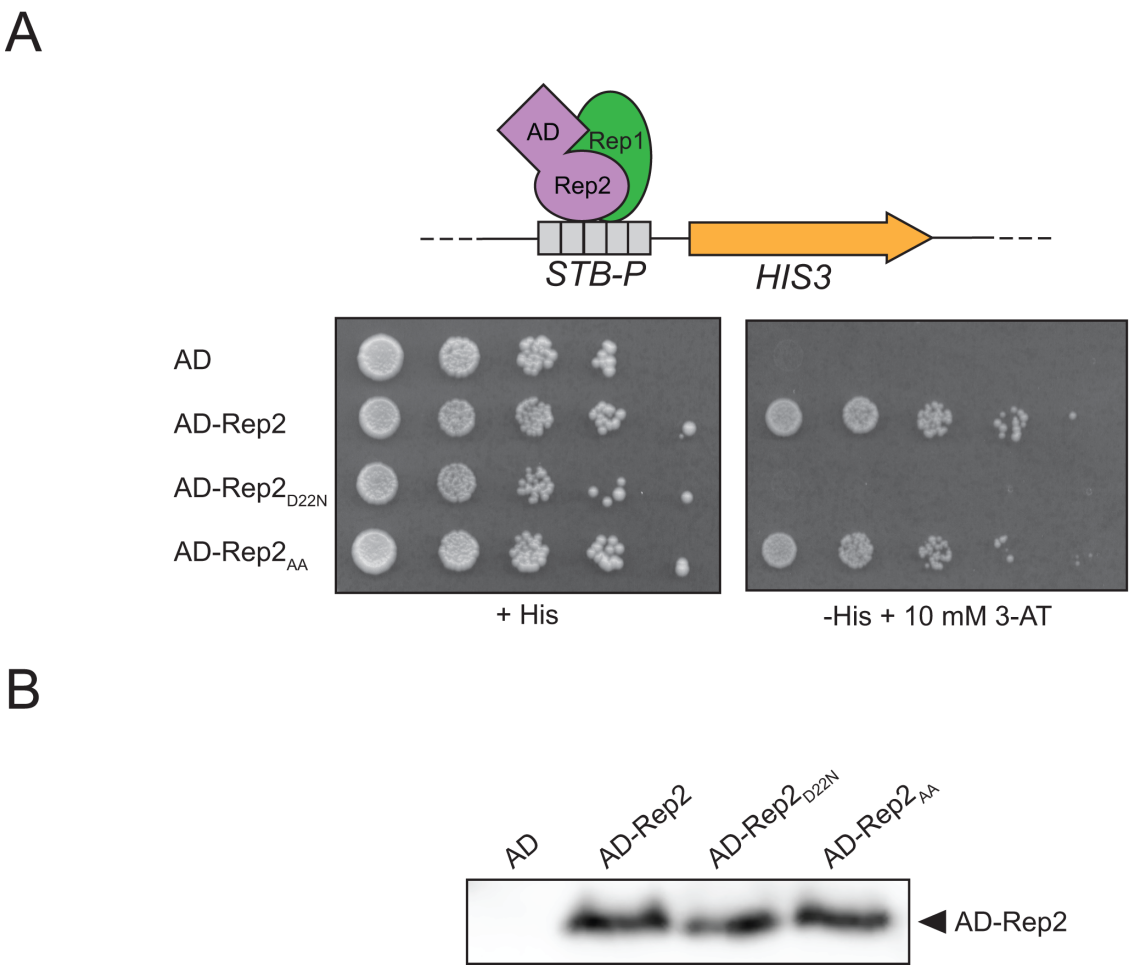


Figure 7. Association of Rep2$_{D22N}$, but not Rep2$_{AA}$, with *STB-P* is impaired *in vivo*. A *cir*^{0} strain carrying *STB-P* integrated in the chromosome upstream of a *HIS3* reporter gene was co-transformed with two plasmids, one expressing Rep1 from the *GAL1/10* promoter, and the other expressing B42$_{AD}$-HA alone or fused to wild-type or mutant versions of Rep2. "AD" denotes B42$_{AD}$-HA. (A) Co-transformants were serially diluted and spotted onto matched plates either containing histidine (left) or lacking histidine and containing 10 mM 3-AT (right). (B) Equal amounts of total protein extracts from the co-transformants shown in (A) were analyzed by western blotting with an anti-HA antibody, which detects the AD (B42$_{AD}$-HA).

efficiently partitioned in *cir*^{0} yeast and that the fraction of cells capable of forming colonies on solid medium containing G418 is a sensitive measure of plasmid partitioning function (Pinder *et al.*, 2013).

Plasmid inheritance assays showed that pKan-based plasmids encoding either Rep2$_{D22N}$ or Rep2$_{AA}$ were inherited with a similar efficiency to a pKan plasmid encoding wild-type Rep2 (~0.7), but that inheritance of the pKan plasmid encoding Rep2$_{NAA}$ was impaired (~0.3; Figure 8 A). This suggests that Rep2 association with either Rep1 or Rep2 is sufficient for partitioning function, but only if the other association is intact, and further implies that Rep2 association with Rep1 and Rep2 self-association may serve an overlapping purpose in plasmid partitioning.

Loss of partitioning function associated with the Rep2$_{NAA}$ mutant could be due to lower steady-state levels for this mutant protein, which would in turn be expected to lead to lower Rep1 levels as well, given the role of Rep2 in stabilizing the Rep1 protein (Pinder *et al.*, 2013). Western blotting analysis showed that Rep1 protein levels were moderately elevated in strains expressing Rep2$_{D22N}$ or Rep2$_{AA}$, and highly elevated in strains expressing Rep2$_{NAA}$, compared to strains expressing wild-type Rep2 (Figure 8 B). Rep2$_{D22N}$ levels were similar to wild-type, while Rep2$_{AA}$ and Rep2$_{NAA}$ levels were moderately elevated. The decrease in Rep2 protein levels relative to Rep1 for Rep2$_{D22N}$ and Rep2$_{NAA}$ suggest that the level of Rep2 protein per plasmid copy may be reduced for these two mutants.

Elevation of both Rep1 and Rep2 protein levels are observed when plasmid copy number in cell populations becomes elevated due to the combined effects of missegration and selection for the presence of the *kanMX4* gene on the plasmid (Pinder *et al.*, 2013). The slight increase in Rep1 levels when co-expressed with Rep2$_{D22N}$ and Rep2$_{AA}$ suggests that, although the fraction of plasmid-bearing cells was similar to those with wild-type Rep2, the partitioning function of Rep2$_{D22N}$ and Rep2$_{AA}$ may have been slightly impaired, but compensated for by the accumulation of cells with higher plasmid copy number than wild type; higher plasmid copy number would, in turn, render the generation of plasmid-free cells less likely. The fact that Rep1 protein levels are elevated when Rep2$_{D22N}$ or Rep2$_{NAA}$ is present, while Rep2$_{D22N}$ levels are not and Rep2$_{NAA}$ levels are elevated to a much lesser extent than those of Rep1, suggests that the stability of the

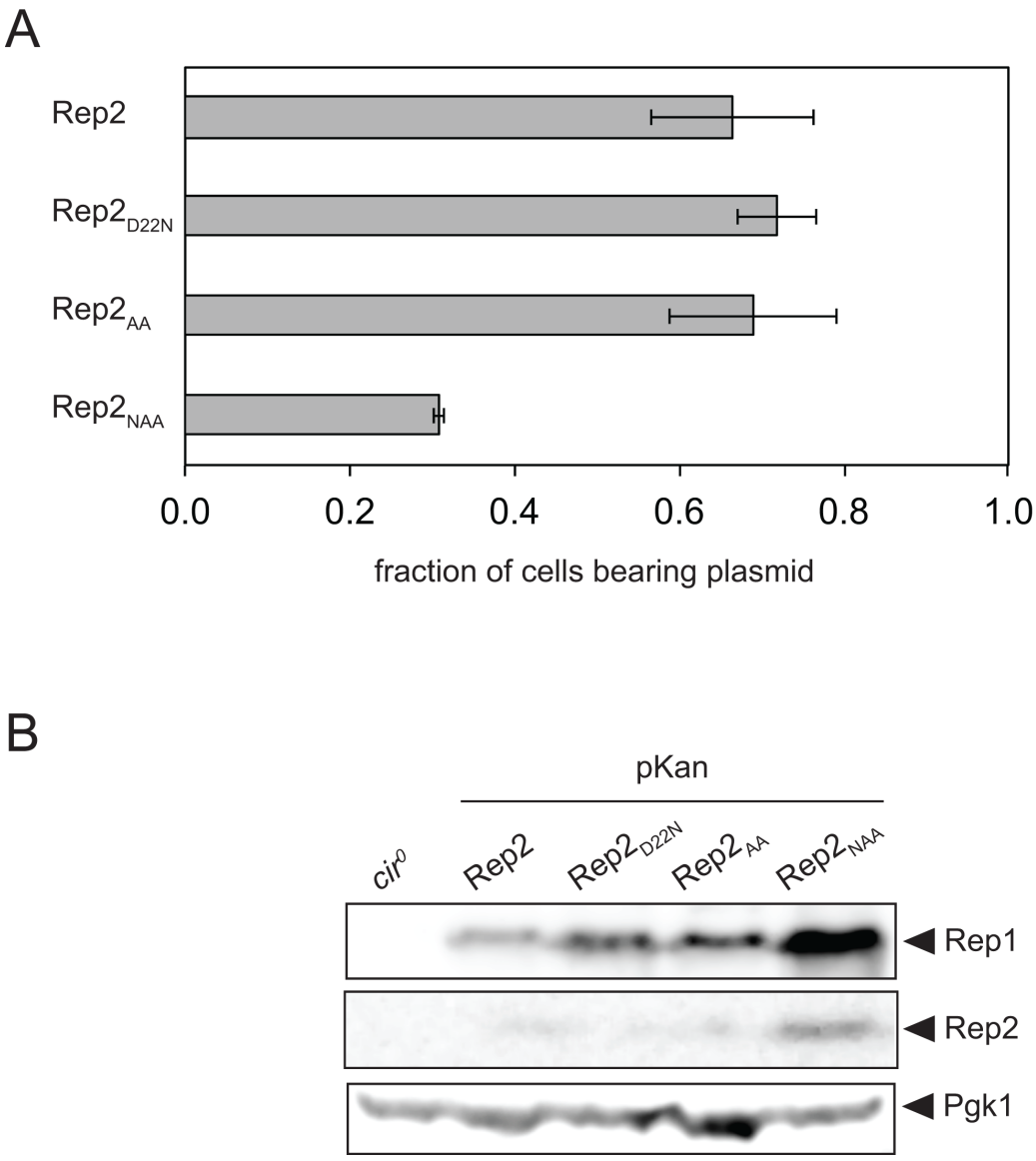


Figure 8. Plasmid inheritance is impaired for $Rep2_{NAA}$, but not $Rep2_{D22N}$ or $Rep2_{AA}$. A *cir*^{0} strain was transformed with a 2µm-based pKan plasmid encoding either wild-type Rep2, or mutants $Rep2_{D22N}$, $Rep2_{AA}$ or $Rep2_{NAA}$. (A) Transformants were cultured overnight (6 to 8 generations) in selective medium (YPAD+G418). Results represent average of a minimum of 4 isolates of each transformant (± standard deviation). (B) Protein extracted from transformants in (A) was analyzed by western blotting with antibodies specific for Rep1, Rep2 and, as a loading control, 3-phosphoglycerate kinase (Pgk1).

Rep2 protein or regulation of transcription on the 2µm plasmid by Rep1 and Rep2 (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988) may be altered by the D22N substitution. For the $Rep2_{NAA}$ mutant, the combined effects of slight plasmid missegregation due to the D22N and AA substitutions, and of potentially reduced steady-state levels due to the D22N substitution, may have been responsible for the decrease in the fraction of plasmid-bearing cells observed; however, further study will be required to determine whether this is the case.

3.1.5 $Rep2_{D22N}$ Is Unable To Mediate Repression Of The *FLP* Gene Promoter

The effect of the D22N substitution in Rep2 on the relative protein levels of Rep1 and Rep2 described above prompted an examination of the ability of $Rep2_{D22N}$ and $Rep2_{AA}$ to mediate transcriptional repression. In addition to its essential role in plasmid partitioning, Rep2 represses transcription driven by 2µm plasmid promoters when co-expressed with Rep1 (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988). To test whether Rep2 association with Rep1 or Rep2 self-association are required for Rep protein-mediated transcriptional repression, the ability of $Rep2_{D22N}$ and $Rep2_{AA}$ to repress transcription from the *FLP* promoter (*FLPp*) was assayed. For this assay, a reporter consisting of *FLPp* positioned in-frame with the *lacZ* coding region, which expressed ß-gal with the first four amino acids of Flp fused to the amino terminus, was integrated into the genome in a cir^0 yeast strain. Wild-type Rep1 and either wild-type or mutant Rep2 proteins were then expressed from the *GAL1/10* promoter on a single-copy (*ARS/CEN*) plasmid (pGAL-TRP), and the effects of this expression on the activity of the *FLPp-lacZ* reporter were monitored. As previously reported, the co-expression of wild-type Rep1 and Rep2 reduced expression directed by *FLPp* (Figure 9 A; (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988)). Upon co-expression of Rep1 with $Rep2_{D22N}$, no reduction in reporter activity was observed, while co-expression of Rep1 with $Rep2_{AA}$ resulted in a decrease in reporter activity similar to that observed with wild-type Rep1 and Rep2. These results suggest that association of Rep2 with Rep1 is required, but Rep2 self-association is dispensable, for Rep protein-mediated transcriptional repression of *FLPp-lacZ*.

The loss of repression observed for the $Rep2_{D22N}$ mutant could be due to physical interaction of Rep1 with Rep2 being required to form a functional repressor complex.

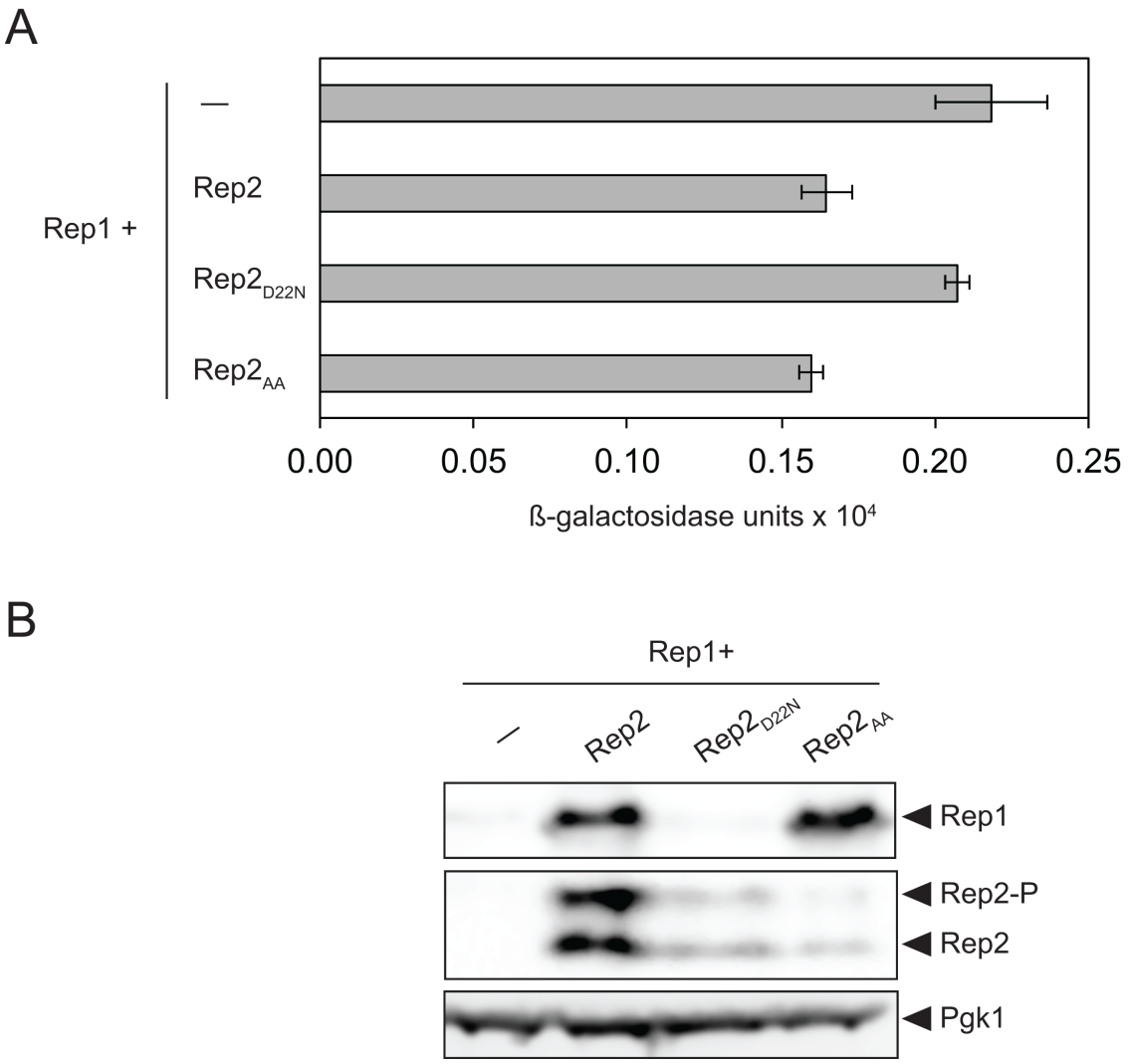


Figure 9. Ability of $Rep2_{D22N}$, but not $Rep2_{AA}$, to mediate transcriptional repression of *FLP* promoter is impaired. A *cir*^{0} strain with a *lacZ* reporter gene fused in-frame downstream of the *FLP* promoter integrated into the genome was transformed with pGAL-TRP plasmids expressing wild-type Rep1 alone, or wild-type Rep1 with wild-type or mutant versions of Rep2, as indicated. (A) Expression of the Flp-ß-gal fusion protein was monitored by assaying for its activity after 24 hours of growth in selective medium containing galactose, using the substrate ONPG, which is cleaved to give a yellow product that can be measured spectrophotometrically. Results represent a minimum of 4 isolates of each transformant (± standard deviation). (B) Total protein extracted from strains in (A) was analyzed by western blotting with antibodies specific for Rep1, Rep2 and Pgk1. Rep2-P is hyperphosphorylated form of Rep2.

Alternatively, this loss of repression could be a result of a reduction in Rep protein levels. Upon examination of Rep protein levels by western blotting, two Rep2 species were detected, as expected for native Rep2 expressed from a galactose-inducible promoter, one at ~35kDa and another at ~37kDa, which was previously shown to be a hyperphosphorylated form of Rep2 (Pinder, 2011). Rep1 levels were found to be highly reduced in the reporter strain expressing $Rep2_{D22N}$, and Rep2 levels moderately reduced by both the D22N and AA substitutions (Figure 9 B). A previous observation that Rep1 mutants impaired for association with Rep2 showed decreased protein levels compared to wild-type Rep1 suggested that stabilization of Rep1 by Rep2 may require a physical interaction between these two proteins (A. Sengupta, unpublished results). The decreased level of Rep1 protein in the presence of $Rep2_{D22N}$ now provides further evidence that stabilization of Rep1 requires a physical interaction with Rep2. These results also suggest that Rep2 association with Rep1 and Rep2 self-association both contribute to Rep2 protein stability, as the steady-state protein levels of both the $Rep2_{D22N}$ and $Rep2_{AA}$ mutants were reduced compared to wild-type Rep2. This reduction in $Rep2_{D22N}$ and $Rep2_{AA}$ protein levels was not observed when these proteins were expressed as $LexA_{BD}$ (Figure 6 B) or $B42_{AD}$-HA (Figure 7 B) fusions, suggesting that these fusions may reduce the turnover rate of $Rep2_{D22N}$ and $Rep2_{AA}$.

3.1.6 Wild-Type Rep1 And Rep2 Protein Levels Are Increased In The Presence Of Raf

In the analyses of Rep1 and Rep2 protein levels by western blotting described above, the effect of both the D22N and AA substitutions in Rep2 differed depending on whether proteins were expressed from their native promoters on pKan plasmids (Figure 8 B), or from the *GAL1/10* promoter on pGAL-TRP plasmids (Figure 9 B). Rep1 and $Rep2_{D22N}$ levels were reduced upon co-expression from a pGAL-TRP plasmid, but not a pKan plasmid. Similarly, $Rep2_{AA}$ mutant protein levels were only lower than wild-type Rep2 levels when expressed from the pGAL-TRP plasmid. This was unexpected, as the *GAL1/10* promoter directs considerably more transcription upon induction than the native 2µm plasmid promoters (data not shown), and is also not subject to Rep protein-mediated repression as the native 2µm promoters are. Another difference between pKan and

pGAL-TRP plasmids is that pKan plasmids encode Raf as well as Rep1 and Rep2, whereas pGAL-TRP plasmids encode no 2μm proteins other than Rep1 and Rep2.

Raf is involved in the regulation of transcription of 2μm plasmid-encoded genes, counteracting the repressive effects of Rep1 and Rep2 on plasmid gene transcription (Murray *et al.*, 1987). Additionally, overexpression of Raf was previously shown to increase Rep1 protein levels when Rep1 and Rep2 were co-expressed from either their native promoters on a pKan plasmid or the *GAL1/10* promoter on a *CEN* plasmid (Pinder, 2011), suggesting that Raf may also regulate Rep protein levels post-transcriptionally. Overexpression of Raf also led to a shift in the balance of Rep2 species from the 37-kDa hyperphosphorylated form toward the 35-kDa form, which is the predominant species observed in wild-type cells in logarithmic growth (data not shown); however, Raf expression did not appear to have an effect on overall Rep2 protein levels (Pinder, 2011). A shift of Rep2 from the 35-kDa species to the 37-kDa hyperphosphorylated form has been observed prior to Rep2 degradation (Pinder *et al.*, 2013). It is therefore possible that the shift in the balance of Rep2 species observed in the presence of Raf is related to a role for Raf in the stabilization of Rep2 protein levels, despite the absence of a change of overall Rep2 protein levels upon overexpression under these conditions.

To fully investigate the effects of Raf on Rep1 and Rep2 protein levels, protein was extracted from *cir*^{0} yeast expressing various combinations of Rep1, Rep2 and Raf from *GAL1/10* promoters, and analyzed by western blotting. Rep1 protein levels were found to be increased in the presence of Rep2, as previously reported (Figure 10; (Pinder *et al.*, 2013)); however, Rep2 protein levels were also increased in the presence of Rep1, which had not been observed previously. Expression of Raf increased both Rep1 and Rep2 protein levels, both in the presence and absence of the second Rep protein. Rep1 protein levels showed a greater increase in the presence of Raf alone than in the presence of Rep2 alone, while total Rep2 protein levels in the presence of Raf alone were similar to those observed in the presence of Rep1 alone. Simultaneous expression of Rep1, Rep2 and Raf led to the largest increase in protein levels for both Rep1 and Rep2. Additionally, a shift in the balance of the Rep2 species toward the lower 35-kDa form was observed upon Raf expression, as previously reported (Pinder, 2011).

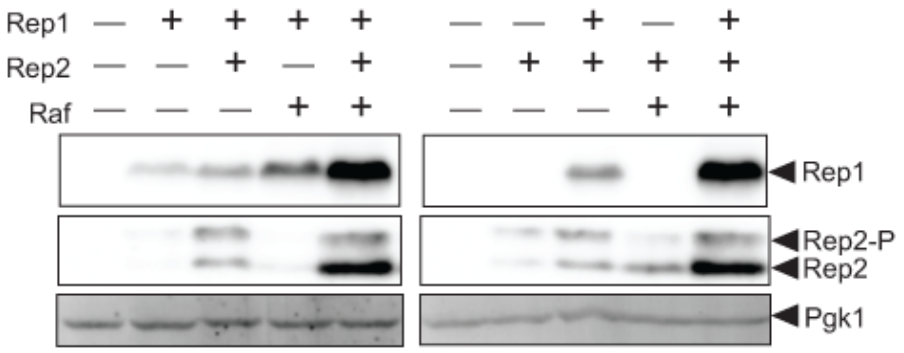


Figure 10. Steady-state levels of Rep1 and Rep2 proteins increased by Raf overexpression. A *cir*^{0} yeast strain was co-transformed with two plasmids: one, either empty pBM272, pBM272-REP1, pBM272-REP2 or pBM272-R1R2; and the other either the empty pGAL-TRP vector or pGAL-TRP-RAF. Transformants were cultured overnight (16 to 24 hours) in selective medium containing galactose, and total protein was extracted and analyzed by western blotting with antibodies specific for Rep1, Rep2 and Pgk1.

Overall, these results confirm the preliminary observation that Raf stabilizes Rep1 protein levels, and suggest that Raf performs a similar function for Rep2. This Raf-associated increase in Rep protein levels occurs in the absence of the 2µm plasmid gene promoters or *STB-P* sequences, where Raf is known to function as a transcriptional anti-repressor. The role of Raf in increasing Rep protein levels is therefore likely independent of its role in transcriptional regulation (Pinder *et al.*, 2013).

These results further suggest that the reason decreases in Rep protein levels were observed in the presence of the Rep2 D22N and AA substitutions when these proteins were expressed from the pGAL-TRP plasmid (Figure 9 B), but not the pKan plasmid (Figure 8 B), may be that Raf, which is expressed by pKan but not pGAL-TRP, is stabilizing Rep proteins expressed from pKan plasmids. It is also possible that the association of Rep proteins with the *STB* locus or 2µm plasmid gene promoter DNA on the pKan plasmids could contribute to Rep protein stability independently of Raf.

3.1.7 $Rep2_{D22N}$ And $Rep2_{AA}$ Are Unable To Mediate Plasmid Inheritance In The Absence Of Raf

To establish whether Raf-mediated increases in Rep1 and Rep2 protein levels have functional relevance, the plasmid inheritance assays described above (Section 3.1.4) were repeated in the absence of Raf. To prevent Raf expression from pKan plasmids, a point mutation was introduced into the *RAF* gene to convert the first codon of *RAF* to a stop codon. As previously reported (Jayaram *et al.*, 1983), the absence of Raf had no effect on inheritance of a pKan plasmid expressing wild-type Rep2 (Figure 11 A). However, for pKan plasmids expressing either $Rep2_{D22N}$ or $Rep2_{AA}$, the fraction of plasmid-bearing cells was drastically reduced in the absence of Raf. This suggests that both in the absence of Rep2 association with Rep1 or in the absence of Rep2 self-association, Raf is able to play a compensatory role in 2µm plasmid partitioning. Raf is able to increase both Rep1 and Rep2 protein levels when expressed from the *GAL1/10* promoter (Figure 10; (Pinder, 2011)), suggesting that the role of Raf in partitioning may be to maintain Rep1 and Rep2 protein levels when levels of either of these proteins are low. Raf could also be interacting with the partitioning complex at *STB-P* to perform a more direct role in partitioning.

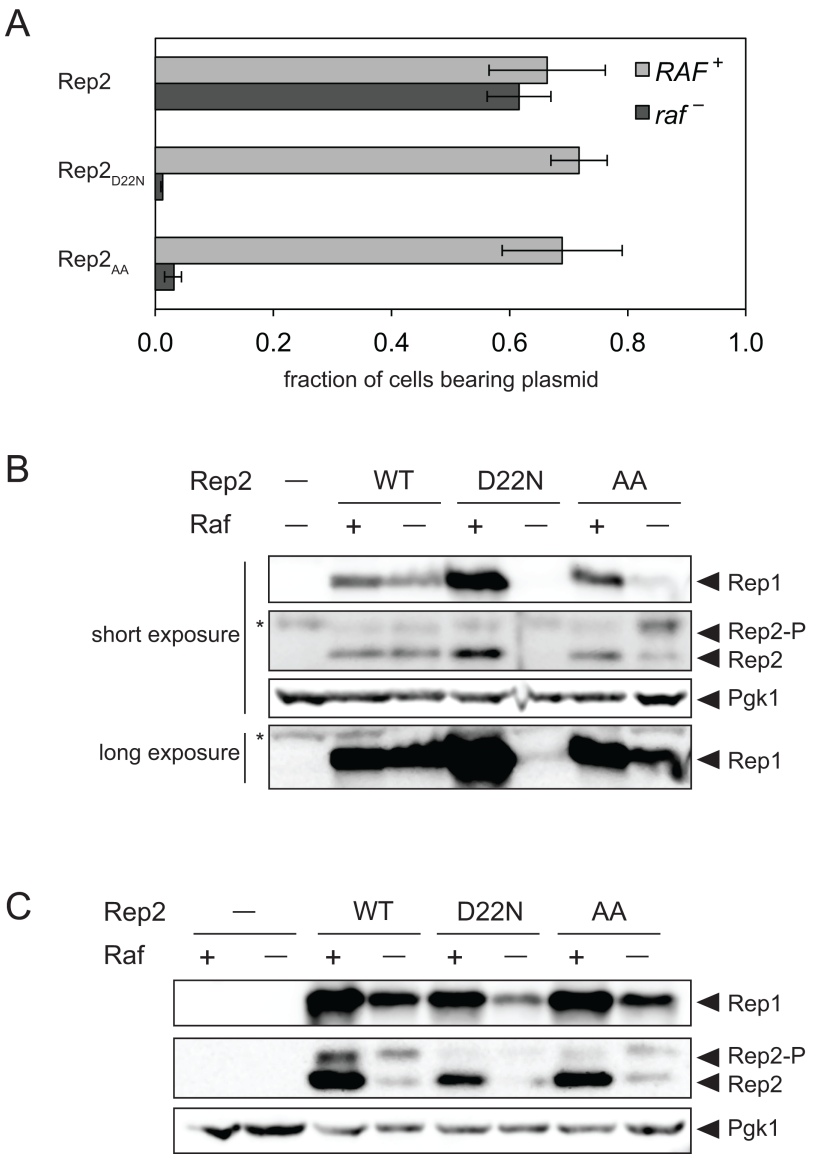


Figure 11. The absence of Raf impairs partitioning function of $Rep2_{D22N}$ and $Rep2_{AA}$, and alters steady-state Rep protein levels in strains expressing these proteins. (A) A *cir*^{0} yeast strain was transformed with a pKan plasmid encoding wild-type Rep1; wild-type Rep2, $Rep2_{D22N}$ or $Rep2_{AA}$; and either a wild-type *RAF* gene or a *RAF* gene containing a premature stop codon (*raf*$^{-}$), as indicated. The fraction of plasmid-bearing cells after overnight growth (6 to 8 generations) in selective medium (YPAD+G418) was determined for at least 5 isolates of each transformant (± standard deviation) by a plating assay. (B) Total protein from strains in (A), as well as an untransformed *cir*^{0} yeast strain ($Rep1^{-}$, $Rep2^{-}$, Raf^{-}), was analyzed by western blotting with antibodies specific to Rep1, Rep2 and Pgk1. (C) A *cir*^{0} yeast strain was co-transformed with two plasmids: one, either empty pBM272, or versions of pBM272-R1R2 expressing wild-type or mutant $Rep2_{D22N}$ or $Rep2_{AA}$; and the other either the empty pGAL-TRP vector or pGAL-TRP-RAF. Total protein was extracted from co-transformants and analyzed by western blotting with antibodies specific to Rep1, Rep2 and Pgk1.

To determine whether Raf was able to increase Rep protein levels when expressed from its native promoter, total protein was extracted from strains carrying pKan plasmids and analyzed by western blotting. The absence of Raf had no effect on Rep1 or Rep2 protein levels when both proteins were wild type (Figure 11 B). This suggests that when wild-type Rep1, Rep2 and Raf are expressed from their own promoters in a 2µm plasmid context, the effect of Raf on Rep1 and Rep2 protein levels is negligible, and is consistent with the observation that loss of Raf does not impair plasmid partitioning function when Rep1 and Rep2 are wild type (Figure 11 A; (Jayaram *et al.*, 1983)). When Rep2 carried either the D22N or AA substitution, the absence of Raf resulted in a decrease in the levels of both Rep1 and Rep2. A shift in the predominant Rep2 species from the 35-kDa form to the 37-kDa form was observed for the $Rep2_{AA}$ mutant in the absence of Raf. This shift may also have occurred for $Rep2_{D22N}$; however, the level of $Rep2_{D22N}$ in the absence of Raf was too low for observations regarding the ratio of Rep2 species to be made.

When Rep1 and Rep2 are expressed from 2µm-based plasmids, Rep1 and Rep2 protein levels can be confounded by plasmid copy number variations, as noted above. Therefore, although Rep1 and Rep2 levels were low in strains containing pKan plasmids encoding $Rep2_{D22N}$ or $Rep2_{AA}$ in the absence of Raf (Figure 11 B), this could be due to the low fraction of plasmid-bearing cells in these strains (Figure 11 A). To eliminate the variable of plasmid copy number variation, Rep1, Rep2 and Raf were expressed from *GAL1/10* promoters on pBM272 plasmids. The pBM272 plasmids give the maximal level of expression we can achieve from a heterologous promoter, with expression of Rep1 and Rep2 being higher than their expression from the pGAL-TRP plasmids, likely due to differences in the linker restriction site sequences immediately upstream of the *REP* initiation codon that affect translational efficiency (data not shown).

Western blotting showed that upon expression from pBM272 plasmids, Rep1 and Rep2 levels were decreased when Rep2 carried the D22N substitution, but not when Rep2 carried the AA substitution (Figure 11 C); this is in contrast to expression from pGAL-TRP plasmids (Figure 9 B), which resulted in a decrease in both Rep1 and Rep2 levels when Rep2 carried either the D22N or the AA substitution. This suggests that Rep1 and Rep2 are less stable when Rep2 carries the D22N substitution than when Rep2 carries the AA substitution, as the higher level of expression mediated by pBM272

plasmids was sufficient to maintain Rep protein levels when Rep2 carried the AA, but not the D22N substitution. This result also suggests that the association of Rep1 and Rep2 is more important to the stability of Rep2 than Rep2 self-association.

Expression of Raf resulted in an increase in the levels of Rep1 and Rep2, whether Rep2 was wild-type or carried the D22N or AA substitution (Figure 11 C). As $Rep2_{AA}$ is impaired for association with Raf in a two-hybrid assay (data not shown), the ability of Raf to maintain $Rep2_{AA}$ protein levels suggests that Raf is able to stabilize Rep2 protein levels indirectly, likely through stabilization of Rep1 protein levels. A shift in the balance of Rep2 species from the 37-kDa species toward the 35-kDa species was observed upon expression of Raf in all three cases.

Taken together, these results show that Raf is required for the plasmid partitioning function of $Rep2_{D22N}$ and $Rep2_{AA}$. Stabilization of Rep1 and Rep2 protein levels by Raf may partially account for this effect. For the $Rep2_{D22N}$ mutant, reductions in Rep1 and Rep2 protein levels correlate with loss of both transcriptional repression (Figure 9) and partitioning (Figure 11 A) functions. However, this straightforward correlation is not observed for $Rep2_{AA}$. Partitioning function, but not repression function, of $Rep2_{AA}$ is compromised in the absence of Raf, despite the fact that, when expressed from the pGAL-TRP plasmids used to test repression, Rep1 and Rep2 levels are reduced compared to wild type when Rep2 carries the AA substitution. This suggests that either lower levels of Rep1 and Rep2 are sufficient for transcriptional repression than are sufficient for plasmid partitioning, or that the AA substitution in Rep2 impairs partitioning function without impairing repression for a reason unrelated to protein levels. It also implies that Raf, in addition to stabilizing Rep1 and Rep2 protein levels, can compensate for the impairment of Rep2 self-association, and therefore may play a more direct role in 2µm plasmid partitioning. Better defining this potential novel role for Raf will be the focus of a future investigation.

3.1.8 Raf Associates With Both Rep1 And Rep2 *In Vivo* Through The Same Protein Termini As Rep2

The observation that Raf is able to stabilize both Rep1 and Rep2 protein levels suggests that Raf may physically interact with these proteins. To determine whether this was the case, Raf was tested for its ability to associate with Rep proteins in a two-hybrid

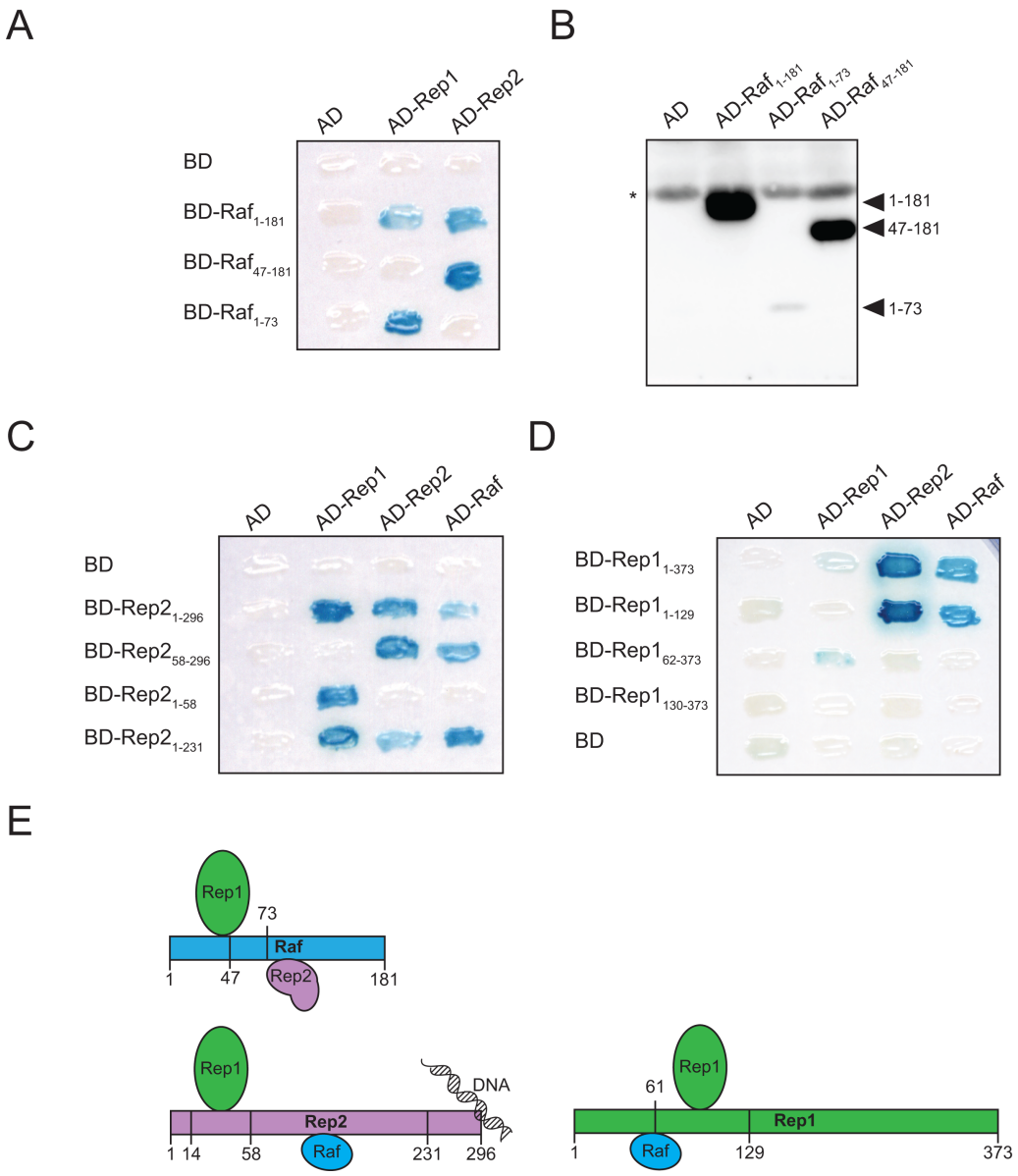


Figure 12. Raf associates with Rep1 and Rep2 independently; residues required for these associations are similar to those required by the Rep2 protein. A *cir*^{0} two-hybrid reporter strain was co-transformed with two plasmids, one expressing $Gal4_{AD}$ (A, B and C) or $B42_{AD}$-HA (D) alone or fused to Rep1, Rep2 or Raf; and the other $LexA_{BD}$ alone or fused to full-length ($Rep1_{1-373}$, $Rep2_{1-296}$, Raf_{1-181}) or truncated versions of Rep1, Rep2 or Raf. (A), (C) and (D) Co-transformants were assayed for protein-protein association as described in the legend of Figure 6. (B) Total protein from the co-transformants shown in (A) was extracted and analyzed by western blotting with an anti-LexA antibody, which detects the BD ($LexA_{BD}$). An asterisk (*) denotes a non-LexA host protein detected by the antibody. "AD" denotes $Gal4_{AD}$ in (A), (B) and (C), and $B42_{AD}$-HA in (D); "BD" denotes $LexA_{BD}$ in panels (A) through (D). (E) Cartoon summary of results in panels (A), (C) and (D). Interaction of Rep2 with Rep1 and Rep2 truncations is not shown; see Figure 2 for comparison.

assay (Figure 12 A). Raf was found to associate with both Rep1 and Rep2 independently, and residues 1 to 73 of Raf were sufficient for association with Rep1, but not Rep2, while residues 47 to 181 were sufficient for association with Rep2, but not Rep1. This suggests that the domains of Raf required for interaction with Rep1 and Rep2 are distinct, with an amino-terminal domain of Raf being responsible for association with Rep1, and carboxy-terminal domain being responsible for association with Rep2. The truncated versions of Raf showed stronger two-hybrid interactions than full-length Raf. Western blotting analysis showed that the levels of both truncated two-hybrid fusions of Raf were reduced compared to full-length Raf, with levels of Raf_{1-73} in particular being highly reduced (Figure 12 B), demonstrating that the apparent increase in association of these truncated proteins with Rep1 and Rep2 is not a result of increased protein levels, and could reflect some inhibition of each interaction by the non-interacting domain. A similar phenomenon is seen for Rep1, where interaction with Rep2 and Rep1 self-association is improved upon the removal of the carboxy-terminal domain of Rep1 ($Rep1_{130-373}$), both *in vivo* in a two-hybrid assay and *in vitro*, despite a decrease in protein levels for $Rep1_{1-129}$ compared to full-length Rep1 (Sengupta *et al.*, 2001).

To determine which regions of Rep1 and Rep2 associate with Raf, two-hybrid assays were performed using full-length Raf and truncated forms of Rep1 and Rep2. As previously reported, a truncation of Rep1 consisting of residues 1 to 129 ($Rep1_{1-129}$), but not residues 62 to 373 ($Rep1_{62-373}$), was sufficient for association with Rep2 (Figure 12 D; (Sengupta *et al.*, 2001); J. Chew, unpublished results). Two-hybrid association of full-length Rep1 with full-length Rep1 and the $Rep1_{62-373}$ truncation was observed, but only weakly, likely due to the low levels of Rep1 protein in cir^0 cells in the absence of Rep2 (data not shown). Neither the $Rep1_{1-129}$ nor $Rep1_{130-373}$ truncation was seen to associate with full-length Rep1; however, $Rep1_{1-129}$ has been seen to associate with full-length Rep1 both in a two-hybrid assay in the presence of native 2µm plasmid and *in vitro* (Sengupta *et al.*, 2001), suggesting that the absence of an interaction in this assay system may be the result of reduced levels of the $Rep1_{1-129}$ truncation. Rep2 residues 1 to 58 were required and sufficient for association with Rep1. Rep2 truncations consisting of residues 1 to 231 ($Rep2_{1-231}$) or 58 to 296 ($Rep2_{58-296}$) were sufficient for self-association (Figure 12 C; (Sengupta *et al.*, 2001); J. Chew, unpublished results), suggesting that a

motif encompassed by residues 58 to 231 of Rep2 is responsible for self-association. For Raf association, $Rep1_{1\text{-}129}$, but not $Rep1_{62\text{-}373}$, was sufficient (Figure 12 D), while $Rep2_{1\text{-}231}$ and $Rep2_{58\text{-}296}$ were both sufficient (Figure 12 C).

The two-hybrid assay results suggest that Raf associates with Rep1 and Rep2 in a manner similar to Rep2: both Rep2 and Raf associate with an amino-terminal domain of Rep1 via domains near their amino termini, and with a central domain of Rep2 via central, or central and carboxy terminal, domains, respectively. No truncations of Raf lacking carboxy-terminal residues were seen to associate with Rep2 (data not shown), which could indicate that Raf, which is a shorter protein than Rep2, lacks a Rep2-specific carboxy-terminal domain that is dispensable for association with Rep2. The only other evident difference between the two-hybrid associations observed for Rep2 and Raf is that Rep2 is able to self-associate, while Raf is not (data not shown). This difference could again be attributed to the shorter length of Raf compared to Rep2; however, as residues 1 to 231 of Rep2 are sufficient for interaction of Rep2 and Raf with Rep2 (Figure 12 C), and substitution of leucines 185 and 186 of Rep2 for alanines impairs Rep2 association with Rep2 and Raf (Figure 6 A, data not shown), differences between the central domains of Rep2 and Raf likely play a role in functionally distinguishing Rep2 and Raf as well.

Finally, it is also possible that the amino-terminal fusions of DNA-binding and transcriptional activation domains used in the two-hybrid assay interfere with interactions among 2µm-encoded proteins. The full-length Rep proteins fused to $LexA_{BD}$ and $Gal4_{AD}$ have been shown to be functional (data not shown), suggesting that they adopt the correct conformation despite the presence of N-terminal fusions. Additionally, all truncated fusion proteins can be detected by western blotting (Figure 12 B, data not shown), suggesting that these proteins are expressed and stable. However, whether the Raf fusion protein or the truncated fusion proteins adopt functional conformations is unknown. Differences between the modes of association of Rep2 and Raf could therefore be due to improper folding of the full-length Raf fusion protein, or of the truncated Rep2 or Raf fusion proteins.

3.1.9 Raf May Be A Rep2 Homolog

The results of two-hybrid analyses (Figure 12) suggest that Rep2 and Raf may share some structural similarities. The results of plasmid inheritance assays and western

blotting analysis performed in the presence and absence of Raf (Figures 10 and 11) provide evidence that Rep2 and Raf may also be functionally similar. Rep2 and Raf were both shown to increase Rep1 and Rep2 protein levels when proteins were expressed from *GAL1/10* promoters (Figure 10), suggesting that Rep2 and Raf act post-translationally to stabilize Rep1 and Rep2 levels. Raf was also shown to be required for partitioning of the 2µm-based pKan plasmid when Rep2 was unable to associate with Rep1 or unable to self-associate (Figure 11 A). This partitioning role of Raf could involve its ability to stabilize Rep1 and Rep2 protein levels, but might also involve a more direct role for Raf at the plasmid partitioning complex.

Raf has also been shown to antagonize activities of Rep2. Raf functions to relieve transcriptional repression mediated by co-expression of Rep1 and Rep2 (Murray *et al.*, 1987). When overexpressed, Raf has also been shown to impair the association of Rep2 with Rep1 and with Rep2 in a two-hybrid assay, while enhancing Rep1 self-association (Pinder, 2011). This suggests that although Raf enhances both Rep1 and Rep2 protein levels, it might also either compete with Rep2 for interacting partners, or favour Rep1 self-association over Rep1 association with Rep2 by other mechanisms.

Overall, these results raise the possibility that Raf may be a Rep2 homolog, with the *RAF* gene having perhaps evolved from a duplication of the *REP2* gene on the 2µm plasmid. Of the 2µm-like plasmids, only pSM1 from *Zygosaccharomyces fermentati* (Utatsu *et al.*, 1987) and pKW1 from *Kluveromyces waltii* (Chen *et al.*, 1992) encode an ORF in the position of *RAF*, known as the *B* ORF in both cases (Figure 4). Insertions in the *B* ORF of pKW1 do not abolish plasmid inheritance, suggesting that, like *RAF* on the 2µm plasmid, this ORF is not required for partitioning or amplification of pKW1. An alignment of Raf and conceptual translations of these two *B* ORFs with Rep2 and conceptual translations of *REP2*-positioned ORFs shows no significant sequence similarity, and therefore does not support the hypothesis that Rep2 and Raf are homologs (Figure 13). However, the features noted in the alignment of Rep2 and Rep2-equivalent proteins (Figure 5) as being of potential functional relevance, including the cluster of hydrophobic amino acids at the amino-termini and the regularly-spaced hydrophobic residues in the central regions of these proteins, were seen to be conserved in Raf and conceptual translations of the *B* ORFs. Raf and the conceptual translation of the *B* ORF

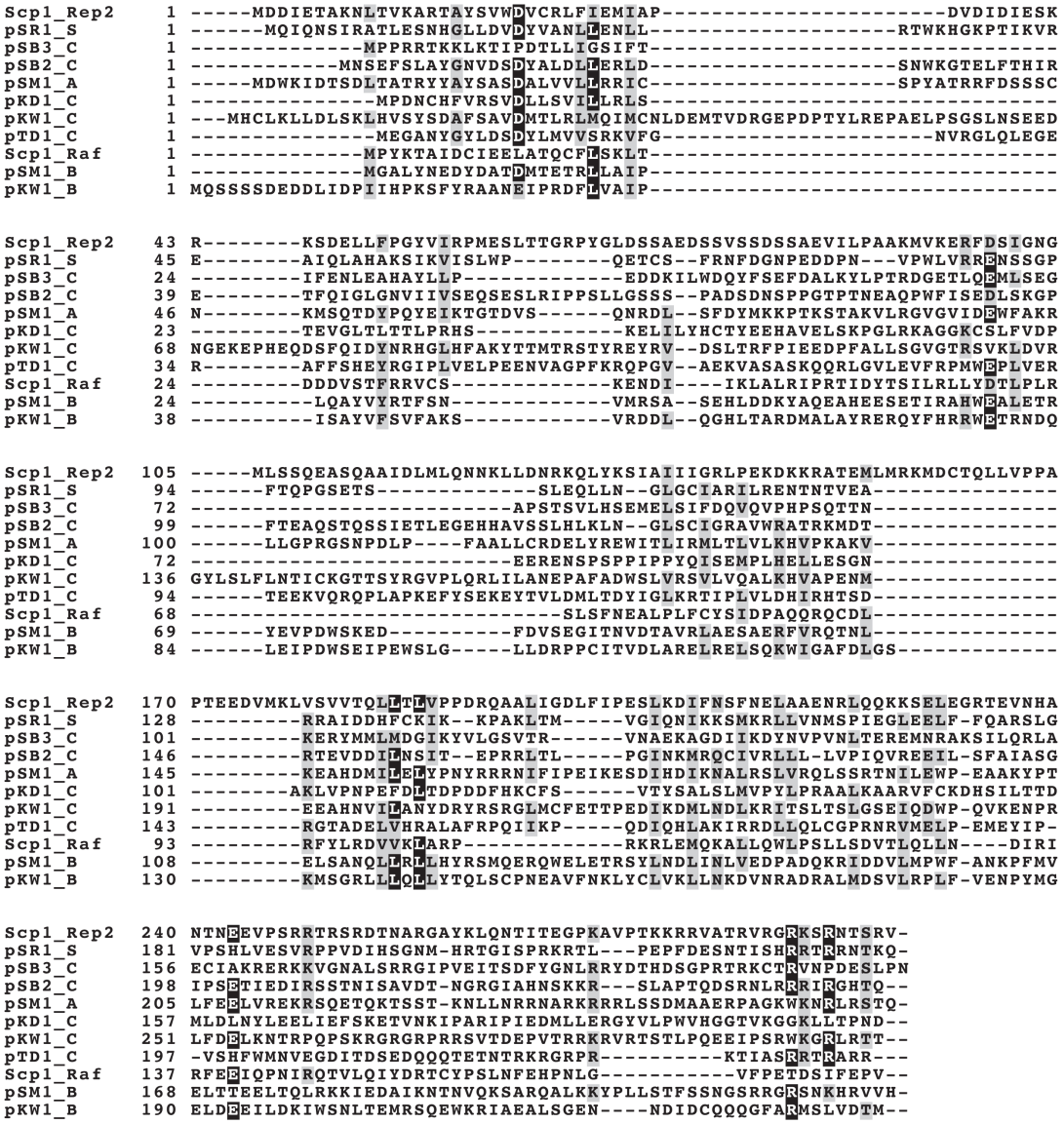


Figure 13. Alignment of Rep2 and Raf with conceptual translations of ORFs in the position of *REP2* and *RAF* on 2µm-like plasmids. Protein sequences were aligned, shaded and labeled as described in the legend of Figure 5.

Table 5. Predicted isoelectric points (pI) for Rep2, Raf and conceptual translations of ORFs in the positions of *REP2* and *RAF* on 2µm-like plasmids. Isoelectric points of amino acids 1 to 58 (the residues of Rep2 that are required and sufficient for association with Rep1 in a 2-hybrid assay, Figure 12) and the 65 amino acids closest to the C-terminus (the residues of Rep2 shown to be sufficient for DNA-binding *in vitro*) are also indicated.

	pI		
Plasmid Protein	Full-length	N-terminal 58 amino acids	C-terminal 65 amino acids
Scp1 Rep2	9.89	4.67	11.50
pSR1 S	10.42	10.66	10.92
pSB2 C	6.09	4.10	10.92
pSB3 C	7.70	5.14	10.40
pTD1 C	9.40	4.56	10.42
pSM1 A	10.24	7.70	11.25
pKW1 C	8.38	4.60	11.39
pKD1 C	5.67	6.40	4.89
pSM1 B	5.46	4.26	11.26
pKW1 B	4.77	4.77	4.14
Scp1 Raf	7.80	7.70	4.37

of pKW1 lack the basic residues that characterize the carboxy-termini of most Rep2-equivalent proteins and the conceptual translation of ORF *B* of pSM1 (Table 5). This suggests that Raf and the protein encoded by the pKW1 *B* ORF may play a different or more limited role in plasmid maintenance than the Rep2-equivalent proteins, perhaps due to loss of a carboxy-terminal DNA binding domain.

3.1.10 Raf associates with *STB-P* in vivo in the presence of Rep1

Rep2 has previously been shown to associate with *STB-P* in a manner that is largely dependent on physical association with Rep1 (Figure 7; (Pinder *et al.*, 2013)). Theabove inference, that, upon impairment of Rep2 association with Rep1 or with itself, Raf might have a role in plasmid partitioning beyond stabilizing Rep1 and Rep2 protein levels, together with the observation that Raf associates with Rep1 in a manner similar to Rep2, suggest that Raf might also associate with *STB-P*. Additionally, a previous study found that the micrococcal nuclease digestion pattern of the *STB-P* locus was altered in the presence of Raf (Fagrelius *et al.*, 1987), further suggesting a physical interaction of Raf with this locus.

To determine whether Raf is able to associate with *STB-P in vivo*, one-hybrid assays were performed. $B42_{AD}$-HA-Raf was expressed in a cir^0 strain containing the chromosomally-integrated *STB-P-HIS3* reporter (Section 3.1.3), as well as a *CEN* plasmid (pGAL-LEU) expressing either no Rep proteins, Rep1 protein only or Rep2 protein only from a galactose-inducible promoter. Raf was seen to associate with *STB-P*, and this association was dependent on the presence of Rep1 (Figure 14), as is the case for Rep2 association with *STB-P* (Pinder *et al.*, 2013). This further supports the hypotheses that Rep2 and Raf are related proteins, and that Raf can compensate for some roles of Rep2 in 2µm plasmid partitioning beyond that of improving Rep protein stability.

3.2 Functional Analysis Of The *STB-P* Locus

The *STB-proximal* (*STB-P*) locus, in the presence of Rep1 and Rep2, is sufficient to confer plasmid partitioning function in most sequence contexts. *STB-P* has been shown to associate not only with Rep1 and Rep2, but also with host proteins including the Kip1 motor protein, subunits of the RSC2 chromatin remodeling complex, the centromere-

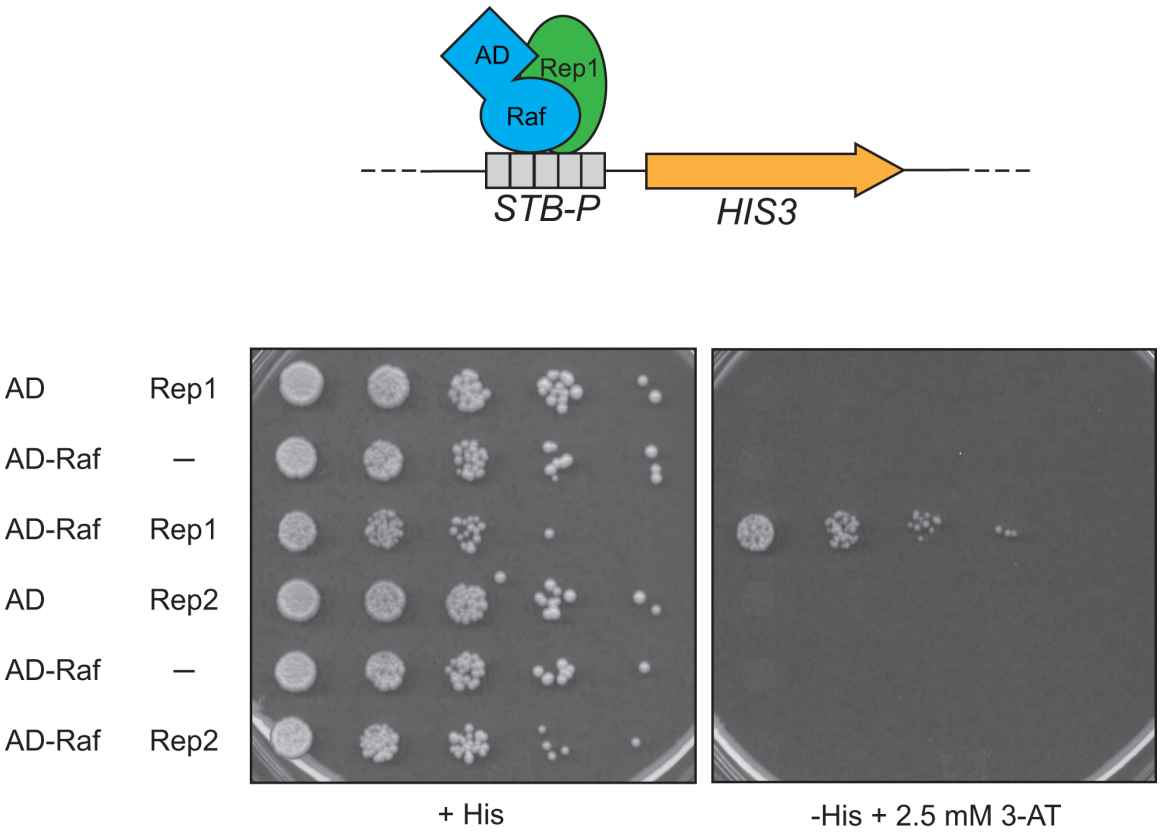


Figure 14. Raf associates with *STB-P in vivo* in a Rep1-dependent manner. A *cir*^{0} strain carrying *STB-P* integrated in the chromosome upstream of a *HIS3* reporter gene was co-transformed with two plasmids: one expressing either no Rep proteins, Rep1, or Rep2 from the *GAL1/10* promoter; and the other expressing either B42$_{AD}$-HA or B42$_{AD}$-HA-Raf. Co-transformants were serially diluted and spotted onto matched plates either containing histidine (left) or lacking histidine and containing 2.5 mM 3-AT (right). "AD" denotes B42$_{AD}$-HA.

specific histone H3 Cse4, and subunits of cohesin. Despite its key roles in mediating equal partitioning of the 2µm plasmid, and as an interface between the plasmid and host components, the DNA sequences required at *STB-P* to perform these functions are unknown.

3.2.1 Two Copies Of A Synthetic *STB* Repeat Are Sufficient To Confer 2µm Plasmid Partitioning Function

The native *STB-P* locus of the commonly studied Scp1 2µm plasmid variant consists of five tandemly arrayed direct repeats of a 62- to 63-bp sequence (Figure 3); however, observations outlined above (Section 1.3.1) suggest that three or fewer repeats are sufficient to confer Rep protein-dependent partitioning function to a plasmid. To determine the minimum number of repeats required for function, a synthetic *STB* repeat based on a single 63-bp stretch of the *STB-P* sequence was generated, and used to build arrays of one to five *STB* repeats (Table 6). These repeat arrays differed from the native *STB-P* in two main respects: first, all repeats within a synthetic array were 100% identical, while repeats within the native *STB-P* show only 65% to 98% identity; and second, the linker sequence (GGATC) added between the repeats to facilitate ligation resulted in arrays in which the repeat sequences were spread 5 bp further apart than in the native *STB-P*.

The synthetic *STB* arrays were introduced into a 2µm-based plasmid from which the native *STB-P* sequence had been removed and replaced with a 21-bp linker sequence (pKanΔSTB), to generate a series of plasmids with one to five copies of the synthetic *STB* repeat directly-arrayed in the position of the native *STB-P* (pKan-1-WT through pKan-5-WT). The native *STB-P* sequence, with ~35 bp of flanking sequence on either end, was also inserted into the linker sequence in pKanΔSTB to assess the impact of the differences between the native and synthetic *STB* repeat arrays (pKan-STB-P). A pKan plasmid with a native *STB* region (pKan) was used to determine the effect of the linker and flanking sequences, and a pKan-based plasmid that does not express Rep1 (pKanΔREP1) was used to assess whether any sequences outside of *STB-P* are able to confer Rep protein-dependent partitioning function.

A 2µm-based plasmid bearing a single copy of the synthetic *STB* repeat in place of native *STB-P* (pKan-1-WT) was inherited in a manner similar to a 2µm-based plasmid

Table 6. Sequences of DNA duplexes used for functional analysis of *STB-P*. The sequence of the origin-to-*STB-distal* (*STB-D*) orientation strand for each duplex is shown. "WT" is the duplex intended to be representative of the *STB-P* repeat closest to *STB-D*; the remaining duplexes contain alterations to the sequence of the WT duplex intended to probe the function of specific sequence elements. "m" and "j" stand for "middle" and "junction," respectively. Nucleotides added for restriction site overhangs are in lower case. Sequence elements chosen for mutational analysis are shown in top row of table. Residues altered in mutant duplexes are in bold.

duplex name	sequence (5'->3')
	TGCA CGCG TGCATTTTT
WT	gatcTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGTTGCATTTTTGTTCTACAAAATg
TGTG	gatcTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCT**T**G**T**GTTGCATTTTTGTTCTACAAAATg
TTT	gatcTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCT**T**TCGCGTTGCATTTTTGTTCTACAAAATg
CG3	gatcTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTC**G**CGCGTTGCATTTTTGTTCTACAAAATg
TGTA	gatcTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGTTG**T**ATTTTTGTTCTACAAAATg
TGAA	gatcTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGTTG**A**ATTTTTGTTCTACAAAATg
CTAGm	gatcTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT**CTAG**TTTTTGTTCTACAAAATg
TCAGj	gatct**CAG**GCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGTTGCATTTTTGTTCTACAAAATg
2CTAG	gatc**CTAG**GCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGT**CTAG**TTTTTGTTCTACAAAATg
TAATT	gatcTGCAGCTCAGATTCTTTGTTTGAAAAATTAGCGCTCTCGCGTTGCAT**AA**TTGTTCTACAAAATg

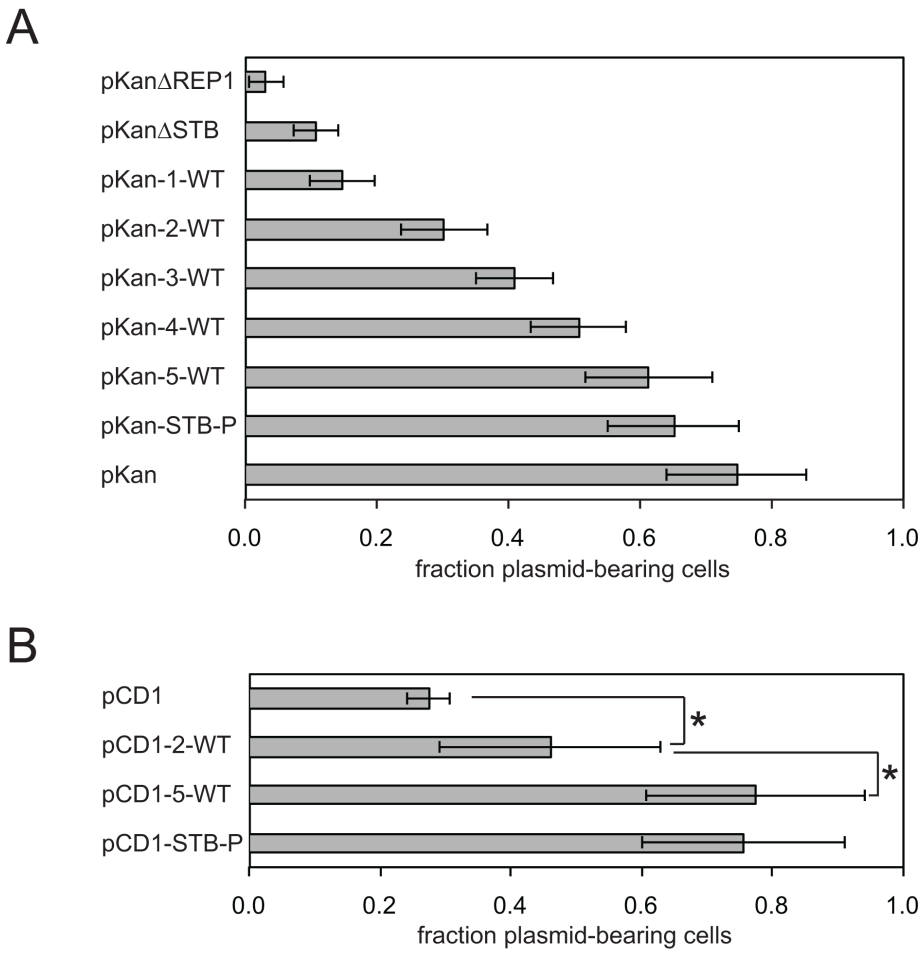


Figure 15. Two or more directly-arrayed copies of the synthetic *STB* repeat provide partitioning function. (A) A *cir*^{0} yeast strain was transformed with a tagged 2μm-based plasmid, either pKan, pKanΔREP1 (which lacks the *REP1* gene), or a pKanΔSTB-based plasmid (in which the native *STB-P* was replaced by a 21-bp linker sequence into which either no sequences, direct arrays of a WT synthetic *STB* repeat or the native *STB-P* flanked flanked by ~35-bp of linker sequences on either side was inserted), as indicated. Transformants were cultured overnight (6 to 8 generations) in selective medium (YPAD+G418), and fraction of plasmid-bearing cells determined by a plating assay. (B) A *cir*$^{+}$ yeast strain was transformed with pCD1 or a pCD1-based plasmid carrying a direct array of synthetic *STB* repeats or native *STB-P*, as indicated. Transformants were cultured overnight (6 to 8 generations) in selective medium (SD-trp). Results in (A) and (B) represent an average of a minimum of 4 isolates of each transformant (± standard deviation). Full sequence of synthetic *STB* repeat is given in Table 6. *$p<0.005$

bearing no *STB-P* sequences (pKanΔSTB; Figure 15 A); however, the presence of two or more synthetic *STB* repeats on a 2µm-based plasmid in place of native *STB-P* (pKan-2-WT through pKan-5-WT) was sufficient to confer an improvement in inheritance for these plasmids over pKanΔSTB and pKan-1-WT (Figure 15 A). This suggests that two *STB* repeats is the minimum required for recruitment of partitioning proteins and conversion to a partitioning-competent locus.

An approximately linear improvement in plasmid inheritance was observed upon each addition of a single plasmid repeat, up to five repeats (the number found in the native array), with the largest improvement occurring between one and two *STB* repeats (Figure 15 A). This trend did not appear to continue above five repeats, as a plasmid containing eight synthetic *STB* repeats showed similar inheritance to a plasmid containing five (data not shown). This suggests that there is an upper limit to the improvement that additional synthetic *STB* repeats confer to plasmid partitioning in this assay system.

Inheritance of a 2µm-based plasmid containing an array of five *STB* repeats in place of *STB-P* (pKan-5-WT) was similar to that of a 2µm-based plasmid on which the native *STB-P* sequence was removed and re-inserted, so that the re-inserted *STB-P* was flanked by the same 21-bp of linker sequence as the synthetic *STB* repeats (pKan-STB-P; Figure 15 A). This demonstrates that arrays of synthetic *STB* repeats are a valid tool for testing the effects of *STB* repeat number and sequence composition on function. It also suggests that slight alterations in the spacing of the repeats do not significantly impact function, and that the 63-bp region of *STB-P* used as the basis for the synthetic repeat contains all sequence elements required for partitioning. Other synthetic *STB* repeats were created, in which the starting nucleotide was shifted upstream or downstream relative to the 63-bp *STB-P* stretch originally chosen as the basis for the *STB* repeat, and were found to confer the same degree of plasmid partitioning function as the original synthetic *STB* repeat (data not shown); therefore, the sufficiency of two tandemly-arrayed *STB* repeats is likely not unique to the repeat frame chosen.

The inheritance of the 2µm-based plasmid in which the *STB* region had not been disrupted (pKan) was slightly improved compared to pKan-5-WT or pKan-STB-P (Figure 15 A). This suggests that the linker sequences used to facilitate the re-insertion of synthetic and native *STB-P* sequences are detrimental to *STB* partitioning efficiency,

although this detriment does not invalidate comparisons made between the partitioning efficiencies of sequences inserted in this linker region.

A 2µm-based plasmid lacking *STB-P* (pKanΔSTB) showed a small but noteable improvement in plasmid inheritance over a 2µm-based plasmid with a native *STB* region but lacking a *REP1* gene (pKanΔREP1; Figure 15 A). This suggests that 2µm plasmid sequences other than *STB-P* provide Rep protein-dependent partitioning function.

3.2.2 Synthetic *STB-P* Sequences Mediate Partitioning In The Absence Of Residual Partitioning Function Provided By Non-*STB-P* 2µm Plasmid Sequences

To determine whether the non-*STB-P* 2µm sequences responsible for the improvement in plasmid partitioning function observed for pKanΔSTB over pKanΔREP1 are required for the function of synthetic *STB-P* sequences, synthetic and native *STB-P* sequences were placed on a *TRP1*-tagged *ARS* plasmid (pCD1). The partitioning function of this pCD1-based series of plasmids was assessed by plasmid inheritance assays. Assays were performed in a *cir*$^+$ yeast strain containing native 2µm plasmid, which supplied the Rep1 and Rep2 proteins *in trans*. The inheritance of a pCD1 plasmid carrying two synthetic *STB* repeats was significantly improved ($p < 0.005$) over that of a pCD1 plasmid carrying no repeats (Figure 15 B). This demonstrates that, despite the finding that sequences outside the *STB-P* repeats do confer some Rep protein-dependent partitioning function to the 2µm plasmid (Figure 15 A, pKanΔREP1 vs. pKanΔSTB), the ability two copies of the synthetic *STB* repeat to mediate plasmid partitioning is not dependent on these non-*STB-P* 2µm plasmid sequences (Figure 15 B, pCD1 vs. pCD1-2-WT).

No significant difference in inheritance was observed between pCD1 plasmids carrying five arrayed copies of the synthetic *STB* repeat or native *STB-P*, with both plasmids giving a similar fraction of plasmid-bearing cells (Figure 15 B, pCD1-5-WT vs. pCD1-STB-P). This suggests that the synthetic *STB* sequences are no more reliant on a 2µm sequence context for function than the native *STB-P* sequence.

3.2.3 Sequence Elements Within The *STB* Repeat Required For Partitioning Function

With two copies of the synthetic *STB* repeat having been shown to be sufficient to confer partitioning function, synthetic repeats with alterations in specific sequence elements within the repeat were designed to assess the importance of these elements. The first sequence targeted for mutational analysis was TGCATTTTT, a putative Rep protein binding motif identified bioinformatically in two previous studies (Veit and Fangman, 1988; Som *et al.*, 1988). The TGCATTTTT sequence occurs in the core of three of the five native *STB-P* repeats, and also in two of the native repeat junctions in the inverse orientation (Figure 3). The 63-bp region of *STB-P* used as the basis for the synthetic *STB* repeat contains one of the core TGCATTTTT sequences and a junction TGCA element (Table 6).

To determine whether either of the TGCATTTTT sequences is required for *STB-P* function, a series of 2 μm-based plasmids was created. Each of these plasmids contained two directly-arrayed copies of the synthetic *STB* repeat, in which a portion of one of the TGCATTTTT sequences was mutated in an identical manner in both copies of the repeat. The ability of these plasmids to be partitioned was tested using a plasmid inheritance assay (Figure 16). Mutation of the TGCA sequence in the core TGCATTTTT to TGTA (pKan-2-TGTA) had no effect on plasmid inheritance, while a single base change to TGAA (pKan-2-TGAA) led to a decrease in the fraction of plasmid-bearing cells. Scrambling of the nucleotides in this sequence to CTAG (pKan-2-CTAGm) led to a further drop in plasmid inheritance. This suggests that the TGCA sequence in the *STB* repeat core is required for partitioning function, and that a pyrimidine but not a purine substitution is tolerated at the third position in this sequence.

To investigate whether the TGCA sequence found at the junction of the *STB* repeats might also contribute to partitioning function, synthetic repeats were generated in which this sequence was scrambled to TCAG (pKan-2-TCAGj). The TCAGj mutation led to a decrease in the fraction of plasmid-bearing cells similar to that observed when the core TGCA was scrambled, suggesting that the junction TGCA is also required for plasmid partitioning function. Scrambling both core and junction TGCA sequences simultaneously (pKan-2-CTAG2) led to a further decrease in the fraction of plasmid-

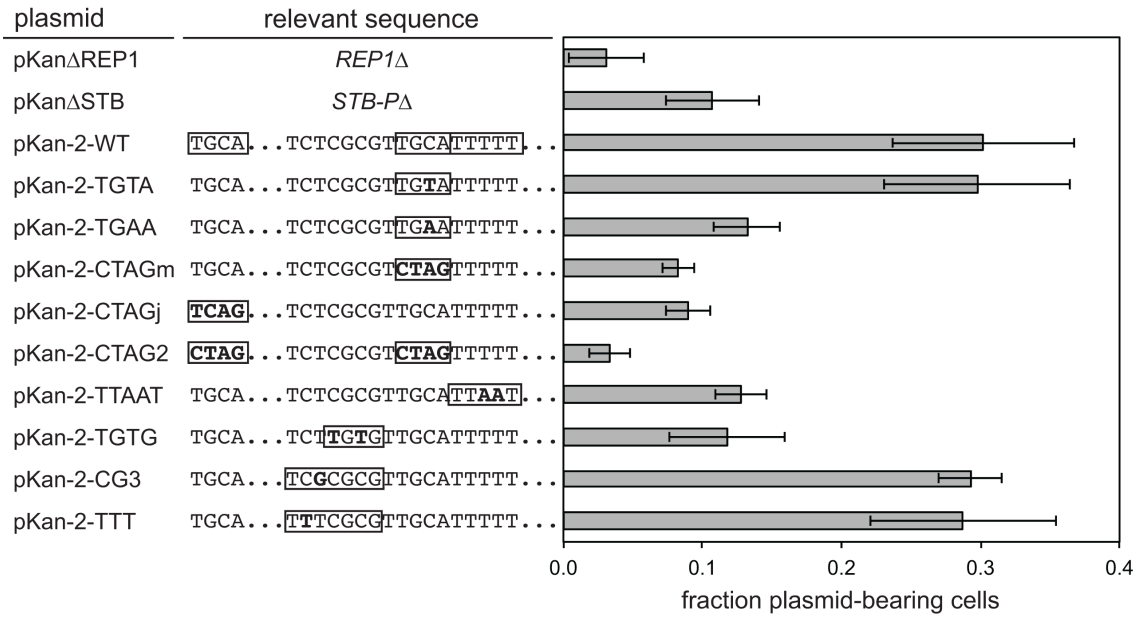


Figure 16. Two directly-arrayed copies of the synthetic *STB-P* repeat provide partitioning function if TGCATTTTT and CGCG sequences are intact. A *cir*^{0} yeast strain was transformed with a tagged 2µm-based plasmid, either pKanΔREP1 (which lacks the *REP1* gene), or a pKanΔSTB-based plasmid (in which the native *STB-P* was replaced by a 21-bp linker sequence into which either no sequences (pKanΔSTB), or two directly-arrayed copies of a WT (pKan-2-WT) or mutant (other pKan-2 series plasmids) synthetic *STB* repeat were inserted), as indicated. Strains were assayed for partitioning function as described in the legend of Figure 15. Results represent an average of a minimum of 4 isolates of each transformant (± standard deviation). Sequence elements targeted for analysis are boxed, and mutated residues are in bold. Full sequences of WT and mutant synthetic *STB* repeats are given in Table 6.

bearing cells, implying that each the core and junction TGCA provide a contribution to plasmid partitioning function in the absence of the other. The fraction of plasmid-bearing cells when both TGCA sequences were scrambled (pKan-2-CTAG2) was significantly lower than that observed when no native or synthetic *STB-P* sequences were present (pKanΔSTB), but similar to that observed when the *REP1* gene was deleted (pKanΔREP1), suggesting that replacement of native *STB-P* with sequences lacking any TGCA motifs may eliminate the Rep protein-dependent partitioning function conferred by non-*STB-P* 2µm plasmid sequences.

The importance of the T-tract in the core TGCATTTTT sequence was also investigated. Replacement of two of the thymines with adenines (pKan-2-TTAAT) led to a decrease in the fraction of plasmid-bearing cells, although this decrease was not as great as that observed when either of the TGCA sequences were scrambled. This demonstrates that the T-tract of the core TGCATTTTT also contributes to efficient plasmid partitioning.

The second sequence element investigated was a CGCG sequence adjacent to the core TGCATTTTT sequence. The recognition motif for the DNA-binding Rsc3 and Rsc30 subunits of the RSC2 chromatin remodeling complex is GCGCGCG, with the most highly-conserved portion being the CGCG residues in positions two to five of this motif (Badis *et al.*, 2008; Zhu *et al.*, 2009). Several subunits of the RSC2 complex associate with *STB-P in vivo* and are required for efficient 2µm plasmid partitioning (Wong *et al.*, 2002; Huang *et al.*, 2004; Ma *et al.*, 2012). CGCG is also the core of the binding motif preferred by homologs Mbp1 and Swi4, the DNA-binding components of the cell-cycle regulated transcription factors MBF and SBF, respectively (Iyer *et al.*, 2001; Badis *et al.*, 2008). MBF and SBF regulate the expression of an overlapping set of genes at the G1-to-S transition (Koch *et al.*, 1993; Iyer *et al.*, 2001). In *STB-P*, the CGCG sequence in one of the five repeats is a match to the consensus Swi4 site, CGCGAAA, while in three other repeats, including the one on which the synthetic repeat is based, a close match, CGCGAGA, is found. The most divergent, *ORI*-proximal repeat lacks the CGCG sequence.

To gauge the importance of the CGCG element to *STB-P* function, CGCG sequences were changed to TGTG in a 2µm-based plasmid carrying two copies of the

synthetic repeat in place of native *STB-P* (pKan-2-TGTG). The TGTG mutation led to a significant decrease in the fraction of plasmid-bearing cells (Figure 16), suggesting that CGCG is required for *STB-P* function.

In an effort to determine whether either Rsc3/Rsc30 or Swi4 association with the CGCG element might be required for *STB-P* partitioning function, residues flanking this element were mutated to either improve the Rsc3/Rsc30 consensus at the detriment of the Swi4 consensus or vice versa. It was hypothesized that making the element a better match to the GC-rich Rsc3/Rsc30 consensus recognition site, GCGCGCG (Badis *et al.*, 2008), would either have no effect on or improve plasmid partitioning if the CGCG element is required for association of the RSC2 complex; however, these mutations might impair partitioning if Swi4 recognition of the CGCG element is required. Conversely, converting the CGCG flanking sequences to a better match to the Swi4 consensus recognition site, TTTCGCG (Badis *et al.*, 2008), might improve plasmid partitioning if the CGCG element is required for Swi4 association; however, conversion to TTTCGCG might impair partitioning if recognition of CGCG by the RSC2 complex is required. To make the context of the CGCG element more similar to the Rsc3/Rsc30 consensus site, the TCTCGCG sequence in the *STB-P* repeat was changed to TCGCGCG (pKan-2-CG3). To create a Swi4 consensus site, the TCTCGCG sequence was changed to TTTCGCG (pKan-2-TTT). Neither change had a significant impact on the fraction of plasmid-bearing cells observed (Figure 16), suggesting either that the core CGCG sequence is sufficient for association of one of these proteins, regardless of changes made to the nearby sequences, or that the CGCG element is required for partitioning for a reason other than mediating association of one of these two known factors.

3.2.4 Increasing The Number Of Tandemly-Arrayed Copies Of Synthetic *STB* Repeat Improves Partitioning Function For Some But Not All Mutant Repeats

As partitioning was seen to improve as a function of the number of arrayed unmutated (wild-type, WT) synthetic *STB* repeats, a selection of mutant *STB* repeats were assessed to determine whether they showed a similar trend. Plasmid inheritance assays were performed using 2µm-based plasmids carrying two, three, four or five directly-arrayed copies of a particular mutant *STB* repeat in place of native *STB-P* (Figure 17). For

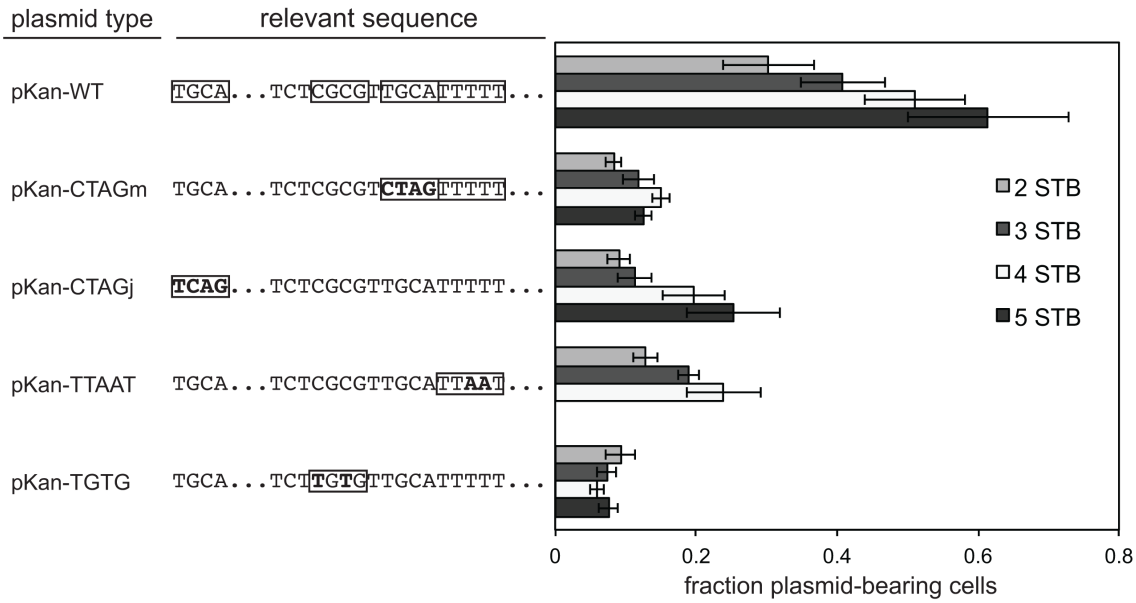


Figure 17. Partitioning defect associated with mutation of the core TGCA and CGCG sequences not alleviated by increasing repeat number. A *cir*^{0} yeast strain was transformed with a tagged 2µm-based plasmid, in which the native *STB-P* sequence was replaced by a 21-bp linker sequence into which two to five directly-arrayed copies of a WT or mutant synthetic *STB* repeat were inserted. Strains were assayed for partitioning function as described in the legend of Figure 15. Results represent an average of a minimum of 4 isolates of each transformant (± standard deviation). Sequence elements targeted for analysis are boxed, and mutated residues are in bold. Full sequences of WT and mutant synthetic *STB* repeats are given in Table 6.

plasmids carrying the TCAGj and TTAAT mutant repeats, partitioning improved as a function of repeat number, with five copies of TCAGj or four copies of TTAAT giving plasmid inheritance similar to that observed with two copies of the WT repeat.

Plasmids carrying the CTAGm and TGTG mutant repeats did not show a consistent improvement in partitioning as a function of repeat copy number. The CTAGm plasmids showed a slight improvement between two and four repeats, but no further improvement with five repeats. For the TGTG plasmids, similar partitioning function was conferred by two, three, four and five repeats.

These findings imply that mutations in the junction TGCA and T-tract sequences can be compensated for by increasing repeat copy number, while mutations within the core TGCA and TGTG sequences cannot, which in turn suggests that the core TGCA and CGCG sequences are of greater functional importance than the junction TGCA or the T-tract in the synthetic *STB* repeat arrays.

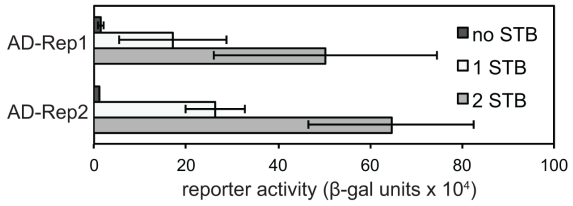
3.2.5 Mutation Of Either TGCA Sequence Impairs Association Of Rep Proteins With *STB* Repeats

As mutations in the core and junction TGCA sequences, the T-tract and the CGCG sequence of the synthetic *STB* repeat all impaired partitioning function, the effect of these mutations on Rep protein association with *STB* repeats *in vivo* was of interest. We have previously shown that Rep protein association with *STB-P*, as detected using a one-hybrid assay, reflects the association of native Rep proteins with *STB-P* observed in a ChIP assay (Pinder *et al.*, 2013). This suggests the one-hybrid assay can be used to assess the effect of mutations in the *STB* repeats on Rep protein association with these sequences.

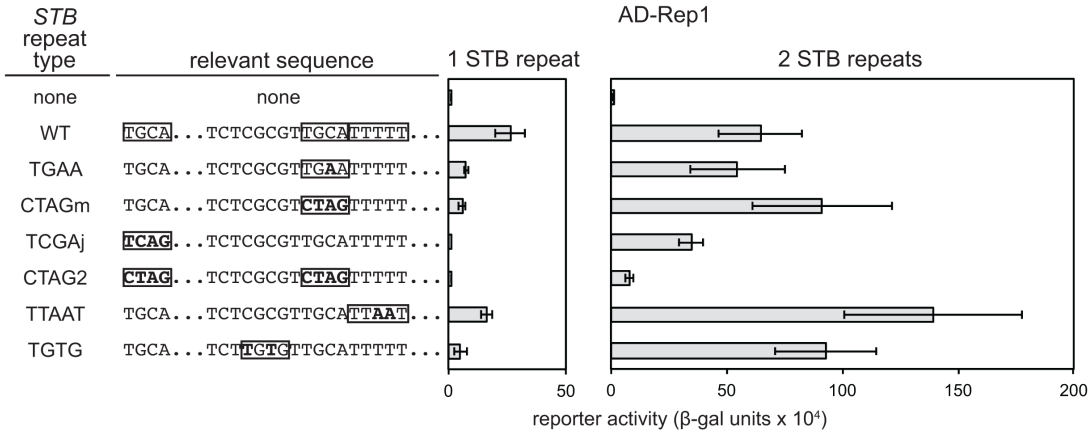
For the one-hybrid assay, Rep proteins were expressed as $Gal4_{AD}$ fusions in cir^{0} strains where no, one or two copies of WT or mutant synthetic *STB* repeats had been inserted upstream of a chromosomally-integrated *lacZ* reporter gene. For each $Gal4_{AD}$-Rep fusion protein being assayed, the partner Rep protein was expressed from a galactose-inducible promoter on a second plasmid. In the case of $Gal4_{AD}$-Rep1, Rep2 stabilizes Rep1 protein levels, resulting in a stronger one-hybrid signal, although Rep2 is not required to observe association of Rep1 with *STB-P* in a one-hybrid assay

Figure 18. Association of Rep1 and Rep2 with synthetic *STB-P* sequences impaired by mutations in the TGCATTTTT and CGCG sequences. A series of *cir*^{0} strains carrying no, one or two copies of WT or mutant synthetic *STB* repeats integrated in the chromosome upstream of a *lacZ* reporter gene were generated. Strains were co-transformed with two plasmids, either one expressing Gal4$_{AD}$-Rep1 (AD-Rep1) and the other expressing Rep2, or one expressing Gal4$_{AD}$-Rep2 (AD-Rep2) and the other expressing Rep1. Co-transformants were assayed for activity of the *lacZ* gene product, ß-gal, which indicates interaction between the Gal4$_{AD}$ fusion protein and the synthetic *STB* sequences of interest. Assays were performed after 24 hours of growth in selective medium containing galactose, using the substrate ONPG, which is cleaved to give a yellow product that can be measured spectrophotometrically. Results represent an average of a minimum of 4 isolates of each transformant (± standard deviation). Results using WT synthetic *STB* repeats are shown in (A); results using mutant synthetic *STB* repeats are shown in (B) for Gal4$_{AD}$-Rep1 and (C) for Gal4$_{AD}$-Rep2. Sequence elements targeted for analysis are boxed, and mutated residues are in bold. Full sequences of WT and mutant synthetic *STB* repeats are given in Table 6.

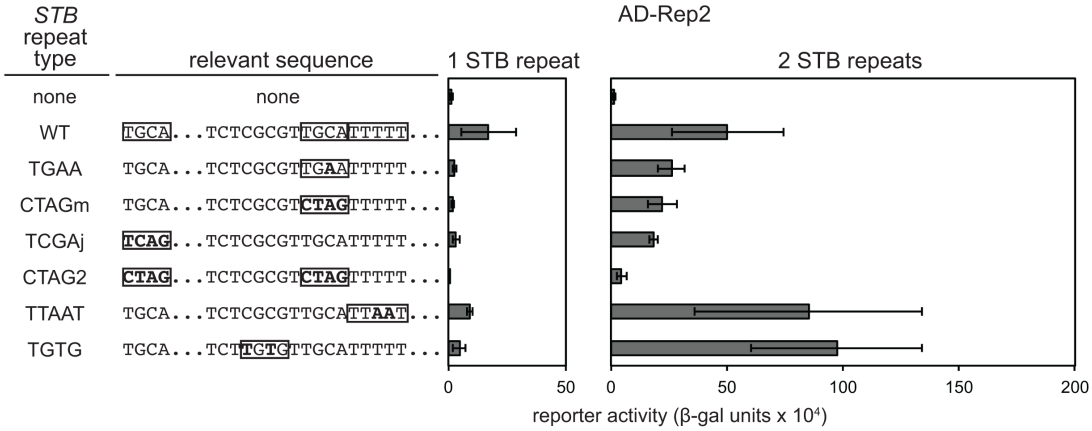
A


AD-Rep1
AD-Rep2
no STB
1 STB
2 STB
0
20
40
60
80
100
reporter activity (β-gal units x 10⁴)

B


AD-Rep1
STB repeat type
relevant sequence
1 STB repeat
2 STB repeats
none
none
WT
TGCA...TCTCGCGTTGCATTTTT...
TGAA
TGCA...TCTCGCGTTGAATTTTT...
CTAGm
TGCA...TCTCGCGTCTAGTTTTT...
TCGAj
TCAG...TCTCGCGTTGCATTTTT...
CTAG2
CTAG...TCTCGCGTCTAGTTTTT...
TTAAT
TGCA...TCTCGCGTTGCATTAAT...
TGTG
TGCA...TCTTGTGTTGCATTTTT...
0
50
0
50
100
150
200
reporter activity (β-gal units x 10⁴)

C


AD-Rep2
STB repeat type
relevant sequence
1 STB repeat
2 STB repeats
none
none
WT
TGCA...TCTCGCGTTGCATTTTT...
TGAA
TGCA...TCTCGCGTTGAATTTTT...
CTAGm
TGCA...TCTCGCGTCTAGTTTTT...
TCGAj
TCAG...TCTCGCGTTGCATTTTT...
CTAG2
CTAG...TCTCGCGTCTAGTTTTT...
TTAAT
TGCA...TCTCGCGTTGCATTAAT...
TGTG
TGCA...TCTTGTGTTGCATTTTT...
0
50
0
50
100
150
200
reporter activity (β-gal units x 10⁴)

(Pinder *et al.*, 2013). In the case of $Gal4_{AD}$-Rep2, Rep1 was expressed because Rep1 is required for robust association of Rep2 with *STB-P in vivo* (Pinder *et al.*, 2013). Expression of either Rep1 or Rep2 fused to $Gal4_{AD}$ resulted in reporter gene activity when a single copy of the WT synthetic *STB* repeat was inserted upstream of the reporter gene (Figure 18 A and B). This indicated that a single copy of the 63-bp *STB-P* sequence was sufficient for recognition by the Rep proteins, despite one copy of this sequence being insufficient to mediate partitioning when inserted in place of the native *STB-P* on the pKanΔSTB plasmid. Reporter activity increased approximately two-fold for both $Gal4_{AD}$-Rep1 and $Gal4_{AD}$-Rep2 when two copies of the repeat were inserted, consistent with the number of Rep protein association sites being doubled.

To determine whether any of the mutations in the *STB* repeat that led to loss of partitioning function also impaired Rep1 or Rep2 association, further one-hybrid assays were performed. One-hybrid associations of the Rep proteins with synthetic *STB* repeats containing mutations that did not compromise partitioning function were similar to those observed with the WT synthetic repeat (data not shown).

Reporter gene activity upon $Gal4_{AD}$-Rep1 or $Gal4_{AD}$-Rep2 expression was reduced for a single copy of all synthetic *STB* sequences with mutations in TGCA elements that had impaired partitioning function (Figure 18 B and C, left). Repeats in which the junction TGCA was scrambled, either alone (CTAGj) or in conjunction with the core TGCA (CTAG2), resulted in reporter gene activity similar to that observed when no *STB* sequences were present upstream of the reporter gene, whether $Gal4_{AD}$-Rep1 or $Gal4_{AD}$-Rep2 was expressed. This suggests that the junction TGCA sequence is required for Rep protein association with a single copy of the *STB-P* sequence, and that the core TGCA sequence can contribute to this association, but is not sufficient to mediate association of Rep proteins in the absence of the junction TGCA sequence. Upon $Gal4_{AD}$-Rep2 expression, strains carrying synthetic *STB* repeats with either TGCA sequence scrambled or the core TGCA mutated to TGAA also gave reporter gene activity similar to that observed with no *STB* sequences; these strains showed reduced reporter gene activity upon $Gal4_{AD}$-Rep1 expression, but not to the extent observed when no *STB* repeats were present upstream of the reporter gene. These results suggest that Rep2 may have more stringent sequence requirements than Rep1 for association with a single *STB* repeat, as

both the core and junction TGCA sequences are required to detect Rep2 association with a single repeat in this one-hybrid assay system, while only the junction TGCA sequence is required to detect Rep1 association in this context.

When Rep protein associations with two directly-arrayed copies of the mutant synthetic *STB* repeats were tested by one-hybrid assay (Figure 18 B and C, right), reporter gene activity observed upon expression of $Gal4_{AD}$-Rep1 was decreased when the junction TGCA sequence was scrambled, but not when the core TGCA contained a single point mutation (TGAA) or was scrambled alone (CTAGm), in contrast to the reduced activity seen for all three of these mutant *STB* repeats when present at a single copy. When $Gal4_{AD}$-Rep1 was expressed and both TGCA sequences were scrambled, reporter gene activity was decreased below that observed when the junction TGCA alone was scrambled, which suggests that both core and junction TGCA sequences can contribute individually to Rep1 interaction with two *STB* repeats.

Upon $Gal4_{AD}$-Rep2 expression in strains with two copies of the synthetic *STB* repeat, reporter gene activity was reduced when the repeats had mutations in either the core or junction TGCA sequence, but not to the level observed when no *STB* sequences were present, suggesting that, unlike a single copy of these mutant *STB* repeats, two copies allowed some Rep2 association. However, when both the core and junction TGCA motifs were scrambled simultaneously, $Gal4_{AD}$-Rep2 failed to activate reporter gene expression above the level observed when no *STB* repeats were present. These results suggest that both the core and junction TGCA motifs contribute similarly to Rep2 association with *STB*, and that Rep2 requires a minimum of two intact TGCA motifs for association with *STB* that can be detected in this assay system. These results also imply that, similar to results obtained with a single copy of the *STB* repeat, sequence requirements for Rep2 association with *STB* are stricter than those of Rep1.

Mutation of the T-tract (TTAAT) or CGCG (TGTG) sequence in strains bearing a single copy of the synthetic *STB* repeat reduced reporter activity when either $Gal4_{AD}$-Rep1 or $Gal4_{AD}$-Rep2 was expressed (Figure 18 B and C, left); however, in strains with two copies of the synthetic *STB* repeat, neither the TTAAT or TGTG mutations reduced reporter gene activity upon expression of either $Gal4_{AD}$-Rep1 or $Gal4_{AD}$-Rep2 (Figure 18 B and C, right). As synthetic *STB* repeats are functional when arrayed as two direct

repeats, the results of these one-hybrid assays suggest that the partitioning defects associated with the TTAAT and TGTG mutations, unlike those associated with mutations of the TGCA sequence, are not due to loss of Rep protein association with these mutant repeats, and may be related to other requirements of the partitioning complex. Alternatively, the T-tract and CGCG sequence may contribute to nuances of Rep protein associations with *STB-P*, or higher-order interactions that would not be measured in this assay. In this context, it is worth noting that activation of the reporter gene by $Gal4_{AD}$-Rep1 and $Gal4_{AD}$-Rep2 showed a slight but consistent increase with two copies of the T-tract or TGTG mutant repeats compared to WT. This could indicate that Rep1 associates with *STB* aberrantly, more stably or more abundantly when the T-tract or CGCG sequences are mutated.

Rep1 association with the mutant *STB* repeats in the absence of Rep2 was also assessed by one-hybrid assay, and although the activity of the reporter gene was greatly reduced, as expected from the lower steady-state levels of the $Gal4_{AD}$-Rep1 fusion protein in the absence of stabilization by Rep2 (data not shown), the effect of the mutations in the synthetic *STB* repeats was similar to that observed in the presence of Rep2 (data not shown).

3.2.6 CGCG Sequence And Rsc2 Protein Provide Independent Contributions To Plasmid Partitioning Function

To determine whether the partitioning defect observed when the CGCG element in the synthetic *STB* repeat was mutated to TGTG was due to loss of RSC2 complex association with *STB*, the effect of *RSC2* gene deletion on plasmid partitioning mediated by synthetic *STB* repeats was examined. The Rsc2 protein is the only subunit of the RSC2 complex that is not shared by the RSC1 isoform, and deletion of the *RSC2* gene is presumed to inactivate the RSC2 complex while leaving function of the RSC1 complex intact (Cairns *et al.*, 1999). If the CGCG element in the *STB-P* repeats is required for association of the RSC2 complex with *STB-P*, then 2μm-based plasmids carrying either two WT (pKan-2-WT) or two TGTG mutant (pKan-2-TGTG) *STB* repeats in place of native *STB-P* should be inherited similarly upon deletion of the *RSC2* gene. Alternatively, if the CGCG element is required for partitioning functions other than mediating the

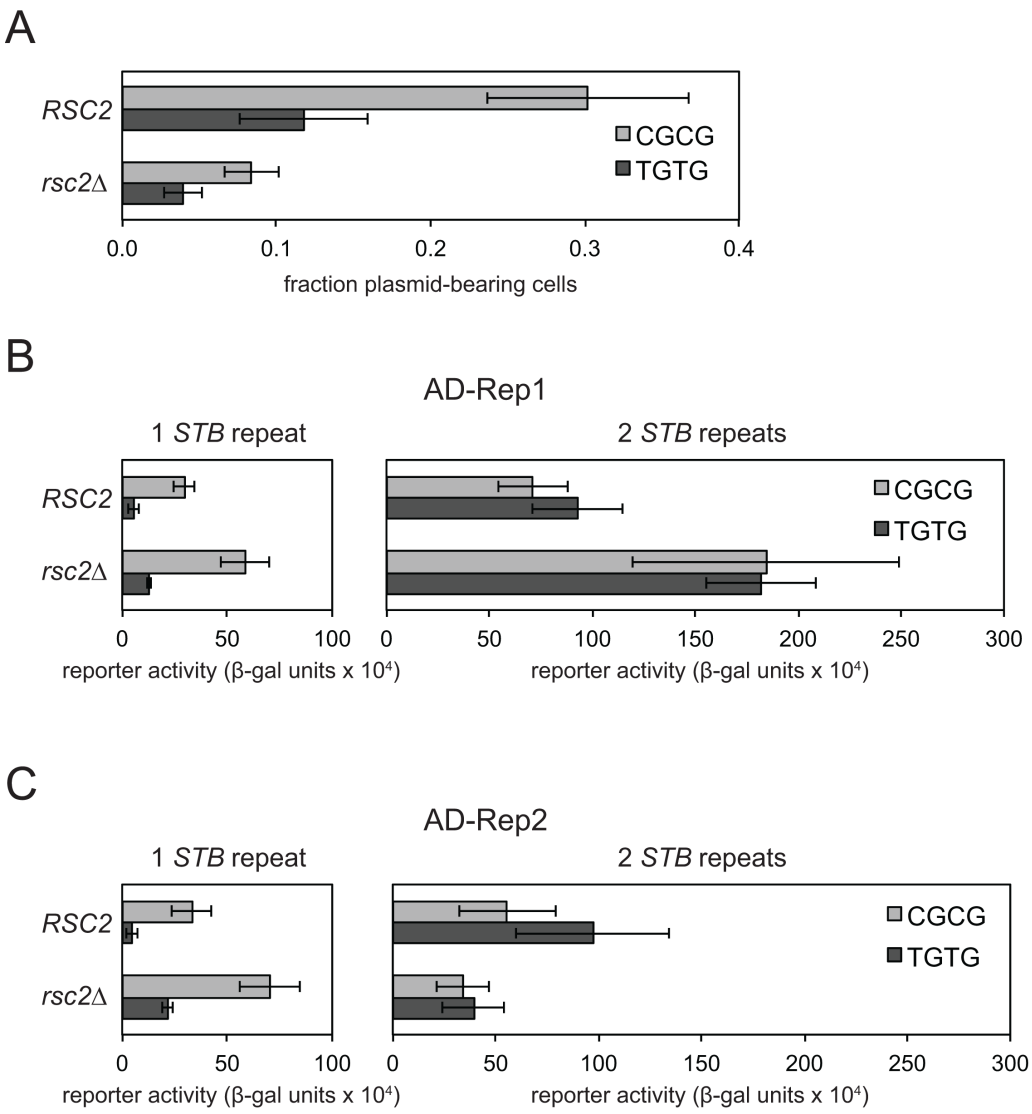


Figure 19. Absence of the Rsc2 protein impairs plasmid partitioning and alters Rep protein association with synthetic *STB* repeats independently of CGCG sequence. (A) Isogenic wild-type *RSC2* and *rsc2*Δ *cir*^{0} strains were transformed with a tagged 2µm-based plasmid in which the native *STB-P* was replaced by a 21-bp linker sequence, into which two directly-arrayed copies of a WT (pKan-2-WT) or TGTG mutant (pKan-2-TGTG) synthetic *STB* repeat were inserted. Strains were assayed for partitioning function as described in the legend of Figure 15. Results represent an average of a minimum of 4 isolates of each transformant (± standard deviation). (B) and (C) One-hybrid assays for the association of Gal4$_{AD}$-Rep1 (B) and Gal4$_{AD}$-Rep2 (C) with synthetic *STB* repeats were performed as described in the legend of Figure 18, in isogenic wild-type *RSC2* and *rsc2*Δ strains, for a minimum of 4 isolates of each transformant (± standard deviation). "AD" denotes Gal4$_{AD}$. Full sequences of WT and mutant synthetic *STB* repeats are given in Table 6.

association of the RSC2 complex with *STB-P*, a plasmid carrying WT repeats should be better inherited than a plasmid carrying TGTG repeats upon deletion of the *RSC2* gene.

As expected, deletion of the *RSC2* gene greatly reduced the inheritance of a 2µm-based plasmid carrying two WT synthetic *STB* repeats (pKan-2-WT, Figure 19 A). Deletion of *RSC2* was also found to reduce the inheritance of a 2µm-based plasmid carrying two TGTG mutant repeats (pKan-2-TGTG), which suggests that the RSC2 complex contributes to efficient plasmid partitioning in a manner that does not require the CGCG sequence. As the RSC2 complex is known to be involved in ensuring accurate chromosome segregation (Tsuchiya *et al.*, 1998; Hsu *et al.*, 2003; Baetz *et al.*, 2004), and the 2µm plasmid has been shown to missegregate with the chromosomes (Velmurugan *et al.*, 2000; Mehta *et al.*, 2002; Scott-Drew *et al.*, 2002; Liu *et al.*, 2013), it is possible that the non-CGCG-mediated effect of *RSC2* deletion on plasmid inheritance is an indirect result of chromosome missegregation.

The TGTG mutation (pKan-2-TGTG) led to a decrease in plasmid inheritance in the presence of a wild-type *RSC2* gene, as previously shown (Figure 16). In the absence of *RSC2*, inheritance of the pKan-2-TGTG plasmid remained less efficient than that of the pKan-2-WT plasmid. This implies that the TGTG mutation leads to a defect in plasmid inheritance that is not mediated by the RSC2 complex, and further suggests that another factor instead of, or in addition to, the RSC2 complex may recognize the CGCG sequence. One potential candidate for this role is the RSC1 complex, as the Rsc3 and Rsc30 DNA-binding subunits are shared by RSC1 and RSC2 (Cairns *et al.*, 1999), and the RSC1 complex-specific subunit, the Rsc1 protein, has been found in association with Rep1 *in vivo* (Ma *et al.*, 2012).

Further study will be necessary to more conclusively identify the non-CGCG-mediated role of the RSC2 complex and the non-RSC2-mediated role of the CGCG element in 2µm plasmid partitioning.

3.2.7 Association Of Rep Proteins With Synthetic *STB* Repeats Is Altered In The Absence Of Rsc2

As 2µm plasmid partitioning is impaired in the absence of the Rsc2 protein (Figure 19 A; (Wong *et al.*, 2002)), the effect of *RSC2* gene deletion on Rep protein association with the synthetic *STB* repeat was investigated using one-hybrid assays.

Reporters consisting of one or two copies of WT or TGTG mutant synthetic *STB* repeats upstream of a *lacZ* reporter gene were chromosomally inserted in isogenic *RSC2* wild-type and *rsc2*Δ strains, as indicated, and Rep proteins with and without amino-terminal $Gal4_{AD}$ fusions were expressed as previously described (Section 3.2.5).

In strains expressing $Gal4_{AD}$-Rep1, with one or two copies of the WT or TGTG mutant repeats upstream of *lacZ*, reporter gene activity was greater for those strains in which the *RSC2* gene was deleted (Figure 19 B). In strains expressing $Gal4_{AD}$-Rep2, with one copy of the WT or TGTG mutant repeats upstream of *lacZ* only, reporter gene activity was again greater for strains in which the *RSC2* gene was deleted (Figure 19 C, left). However, in strains expressing $Gal4_{AD}$-Rep2 with two copies of the WT or TGTG mutant repeats upstream of *lacZ*, reporter gene activity was less for strains in which the *RSC2* gene was deleted (Figure 19 C, right).

These findings support a model in which the presence of Rsc2 negatively impacts association of Rep1, but promotes association of Rep2 with a partitioning-competent *STB-P* sequence (i.e., two or more directly-arrayed synthetic *STB* repeats). The reason that Rsc2 negatively affects Rep2 association with the synthetic *STB* repeat at a single copy, but contributes positively at two copies, could be related to differences in the manner in which Rep2 associates with these sequences. For example, association of Rep2 with a single copy of the *STB* repeat may be entirely bridged through Rep1, but association of Rep2 with two copies of the repeat, which results in a functional partitioning complex, may depend on other factors in addition to Rep1, such as the presence of the RSC2 complex. The negative effect of Rsc2 on the association of Rep1 with the *STB* repeats could reflect a role for the RSC2 complex in preventing non-productive associations of Rep1 with *STB-P*, or in favouring the association of Rep2 with *STB-P* at the detriment of the association of Rep1 with *STB-P*.

Western blotting showed that deletion of the *RSC2* gene did not alter levels of either Rep1 or Rep2 proteins, or their $Gal4_{AD}$-fused versions, suggesting that any changes observed in one-hybrid associations are an effect of Rsc2 on the *STB-P* partitioning complex and not on the expression or stability of the Rep proteins (data not shown). Further investigations will be required to determine the role of the RSC2 complex in the association between Rep proteins and *STB-P*.

Although both the WT and TGTG mutant repeats showed similar trends in the one-hybrid associations they mediated in the presence and absence of the Rsc2 protein, there was a slight improvement in Rep1 and Rep2 association with two copies of the TGTG mutant over two copies of the WT synthetic *STB* observed in the presence of wild-type *RSC2*, but abrogated in the absence of *RSC2*. Along with the results of the plasmid inheritance assays performed both in the presence and absence of Rsc2 (Figure 19 A), these results suggest that there may be some interaction between the RSC2 complex and the CGCG motif, but that a role of the RSC2 complex in 2µm plasmid partitioning exists that is not mediated through the CGCG motif in the *STB-P* repeats.

3.2.8 Plasmid Inheritance Is Impaired By D22N Substitution In Rep2 In The Absence Of Rsc2

As the above results suggest that Rsc2 may be required to facilitate association of Rep2 with *STB-P*, the inheritance of pKan plasmids expressing mutant Rep2 proteins $Rep2_{D22N}$ and $Rep2_{AA}$ were assayed in an *rsc2*Δ strain. $Rep2_{D22N}$, in which asparagine is substituted for aspartic acid 22, is unable to associate with Rep1, while $Rep2_{AA}$, in which alanine residues are substituted for leucine residues at positions 185 and 186, is unable to associate with Rep2 (Figure 6 A). Both $Rep2_{D22N}$ and Rep_{AA} are able to mediate plasmid inheritance with an efficiency similar to wild-type Rep2 in the presence of the Raf protein, but are severely compromised in their ability to mediate plasmid partitioning in the absence of Raf (Figure 11 A). We therefore aimed to determine whether the absence of Rsc2 would also reveal limitations in the partitioning function of these mutant Rep2 proteins.

In a strain in which the *RSC2* gene was deleted, inheritance of a pKan plasmid expressing $Rep2_{D22N}$ was reduced compared to a pKan plasmid expressing wild-type Rep2, while inheritance of a pKan plasmid expressing $Rep2_{AA}$ was unchanged compared to a pKan plasmid expressing wild-type Rep2 (Figure 20 A). This suggests that the RSC2 complex can compensate for the absence of the association of Rep1 with Rep2. As western blotting analyses have shown that neither Rep1 nor Rep2 protein levels are reduced by either the D22N or AA mutations in Rep2 to an extent that would compromise partitioning function (Figure 8), Rep1-Rep2 association and the RSC2 complex must be performing a function in partitioning that does not involve stabilizing

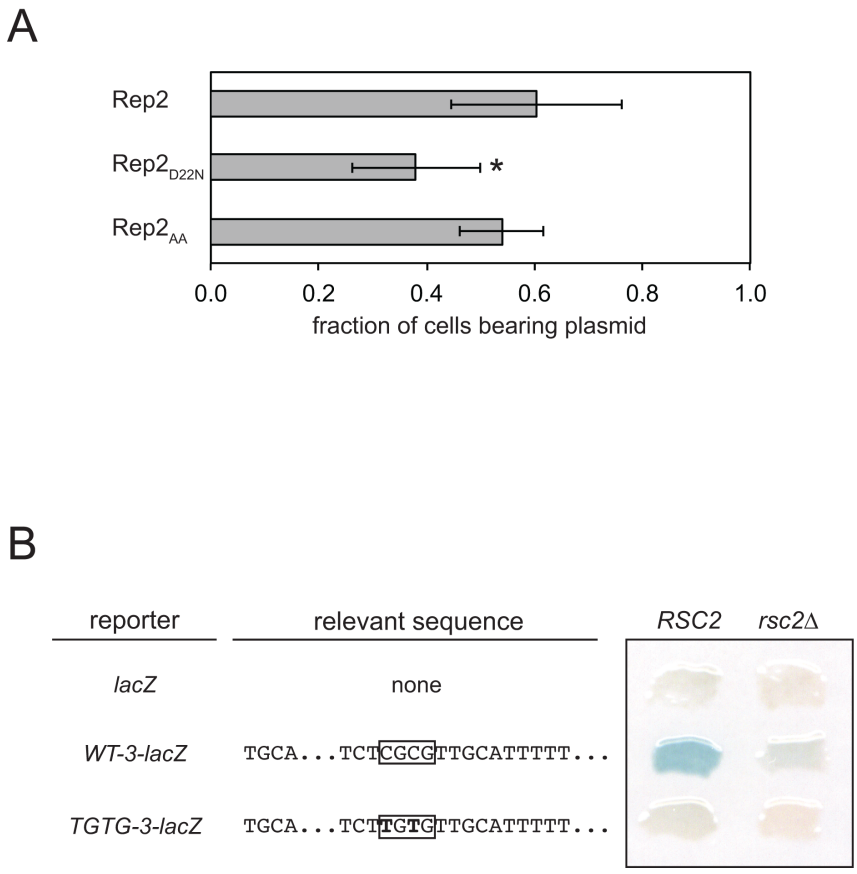


Figure 20. Deletion of *RSC2* impairs partitioning function of $Rep2_{D22N}$; both deletion of *RSC2* and TGTG mutation lead to a decrease in transcription driven by synthetic *STB* repeats. (A) An *rsc2*Δ *cir*^{0} strain was transformed with a pKan plasmid expressing either wild-type Rep2, $Rep2_{D22N}$ or $Rep2_{AA}$, and assayed for partitioning function as described in the legend of Figure 15. Results represent an average of a minimum of 6 isolates of each transformant (± standard deviation). *<0.01. (B) Three directly-arrayed copies of WT or TGTG mutant synthetic *STB* repeats were integrated in the chromosome upstream of a *lacZ* reporter gene in wild-type and *rsc2*Δ strains. Activity of the *lacZ* gene product, ß-gal, was monitored by growing strains in patches on a nitrocellulose filter overnight (16 to 24 hours), and assaying using the substrate X-gal, which is cleaved by ß-gal to produce a blue precipitate. Full sequences of WT and mutant synthetic *STB* repeats are given in Table 6.

Rep protein levels. This is further evidence that the association of Rep1 and Rep2 performs a function in partitioning beyond stabilizing Rep protein levels.

The apparently modest effect of loss of functional RSC2 complex on inheritance of the pKan plasmid with a wild-type copy of Rep2 (~0.7 being the fraction of cells bearing plasmid in both cases; Figure 8 A and Figure 20 A) differs from the more severe defect reported previously (Wong *et al.*, 2002). We and others have found that the partitioning defect associated with deletion or mutation of genes encoding RSC2 complex components is not as severe as originally reported (Ma *et al.*, 2012; Pinder *et al.*, 2013). In the original assays, inheritance of a marker gene-tagged *STB*-containing plasmid was measured in the presence of the native 2μm plasmid, which has been shown to lead to a decrease in copy number for both the native and tagged plasmid, and therefore an increased rate of loss for the tagged plasmid (Jayaram *et al.*, 1983; Kikuchi, 1983). This suggests that the deletion of *RSC2* does impair plasmid partitioning function, but perhaps to an extent that is not detectable in our assay system.

3.2.9 Mutation Of The CGCG Motif And Deletion Of *RSC2* Impair *STB-P*-Driven Transcription

In addition to being a partitioning locus, *STB-P* promotes transcription of a 1950-nt transcript in the direction of *STB-D*, anti-sense through the *RAF* gene and co-terminal with *REP1* (Jayaram *et al.*, 1985). As one of the primary roles of the RSC2 complex is mediating transcriptional activation (Cairns *et al.*, 1999; Angus-Hill *et al.*, 2001), the effects of mutation of the putative RSC2 complex recognition site in the synthetic *STB* repeat (CGCG) and of deletion of the *RSC2* gene on transcription driven by the synthetic *STB* repeats were examined.

To assess the role of the CGCG element in transcription directed by *STB-P*, three directly-arrayed copies of either the WT or TGTG mutant *STB* repeat were inserted upstream of a chromosomally-integrated *lacZ* reporter gene with a basal promoter, so that the *lacZ* gene was in the position of the DNA transcribed to generate the 1950-nt transcript. Expression of the *lacZ* reporter gene was monitored by a filter assay for activity of the *lacZ* gene product, ß-gal (Figure 20 B). Presence of the WT *STB* repeats upstream of *lacZ* resulted in activation of *lacZ* expression, while very little expression

was seen when the TGTG mutant *STB* repeats were in this position, suggesting that the CGCG element in the *STB* repeat is required for the promoter function of *STB-P*.

To examine the effect of *RSC2* deletion on transcription driven by the *STB* repeats, these reporters were inserted into the chromosome of an *rsc2*Δ strain. Expression of both the WT and TGTG *STB* reporters was reduced in the *rsc2*Δ strain compared to the wild-type *RSC2* strain. This suggests that the RSC2 complex does contribute to the ability of *STB-P* to drive transcription from a downstream promoter, but that the CGCG sequence in the *STB* repeat contributes to this transcription in a manner that is independent of the RSC2 complex.

3.3 2µm Plasmid Promoters

In addition to their role in plasmid partitioning, the Rep1, Rep2 and Raf proteins serve to regulate transcription driven by 2µm plasmid promoters. Co-expression of Rep1 and Rep2 is required to repress transcription driven by the *FLP/REP2* promoter (*FLP/REP2p)*, the *REP1/RAF* promoter (*REP1/RAFp*) and *STB-P* (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988; Veit and Fangman, 1988). Expression of Raf relieves this repression (Murray *et al.*, 1987). This regulation is presumably achieved by association of at least the Rep1 and Rep2 proteins with the DNA of the promoters, although Rep1 and Rep2 have only been shown to associate with the DNA of the *STB-P* locus (Velmurugan *et al.*, 1998). Analysis of *STB-P* sequences (Section 3.2) showed that the TGCA component of the TGCATTTTT sequence was required for Rep protein association with *STB-P* and *STB-P*-mediated plasmid partitioning function; however, whether association of Rep proteins with plasmid gene promoters and Rep protein-mediated repression of transcription had the same sequence requirements was unknown.

3.3.1 Rep1, Rep2 And Raf Associate With 2µm Plasmid Gene Promoters *In Vivo* To Regulate Transcription

To determine whether Rep1, Rep2 and Raf are able to associate with the two divergent plasmid gene promoter regions, *FLP/REP2p* and *REP1/RAFp*, ChIP assays were performed (Figure 21). The association of Rep1, Rep2 and Raf with *STB-P* was also examined, as Raf had not previously been shown to associate with this locus. As antibodies directed against native Raf are not available, an amino-terminally FLAG-

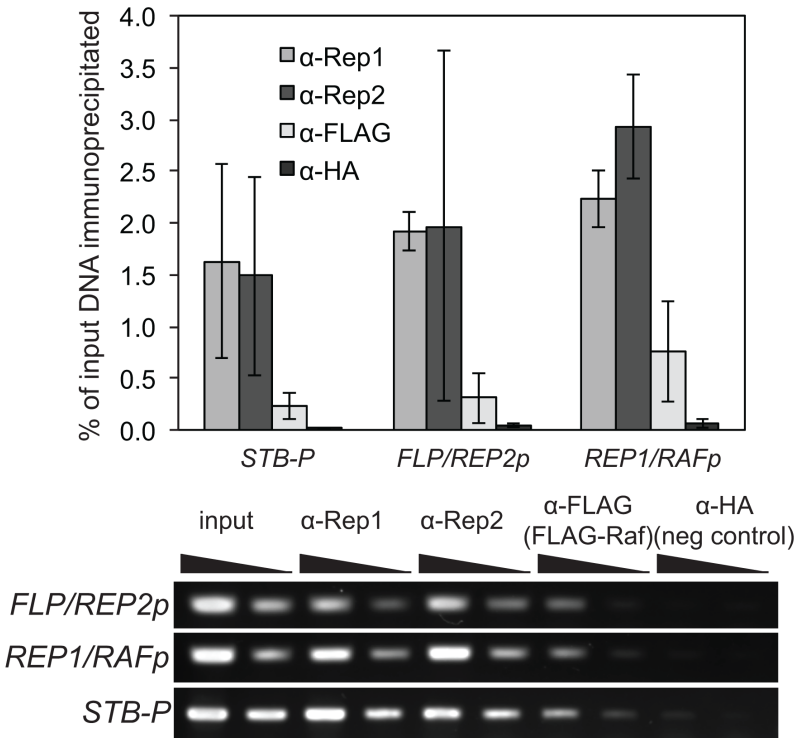


Figure 21. 2µm proteins associate with *STB-P* and 2µm gene promoters. A *cir*$^{+}$ yeast strain expressing FLAG-Raf from a *CEN* plasmid was assayed for association of Rep1, Rep2 and FLAG-Raf with *STB-P*, the *FLP/REP2* promoter and the *REP1/RAF* promoter by ChIP. Amount of immunoprecipitated DNA relative to input DNA was assessed by semi-quantitative PCR. PCR products were visualized by electrophoresis on 1.5% agarose gels and quantified by densitometry (Section 2.8). The average of 3 replicates (± standard deviation) is represented in the bar graph (top). The PCR products from a representative replicate, visualized by agarose gel electrophoresis are shown (bottom).

tagged version of Raf was expressed from a heterologous promoter. Chromatin was precipitated using antibodies specific for native Rep1 and Rep2, and for the FLAG epitope. Upon purification, immunoprecipitated DNA was found to be specifically enriched for *FLP/REP2p*, *REP1/RAFp* and *STB-P* when compared to input DNA. A sequence not expected to be associated with Rep1, Rep2 or Raf (the chromosomal *TRP1* locus) was not enriched (data not shown), and none of these loci were enriched in DNA precipitated with an antibody specific for an epitope not present in the cell (anti-HA).

These results demonstrate that Rep1, Rep2 and Raf associate with the DNA of the 2µm plasmid gene promoters *in vivo*, and that Raf, like Rep1 and Rep2, associates with the *STB-P* locus. To determine whether recognition of the plasmid gene promoters requires the presence of any of the 2µm plasmid proteins, as is the case for Rep2 and Raf association with *STB-P* (Figure 14; (Pinder *et al.*, 2013)), or the sequence context of the native 2µm plasmid, one-hybrid assays were performed. Isogenic cir^{+} and cir^{0} strains were generated that had chromosomally-integrated reporters consisting of 2µm plasmid gene promoter sequences upstream of the *HIS3* gene, which encodes an enzyme in the histidine biosynthetic pathway. Either Rep1, Rep2 or Raf was expressed in these strains as a $Gal4_{AD}$ fusion, so that association of the protein of interest with the promoter-*HIS3* reporter would lead to activation of *HIS3* transcription. Activity of the *HIS3* gene product was visualized by growth on solid medium lacking histidine and containing a competitive inhibitor of the *HIS3* gene product, 3-AT. The *HIS3* expression driven by the 2µm promoters in the presence of unfused $Gal4_{AD}$ was titrated using 3-AT to give no growth on medium lacking histidine, so that growth in this case was reflective of protein association only and not promoter activity. Although promoter sequences were positioned in the reporters so that the *HIS3* gene was in the position of either the *FLP* (for the *FLPp-HIS3* reporter) or the *REP1* (for the *REP1p-HIS3* reporter) gene, the *FLPp* and *REP1p* sequences upstream of *HIS3* encompassed nearly the entire divergent *FLP/REP2p* and *REP1/RAFp* regions, respectively; one-hybrid results are therefore reflective of associations that may be impacting transcriptional activity in either direction.

In cir^{+} cells bearing *FLPp-HIS3* or *REP1p-HIS3* reporters, expression of $Gal4_{AD}$-Rep1, -Rep2 or -Raf led to reporter gene activation, as evidenced by colony growth on medium lacking histidine and containing 2.5 mM 3-AT (Figure 22). This indicates that

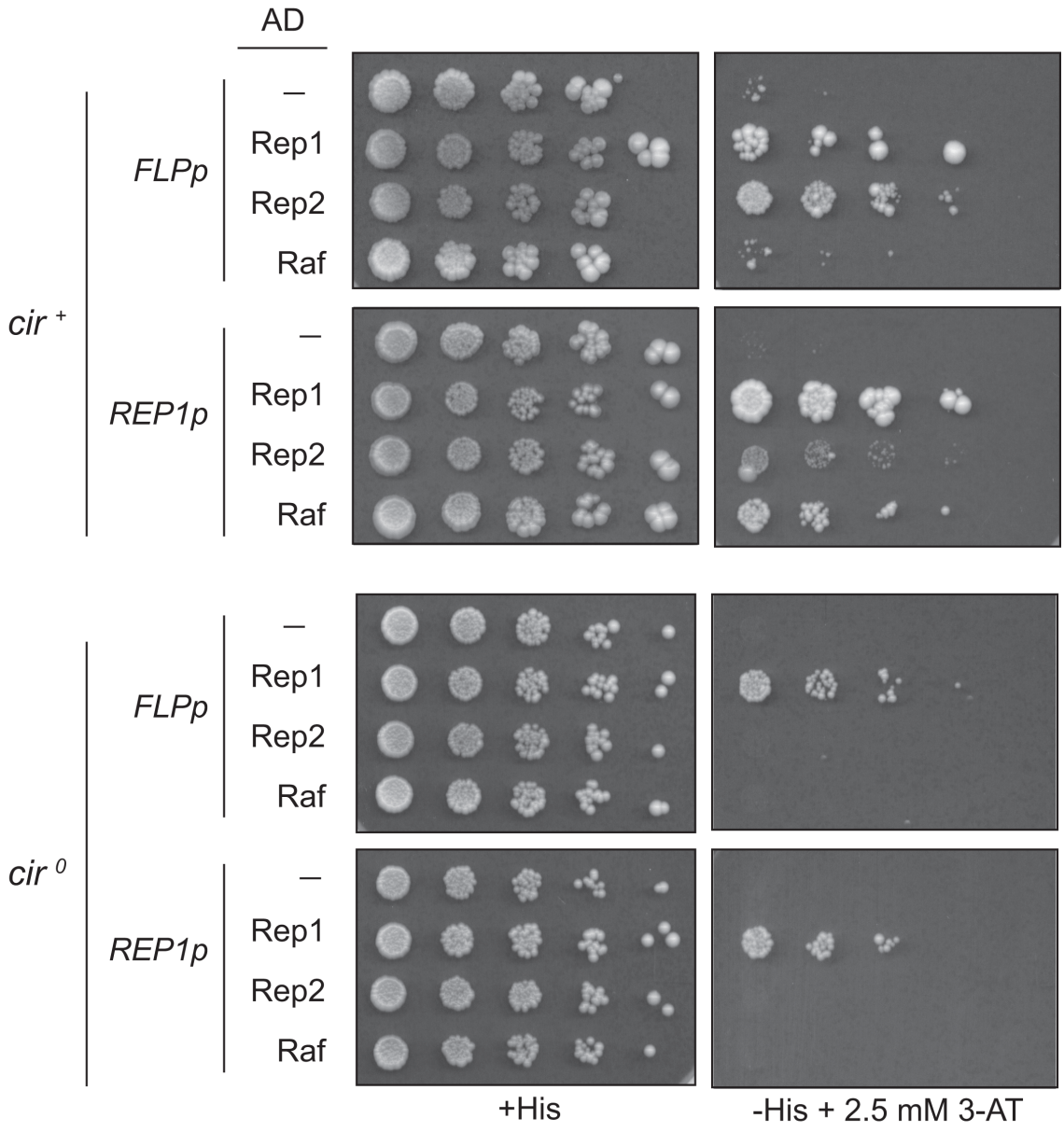


Figure 22. Rep1 associates with 2µm plasmid gene promoters in the absence of native 2µm plasmid, while Rep2 and Raf do not. A series of *cir*$^{+}$ and *cir*^{0} yeast strains containing a *lacZ* reporter gene fused in-frame downstream of the either the *FLP* or *REP1* promoter integrated into the genome were transformed with a plasmid expressing either $Gal4_{AD}$ alone, or $Gal4_{AD}$-Rep1, -Rep2 or -Raf. "AD" denotes $Gal4_{AD}$. Transformants were serially diluted and spotted onto matched plates either containing histidine (left) or lacking histidine and containing 2.5 mM 3-AT (right).

association of Rep1, Rep2 and Raf with the 2µm promoters does not require the 2µm promoters to be in their native sequence context on the 2µm plasmid. In *cir*^{0} cells, only expression of Gal4$_{AD}$-Rep1 led to reporter gene activation. The failure of Rep2 and Raf to activate the *FLPp-HIS3* and *REP1p-HIS3* reporters in the absence of native 2µm plasmid suggests that a protein product of the 2µm plasmid – either Flp, Rep1, Rep2 or Raf – may be required by Rep2 and Raf for association with 2µm plasmid promoters. As association of Rep2 and Raf with *STB-P* has been shown to be dependent on the presence of Rep1, Rep1 may also be required for Rep2 and Raf association with 2µm plasmid promoters.

Although the above results demonstrate that Rep1, Rep2 and Raf associate with 2µm plasmid promoters, they provide no information about whether these associations are required for promoter regulation. To address this, the ability of a Rep1 protein impaired for association with *FLPp* to mediate repression of transcription driven by *FLPp* was assessed. A Rep1 mutant that is unable to associate with *STB-P*, Rep1$_{S330Y}$ has been previously described (Yang *et al.*, 2004). A similar mutant, Rep1$_{S330A}$, was identified in a screen for Rep1 mutants unable to mediate plasmid inheritance (A. Sengupta, unpublished data). In one-hybrid assays using *HIS3* reporters belonging to the series described above, Rep1$_{S330A}$ was found to be unable to activate expression of *STB-P-HIS3* (data not shown) or *FLPp-HIS3* (Figure 23 A), indicating that Rep1$_{S330A}$ is unable to associate with either of these 2µm plasmid sequences, despite steady-state levels of the wild-type Rep1 and Rep1$_{S330A}$ activation domain fusion proteins being similar in these strains (Figure 23 B; data not shown).

To determine whether Rep protein association with *FLPp* is required for repression of *FLPp*-driven transcription, the Rep1$_{S330A}$ mutant was assayed for its ability to mediate repression of a *FLPp-lacZ* reporter (as described in Figure 9), which is identical to the *FLPp-HIS3* promoter except that it carries the *lacZ* gene in place of *HIS3*. The *FLPp-lacZ* reporter was integrated into a *cir*^{0} strain in which Rep proteins were expressed from galactose-inducible promoters. Co-expression of wild-type Rep1 and Rep2 reduced expression of the *FLPp-lacZ* reporter, while expression of neither protein alone impaired *FLPp-lacZ* reporter expression (Figure 23 C), as previously seen (Reynolds *et al.*, 1987; Murray *et al.*, 1987; Veit and Fangman, 1988; Som *et al.*, 1988). When Rep1$_{S330A}$ was co-expressed with wild-type Rep2, no reduction in *FLPp-lacZ*

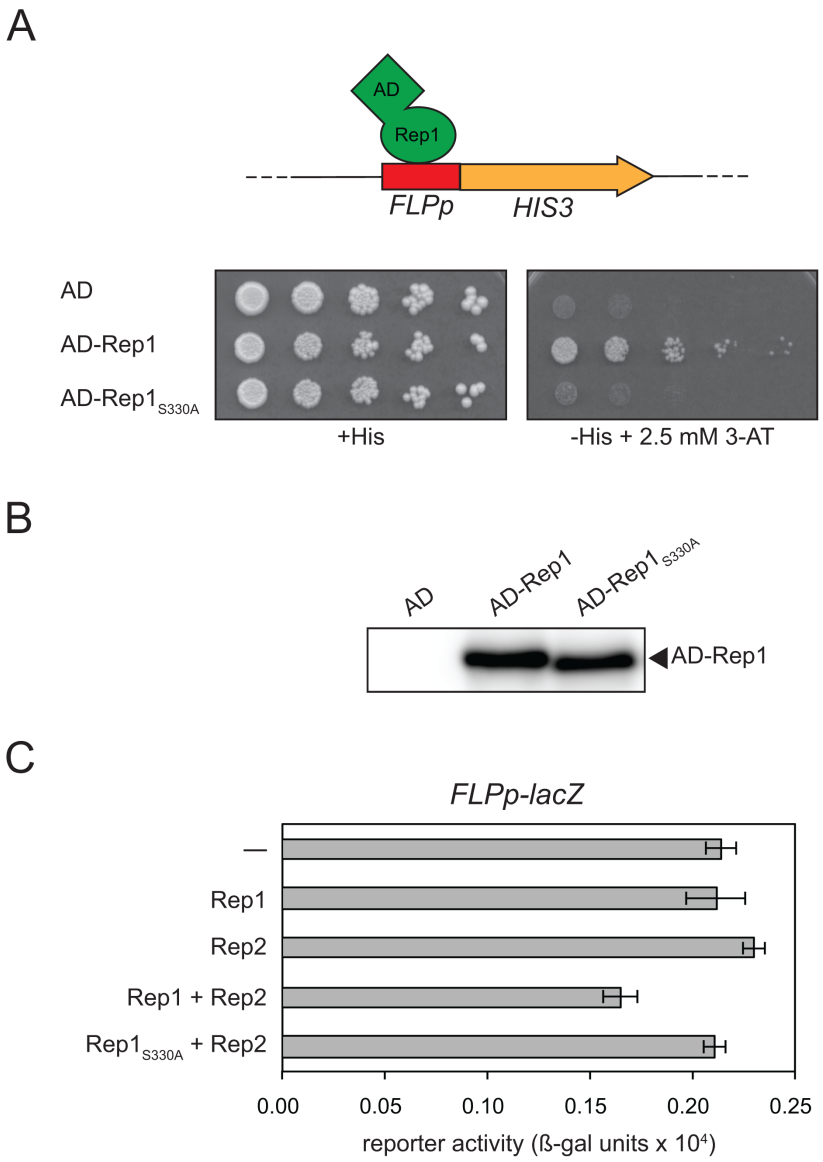


Figure 23. Rep1$_{S330A}$ mutant is unable to associate with *FLP* promoter in a one-hybrid assay or repress transcription driven by this promoter. (A) A *cir*^{0} yeast strain containing a chromosomally-integrated *FLPp-HIS3* reporter was transformed with a plasmid expressing either B42$_{AD}$-HA, B42$_{AD}$-HA-Rep1 or B42$_{AD}$-HA-Rep1$_{S330A}$. Transformants were serially diluted and spotted onto matched plates either containing histidine (left) or lacking histidine and containing 2.5 mM 3-AT (right). (B) Equal amounts of total protein extracts from the transformants shown in (A) were analyzed by western blotting with an anti-Rep1 antibody. (C) A *cir*^{0} strain containing a chromosomally-integrated *FLPp-lacZ* reporter was transformed with a pGAL-TRP plasmid expressing combinations of wild-type Rep1, mutant Rep1$_{S330A}$ and wild-type Rep2, as indicated. Expression of the Flp-ß-gal fusion protein was monitored by assaying for ß-gal activity after 24 hours of growth in selective medium containing galactose, using the substrate ONPG. Results represent an average of a minimum of 4 isolates of each transformant (± standard deviation).

expression was observed. This suggests that $Rep1_{S330A}$ is unable to mediate repression of *FLPp*-driven transcription, and that Rep1 association with promoter DNA is required for this repression.

Presently, only one amino acid substitution in Rep2 has been found to impair its association with *STB-P in vivo* (substitution of aspartic acid 22 for asparagine; Figure 7 A). However, the $Rep2_{D22N}$ mutant, which carries this substitution, is also impaired in its ability to associate with Rep1 (Figure 6 A). Co-expression of $Rep2_{D22N}$ with wild-type Rep1 does not reduce *FLPp-lacZ* reporter activity, with reporter activity being similar to that observed in the absence of Rep2 (Figure 9 A), suggesting that transcriptional repression is lost when association of Rep2 with DNA is impaired; however, Rep1 protein levels are reduced when Rep1 is co-expressed with $Rep2_{D22N}$ (Figure 9 B), and this decrease in the level of Rep1 may be the cause of the observed loss of repression.

3.3.2 CGCG Sequence Required For Transcription, TGCA Sequence Required For Rep-Mediated Repression Of Transcription From *FLP* And *REP2* Promoters

Mutational analysis of the *STB-P* sequence (Section 3.2) revealed three elements in the *STB-P* repeat that contribute to *STB-P* plasmid partitioning function: two TGCA sequences, a TGCA-adjacent T-tract and a CGCG sequence. This CGCG sequence was also found to be required for *STB-P*-driven transcription (Figure 20 B), while the TGCA sequences contributed to the association of Rep1 and Rep2 with *STB-P* (Figure 18). CGCG and TGCA sequences are also found in the 2µm plasmid gene promoters. The *FLP/REP2p* contains two CGCG sequences and two TGCA sequences with adjacent T-tracts, while the *REP1/RAFp* contains two CGCG sequences and a single TGCA sequence with an adjacent T-tract (Figure 24 A).

To determine whether the CGCG sequences and TGCA sequences with adjacent T-tracts were required for transcription or Rep protein-mediated repression of the *FLP* or *REP2* genes, a series of *CEN* plasmid carrying wild-type or mutant versions of either a *FLPp-lacZ* or a *REP2p-lacZ* reporter was used. In the *FLPp-lacZ* reporter, the *FLP/REP2p* sequence was positioned either as above (Figure 23 C), to generate a translational fusion of *lacZ* with the first four codons of *FLP* (Flp-ß-gal), while in the *REP2p-lacZ* reporter, the *FLP/REP2p* sequence was positioned in the opposite

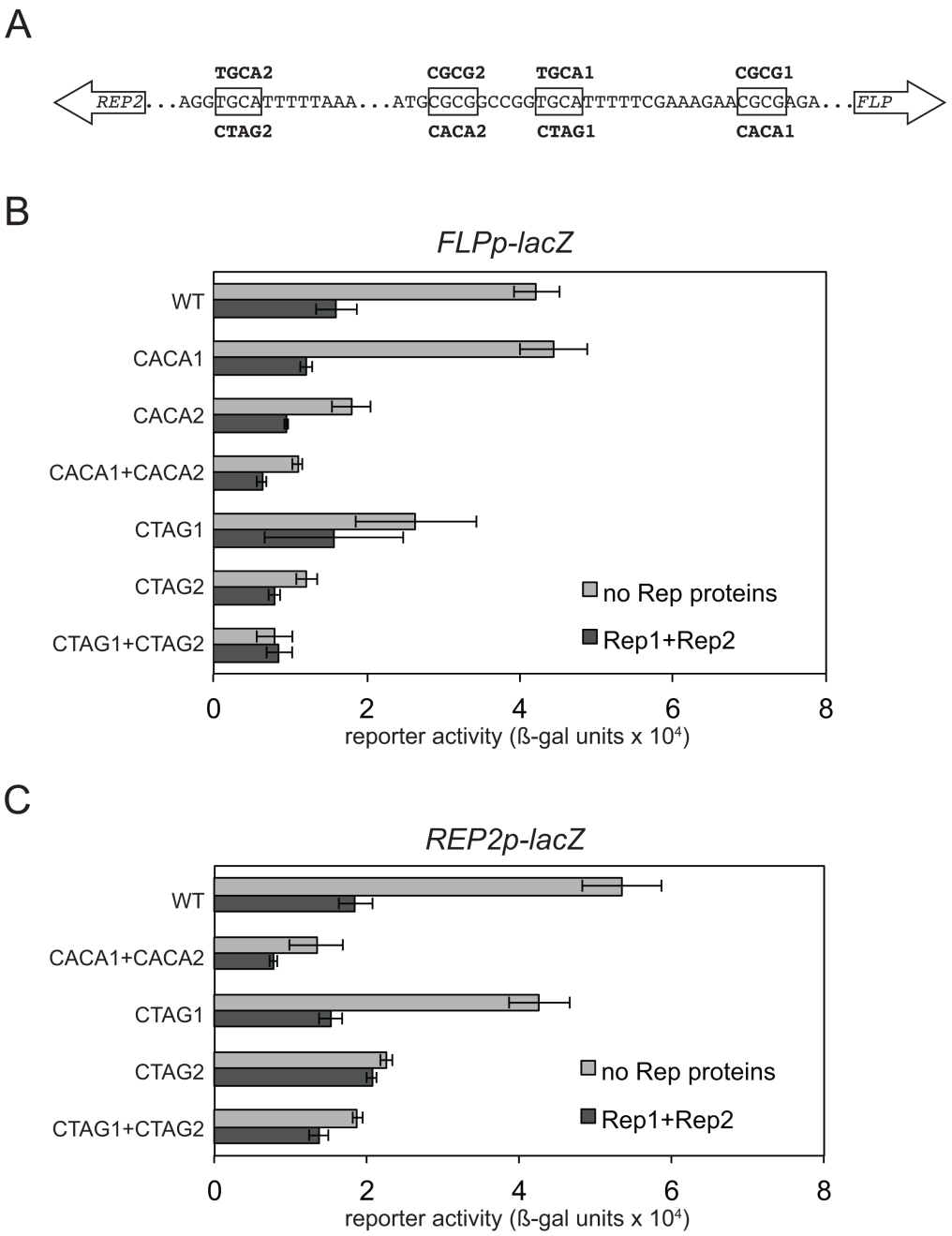


Figure 24. Effect of mutating CGCG and TGCA sequences in the *FLP/REP2* promoter. (A) Relevant sequences in the divergent *FLP/REP2* promoter region. Sequence elements of interest are boxed. Names of wild-type elements are indicated above boxes, while names of mutant versions are indicated below. (B) and (C) A *cir*^{0} yeast strain was co-transformed with a *CEN* plasmid carrying a wild-type or mutant *FLPp-lacZ* (B) or *REP2p-lacZ* (C) reporter, and either empty pGAL-TRP vector (light grey bars) or pGAL-TRP-R1R2 expressing Rep1 and Rep2 (dark grey bars). Expression of the Flp-ß-gal fusion protein was monitored by assaying for its activity after 24 hours of growth in selective medium containing galactose, using the substrate ONPG. Results represent a minimum of 4 isolates of each transformant (± standard deviation).

orientation, to generate a translation fusion with the first two codons of *REP2* (Rep2-ß-gal). In mutant versions of the *FLPp-* and *REP2p-lacZ* reporters, one or both CGCG sequences were changed to CACA, or one or both of the TGCA sequences found in TGCATTTTT elements were changed to CTAG. The CGCG and TGCA sequences were assigned numbers based on their proximity to the *FLP* gene (i.e., CGCG1 is closest to the *FLP* gene, CGCG2 is closest to the *REP2* gene; Figure 24 A). Assays were performed in cir^0 strains, and Rep1 and Rep2 were expressed under the control of heterologous promoters from a second *CEN* plasmid.

Expression from both the *FLPp-lacZ* and *REP2p-lacZ* reporters was observed (Figure 24 B and C). For *FLPp-lacZ*, expression of the plasmid-borne version of the reporter (Figure 24 B) was greater than that observed from the chromosomally-integrated version (Figure 9 A); this may be attributable to the fact that *CEN* plasmids are often present at copy numbers of greater than one, while this is very rarely the case for chromosomes (Futcher and Carbon, 1986). Expression of both *FLPp-lacZ* and *REP2p-lacZ* was decreased upon expression of Rep1 and Rep2, demonstrating that Rep1 and Rep2 are able to mediate transcriptional repression in this assay system.

In the absence of Rep proteins, mutation of the CGCG sequence closest to the *FLP* gene (CACA1) had no effect on expression of *FLPp-lacZ*, while mutation of the CGCG sequence closest to the *REP2* gene (CACA2) led to a dramatic decrease in *FLPp-lacZ* expression (Figure 24 B). Mutation of both CGCG sequences to CACA (CACA1+CACA2) was found to reduce expression from both the *FLPp-lacZ* and *REP2p-lacZ* reporters (Figure 24 B and C). The effect of the mutation of the CGCG sequences to CACA individually was not tested for the *REP2p-lacZ* reporter. These results suggest that the CGCG sequence closest to the *REP2* gene is involved in promoting transcription of the *FLP* gene, while either one or both of the CGCG sequences is involved in promoting transcription of *REP2*.

Upon expression of Rep proteins, both *FLPp-lacZ* and *REP2p-lacZ* reporters with mutations in one or both CGCG sequences showed reduced expression compared to that observed in the absence of Rep proteins, suggesting that Rep protein-mediated repression of these mutant reporters is intact. This result implies that CGCG sequences are not required for Rep protein-mediated repression of *FLP* or *REP2* transcription.

Unexpectedly, mutation of the TGCA sequences in the two TGCATTTTT elements to CTAG resulted in a decrease in both *FLPp-lacZ* and *REP2p-lacZ* expression. While mutation of TGCA1 alone (CTAG1) resulted in a decrease in reporter expression, mutation of TGCA2 (CTAG2) or both TGCA1 and 2 simultaneously (CTAG1+CTAG2) resulted in a much greater decrease. Mutation of the T-tract adjacent to the TGCATTTTT element, but not the TGCA sequence, has been seen to reduce *STB-P*-driven transcription (N. Arumuggam, unpublished results); however, due to differences in sequence context, the TGCA sequences in *FLP/REP2p* could be influencing the function of the T-tract, or playing a separate role in which they contribute positively to *FLP* and *REP2* transcription.

Rep protein-mediated repression of both *FLPp-lacZ* and *REP2p-lacZ* expression was still observed when TGCA1 was mutated to CTAG, suggesting that TGCA1 is not responsible for Rep protein-mediated repression of *FLP* or *REP2*. Upon mutation of TGCA2 to CTAG, *FLPp-lacZ* expression, but not *REP2p-lacZ* expression, was reduced by expression of Rep1 and Rep2, implying that TGCA2 is required for transcriptional repression of *REP2* but not *FLP*. When both TGCA sequences were mutated simultaneously, Rep protein-mediated repression of *FLPp-lacZ* was abolished, but some repression of *REP2p-lacZ* was observed. This suggests that both TGCA1 and TGCA2 contribute to Rep protein-mediated repression of *FLP* transcription, while TGCA2 is mainly responsible for Rep protein-mediated repression of *REP2* transcription, although *REP2* transcription can be influenced by the TGCA1 sequence.

One potential reason for differences in the behavior of the *FLPp-lacZ* and *REP2p-lacZ* constructs is that the DNA sequences upstream of each reporter differ slightly. Each includes the 5' untranslated region (UTR) and immediately adjacent sequences for the downstream gene, but lacks this region for the oppositely-oriented gene. The *FLPp-lacZ* reporter includes a TGCA sequence closer to the 5' end of the *FLP* ORF that is not included in the *REP2p-lacZ* reporter, which, given the results shown here, could be contributing to regulation of *FLPp-lacZ* expression. Preliminary results indicate that mutation of this third TGCA element, which lacks an adjacent T-tract, may be functionally redundant with mutation of the TGCA1 element (data not shown).

Despite differences in sequence context, these results show overall that TGCA1 and TGCA2 both contribute to Rep protein-mediated transcriptional repression at *FLP/REP2p*, although further sequence requirements for transcriptional repression of *FLP* and *REP2* may differ.

3.3.3 The Divergent *FLP/REP2* Promoter Confers Partitioning Function On A Non-2µm-Based Plasmid

The results of the above assays for Rep protein association with and transcriptional regulation of 2µm plasmid promoters suggest that there are several similarities between the *FLP/REP2p* and *STB-P* sequences of the 2µm plasmid. Both *FLP/REP2p* and *STB-P* associate with Rep1, Rep2 and Raf; both promote bi-directional transcription that requires CGCG sequences in at least one direction; and transcription driven by both sequences is repressed by the association of Rep proteins. One major difference between the two sequences is that *STB-P* has a well-documented role in plasmid partitioning, which requires the presence of Rep1 and Rep2, while the *FLP/REP2p* sequence is presumed not to perform this function due to the drastic decrease in the inheritance of a 2µm-based plasmid observed upon deletion of the *STB-P* sequence (Figure 15 A; (Jayaram *et al.*, 1983; Kikuchi, 1983)). In the absence of *STB-P*, a 2µm-based plasmid was seen to retain some degree of Rep protein-dependent partitioning function; however, this function was attributed to cooperation between sequences flanking *STB-P* on either side, as inserting sequences in place of *STB-P* that had no partitioning function, which separated these flanking sequences by 138-bp, resulted in a degree of plasmid inheritance similar to that observed when the Rep1 protein was absent (Section 3.2.3).

To determine whether the divergent promoter of the *FLP* and *REP2* genes is able to confer partitioning function, the *FLPp* sequence from the *FLPp-lacZ* reporter was placed on a non-2µm-based plasmid, pCD1 (as described in Section 3.2.1). The pCD1 plasmid contains the *TRP1* gene with the *ARS* found adjacent to *TRP1* in the genome. The pCD1 plasmid was used as a negative control, while pCD1 containing the *STB-P* sequence (pCD1-STB-P) served as a positive control. When assayed in a cir^+ strain, which provided Rep1 and Rep2 proteins *in trans*, a pCD1 plasmid with *FLPp* sequence

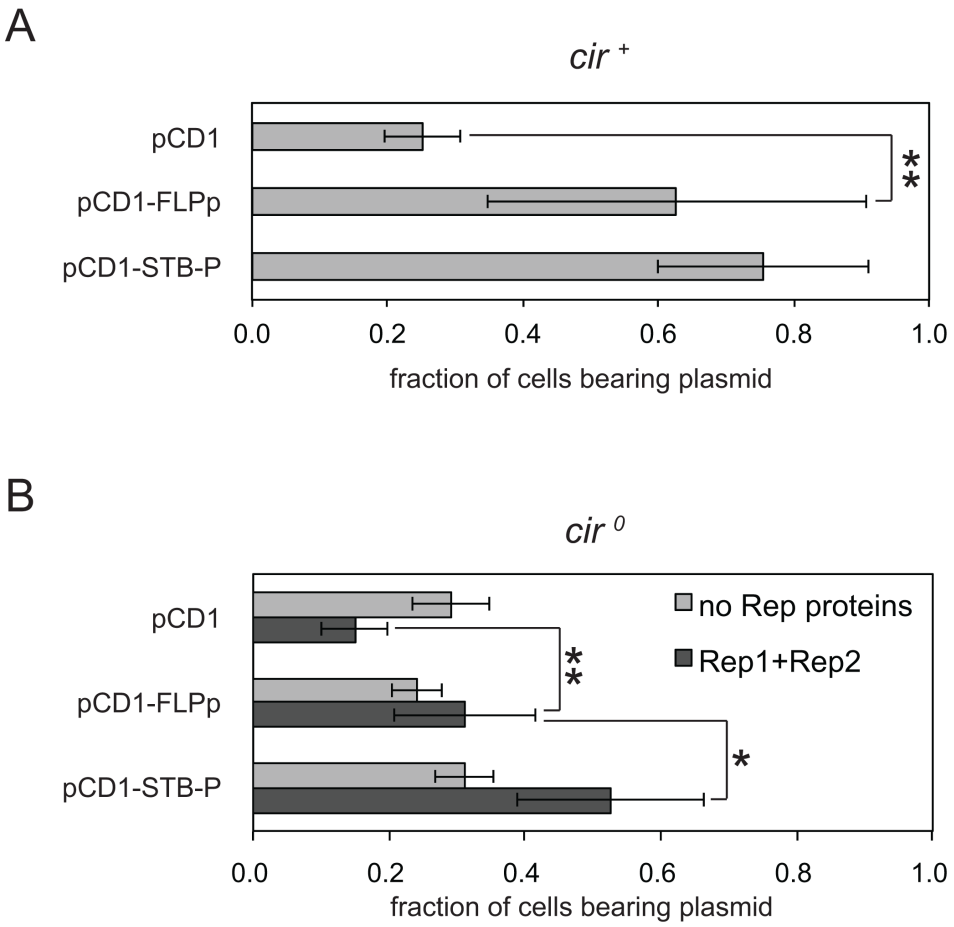


Figure 25. *FLP/REP2* promoter has Rep protein-dependent partitioning function. (A) A *cir*$^{+}$ yeast strain was transformed with either pCD1, pCD1-FLPp or pCD1-STB-P. Transformants were cultured overnight (6 to 8 generations) in selective medium (SD-trp). (B) A *cir*^{0} strain was co-transformed with either pCD1, pCD1-FLPp or pCD1-STB-P and pGAL-LEU-R1R2 expressing Rep1 and Rep2 from a galactose-inducible promoter. Transformants were cultured overnight (6 to 8 generations) in selective medium (SD-leu-trp) containing either glucose (pale grey bars, no Rep proteins) or galactose (dark grey bars, Rep1+Rep2). Results in (A) and (B) represent an average of a minimum of 4 isolates of each transformant (± standard deviation). *$p<0.005$, **$p<0.001$

inserted (pCD1-FLPp) was inherited by a significantly greater fraction of cells than pCD1 (Figure 25 A), suggesting that *FLP/REP2p* can provide partitioning function.

To ensure that the increase in plasmid inheritance observed when *FLP/REP2p* was present was not the result of recombination between the *FLP/REP2p* sequence on the pCD1 plasmid and *FLP/REP2p* on the native plasmid, plasmid inheritance assays were repeated in a cir^0 strain in which *REP1* and *REP2* were present under the control of galactose-inducible promoters on a *CEN* plasmid (Figure 25 B). When cells were grown in glucose, which repressed expression of Rep proteins, no difference was observed between the inheritance of pCD1, pCD1-FLPp and pCD1-STB-P. This demonstrates that the partitioning function of pCD1-FLPp and pCD1-STB-P is dependent upon Rep protein expression.

Upon induction of Rep1 and Rep2 expression by growth in galactose-containing medium, pCD1-FLPp and pCD1-STB-P were inherited by a significantly greater fraction of cells than pCD1, and pCD1-STB-P was inherited by a significantly greater fraction of cells than pCD1-FLPp. This demonstrates that any partitioning function of pCD1-FLPp is not a result of recombination between the *FLP* promoters of pCD1-FLPp and the native 2µm plasmid leading to the acquisition of an *STB* sequence by pCD1-FLPp.

In the cir^0 strain, inheritance of all pCD1-based plasmids in the absence of Rep proteins was greater than that observed for the empty pCD1 plasmid when Rep proteins were expressed from galactose-inducible promoters. High levels of Rep protein expression can be toxic to the host (Scott-Drew and Murray, 1998), so it is possible that expression of Rep1 and Rep2 from galactose-inducible promoters, which drive greater expression than the native 2µm plasmid promoters (data not shown), reduced host fitness in a manner that impaired its ability to retain *ARS* plasmids.

3.4 Screens For Gene Deletions That Alter Functions Of 2µm Plasmid And 2µm Plasmid-Encoded Proteins

The complete collection of viable haploid yeast gene deletion strains is a powerful tool for genetic studies. With the use of a robotic arrayer, genome-wide screens for characteristics such as synthetic lethality with a gene of interest or suppression of an

induced phenotype can be undertaken (Tong *et al.*, 2001; Smith *et al.*, 2004). Such unbiased screens often serve as a useful starting point, identifying genes that have functional overlap or that were previously unknown participants in a cellular process.

Two screens of the EUROSCARF yeast gene deletion collection (Winzeler *et al.*, 1999) were designed and performed. The first aimed to identify gene deletions that altered chromatin structure of a chromosomally-integrated *STB-P* sequence, using the changes in the level of transcription promoted by this sequence as a read-out for chromatin structure. The second examined whether any deletions alleviated the phenotypes of delayed colony formation and lethal sectoring associated with overexpression of the Rep1 and Rep2 proteins. To date, only one category of candidate genes from the *STB-P* chromatin screen has been further analyzed, from among which two candidates have been investigated for their role in plasmid partitioning; however, the data collected from these screens will serve as a basis for future studies.

3.4.1 Screen For Yeast Gene Deletions That Alter *STB-P*-Driven Transcription

The *STB-P* locus is a repeated DNA sequence that is required *in cis* for Rep protein-dependent plasmid partitioning. In addition to the Rep1 and Rep2 proteins, several host proteins, including the motor protein Kip1, the centromere-specific histone H3 Cse4, and subunits of cohesin and the RSC2 complex are known to interact with this locus, and are required for plasmid partitioning (Section 1.2.1).

To determine whether any other host proteins might be interacting with *STB-P* and contributing to chromatin structure at this locus, the EUROSCARF yeast gene deletion strain collection was screened for deletions that altered the level of transcription driven by *STB-P*. *STB-P* has been shown to promote transcription both in the direction of *STB-D*, resulting in the generation of a 1950-nt transcript (Sutton and Broach, 1985), and in the opposite direction, toward the origin (M. Dobson, unpublished results). Although the role of this transcription in plasmid maintenance, if any, is unclear, the fact that it is mediated by the same sequence that is required for plasmid partitioning suggests that transcription and partitioning functions at *STB-P* may have evolved to use some of the same host proteins. This is the case for Rsc2, a non-essential subunit of the chromatin remodeling complex RSC2, which, when absent, leads to a decrease in *STB-P*-driven transcription (Figure 20 B) and in 2μm plasmid inheritance (Wong *et al.*, 2002; Pinder *et*

al., 2013). *STB-P*-driven transcription is also influenced by the presence of the Rep1 and Rep2 proteins, which serve as transcriptional repressors at this locus as they do at the 2µm plasmid gene promoters (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988). It is therefore possible that changes in *STB-P*-driven transcription could be a result of changes in the ability of the Rep proteins to mediate repression.

To monitor transcription driven by *STB-P*, a yeast strain (Y2454/2-STB(O)) was constructed, in which the *STB-P* sequence was chromosomally integrated upstream of a *lacZ* reporter gene, so that *lacZ* expression reflected transcription driven by *STB-P* toward the origin ("O" orientation). This strain was then mated to the complete collection of viable haploid yeast gene deletion strains. The resulting diploids were sporulated, and haploid segregants bearing the *STB-P(O)-lacZ* reporter and the gene deletion of interest were selected. Strains were grown on nitrocellulose filters and assayed for *lacZ* expression using a qualitative assay for enzyme activity of the *lacZ* gene product, ß-gal, in which the level of activity is correlated with the production of a blue precipitate.

When assayed, the majority of colonies on the filters gave a similar colour of blue; however, a number of paler and darker colonies were also observed. Of the gene deletion strains that gave shades of blue that differed from the majority of the collection when assayed, 124 deletion strains were initially identified as being paler blue (Appendix Table 8). The proteins encoded by these deleted genes have roles in a range of cellular processes; mitochondrial function was predominant, but genes encoding proteins with roles in biosynthesis, transport and gene expression, among others, were also identified (Figure 26 A). Of these, genes encoding proteins involved in gene expression are the most likely candidates for a direct role at the *STB-P* locus, while the others may affect *STB-P*-driven transcription or partitioning indirectly.

A number of deletion strains were also identified as being darker blue than the rest in the filter assay, suggesting that they had increased expression of the *STB-P(O)-lacZ* reporter. Because Rep1 and Rep2 act together to repress *STB-P*-promoted transcription, strains that lack 2µm plasmid, and consequently do not express Rep1 or Rep2, would be expected to have increased *STB-P*-mediated transcription, and therefore appear darker blue. As the query strain used for this screen was cir^0, any deletion collection strains that lacked the 2µm plasmid prior to mating remained cir^0. To identify

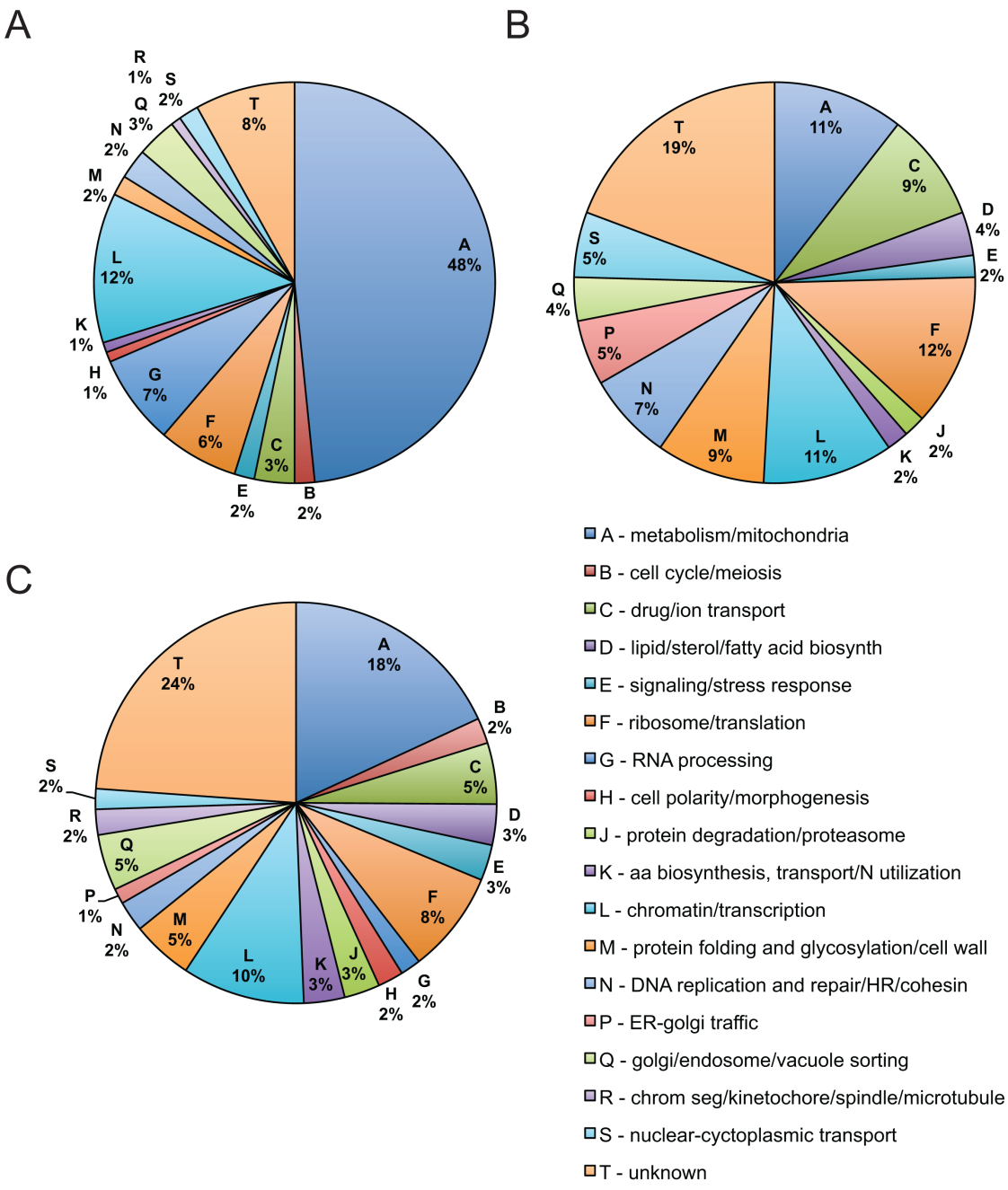


Figure 26. Classification of genes identified in screen for host proteins regulating *STB-P*-driven transcription. Strains showing reduced reporter gene expression (A), or increased reporter gene expression and either no detectable hybridization signal (B) or a detectable hybridization signal (C) were classified according to the assignments described in (Costanzo *et al.*, 2010). "N" stands for nitrogen, "chrom seg" for chromosome segregation.

strains that had potentially lost the 2µm plasmid, thereby facilitating the interpretation of the results of this screen, data from two colony hybridization assays performed with a 2µm DNA-specific radiolabeled probe were also examined (J. Pinder, unpublished results). One of these colony hybridization assays was performed using the *STB-P(O)-lacZ* reporter gene deletion haploids that had been screened for altered *STB-P*-driven transcription, while the other used the original unmated deletion collection strains. Upon cross-referencing the strains that gave dark blue colonies with the colony hybridization assay performed on the *STB-P(O)-lacZ* reporter strains, it was noted that many of these strains had failed to hybridize with the 2µm plasmid DNA probe, suggesting that they did not contain 2µm plasmid.

Among these were presumed to be colonies that were the non-G418-resistant parental Y2454/2-STB(O) strain, which lacked the *kanMX4* gene. The presence of these Y2454/2-STB(O) colonies was likely due to insufficient passages on selective media prior to pinning to the filter, which was overlaid on non-selective YPAD medium, allowing the small number of remaining wild-type Y2454/2-STB(O) cir^0 haploid cells to grow. To eliminate as many of these putative Y2454/2-STB(O) colonies as possible, filters were cross-referenced with plates onto which the non-mated deletion strain collection had been pinned, to verify that each dark blue colony represented a viable deletion strain. Filters were also cross-referenced with the colony hybridization screens performed using the non-mated haploid strains, and any dark blue colonies that corresponded to positions where hybridization was observed for the non-mated haploids, but gave no signal for the mated haploids, were discounted, as this suggested a failed mating and replacement by the parental Y2454/2-STB(O) cir^0 strain at that position, rather than increased *STB-P* expression from the gene deletion strain expected to be present at that position.

After elimination of strains fulfilling either of these criteria, 308 deletion strains giving dark blue colonies remained. These were further subdivided into strains that did not give hybridization signal with the 2µm DNA probe in either the mated or non-mated screens, suggesting that they were cir^0 (63 strains; Appendix Table 9); and strains that did give hybridization signal in either the mated or both the mated and non-mated screens,

suggesting that their increased reporter activity was not due to the absence of Rep protein-mediated repression (245 strains; Appendix Table 10).

Among the putative *cir*^{0} strains were a number whose deleted gene products are involved in transport, biosynthesis and protein degradation (Figure 26 B); any role of these proteins in 2µm plasmid maintenance is likely indirect, as these functions occur mainly outside the nucleus while the 2µm plasmid is nuclear. Several transcription factors and chromatin modifiers, as well as subunits of the nuclear pore complex, were also among the putative *cir*^{0} strain gene deletion products; these may play a more direct role in 2µm plasmid maintenance, as they act on DNA or in the nucleus, and therefore merit further analysis. Additional hybridization screens to verify the absence of 2µm plasmid from these strains is needed to establish their role in 2µm plasmid maintenance.

Similar to those of the putative *cir*^{0} strains, products of the genes deleted in strains that were dark blue in the filter assay, but contained 2µm plasmid on the basis of hybridization signal, were found to be involved in transport and biosynthesis, as well as ribosomal and mitochondrial functions (Figure 26 C). Products of deleted genes that have the potential to play a more direct role in 2µm plasmid function include transcription factors, chromatin modifiers and those involved in DNA repair.

3.4.2 Further Analysis Of Gene Deletions Giving Decreased *STB-P*-Driven Transcription

With the aim of identifying host factors required for the transcription driven by *STB-P*, deletion strains that gave decreased *STB-P(O)-lacZ* reporter activity were chosen as the focus of further analyses. To determine whether gene deletions were specifically affecting *STB-P*-driven transcription, or merely having a general effect on gene expression, 20 representative gene deletion strains were chosen to assay for their effect on *lacZ* expression directed by a different promoter, that of the yeast *CYC1* gene (*CYC1p*; Table 7). *CYC1* encodes isoform 1 of cytochrome c of the mitochondrial electron transport chain (Sherman, 1990; Volkov *et al.*, 2011).

Many strains showed a level of *CYC1p-lacZ* activity similar to the wild-type strain; however, several strains did show altered *CYC1p-lacZ* activity. Strains in which either one of the mitochondrial genes *COX10*, *MRPS5* or *PET309*, or the ribosomal gene *RLP22A* was deleted showed a decrease in *CYC1p-lacZ* activity compared to that

Table 7. Reporter expression in strains of the yeast gene deletion collection selected for further analysis. Selected BY4741-based strains from the EUROSCARF yeast gene deletion collection were transformed with a *CEN* plasmid carrying either the *STB-P(O)-lacZ* or *CYC1p-lacZ* reporter. Activity of the *lacZ* gene product, ß-gal, was measured qualitatively using an X-gal filter. Strains were classified based on time required for colour development in the filter assay, as 5 to 10 minutes (++++++), 10 to 15 minutes (+++++), 15 to 30 minutes (++++), 30 minutes to 1 hour (+++), 1 to 2 hours (++), or 2 to 8 hours (+) for colour development, or no colour development after 8 hours (-).

	Reporter expression	
BY4741 Strain	***STB-lacZ***	***CYC1p-lacZ***
WT	+++	++++++
***yap1802*Δ**	+++	++++++
***pat1*Δ**	+++	++++++
***msa2*Δ**	+++	++++++
***swi4*Δ**	+	++++++
***eaf3*Δ**	+++	++++++
***nup170*Δ**	+++	++++++
***swc5*Δ**	+++	++++++
***mbp1*Δ**	+++	++++++
***thp2*Δ**	–	++++
***lsm1*Δ**	+++	++++++
***pdf1*Δ**	++	++++++
***rtt109*Δ**	+++	++++++
***ixr1*Δ**	+	+++++
***rpl22a*Δ**	+	+++++
***eaf7*Δ**	++	+++++
***cox10*Δ**	++	+++++
***deg1*Δ**	+++	++++++
***mrps5*Δ**	+++	+++++
***pet309*Δ**	+++	+++++

observed in a wild-type strain; this may have been due to a general effect of deletion of mitochondrial and ribosomal genes on growth or gene expression. Deletion of the genes encoding the transcriptional repressor Ixr1, the NuA4 and Eaf3/5/7 complex subunit Eaf7, or the THO complex subunit Thp2 also resulted in a decrease in *CYC1p-lacZ* expression. Ixr1 targets genes related to hypoxia (Vizoso-Vazquez *et al.*, 2012), and may therefore have effects on the overall growth rate of the cell. NuA4, of which Eaf7 is a subunit, is an essential histone acetyltransferase targeting histones H4, H2A and H2A.Z, and has been found to activate transcription in a gene-specific manner (Lu *et al.*, 2009). However, Eaf7 is also part of Eaf3/5/7, a recently-identified subcomplex of NuA4 that regulates transcriptional elongation and mRNA processing independently of NuA4 (Bhat *et al.*, 2015); the absence of Eaf7 from this subcomplex may therefore result in the impairment of *lacZ* expression from any promoter, as the *lacZ* transcript is longer than most yeast mRNAs. The THO complex is also involved in transcriptional elongation and mRNA export (Straszer *et al.*, 2002), and deletion of the *THP2* gene has previously been shown to impair expression of the *lacZ* gene specifically (Chávez *et al.*, 2000).

Many of the gene deletion strains chosen for further analysis did not show a decrease in *STB-P*-promoted *lacZ* reporter activity as initially observed in the genome-wide *STB-P(O)-lacZ* reporter screen. This difference could be due to this secondary analysis being carried out by introducing the *STB-P(O)-lacZ* and *CYC1p-lacZ* reporters into the strains of interest on *CEN* plasmids instead of the reporters being chromosomally integrated as they had been for the initial screen. Reporter genes present on *CEN* plasmids are more highly and more variably expressed than those integrated in the chromosome, and subtle effects on gene expression observed with chromosomally-integrated reporters are not always reproducible with *CEN* plasmid-borne reporters (unpublished observations). These differences may be attributable to the fact that *CEN* plasmids can be maintained at copy numbers greater than one (Futcher and Carbon, 1986), or to changes in the chromatin environment of the reporter that could be imposed by the proximity of the centromere to the reporter on the *CEN* plasmids (Choy *et al.*, 2012).

Of the gene deletion strains chosen for further analysis, BY4741 *swi4*Δ was one of the only strains in which reporter activity was decreased for *STB-P(O)-lacZ*, but

remain unchanged for *CYC1p-lacZ*, compared to reporter activity in wild-type BY4741. The *SWI4* gene encodes the DNA-binding subunit of the cell-cycle regulated transcription factor SBF, which consists of Swi4 and Swi6 (Primig *et al.*, 1992). Swi4 has a paralog, Mbp1, that arose during the *S. cerevisiae* whole genome duplication. Like Swi4, Mbp1 binds Swi6, forming the MBF transcription factor (Dirick *et al.*, 1992). SBF and MBF activate an overlapping but distinct set of genes at the G1-to-S transition (Iyer *et al.*, 2001). The colonies of the *swi4*Δ and *swi6*Δ *STB-P(O)-lacZ* reporter strains were pale blue in the initial ß-gal filter assay screen, while those of the *mbp1*Δ *STB-P(O)-lacZ* reporter strain were not. When the *STB-P(O)-lacZ* reporter was re-introduced into the *swi4*Δ and *mbp1*Δ strains on a *CEN* plasmid, the *swi4*Δ strain again gave less reporter gene activity than a wild-type strain, while the *mbp1*Δ strain gave similar reporter gene activity to the wild-type strain. These results suggest that inactivation of SBF specifically, and not MBF, was responsible for the observed decrease in *STB-P(O)-lacZ* expression. SBF regulates expression of a number of genes, so altered *STB-P*-driven transcription could be an indirect effect of the absence of SBF; however, a perfect match to the Swi4 consensus recognition motif, CGCGAAA (Iyer *et al.*, 2001; Badis *et al.*, 2008), is found in one of the five directly-arrayed repeats at *STB-P*, while the sequence CGCGAGA, which differs from the consensus motif by only one nucleotide, is found in three of the other repeats, suggesting that a direct role for SBF at *STB-P* is also possible (Figure 3).

The divergent 2µm plasmid promoters also contain potential Swi4 recognition sites – the perfect CGCGAAA recognition site in the *REP1*/*RAF* promoter (nucleotides 2088 to 2082 in Scp1 2µm A form, NCBI GenBank version J01347.1, GI: 172190), and the variation CGCGAGA in the *FLP*/*REP2* promoter (nucleotides 5448 to 5454 in Scp1 2µm A form, NCBI GenBank version J01347.1, GI: 172190). This means that in strains containing native 2µm plasmid, Swi4 could affect transcription from *STB-P* indirectly by regulating Rep protein transcription. However, because a decrease, not an increase, in *STB-P(O)-lacZ* reporter activity was observed for strains in which *SWI4* was deleted, it is more likely that, if anything, Rep protein levels were increased in these strains relative to a wild-type strain, as SBF is a transcriptional activator. The effect of Swi4 on *STB-P(O)-lacZ* reporter activity is therefore unlikely to be mediated by the Rep protein levels in this manner.

3.4.2.1 Deletion Of SBF Complex Subunit Genes *SWI4* And *SWI6* Alters *STB-P*-Driven Transcription

To investigate the possibility that Swi4 and Swi6 are required for *STB-P*-driven transcription, and to determine whether transcription was influenced by the presence of 2µm plasmid-encoded proteins, *SWI4* and *SWI6* genes were deleted in isogenic cir^+ and cir^0 strains from the W303 background, and activity of *STB-P*-driven reporters was monitored (Figure 27 A). In addition to the *STB-P(O)-lacZ* reporter, in which *STB-P* is oriented so that *lacZ* expression is a measure of transcription directed toward the origin in the native context, a reporter containing *STB-P* in the opposite orientation, in which *lacZ* expression is a measure of transcription directed toward *STB-D* ("D" orientation, *STB-P(D)-lacZ*), was also generated, to reflect transcription of the 1950-nt RNA promoted by *STB-P* in the direction of *STB-D* on the native 2µm plasmid.

Activity of the *STB-P-lacZ* reporters in the W303 background was similar to that observed for the strains of the gene deletion collection (BY4741 background), but not all observations were recapitulated. Activity of both *STB-P-lacZ* reporters was reduced in cir^+ strains compared to cir^0 strains, irrespective of whether strains were wild type or had a deletion of *SWI4* or *SWI6*, likely due to Rep protein-mediated repression of *STB-P*-driven transcription (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988). *STB-P(D)-lacZ* expression, but not *STB-P(O)-lacZ* expression, was decreased in both cir^+ and cir^0 strains upon deletion of the *SWI4* or *SWI*6 gene. This differs from the results obtained with the gene deletion collection strains, which showed that *STB-P(O)-lacZ* expression was decreased in the absence of Swi4 or Swi6 (Table 7 and Appendix Table 8). Despite these strain-specific differences regarding the direction of the *STB-P*-driven transcription that is affected by *SWI4* or *SWI6* deletion, these results suggest overall that the SBF complex is involved in mediating *STB-P*-driven transcription, and, based on repression of this transcription in cir^+ yeast, that Rep1 and Rep2 are not dependent on SBF for association with *STB-P*.

3.4.2.2 Mutation Of A Putative SBF Recognition Motif Alters *STB-P*-Driven Transcription

To provide insight into whether SBF directly activates *STB-P*-driven transcription by recognizing the CGCGAAA sequence in *STB-P*, synthetic versions of the *STB-P*

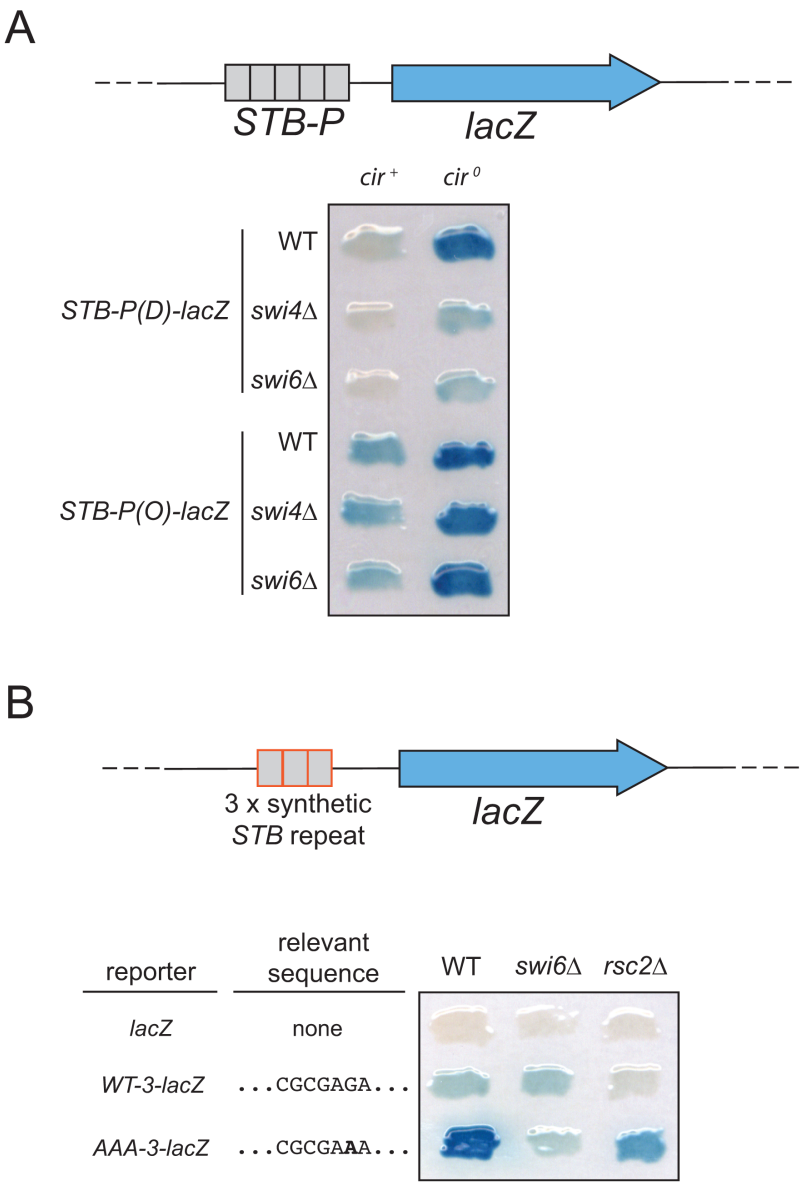


Figure 27. Effect of deletion of *SWI4* and *SWI6*, and of mutations in synthetic *STB* repeat sequence, on *STB*-driven transcription. (A) The native *STB-P* sequence was integrated in the chromosome upstream of a *lacZ* reporter gene oriented to place the *ORI*-proximal (O) or *STB-D*-proximal (D) end of *STB-P* closest to *lacZ*, in wild-type, *swi4*Δ and *swi6*Δ *cir*$^{+}$ and *cir*^{0} strains. (B) 3 directly-arrayed copies of wild-type or AAA mutant synthetic *STB* repeats were integrated in the chromosome upstream of a *lacZ* reporter gene in wild-type, *swi6*Δ or *rsc2*Δ strains. In (A) and (B), activity of the *lacZ* gene product, ß-gal, was monitored by growing patches of yeast on a filter and incubating with the substrate X-gal, which is cleaved to produce a blue precipitate in the presence of ß-gal activity.

sequence were used in which sequence composition could be more easily manipulated (Section 2.2.5). The native *STB-P* sequence is made up of five directly-arrayed tandem imperfect repeats of 62- to 63-bp in length. Previous analyses showed that tandem arrays of a synthetic *STB* repeat, designed to match a single 63-bp stretch of *STB-P*, provide partitioning function similar to the native *STB-P* sequence (Figure 15). A synthetic *STB-P* sequence consisting of three identical synthetic *STB* repeats was inserted upstream of the *lacZ* gene in the "D" orientation (i.e., with the *STB-D*-proximal end of the synthetic *STB* repeat sequence positioned closest to the *lacZ* gene; *WT-3-lacZ*). The *WT-3-lacZ* reporter gave sufficient reporter gene activity to be detected in a filter assay, although this activity was less than that observed for the *STB-P(D)-lacZ* reporter (data not shown).

The *STB-P* repeat on which the synthetic *STB* repeat was based is not the repeat that contains the SBF consensus motif, CGCGAAA; however, the synthetic repeat does contain a similar sequence, CGCGAGA, in this position. When the CGCG portion of the CGCGAGA sequence in the synthetic *STB* repeat was changed to CACA in all three copies of the repeat (*CACA-3-lacZ*), transcription of the reporter was effectively abolished (Figure 20 B). This suggests that SBF may be responsible for transcription driven by *STB-P*; however, another host factor recognizes the sequence CGCG – the RSC2 complex (Badis *et al.*, 2008; Zhu *et al.*, 2009). The RSC2 complex has previously been shown to associate with the *STB-P* locus by ChIP and contribute to efficient plasmid partitioning (Wong *et al.*, 2002; Ma *et al.*, 2012). Deletion of the *RSC2* gene, encoding a subunit of the RSC2 chromatin remodeling complex, decreased transcription of *WT-3-lacZ*, demonstrating that RSC2 is required for efficient *STB-P* driven transcription (Figure 20 B); however, transcription of *CACA-3-lacZ* was also decreased upon deletion of *RSC2*, suggesting that the RSC2 complex may contribute to *STB-P*-driven transcription independently of the CGCG sequence (see Section 3.2.8).

To determine whether SBF is able to contribute to *STB-P*-driven transcription, the CGCGAGA sequence was mutated to more closely resemble the actual SBF consensus motif CGCGAAA in all three copies of the synthetic repeat (*AAA-3-lacZ*; Figure 27 B). In a wild-type strain, activity of the *AAA-3-lacZ* reporter was increased compared to that of the *WT-3-lacZ* reporter, indicating that the presence of the perfect consensus SBF recognition motif increased transcription driven by the synthetic *STB* repeats. To

determine whether this increased transcription was dependent on the SBF complex, the synthetic *STB-P* reporters were introduced into yeast strains in which either *SWI6* or *RSC2* genes were deleted (Figure 27 B). In yeast strains in which the *SWI6* gene was deleted, but not strains in which the *RSC2* gene was deleted, the enhanced expression of *AAA-3-lacZ* was lost, with *lacZ* expression in the strain lacking *SWI6* being similar to that observed for *WT-3-lacZ*. Overall, these results suggest that SBF is capable of associating with the synthetic *STB* repeat at least when the CGCGAGA sequence is mutated to CGCGAAA.

3.4.2.3 Deletion Of *SWI6* Has A Slight Effect On 2µm Plasmid Inheritance

The role of *STB-P*-driven transcription in 2µm plasmid maintenance, if any, is unknown; however, the fact that *STB-P* also functions as the partitioning locus for the plasmid suggests that there may be a correlation between *STB-P*-driven transcription and partitioning. To determine whether SBF is involved in plasmid partitioning, the effect of deletion of the *SWI6* gene on inheritance of a *kanMX4*-tagged 2µm plasmid, pKan, was assayed. The effect of the absence of Swi4 on pKan inheritance was not determined, as *swi4*Δ strains were significantly more impaired for cell cycle progression than *swi6*Δ strains, resulting in the rapid appearance of suppressor mutations in the *swi4*Δ strain when cultured in logarithmic growth phase for an extended period of time (data not shown).

No difference in the fraction of pKan-bearing cells between *SWI6* wild-type and *swi6*Δ yeast was observed when the strains were cultured in G418 (data not shown). However, the delay in cell cycle progression for the *swi6*Δ strains relative to *SWI6* wild type strains may have prevented sufficient doublings during the time period of the assay to allow differences in the rates of plasmid loss between *SWI6* and *swi6*Δ strains to be observed from the fraction of plasmid-bearing cells. To circumvent this problem, the rate of generation of plasmid-free cells per doubling, under conditions that do not select for the presence of the plasmid, was determined for *SWI6* and *swi6*Δ yeast. The *swi6*Δ strains were found to generate plasmid-free cells at a greater rate (10% per doubling) than strains containing wild-type Swi6 (5% per doubling; Figure 28 A). This change in the rate of generation of plasmid-free cells suggests that Swi6, and by extension SBF, is required for efficient plasmid partitioning function. The role of SBF in plasmid partitioning may be

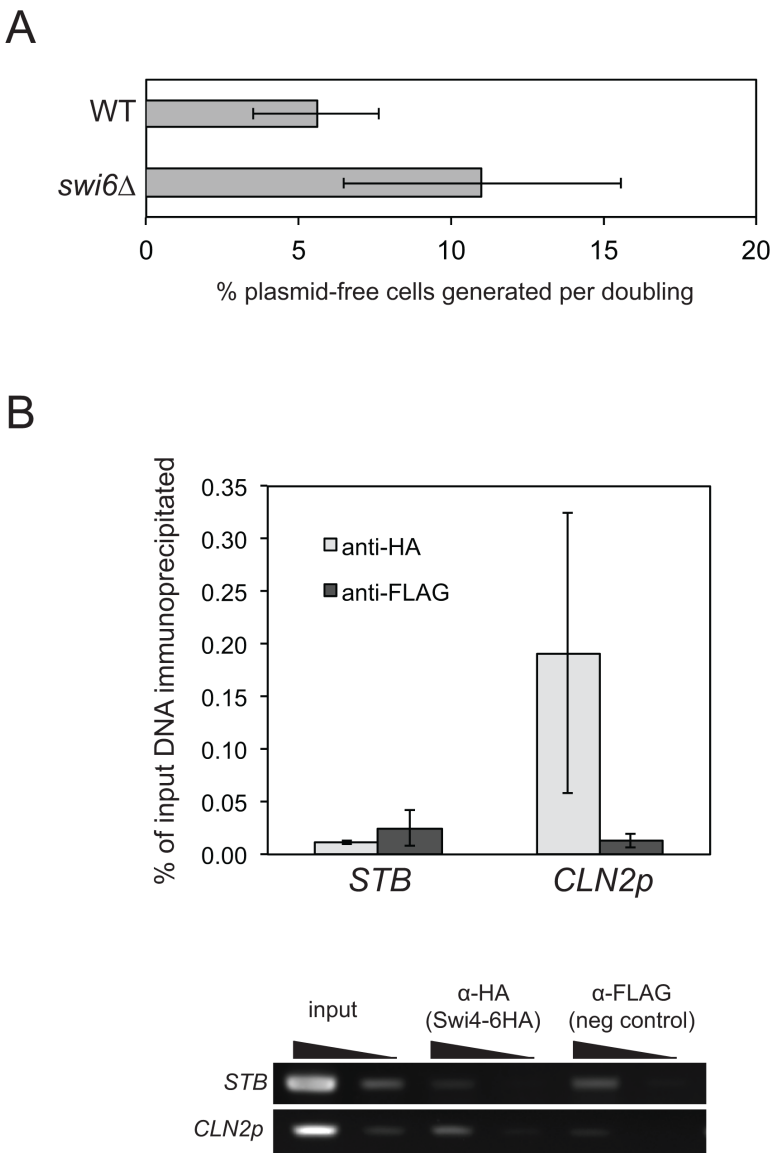


Figure 28. Deletion of *SWI6* increases rate of generation of plasmid-free cells; association of Swi4 with *STB-P* not detected by ChIP. (A) Isogenic *SWI6* and *swi6*Δ *cir*^{0} strains were transformed with the pKan plasmid. Transformants were cultured for 6 to 8 generations in selective medium (YPAD+G418), then cultured for 10 to 12 generations in non-selective medium (YPAD). The rate of generation of plasmid-free cells was determined from the fraction of plasmid-containing cells before and after culturing in non-selective medium and the number of doublings undergone. (B) A *cir*$^{+}$ yeast strain expressing Swi4-6-HA from the native *SWI4* gene promoter at the genomic *SWI4* locus was assayed for association of Swi4-6-HA with *STB-P* and the *CLN2* promoter by ChIP. PCR products were visualized by electrophoresis on 1.5% agarose gels and quantified by densitometry (Section 2.8). The average of 3 replicates (± standard deviation) is represented in the bar graph (top). The PCR products from a representative replicate, visualized by agarose gel electrophoresis are shown (bottom).

indirect, perhaps through activation of genes required for 2µm plasmid partitioning or chromosome segregation.

3.4.2.4 Association Of Swi4 With *STB-P* Not Observed In Chip Assay

To determine whether SBF associates directly with native *STB-P*, a ChIP assay was performed using a *cir*$^+$ strain expressing HA-tagged Swi4 at its native chromosomal locus under the control of its own promoter (Figure 28 B). Upon immunoprecipitation with anti-HA antibodies, the promoter of the *CLN2* gene, which has previously been shown to be regulated by Swi4 (Koch *et al.*, 1993), was enriched in the chromatin fraction, but *STB-P* was not. The absence of *STB-P* enrichment suggests that Swi4 either does not associate with the 2µm *STB-P* locus, or does so transiently or less efficiently than with the *CLN2* promoter. If this is the case, cell cycle arrest and release, followed by ChIP at time points of maximum SBF expression, might be required to reveal the association of Swi4 with *STB-P*. However, given the modest effect of *SWI6* deletion on 2µm plasmid inheritance, the possibility that SBF does not interact with *STB-P* directly may be more likely.

3.4.3 Screen For Yeast Gene Deletions That Alter Toxicity Caused By Presence Of High Levels Of Rep1 And Rep2 Proteins

A second screen of the EUROSCARF yeast gene deletion strain collection was performed, this time monitoring the effect of Rep protein overexpression on colony morphology. Simultaneous overexpression of Rep1 and Rep2 results in slowed colony growth and the appearance of colonies with uneven "nibbled" edges due to lethal sectoring (Scott-Drew and Murray, 1998). Cells in the "nibbled" sectors have altered budding patterns and multiple buds, consistent with G2/M cell cycle defects. This phenotype is observed whether the native 2µm plasmid is present or absent. The reason for this phenotype is unknown; however, it may reflect sequestration by the Rep proteins of host factors needed for normal cell cycle progression. It is also possible that excess Rep1 and Rep2 inappropriately target host genes for transcriptional repression. Co-expression of Rep1 and Rep2 has been shown to repress transcription driven by 2µm plasmid gene promoters in addition to that driven by *STB-P* (Murray *et al.*, 1987;

Reynolds *et al.*, 1987; Som *et al.*, 1988); therefore, repression of host gene transcription could be an extension of this native function.

To first verify that this slow growth and nibbled colony phenotype would be recapitulated in the BY4741 background of the yeast deletion strains, the pNAT-R1R2 plasmid carrying both the *REP1* and *REP2* genes under the control of a galactose-inducible promoter and the empty vector, pNAT, were used to transform both cir^+ and cir^0 strains of wild-type BY4741 yeast to nourseothricin resistance (Figure 29 A). Upon serial dilution on solid galactose medium, the presence of the pNAT-R1R2 plasmid severely delayed the appearance of colonies for both the cir^+ and cir^0 strains relative to those transformed with the pNAT vector. Once formed, the colonies of pNAT-R1R2 strains, but not pNAT strains, displayed lethal sectoring. This phenotype was more pronounced for the strain that lacked native 2µm plasmid (cir^0), perhaps due to the expression of Raf from the native 2µm plasmid in cir^+ strains, although the toxic phenoteype was readily observable for both strains. This difference between the toxic phenotype observed in cir^+ and cir^0 strains was not observed in the W303 background (data not shown), perhaps because W303 strains have lower 2µm plasmid copy number levels than BY4741 strains (data not shown), which could in turn lead to insufficient Raf expression in W303 cir^+ strains to impact Rep protein-mediated toxicity. Overall, these results demonstrate that the Rep protein-mediated toxicity documented in W303 and other yeast strain backgrounds also occurs in the BY4741 background, and therefore that assays for changes to this phenotype can be performed using the EUROSCARF complete collection of viable haploid yeast gene deletion mutant strains.

The pNAT-R1R2 plasmid was introduced into the deletion strain collection by mating the strains of the collection with a strain (Y2454 cir^+) transformed with the pNAT-R1R2 plasmid. Haploid segregants bearing the plasmid and the gene deletion of interest were selected, and the ability of strains to form colonies on solid medium containing galactose and solid medium containing glucose was compared. Growth on galactose medium activated expression of *REP1* and *REP2*, under the control of a galactose-inducible promoter on pNAT-R1R2, leading to delayed colony growth and a nibbled colony phenotype for the majority of strains. Glucose plates served as a control to verify that strains were able to form colonies in the absence of Rep protein

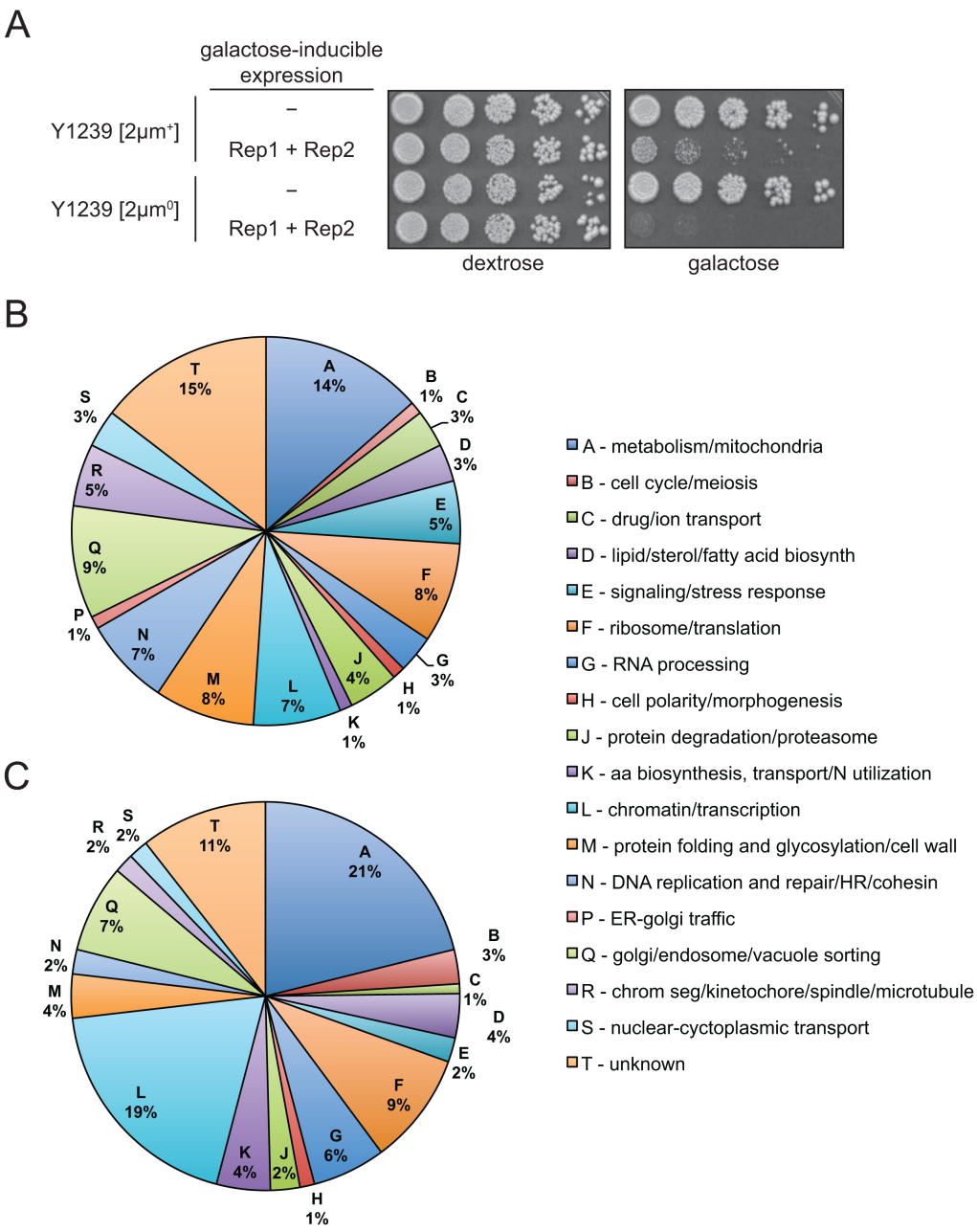


Figure 29. Summary of screen for gene deletions that affect Rep protein overexpression-mediated toxicity. (A) Isogenic BY4741 *cir*$^{+}$ and *cir*^{0} strains were transformed with either pNAT or pNAT-R1R2, as indicated, and serially diluted and spotted onto matched plates either containing glucose (left) or galactose (right) to induce the expression of Rep1 and Rep2 from the *GAL1/10* promoter on pNAT-R1R2. (B) and (C) Strains that gave small (B) or large, smooth colonies (C) were classified according to the assignments described in (Costanzo *et al.*, 2010). "N" stands for nitrogen, "chrom seg" for chromosome segregation.

overexpression after the mating, sporulation and passages required to select the haploids of interest.

A number of strains were able to grow on dextrose medium but not galactose medium, suggesting that they were either unable to metabolize galactose, or particularly sensitive to high levels of Rep1 and Rep2. A control screen of the yeast gene deletion collection, in which deletion strains containing an empty *CEN* vector (pEMBL) had been cultured on galactose medium, was used to identify strains that were unable to form colonies when the carbon source was galactose (A. Flamin, unpublished results). After cross-referencing, 96 strains were identified as being able to form colonies when glucose was the carbon source, but either were not able to form colonies or had reduced colony size when galactose was the carbon source only when carrying the pNAT-R1R2 Rep protein-overexpressing plasmid (Appendix Table 11). If the Rep proteins are preventing host proteins from performing their normal roles in the host, either directly, by sequestration, or indirectly, by transcriptional repression, then genes deleted in these strains, which show particularly small or absent colonies upon Rep protein overexpression, may be those that function in similar processes to proteins that are sequestered or repressed by the Rep proteins.

The majority of the genes deleted in strains identified with this phenotype had products involved in biosynthesis, metabolism and transport. As these processes occur mainly outside the nucleus, any interference of the Rep proteins in these processes would likely be indirect, for example, through transcriptional repression of the genes involved, as Rep proteins are normally localized to the nucleus. However, when overexpressed, Rep1 has been observed by immunofluorescence to localize throughout the cell, and Rep2 to the area just beyond the nuclear periphery (Scott-Drew and Murray, 1998), suggesting that Rep proteins could interfere with cellular processes outside the nucleus in a more direct manner when present at high levels. In addition to genes involved in biosynthesis, metabolism and transport, several of the strains identified had deletions of genes with roles related to transcription and chromatin modification; the products of these genes could be in pathways that are targeted by Rep proteins more directly.

In addition to strains that showed increased sensitivity to Rep protein overexpression, this screen also identified strains that were able to form larger, smoother

colonies than most (Appendix Table 12), suggesting that Rep protein overexpression was not as detrimental to these strains. Like those of the strains identified above as showing little or no growth in the presence of Rep protein overexpression, the protein products of the genes deleted in these strains may be present in similar pathways to those targeted either directly or indirectly by the Rep proteins. However, in contrast to the proteins that exacerbate the Rep protein-mediated toxicity when absent, proteins that relieve Rep protein-mediated toxicity when absent are likely those that antagonize the cellular functions inhibited by Rep proteins. Alternatively, some of the genes deleted in these strains may be those required by the Rep proteins to mediate their toxic effects, for example, histone deacetylases or chromatin remodelers associated with transcriptional repression. Strains lacking the proteins necessary for Rep-mediated toxicity would be expected to show similar growth in both the presence and absence of Rep protein overexpression. If any of these putative Rep protein targets were required for normal growth, suppressor mutations accumulating in the gene deletion strain over many generations of culturing in the absence of this protein might account for the normal size and smooth appearance of these colonies.

Among the genes deleted in the 246 strains identified with large, smooth colonies were many encoding transcription factors and chromatin remodelers, which could be involved in modulating Rep protein-mediated transcriptional repression. Strains with deletions of genes involved in biosynthesis, metabolism, transport and signaling were also identified; the products of these genes are candidates for those that may be involved in pathways targeted directly or indirectly by the Rep proteins.

Further work is needed to validate the gene deletions identified in this genome-wide screen as having functional overlap with the 2μm plasmid Rep proteins.

Chapter 4 DISCUSSION

4.1 General Overview

Maintenance of the yeast 2µm plasmid involves equal partitioning of plasmid copies during host cell division, amplification of plasmid copy number, and strict transcriptional regulation of the plasmid-encoded proteins required for these activities. Partitioning is dependent on the association of the Rep1 and Rep2 proteins encoded by the plasmid with a region of repeated sequence at the plasmid *STB* locus called *STB-proximal* (*STB-P*); amplification requires the plasmid-encoded Flp recombinase; and transcriptional regulation is mediated by Rep1, Rep2 and plasmid-encoded Raf.

In this thesis, I have presented evidence that improves our understanding of the partitioning complex formed at the *STB-P* locus. I show that association of Rep1 with Rep2 and Rep2 self-association contribute to efficient plasmid partitioning, but that partitioning can still occur if one of these two interactions is impaired, provided Raf is present. I demonstrate that Raf is able to stabilize both Rep1 and Rep2 protein levels, and may also play a more direct role in partitioning by complementing functions of Rep2 other than that of Rep protein stabilization.

I also investigated the DNA of *STB-P*, to better understand the sequence requirements for a Rep protein-mediated partitioning locus. I show that two directly-arrayed copies of a single 63-bp stretch of *STB-P* are sufficient to confer partitioning function. Mutation of either of two TGCA sequences, a TGCA-adjacent T-tract or a CGCG sequence found in the synthetic *STB* repeat was seen to impair partitioning function. Mutation of either TGCA sequence also impaired Rep protein association with the *STB* repeats, while mutation of the CGCG sequence impaired synthetic *STB* repeat-driven transcription.

I expanded my investigation of 2µm plasmid sequences to the divergent promoter region that controls transcription of the *FLP* and *REP2* genes (*FLP/REP2p*), and found that it bore several similarities to the *STB-P* sequence. Mutation of CGCG sequences in *FLP/REP2p* impaired its ability to promote transcription, while mutation of TGCA sequences impaired Rep protein-mediated repression of *FLP/REP2p*, consistent with loss

of Rep protein recognition sequences from this promoter. I also showed that *FLP/REP2p* was able to mediate partitioning of an *ARS* plasmid in a Rep protein-dependent manner, suggesting that there may be functional similarities between promoter regions and the *STB-P* locus.

The 2µm plasmid requires several host proteins for its maintenance. I found that the RSC2 chromatin remodeling complex, which has a well-established role in plasmid partitioning, is involved in *STB-P*-driven transcription and efficient association of Rep2 with *STB-P*. I also performed two genome-wide screens, through which I have identified candidate genes involved in regulating chromatin structure at *STB-P*, and Rep protein-mediated toxicity. These screens provide a foundation for future studies of the relationship between the 2µm plasmid and its host.

Overall, my findings improve our understanding of the requirements for Rep protein-mediated plasmid partitioning, and contribute to a more developed model of the partitioning complex formed at the 2µm plasmid *STB-P* locus. My findings also further our knowledge of other roles for 2µm plasmid-encoded proteins in 2µm plasmid maintenance and in other functions in the host cell.

4.2 Association Among 2µm Plasmid-Encoded Proteins, The *STB-P* Locus And Plasmid Gene Promoters

4.2.1 Rep Proteins

We have known since the 1980s that the absence of either Rep1 or Rep2 results in missegregation of the 2µm plasmid (Kikuchi, 1983), and that expression of both Rep1 and Rep2 proteins is required to repress transcription driven by the 2µm plasmid gene promoters and *STB-P* (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988). What is physically required of the Rep proteins to achieve these effects has not been established. Rep1 and Rep2 have been shown to self-associate and to associate with each other both *in vivo* and *in vitro*. Rep1 and Rep2 have also been shown to associate with *STB-P in vivo*, with the presence of Rep1 being required for efficient Rep2 association; however, *in vitro*, only Rep2 has been shown to have DNA binding activity in the absence of other 2µm proteins or host proteins (Sengupta *et al.*, 2001). In my studies, I have shown that Rep1 and Rep2 associate with the divergent *FLP/REP2* and *REP1/RAF*

promoters, but that Rep2 only does so in the presence of the native 2µm plasmid (Figures 21 and 22), suggesting that Rep1 may also be required for efficient association of Rep2 with 2µm plasmid gene promoters.

Although both Rep proteins can associate with *STB-P* (Yang *et al.*, 2004) and the plasmid gene promoters, this does not mean that both associations are required for Rep protein-mediated plasmid partitioning and transcriptional repression. However, there is support for association of Rep1 with *STB-P* being functionally important, as mutant Rep1 proteins that fail to associate with *STB-P* fail to mediate plasmid partitioning (Yang *et al.*, 2004), and, as I have shown here, a mutant Rep1 protein that is unable to associate with *FLP/REP2p* does not mediate repression of transcription from the *FLP* promoter (Figure 23 A), despite being present at steady-state levels similar to those of wild-type Rep1 (Figure 23 C).

The role of the Rep2 protein in plasmid partitioning is less clear. We have shown that, in the absence of Rep2 (Pinder *et al.*, 2013), or when Rep2 is unable to associate with Rep1 (Figures 9 B and 11 C), Rep1 protein levels are reduced. This suggests that Rep2 protects the Rep1 protein from degradation, which could be the only role of Rep2 in plasmid partitioning. No mutant Rep2 proteins have been identified for which association with *STB-P* is impaired specifically. However, I identified a Rep2 mutant that was impaired for association with both Rep1 (Figure 6 A) and *STB-P* (Figure 7 A), informed by an alignment of conceptual translations of ORFs in the position of *REP2* on 2µm-like plasmids identified in other yeast species (Figure 5), as well as the findings from previous truncation analyses (Sengupta *et al.*, 2001). This mutant, $Rep2_{D22N}$, in which aspartic acid 22 is replaced by asparagine, was unable to mediate efficient plasmid partitioning in the absence of the Raf protein (Figure 11 A), or to mediate transcriptional repression (Figure 9 A). However, upon examining the steady-state protein levels of Rep1 and Rep2, I observed that Rep1 protein levels decreased dramatically when Rep1 was co-expressed with $Rep2_{D22N}$ compared to when Rep1 was co-expressed with wild-type Rep2 (Figure 9 B), suggesting that stabilization of Rep1 by Rep2 requires association between these two proteins. The reason for the inability of $Rep2_{D22N}$ to mediate plasmid partitioning in the absence of Raf or transcriptional repression may therefore not be a direct result of its inability to associate with Rep1 or *STB-P*.

Robust association of Rep2 with *STB-P in vivo* requires the presence of Rep1. Rep1 may be needed to bridge the association of Rep2 with *STB-P*, or to modify an aspect of the *STB-P* locus, perhaps by bending the DNA or altering chromatin structure through recruitment of host proteins, that renders *STB-P* more amenable to Rep2 association. The coincidence of the impairment of $Rep2_{D22N}$ association with both Rep1 and *STB-P* is suggestive of the former scenario; however, the latter is also possible, as the low steady-state levels of Rep1 upon co-expression with $Rep2_{D22N}$ may be insufficient to modify *STB-P* in a way that allows robust Rep2 association. Changes in DNA conformation induced by the binding of partner proteins have been shown to improve the association of proteins with their target DNA sequences in other systems. For example, the affinity of the ParB protein of the P1 bacteriophage for its binding sequence at the partitioning locus, *parS*, is increased by the binding of integration host factor (IHF), a host protein that induces a bend in the DNA at *parS*, which allows ParB to contact its two binding sites in *parS* simultaneously (Funnell and Slavcev, 2004).

Prior to this study, no amino acid substitutions in Rep1 or Rep2 were published that impaired the self-association of either protein. This remains the case for Rep1; however, in this study, I generated a Rep2 mutant in which leucines 185 and 186 were replaced by alanines, and, using a two-hybrid assay, demonstrated that this mutant, $Rep2_{AA}$, was unable to self-associate (Figure 6 A). $Rep2_{AA}$ was impaired in its ability to mediate plasmid partitioning in the absence of Raf (Figure 11 A), but was not impaired in its ability to mediate repression of *FLPp*-driven transcription (Figure 9 A), which suggests that Rep2 self-association is required for partitioning, but not transcriptional repression. However, protein levels of $Rep2_{AA}$ were slightly lower than those observed for wild-type Rep2 (Figure 9 B), which suggests that Rep2 self-association helps to stabilize Rep2 protein levels; therefore, an alternative possibility is that $Rep2_{AA}$ partitioning function is impaired because a greater amount of Rep2 protein is required for partitioning than for repression. Rep1 protein levels were not reduced when Rep1 was expressed with $Rep2_{AA}$ (Figure 9 B), suggesting that low levels of Rep1 protein were not likely the reason for impaired partitioning function.

4.2.2 Role For Raf In Partitioning

A novel finding from this study was that the Raf protein was able to complement the partitioning defects associated with both $Rep2_{D22N}$ and $Rep2_{AA}$, which suggests that Raf can compensate for the loss of Rep2 association with *STB-P*, Rep1 and Rep2. These results expand on an earlier finding, that Rep1 steady-state levels were increased in the presence of Raf (Pinder, 2011), showing here that Raf can stabilize Rep1 and Rep2 protein levels, whether one or both Rep proteins is present. Raf expression increased the level of Rep1 when Rep1 was co-expressed with $Rep2_{D22N}$, suggesting that this is at least one way in which Raf compensates for the absence of association of Rep1 with Rep2. However, Rep1 levels were not decreased when Rep1 was co-expressed with $Rep2_{AA}$ at high levels in the absence of Raf, and Raf was unable to associate with $Rep2_{AA}$ in a two-hybrid assay (data not shown). This suggests that the ability of Raf to maintain plasmid partitioning function in the presence of $Rep2_{AA}$ did not involve increasing the steady-state levels of Rep1 or Rep2, and leaves the manner in which Raf compensates for the absence of Rep2 self-association unclear.

Raf may be a part of the functional *STB-P* partitioning complex when Rep2 associations are compromised. Although Raf is not required for efficient plasmid partitioning when Rep1 and Rep2 are wild type and cannot provide partitioning function in the absence of Rep1 or Rep2, I have shown that Raf associates with *STB-P* (Figure 14) and with Rep1 and Rep2 (Figure 12) *in vivo*. I found that an amino-terminal domain of Raf was sufficient for association with Rep1, while removal of this domain did not impair association with Rep2 (Figure 12 A), suggesting that Raf may be able to associate with both Rep1 and Rep2 simultaneously. In the case of $Rep2_{D22N}$, Raf may bridge the association of Rep2 with an *STB-P*-bound Rep1, to recruit Rep2 to *STB-P*. However, $B42_{AD}$-HA-$Rep2_{D22N}$ was not seen to associate with *STB-P* in a one-hybrid assay when Rep1 and Raf were also expressed from their native promoters (data not shown), suggesting that Raf does not bridge an association between Rep1 and Rep2 at *STB-P*. Alternatively, association of Raf with Rep1 may stabilize sufficient Rep1 to allow Rep1 to modify *STB-P* chromatin or DNA topology in a manner that facilitates Rep2 association.

A Rep2 mutant in which the substitutions from both $Rep2_{D22N}$ and $Rep2_{AA}$ were present ($Rep2_{NAA}$) was impaired both for association with Rep1 and for self-association (Figure 6 A). $Rep2_{NAA}$ also showed decreased efficiency of plasmid partitioning in the presence of Raf (Figure 8 A), but this decrease was not as great as that observed for either $Rep2_{D22N}$ or $Rep2_{AA}$ in the absence of Raf (Figure 11 A), suggesting that $Rep2_{NAA}$ does have some plasmid partitioning function in the presence of Raf. Relative to Rep1 protein levels, steady-state levels of $Rep2_{NAA}$ are decreased in comparison to those observed for wild-type Rep2, $Rep2_{D22N}$ and $Rep2_{AA}$ when these proteins are expressed from their native promoters on a 2µm-based plasmid that also expresses Raf (pKan; Figure 8 B). This decrease could be the reason for the reduced efficiency of partitioning of the $Rep2_{NAA}$ mutant protein.

As the $Rep2_{NAA}$ mutant cannot associate with either Rep1 or Raf (Figure 6 A, data not shown), its association with *STB-P* cannot be bridged by either Rep1 or a complex of Rep1 and Raf. This suggests that if association with *STB-P* is required for the residual partitioning function of $Rep2_{NAA}$, Rep1, stabilized by Raf, must be modifying this locus in a way that allows Rep2 association to take place. Alternatively, Rep2 may have a role in partitioning that is not dependent on association with *STB-P* DNA in a manner detectable by one-hybrid assay.

A caveat in all conclusions based on one- two-hybrid analyses is that the fusion of transcriptional activation or DNA binding domains to the amino-termini of the Rep and Raf proteins may have affected interactions monitored using these fusions. However, at least for Rep1 and Rep2, the fusion proteins are able supply partitioning function to 2µm-based plasmids that lack *REP1* and *REP2*, respectively ((Sengupta *et al.*, 2001); data not shown), suggesting that the conformation of the fusion proteins is sufficiently similar to those of the native proteins to participate in interactions required for partitioning function.

4.2.3 TGCA Sequences In The *STB-P* Locus And Plasmid Gene Promoters

Little research has been published regarding what is required of the *STB-P* sequence to allow association of Rep1 and Rep2 with this locus. Previous studies have shown that the three *STB-P* repeats closest to the origin are sufficient to mediate plasmid partitioning (Jayaram *et al.*, 1985; Huang *et al.*, 2011b), and variants of the 2µm plasmid

with anywhere from three to seven copies of the *STB-P* repeat have been identified (Xiao and Rank, 1993), which suggests that Rep proteins may only require three copies of the *STB* repeat to be able to associate with this locus and form a functional partitioning complex. Using synthetic versions of the *STB-P* sequence built of direct tandem arrays of a 63-bp stretch of the native sequence (Table 6), I found that even a single copy of this synthetic *STB* repeat was sufficient to mediate association with both Rep1 and Rep2 in a one-hybrid assay (Figure 18), despite the fact that a single copy was unable to confer plasmid partitioning function (Figure 15 A). When I tested association of Rep proteins with a functional partitioning sequence consisting of two directly-arrayed copies of the synthetic *STB* repeat, the activity of the reporter gene in the one-hybrid assay doubled compared to that observed when a single synthetic repeat was present (Figure 18). This result suggested that twice as many Rep protein molecules were associated with two copies of the synthetic *STB* repeat than were present at a single copy of the repeat. These molecules could be arranged on two copies of the repeat as two separate complexes, each identical to the complex of Rep proteins present at a single copy of the repeat, or could be in a different arrangement that is more conducive to the formation of a partitioning complex.

Previous bioinformatics analyses (Som *et al.*, 1988; Veit and Fangman, 1988) and preliminary mutation analyses (Pinder, 2011) had suggested that the sequence TGCATTTTT might be a Rep protein binding motif. Using mutant versions of the synthetic *STB* repeats (Table 6), I showed that two TGCA sequences present in the sequence chosen as the basis for the synthetic repeat in this analysis were required for the association of Rep1 and Rep2 with a directly-arrayed pair of synthetic *STB* repeats (Figure 16). For the 2μm plasmid variant found in laboratory strains of yeast, Scp1, all five copies of the native *STB-P* repeat contain at least one TGCA sequence (the "core" TGCA) that is followed by a run of three to five Ts, while only two of these also encode a second TGCA sequence (the "junction" TGCA), which is also adjacent to a run of five Ts. Additionally, there are two TGCA sequences in the *REP1/RAF* promoter, one of which is adjacent to a T-tract, and four in the *FLP/REP2* promoter, two of which are adjacent to T-tracts. Although I have not tested the effect of mutation of TGCA sequences in the plasmid gene promoters on Rep1 and Rep2 association with these

sequences, I have shown that mutation of the two TGCA sequences in the *FLP/REP2* promoter with adjacent T-tracts impairs the ability of the Rep proteins to repress transcription promoted toward both the *FLP* and *REP2* genes, which suggests that Rep protein association with this divergent promoter is impaired.

In one-hybrid assays, association of Rep2 with a single copy of wild-type or mutant versions of the synthetic *STB* repeat was reflective of Rep1 association with these sequences (Figure 18), consistent with the association of Rep2 with these sequences being bridged by Rep1. A similar agreement between Rep1 and Rep2 associations was observed with two copies of wild-type and most mutant versions of the synthetic *STB* repeat. However, for the mutant repeat in which central TGCA sequence was scrambled, association of Rep2 was impaired, while association of Rep1 was not. This suggests that, when two copies of the repeat are present, bridging by Rep1 is not sufficient to mediate wild-type levels of Rep2 association with the repeats, and that Rep2 may contribute to recognition of the core TGCA sequence. These findings also support the notion that the arrangement of Rep proteins associated with two copies of the synthetic *STB* repeat may be distinct from their association with a single copy of the repeat. Taken together with the effect of similar mutations on repression of the *FLP/REP2* promoter, these results further suggest that the Rep proteins require the TGCA sequences for recognition of all their target loci on the Scp1 2µm plasmid.

Variant forms of the 2µm plasmid isolated from *S. cerevisiae* strains have been classified as Type-I or Type-II based on sequences found at the *STB* locus. A sampling of Type-I and Type-II *STB* loci have been shown to confer partitioning function in the presence of Rep proteins supplied by other 2µm plasmid variants (Rank *et al.*, 1994a), demonstrating that the Rep protein recognition motif is likely conserved among *STB* sequence variants. The commonly studied 2µm variant used for my studies, Scp1, belongs to the Type-II class of plasmid variants, which encode three to seven copies of the 62- to 63-bp sequence defined as the *STB-P* repeat for the purposes of my study (Xiao and Rank, 1993). The *STB-P* repeats show little sequence variation between members of this class, with the TGCA sequences that I have shown to be required for Rep protein association being conserved in all. The conservation of the TGCA sequences in the 2µm plasmid Type-II variants supports the hypothesis that this is a Rep protein recognition

motif. The sequences of Type-I 2µm plasmid variant *STB* loci are only 70% identical to the Type-II *STB* loci and encode just two regions that show modest similarity to the Type-II variant *STB-P* repeat (Xiao *et al.*, 1991a). When aligned, Type-I and Type-II repeats do have a conserved core of ~23-bp, as described by Rank and co-workers (Rank *et al.*, 1994b). However, Type-I *STB* loci do not contain TGCA sequences in either the central or junction position within their *STB-P* repeats, although this sequence is found in other places within and flanking the *STB* locus. In place of the central TGCA, *STB* Type-I variants carry the sequence TGTA, while in the position of the junction TGCA, *STB* Type-I variants do not show a consensus. This suggests that the Rep protein recognition motif may allow variations at the third position. My analysis using the synthetic *STB* repeat showed that mutating the central TGCA to TGTA, as is seen in the *STB* Type-I variants, did not impair partitioning or Rep protein association, while mutating the central TGCA to TGAA did impair partitioning and Rep protein association. Based on these observations, I suggest that the Rep protein recognition motif is TGYA, where Y is a pyrimidine (C or T). In addition to the two TGCA sequences examined in my studies, there is a TGTA sequence in my wild-type synthetic *STB* repeat (Table 6). No association of Rep proteins with a single copy of the synthetic *STB* repeat in which both TGCA sequences were scrambled, but the TGTA sequence was intact, was observed in a one-hybrid assay (Figure 18). This suggests that either the TGTA sequence at this position does not mediate Rep protein association, or a single copy of the TGYA sequence is unable to mediate a detectable level of Rep protein association in a one-hybrid assay.

4.2.4 Models Of Plasmid Partitioning And Transcriptional Repressor Complexes

Together, the results of my analyses of protein and DNA sequence requirements for associations among Rep1, Rep2 and 2µm plasmid sequences, and the roles of these associations in partitioning and repression, combined with previous studies, allow models for both the Rep protein partitioning and transcriptional repressor complexes to be described (Figure 30). For the purposes of these models, I have assumed that Rep2 association with the DNA of *STB-P* and 2µm gene promoters is functionally relevant to these complexes, although, as outlined above, this is not definitive.

In the models of both partitioning and repression, a dimer of Rep1 molecules could associate with a pair of TGCA sequences (Figure 30 A and B, part (I.)). In a one-

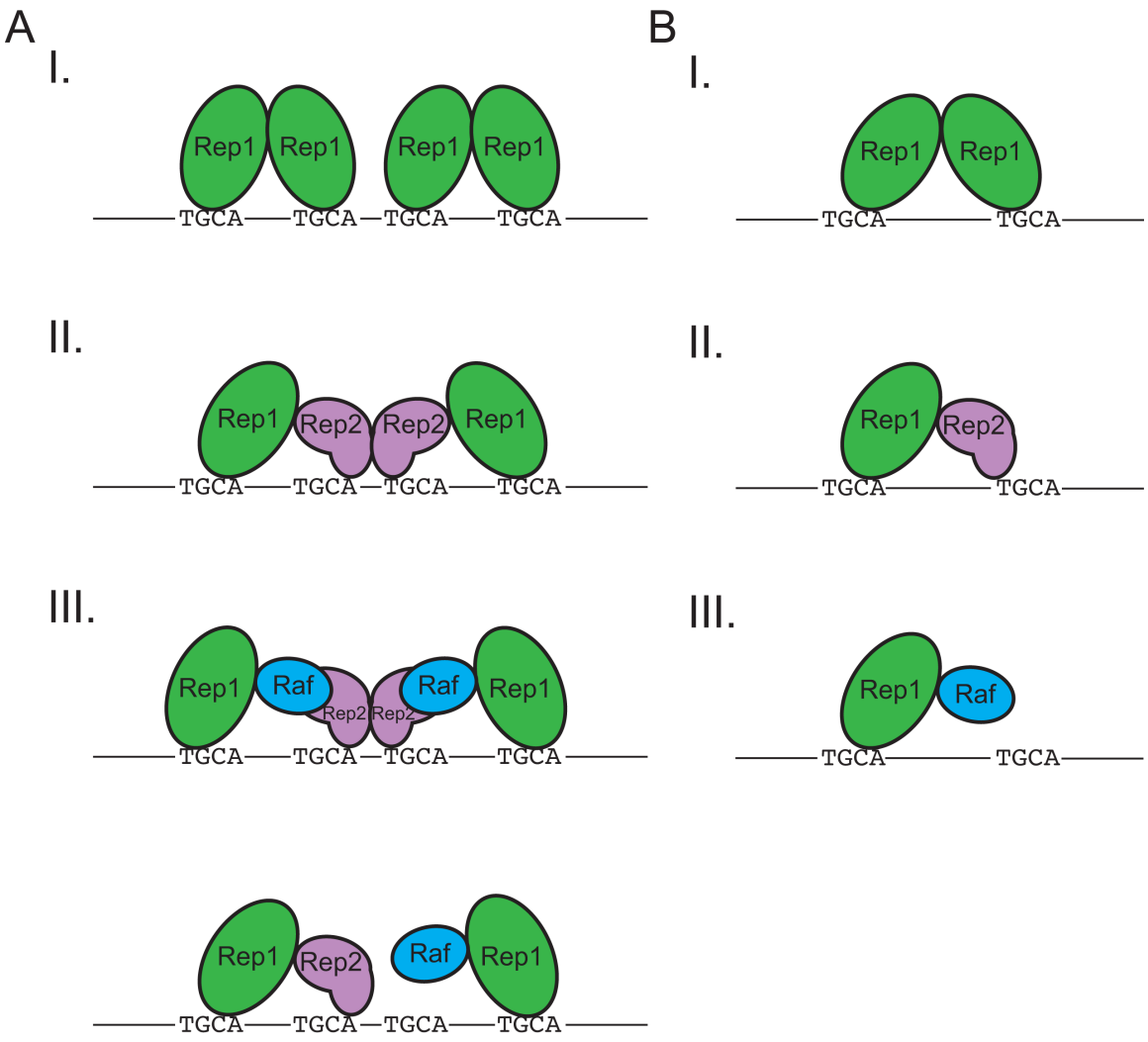


Figure 30. Models of 2µm plasmid partitioning and transcriptional repressor complexes. Partitioning complex models are shown in (A); transcriptional repressor complex models are shown in (B). In both (A) and (B), (I.) shows the initial association of Rep1 with DNA as a homodimer, while (II.) shows the functional complexes made up of both Rep1 and Rep2 molecules. (III.) of (A) and (B) shows how Raf might interact with these complexes. See text for references to experimental evidence.

hybrid assay, activation of the reporter gene by association of Rep1 with a single copy of the synthetic *STB-P* repeat in which one of the two TGCA sequences in the repeat was scrambled, was less than a quarter of that observed with a synthetic *STB* repeat in which both TGCA sequences were intact (Figure 18), suggesting that a pair of TGCA sequences is required for robust interaction. Consistent with this is the observation that Rep1 association with a mutant *STB* repeat having a single TGCA sequence, was significantly improved when this mutant repeat was present as two directly-arrayed copies versus a single copy, although two copies of these mutant repeats were still compromised for partitioning function (Figure 16). Additionally, in one-hybrid assays using truncated versions of Rep1, I have seen that Rep1 association with a single copy of the synthetic *STB* repeat is impaired when the domain required for Rep1 homodimerization is truncated, although not all Rep1 truncations that are able to homodimerize associate with *STB* (data not shown); this suggests that Rep1 may associate with *STB-P* preferentially as a dimer, although Rep1 dimerization and DNA association may be performed by separate domains. Obtaining mutant forms of Rep1 that are impaired for homodimerization would allow this model to be tested. Taken together, these observations could indicate that a dimer of rather than a single Rep1 transcriptional activation domain fusion protein molecule forms a stable association with reporters carrying two TGCA sequences, while only one fusion protein is recruited to reporters with a single TGCA, resulting in a less stable association. These hypothetical stoichiometries suggest that a minimum of four Rep1 molecules, possibly arranged as dimers on each repeat, is needed for establishing the partitioning complex at *STB-P*.

As Rep2 association with *STB-P* and 2µm gene promoters was not observed in one-hybrid assays in this or a previous study in the absence of Rep1 (Pinder *et al.*, 2013), I suggest that Rep2 could associate with *STB-P* and gene promoters only following Rep1 association (Figure 30 A and B, part (II.)). There is some evidence suggesting that Rep2 can associate with *STB-P* in the absence of Rep1. Rep2 has *in vitro* DNA binding activity, which shows a preference for *STB-P* sequences (Sengupta *et al.*, 2001). Additionally, the Jayaram lab has reported robust association of Rep2 with *STB-P* in absence of Rep1 in a ChIP assay (Mehta *et al.*, 2002; Yang *et al.*, 2004), although the results of these assays were not quantified, and used only ChIP performed in the absence

of antibody as a negative control, which I have found to be less reliable for establishing specific association than using an unrelated antibody (data not shown). ChIP analyses in our lab have also suggested that Rep2 may associate weakly with *STB-P* in the absence of Rep1 (Pinder *et al.*, 2013). However, I have observed that our polyclonal Rep2 antibody immunoprecipitates a greater percentage of input DNA compared to other antibodies for all loci tested (data not shown), so if results are not normalized to the percent of input DNA immunoprecipitated for a control DNA locus, false positives can occur; this could potentially be the case for results from our lab, as well as the Jayaram lab, which also used our anti-Rep antibodies in their studies (Mehta *et al.*, 2002; Yang *et al.*, 2004).

These issues with ChIP analyses raise the possibility that Rep2 may not recognize *STB-P* independently *in vivo*. Instead, Rep2 may be recruited to *STB-P* subsequently to or in concert with Rep1, either through direct interaction with Rep1 or by reconfiguration of the DNA by Rep1 to facilitate Rep2 DNA binding. Because $Rep2_{NAA}$ has some partitioning function (Figure 8 A), despite the fact that it does not associate with Rep1 or Raf in a two-hybrid assay (Figure 6 A), and, on this basis, should no longer be recruited to *STB-P* by either of these proteins, I suggest that Rep2 can be recruited to *STB-P* by a combination of both direct interaction with Rep1 and *STB-P* reconfiguration by Rep1. The observation that Rep2 association with two copies of the synthetic *STB* repeat can be impaired by mutations of the core TGCA sequence (Figure 18 C), which do not impair Rep1 association with two copies of the synthetic *STB* repeat (Figure 18 B), provides further evidence that Rep2 association with *STB-P* is not a simple case of bridging by Rep1, and may involve sequence-specific DNA recognition by Rep2. The role of Raf in partitioning when Rep2 is unable to associate with Rep1 is likely to stabilize Rep1 protein levels; however, a role for Raf in the *STB-P* partitioning complex, for example, in destabilizing Rep1 homodimers, is also possible (Figure 30 A, part (III.), bottom).

In vitro competition experiments suggest that Rep1 is unable to associate with itself and Rep2 simultaneously, and that association of Rep1 with Rep2 may be favored over Rep1 homodimerization (Scott-Drew and Murray, 1998). Therefore, upon association of Rep2 with *STB-P* or 2µm gene promoters, one molecule of the Rep1 homodimer may be displaced by a molecule of Rep2 (Figure 30 A and B, part (II.)).

I have shown that Rep2 self-association or the presence of Raf is required for plasmid partitioning (Figure 30 A, part (II.) and (III.)), but not transcriptional repression (Figure 30 B, part (II.)). This suggests that a single heterodimer of Rep1 and Rep2 may be sufficient to mediate transcriptional repression at plasmid gene promoters, but that the arrangement of Rep proteins at *STB-P* for partitioning may be more complex. As two copies of the synthetic *STB* repeat are required for partitioning (Figure 15 A), a functional partitioning complex may consist of two heterodimers of Rep1 and Rep2 oriented to allow the Rep2 molecules to associate with each other, forming a tetramer (Figure 30 A, part (II.)). Association of Rep2 with Rep1 and with itself is able to occur simultaneously and non-competitively *in vitro* (Scott-Drew and Murray, 1998), suggesting that this arrangement is possible. The carboxy-terminal portion of Rep2 is highly basic (Table 5) and has DNA binding activity (Sengupta *et al.*, 2001) that would allow both Rep2 molecules, each in association with a Rep1 molecule, to make appropriate contacts with the *STB-P* DNA. For the $Rep2_{D22N}$ mutant, which is impaired for association with Rep1, the presence of Raf in the partitioning complex may serve to bridge the association of Rep1 and Rep2, or to favour a DNA or protein arrangement at *STB-P* that promotes, and is required for, Rep2 association with *STB-P* in the absence of association between Rep1 and Rep2 (Figure 30 A, part (III.), top). For the $Rep2_{AA}$ homodimerization mutant that cannot provide full partitioning function in the absence of Raf, homodimerization must be compensated for by Raf, perhaps by enabling appropriate placement of the carboxy-terminal region of Rep2 in the partitioning complex. Raf, a smaller protein lacking the basic carboxy-terminal region that Rep2 has, might be able to replace a homodimerization-impaired Rep2 bound to one of the Rep1 proteins, so that the arrangement on *STB-P* is two TGCA-associated Rep1 molecules, one bound to a Rep2 molecule and the other to a Raf molecule, enabling a more functional placement of the sole Rep2 protein in the complex (Figure 30 A, part (III.), bottom).

4.2.5 Role Of Raf In Relieving Rep Protein-Mediated Transcriptional Repression

Prior to my studies, it was known that Raf played a role in relieving Rep protein-mediated transcriptional repression of plasmid-encoded genes and of the 1950-nt transcript driven by *STB-P* (Murray *et al.*, 1987); however, the mechanism by which Rep proteins mediated this repression, and by which Raf counteracted their effects, was

unclear. As described in the model above, the Rep proteins may repress transcription by association with the TGCA sequences, recognized primarily by Rep1, as a heterodimer of Rep1 and Rep2 (Figure 30 B (II.)). Raf may inactivate this complex by binding Rep1 and displacing Rep2, leaving a heterodimer of Rep1 and Raf at the promoters and *STB-P* instead (Figure 30 B, part (III.)). Association of Rep1 with Rep2 in a two-hybrid assay has been seen to decrease upon Raf overexpression (Pinder, 2011), suggesting that Rep2 and Raf compete for association with Rep1. Two-hybrid assays performed using truncated Rep1 proteins show that Rep2 and Raf both associate with the same region of the Rep1 protein (Figure 12 D), which provides further evidence that Rep2 and Raf may be unable to associate with Rep1 simultaneously.

4.2.6 Evolutionary Relationship Of Rep2 And Raf

Due to the similarities I have observed in the interactions of Rep2 and Raf with 2µm proteins and DNA sequences *in vivo*, and due to their involvement in the same plasmid functions, I hypothesize that Rep2 and Raf are paralogs. As the 2µm plasmid forms multimers and undergoes intramolecular recombination events as part of its amplification strategy, it is easy to imagine how a duplication of one of the genes required for partitioning could occur. Since this duplication, Rep2 and Raf could have evolved so that their homology is no longer evident from their amino acid sequences. In this scenario, Raf could have lost its ability to mediate partitioning without assistance from the Rep2 protein, but been maintained as a part of the 2µm genome due to its ability to perform functions not performed by Rep2, for example, relieving Rep protein-mediated transcriptional repression. I suggest that these functional differences between Rep2 and Raf stem from the fact that Raf does not have a basic carboxy-terminal domain, while Rep2 does. A truncation of Rep2 containing only the 65 carboxy-terminal residues is sufficient for DNA binding in a southwestern assay (N. Arumuggam, Dalhousie University, Honours BSc thesis). This region of Rep2 is highly basic (pI = 11.5); therefore, whether the interaction of Rep2 with DNA *in vitro* is sequence specific or merely the result of non-specific associations between the acidic DNA backbone and basic residues in Rep2 is unknown. Raf, which lacks an equivalent basic region, may be unable to bind DNA directly, associating with DNA loci only through interactions

bridged by Rep1, and therefore may also be unable to mediate plasmid partitioning or transcriptional repression.

Of the 2µm-like plasmids described in other yeast species to date, only two encode a fourth ORF in the position of *RAF*. These plasmids, pKW1 and pSM1, were isolated in the yeast species *Kluveromyces waltii* and *Zygosaccharomyces fermentati*, which are more closely related to each other than to any other Saccharomycetaceae family species carrying 2µm-like plasmids (Hedtke *et al.*, 2006). Experimental analysis showed that the *RAF*-positioned ORF of pKW1 is not a partitioning protein, as it did not contribute to plasmid inheritance and was unable to substitute for the *REP1*- or *REP2*-positioned ORF (Chen *et al.*, 1992); the functions of the *REP1*-, *REP2*- or *RAF*-positioned ORFs of pSM1 have not been analyzed experimentally.

The proteins encoded by the *RAF*-positioned ORFs of pKW1 and pSM1 show greater similarity to each other than to their respective *REP2*-position ORF-encoded proteins (data not shown), suggesting that the duplication that gave rise to the *RAF*- and *REP2*-position genes on these plasmids occurred in a common ancestor, prior to plasmid divergence. Several species of 2µm-like plasmid-containing yeast that are more closely related to *S. cerevisiae* than either *Z. fermentati* or *K. waltii* do not encode *RAF*-positioned ORFs. Whether both *RAF* and the *RAF*-positioned ORFs of pKW1 and pSM1 arose from the same duplication in an ancestral form of the 2µm plasmid and were subsequently lost from the rest of the 2µm-like plasmids, or whether these ORFs arose from separate duplication events, is unclear.

4.3 Other Sequences Required For *STB-P*-Mediated Partitioning

Mutational analyses of *STB-P* identified a T-tract adjacent to the core TGCA sequence and a CGCG sequence as contributors to the partitioning function of two directly-arrayed copies of the synthetic *STB* repeat (Figure 16), but not to Rep protein association with this sequence (Figure 18 B and C). Mutation of the T-tract and CGCG sequence did, however, impair association of Rep proteins with a single copy of the synthetic *STB* repeat (Figure 18 B and C), which is non-functional (Figure 15 A). This suggests that the T-tract and CGCG sequence do contribute to Rep protein association with *STB-P*, but that this may not be their main role in partitioning. The impact of the T-

tract and CGCG sequence on Rep protein association with *STB-P* could instead be an indirect effect of a more vital role.

4.3.1 Potential Roles Of T-Tract And CGCG Sequence In DNA Topology

The role of the T-tract and CGCG sequence in partitioning could be to regulate nucleosome positioning at *STB-P*. DNA consisting exclusively of either G and C nucleotides or A and T nucleotides is inherently less flexible than DNA containing all four nucleotides (Simpson and Künzler, 1979). In the case of tracts of GC-rich sequences, this rigidity is attributed to the fact that there is more hydrogen bonding between G and C nucleotides than A and T nucleotides. For AT-rich sequences, this rigidity is likely due to the novel conformation of the double-helix for these sequences, which is distinct from the canonical double-helix, having a greater twist at each base pair that allows non-Watson-Crick cross-strand hydrogen bonds to form (Nelson *et al.*, 1987). AT-rich regions of DNA, in particular homopolymeric runs of A or T, are preferentially associated with nucleosome-free regions, both *in vivo* and *in vitro* (reviewed in (Radman-Livaja and Rando, 2010)). A- and T-tracts as short as four nucleotides in length have been observed to favour nucleosome positioning that places them at the ends of the nucleosomal DNA sequence, which show the least curvature (Satchwell *et al.*, 1986). In addition to translational positioning, nucleotide sequences can show preferences for particular rotational positioning in the nucleosome particle, with A- and T-tracts preferring to be positioned so that the minor groove faces inward toward the histones, and CG-tracts preferring to be positioned so that the minor groove faces outward (Drew and Travers, 1985).

The roles of the T-tract and CGCG sequences in the *STB-P* sequence could be to set the positioning or rotational orientation of the TGCA sequence on the nucleosome particle in a manner that facilitates recognition by Rep1 and Rep2. In particular, the T-tract shown to be required for efficient partitioning may impact the rotational positioning of the central TGCA, as it is directly adjacent to this sequence. Positioning of the TGCA sequence in the nucleosome particle by the T-tract could be necessary for efficient recognition of the TGCA sequence by Rep proteins when a single copy of the synthetic *STB* repeat is present, but not be required when two copies of the synthetic *STB* repeat are

present, perhaps due to the increased number of TGCA sequences leading to cooperativity among Rep protein associations.

The T-tract and CGCG sequence could also contribute to partitioning through nucleosome positioning for purposes other than facilitating Rep protein association with *STB-P*. One possibility is that the T-tract, together with several other T- and A-tracts found in *STB-P*, or the CGCG, in conjunction with a nearby GCGC sequence, helps to destabilize nucleosomes at *STB-P*, which may be important for the roles of host factors in *STB-P* partitioning. Some host factors that have been shown to associate with *STB-P* and may require nucleosome destabilization to perform their partitioning roles include the RSC2 chromatin remodeling complex (Wong *et al.*, 2002; Huang *et al.*, 2004; Ma *et al.*, 2012), which can move or replace histones, and Cse4 (Hajra *et al.*, 2006), the centromere-specific histone that has been shown to replace histone H3 at *STB-P*.

4.3.2 Potential Roles For T-Tract And CGCG Sequence As Host Factor Recognition Motifs

Alternatively, or in addition, to a structural role at *STB-P*, the T-tract and CGCG sequences could act as recognition motifs for host proteins required for partitioning function. Two host proteins have been identified to date that specifically recognize A- or T-tracts. One of these is datin, which has been shown to behave both as a transcriptional activator (Moreira *et al.*, 1998) and repressor (Iyer and Struhl, 1995), and recognizes A- or T-tracts ten to eleven base pairs in length (Reardon *et al.*, 1995). The other is Stb3, a ribosomal RNA processing element binding protein involved in gene repression in response to glucose starvation (Liko *et al.*, 2007; Liko *et al.*, 2010), which recognizes the motif TTTTTCA (Zhu *et al.*, 2009). In both cases, the sequences outside of the 5-bp T-tract in the *STB-P* repeat do not match those of the identified datin or Stb3 recognition sequences; it therefore seems unlikely that the T-tract would be recognized by either datin or Stb3. There is also no evidence to suggest that either datin or Stb3 have roles in 2µm plasmid maintenance. Neither of the strains containing deletions of these genes were identified in either of my genome-wide screens as having altered *STB-P* chromatin or as having altered 2µm copy number. Overall, these observations suggest that the T-tract may be more likely to impose structural constraints on *STB-P* or improve recognition of *STB-P* by Rep proteins, than to act as a host protein recognition sequence.

The sequence CGCG is part of the recognition motifs identified for three host factors: the RSC chromatin remodeling complex (Badis *et al.*, 2008; Zhu *et al.*, 2009), and the cell cycle regulated transcription factors MBF and SBF (Iyer *et al.*, 2001; Badis *et al.*, 2008). One isoform of the RSC complex, RSC2, has previously been shown to contribute to 2µm plasmid partitioning (Wong *et al.*, 2002), although both the RSC1 and RSC2 isoforms may be present at the *STB-P* locus (Ma *et al.*, 2012). In my studies, I have shown that both subunits of the SBF complex, Swi4 and Swi6, but not the MBF-specific subunit Mbp1, contribute to transcription driven by the *STB-P* sequence (Table 7, Figure 27 A), suggesting that the SBF complex influences *STB-P* chromatin structure. A perfect consensus to the SBF recognition motif, CGCGAAA (Iyer *et al.*, 2001; Badis *et al.*, 2008), is found once in *STB-P*, while a sequence similar to the consensus, CGCGAGA, is found three times. Although I observed that 2µm plasmid partitioning was slightly impaired in the absence of Swi6, I was able to detect Swi4, the DNA-binding subunit of SBF, at chromosomal targets only and not at *STB-P* in a ChIP assay. This suggests that the influence of SBF on plasmid partitioning may be indirect, or that its interaction with *STB-P* is transient or unstable.

4.4 The RSC Chromatin Remodeling Complex

With its documented role in plasmid partitioning, the RSC2 complex may be more likely than SBF to interact with the CGCG sequence in *STB-P* in a manner required for partitioning. Both deletion of the *RSC2* gene, which is presumed to inactivate the RSC2 complex, and mutation of CGCG to TGTG in two directly-arrayed synthetic *STB* repeats, result in a similar decrease in plasmid partitioning efficiency (Figure 19 A). However, upon deletion of the *RSC2* gene and mutation of the CGCG sequence simultaneously, a further decrease in plasmid partitioning efficiency is observed. This suggests both that the CGCG sequence plays a role in plasmid partitioning that does not involve the Rsc2 protein, and, conversely, that the Rsc2 protein has a partitioning function that does not require the CGCG sequence.

If RSC2 is not wholly responsible for the partitioning defect associated with mutation of the CGCG sequence, then perhaps this is because the RSC1 complex can perform some of the same plasmid partitioning functions as RSC2. Many of the roles of RSC1 and RSC2 in the host can be carried out by either isoform of the complex, so it is

possible that this is true for some aspects of the role of the RSC2 complex in 2µm plasmid partitioning. The Rsc1 protein, which is found exclusively in the RSC1 isoform, has been shown to co-purify with Rep1 in tandem-affinity purifications (Ma *et al.*, 2012), implying that Rsc1 is capable of interacting with the *STB-P* locus. Deletion of the gene encoding Rsc1 does not impair plasmid partitioning (Wong *et al.*, 2002), suggesting that if the RSC1 complex plays any role in partitioning, it is only in the absence of a functional RSC2 complex.

Although CGCG is the only recognition sequence that has been identified for the RSC2 complex, there is evidence to suggest that this complex may also be able to associate with DNA in a non-sequence-specific manner, which could account for the observation that deletion of the *RSC2* gene impairs partitioning function in the absence of an intact CGCG sequence (Figure 19 A). The sequence-specific DNA binding subunits of RSC, Rsc3 and Rsc30, have been reported to associate preferentially with the RSC1 isoform (Campsteijn *et al.*, 2007), while the Rsc2 protein has been shown to be associated with sub-stoichiometric amounts of Rsc3 and Rsc30 (Chambers *et al.*, 2012). This implies that the recruitment of RSC2 is less dependent on the CGCG sequence than the recruitment of RSC1. Homologs of the RSC complex in other non-fungal eukaryotes do not contain subunits homologous to Rsc3 and Rsc30, but instead associate with chromatin through contacts with histones and DNA in the absence of a specific recognition sequence (reviewed in (Mohrmann and Verrijzer, 2005)); some forms of the RSC2 complex in *S. cerevisiae* could therefore be recruited in this manner. The Rsc2 protein itself contains two bromodomains, which recognize acetylated amino-terminal histone tails, and an AT-hook domain, which binds in the minor groove of AT-rich DNA (Cairns *et al.*, 1999), suggesting that it is possible that the Rsc2 protein itself associates with the *STB-P* locus and has a role in partitioning separate from the RSC2 complex.

Overall, data obtained from experiments involving synthetic *STB* sequences with the CGCG sequence mutated to TGTG and yeast strains lacking the *RSC2* gene (Figure 19) suggest that the RSC complex may have two roles in plasmid partitioning: one that requires the CGCG sequence and can be performed by either the RSC1 or RSC2 isoform, and the other that occurs independently of the CGCG sequence and requires the Rsc2 protein specifically. Roles for either isoform of the RSC complex at the *STB-P* locus may

be similar to their roles in the host, which include involvement in transcription, cohesin recruitment, chromosome segregation and DNA repair (reviewed in (Clapier and Cairns, 2009)). The *STB-P* sequence promotes transcription of a 1950-nt transcript in the direction of *STB-D* (Sutton and Broach, 1985), and I have shown that both the CGCG sequence and the Rsc2 protein are required for efficient transcriptional activation by *STB-P* (Figure 20 B); however, the role of this transcription, if any, in plasmid partitioning is still unknown. As cohesin has been shown to associate with *STB-P* and is required for efficient plasmid partitioning, the RSC complex could be involved in cohesin recruitment to *STB-P*. The 2µm plasmid has been shown to missegregate with the host chromosomes (Mehta *et al.*, 2002; Mehta *et al.*, 2005; Liu *et al.*, 2013), and so the RSC complex could also contribute to partitioning function independently of association with the *STB-P* locus through its involvement in chromosome segregation.

My results suggest that one of the CGCG-independent roles of the RSC2 complex or Rsc2 protein in 2µm plasmid partitioning is in regulating the relative associations of Rep1 and Rep2 proteins with *STB-P*. In a one-hybrid assay using a strain deleted for the *RSC2* gene and carrying a reporter with two copies of the synthetic *STB* repeat upstream of the reporter gene, I observed increased reporter activity in the presence of activation domain-fused Rep1 and decreased reporter activity in the presence of activation-domain fused Rep2, relative to the activities observed when the *RSC2* gene was present. When a single copy of the synthetic *STB* repeat was used in the one-hybrid assay, deletion of *RSC2* resulted in increased reporter gene activation in the presence of activation domain-fused Rep1 and Rep2. These observations support a role for the RSC2 complex or Rsc2 protein in mediating Rep protein association with *STB-P*. They also provide further evidence that the manner in which Rep proteins associate with one versus two copies of the synthetic *STB* repeat is distinct, and suggest that Rep1 may compete with Rep2 for association with two copies of the synthetic *STB* repeat. *In vitro* competition experiments suggest that Rep1 homodimerization and association of Rep1 with Rep2 are mutually exclusive; the RSC2 complex or Rsc2 protein could therefore alter the relative amount of Rep1 and Rep2 association at *STB-P* by favouring heterodimerization of Rep1 and Rep2 over Rep1 homodimerization. An increase in Rep1 homodimerization on *STB-P* in the absence of Rsc2 could explain the increased protection of the *STB* repeats from

micrococcal nuclease digestion observed when yeast lack the Rsc2 protein (Wong *et al.*, 2002), if oligomers of Rep1 provide more protection than heterodimers of Rep1 and Rep2.

4.5 Role Of *STB-P*-Driven Transcription In Partitioning

Mutation of the CGCG sequence to TGTG, or deletion of the *RSC2* or *SWI6* genes leads to a decrease in both plasmid partitioning efficiency and transcription driven by synthetic *STB* repeats. However, whether *STB-P* transcriptional activity has an impact on partitioning function, or is merely a consequence of recruitment of host factors and chromatin remodeling at the *STB-P* locus required for partitioning function, is unclear. The unwinding of DNA in the vicinity of *STB* as a part of the transcriptional initiation process could promote reconfiguration of *STB-P* chromatin. High levels of transcription through both the *STB-P* sequence (Murray and Cesareni, 1986) and host centromeres (Doheny *et al.*, 1993) have been shown to impair plasmid partitioning and chromosome segregation, respectively; however, it has been suggested that a low level of transcription through centromeric sequences is required for efficient chromosome segregation in *S. cerevisiae* (Ohkuni and Kitagawa, 2011). Transcription of centromeric sequences has also been shown to play a role in kinetochore formation in higher eukaroytes, where evidence suggests that transcription by RNA polymerase II is required for incorporation of centromere-specific histone H3 CENP-A at centromeres (reviewed in (Scott, 2013)). As the *S. cerevisiae* CENP-A homolog Cse4 has been shown to be a component of one or both nucleosomes assembled at *STB-P* (Huang *et al.*, 2011a; Huang *et al.*, 2011b) and is required for partitioning function (Hajra *et al.*, 2006; Huang *et al.*, 2011a; Huang *et al.*, 2011b), it is possible that transcription driven by *STB-P* assists in Cse4 recruitment. Although *STB-P*-driven transcription does not span the *STB-P* locus per se, perhaps the distance between the transcribed region and *STB-P* is small enough that it can effect Cse4 recruitment to *STB-P*.

4.6 Similarities Between *STB-P* And 2µm Plasmid Gene Promoters

The *STB-P* locus and the divergent 2µm plasmid gene promoters, *FLP/REP2p* and *REP1/RAFp*, drive transcription in similar ways. Transcription from both the *STB-P* locus and the gene promoters is bi-directional ((Sutton and Broach, 1985); Figure 27 A).

Mutation of CGCG sequences both in synthetic *STB* repeat and in the gene promoters impairs this transcription (Figures 20 B and 24 A; data not shown), as does deletion of the *RSC2* gene (Figure 20 B; data not shown). Additionally, Rep1, Rep2 and Raf have been seen to associate with *STB-P*, *FLP/REP2p* and *REP1/RAFp in vivo* (Figures 14, 21, and 22), likely by recognition of the sequence TGCA, and regulate the transcription driven by these sequences (Murray *et al.*, 1987; Reynolds *et al.*, 1987; Som *et al.*, 1988). Several of these shared transcriptional features, such as CGCG sequences, the Rsc2 protein, and association of Rep1 and Rep2, are also required for *STB-P* mediated partitioning; I therefore tested whether a 2µm plasmid gene promoter, the *FLP/REP2* promoter, could confer any partitioning function to a non-2µm-based plasmid. I found that the *FLP/REP2* promoter was able to confer partitioning function in a Rep protein-dependent manner on a plasmid containing only an origin of replication (*ARS* plasmid), although this partitioning was less efficient than that observed for the native *STB-P* sequence in this context.

Overall, these similarities suggest a relationship between 2µm plasmid gene promoters and the *STB-P* locus, with *STB-P* having perhaps evolved from a gene promoter. On 2µm-like plasmids that do not encode an ORF in the position of *RAF*, the promoter of the gene encoding the Rep1 homolog and the partitioning locus, where defined (Jearnpipatkul *et al.*, 1987b; Bianchi *et al.*, 1991), have been shown to occupy the same region, suggesting that promoter of the gene of the Rep1 homolog and the partitioning locus could be the same sequence. My plasmid inheritance assays showed that a single synthetic *STB* repeat, containing one TGCATTTTT sequence and one TGCA sequence without an adjacent T-tract, provided no partitioning function (Figure 15), while the *FLP/REP2* promoter (Figure 25) and two copies of the synthetic *STB* repeat (Figure 15), each containing two TGCATTTTT sequences and two TGCA sequences without adjacent T-tracts, provided a similar degree of partitioning function in the context of a non-2µm-based *ARS* plasmid. The native *STB-P* sequence in the Scp1 2µm plasmid variant, which contains five TGCATTTTT sequences and two TGCATTT sequences (Figure 3), provides better partitioning function than either the *FLP/REP2* promoter or two copies of the synthetic *STB* repeat (Figure 15, Figure 25). This suggests that the difference between a promoter sequence and a sequence that is both a promoter

and an efficient partitioning locus may be the number of TGCATTTTT or TGCA sequences present, with more copies of these sequences conferring improved partitioning function. I found that, for the synthetic *STB* repeats, improvements in partitioning function reached a plateau at five directly-arrayed copies (data not shown), suggesting that the five copies found in the native *STB-P* sequence may be the optimum number for efficient partitioning function, at least in strains carrying the Scp1 2µm plasmid variant. Only two of the five repeats in Scp1 *STB-P* have a TGCA sequence at the position of the "junction" TGCA in the synthetic *STB* repeat (Figure 3), which suggests that more than two copies of the junction TGCA sequence may not improve plasmid partitioning. This suggestion is supported by the observation that increasing the number of directly-arrayed mutant synthetic *STB* repeats with the junction TGCA sequence scrambled but core TGCA intact led to an improvement in partitioning function, while increasing the number of directly-arrayed repeats with the junction TGCA intact but the core TGCA scrambled did not improve partitioning function to the same extent (Figure 17). This implies that further copies of the core TGCA sequence can improve partitioning function, while further copies of the junction TGCA sequence cannot.

If the point centromeres of the budding yeast Saccharomycetaceae family originated from an ancestral *STB* locus, as hypothesized by Malik and Henikoff (Malik and Henikoff, 2009), this could mean that a gene promoter is the ancestral form of the point centromere. The point centromeres of *S. cerevisiae* do share some characteristics with promoters; for example, one of the sequence-specific centromere-binding components associated with the inner kinetochore, Cbf1, also binds the promoter regions of a number of genes, and, in association with transcription factors, mediates transcriptional activation and repression (Kent *et al.*, 2004); however, neither of the Rep proteins resembles Cbf1 or any other inner kinetochore protein, so this hypothesis remains highly speculative.

4.7 Rep Protein-Mediated Toxicity

Rep1 and Rep2 have been shown to lead to host toxicity when co-expressed at high levels, leading to a cell cycle defect characterized by enlarged, elongated cells and slow-growing colonies displaying lethal sectoring (Scott-Drew and Murray, 1998). The cause of Rep protein-mediated toxicity is unknown, but it has been demonstrated that

overexpression of Raf can relieve this toxicity (Pinder, 2011). As Raf also counteracts the effect of excess Rep proteins in the 2µm plasmid gene regulatory circuit (Murray *et al.*, 1987), this observation suggests that the toxicity may be due to inappropriate repression of host gene transcription.

My findings suggest that a minimum of two TGCA sequences are required for efficient association of Rep proteins with the *STB-P* locus (Figure 18), and for Rep protein-mediated repression of transcription driven by 2µm plasmid gene promoters (Figure 24). The sequences surrounding the TGCA motif and their spacing vary between *STB-P* and promoters (Figures 3 and 24); it is therefore possible that promoters of some host genes, including genes with vital roles in cell cycle progression, contain a pair of TGCA motifs in a context that also makes them targets for Rep protein association and repression. Pairs of TGCA sequences with spacing similar to that observed at the *STB-P* and 2µm plasmid gene promoters (i.e., between 15 and 75 nucleotides apart) are found in thousands of intergenic regions within the yeast genome, suggesting that there may be additional requirements for Rep protein-mediated repression. Indeed, if the requirement for a nearby CGCG sequence, or for an adjacent T-tract, is added, a search of intergenic regions returns less than 100 candidates (data not shown). Further investigation will be necessary to determine whether a sequence-based analysis would be informative regarding genes that may be responsible for Rep protein-mediated toxicity. Alternatively, high-throughput sequencing of DNA that immunoprecipitates with anti-Rep protein antibodies when Rep1 and Rep2 are overexpressed could be carried out to determine potential genomic target sites.

The results of my genome-wide screen for gene deletion strains with altered phenotypes in the presence of Rep protein overexpression may also give a clearer picture of the mechanism of this toxicity. If the toxicity does involve Rep protein-mediated repression of host gene transcription, this screen could identify histone modifiers or other co-repressors that may be recruited by Rep1 and Rep2. My other genome-wide screen, for gene deletions that resulted in altered *STB-P*-driven transcription, may also be helpful in identifying these co-repressors, as strains that contain native 2µm plasmid, but have increased levels of *STB-P*-driven transcription, may be ones in which the gene deleted is required for Rep protein-mediated repression.

If the toxicity associated with Rep protein overexpression does not involve Rep protein-mediated repression, but instead occurs through other mechanisms, such as sequestration of host proteins required for cell cycle progression, then my screen for altered effects of Rep protein overexpression can also be informative. For example, sets of genes that belong to the same pathway and are particularly affected, either positively or negatively, by Rep protein overexpression will inform us as to which pathways host proteins targeted by Rep proteins, either for transcriptional repression or sequestration, might belong. If a host protein targeted by Rep proteins for repression or sequestration is an essential gene, then it will not have been included in my analysis, which covered only viable haploid yeast gene deletion strains. As one or several of the proteins targeted by the Rep proteins for repression or sequestration may be essential, as is suggested by the severity of the phenotype observed, repeating this screen using a collection of yeast strains in which essential genes can be inactivated may be informative.

4.8 Concluding Remarks

The 2μm plasmid was first identified nearly 50 years ago. Since that time, we have gained an understanding of how the plasmid uses efficient partitioning and strictly regulated copy number amplification to ensure its maintenance. Several host proteins with roles in plasmid partitioning have also been identified; however, questions still remain regarding the roles of plasmid-encoded components in plasmid maintenance.

I have identified aspects of the Rep1, Rep2 and Raf proteins that are required for their functions and associations with each other, with *STB-P* and with plasmid gene promoters, as well as short DNA sequences within these regions that are required for function and Rep protein association. My analyses of these associations and sequences, together with previous analyses, have allowed me to propose a model of how plasmid proteins and DNA loci interact to mediate partitioning and repressor functions. However, my results also highlight questions regarding these associations that remain to be answered; for example, are Rep proteins able to physically associate with *STB-P* DNA or do they require the assistance of a host protein? Is Rep1 homodimerization required for any Rep1 functions? And what contributors to partitioning function, both host factors and *STB-P* sequence elements, remain to be identified?

I have also demonstrated that the Raf protein, previously shown to provide no contribution to the plasmid partitioning function of wild-type Rep proteins, can complement the partitioning function of a mutant Rep2 protein that is impaired for association with Rep1 or with itself. This additional function for Raf is perhaps not surprising, as, given the small size and rapid evolution of the 2µm plasmid (Murray *et al.*, 1988), sequences that do not contribute to plasmid maintenance are likely to be rapidly eliminated. This finding highlights the redundancy among the interactions of 2µm plasmid components required for partitioning function that I have observed. Further examples of this redundancy include the observations that association of Rep2 with either Rep1 or itself is sufficient to confer plasmid partitioning function in the presence of Raf, and that Raf and Rep2 are both able to stabilize Rep1 post-transcriptionally. Evolutionarily, this redundancy may be an effective strategy for an extrachromosomal element like the 2µm plasmid, which confers no known selective advantage to its host.

Circular DNA plasmids have been observed in a wide range of organisms, from bacteria to humans. Many of these do not confer a selective advantage to their host, and therefore encode systems to ensure their maintenance, including partitioning systems. As a eukaryotic plasmid that may have a bacterial origin, the 2µm plasmid shows similarities to both the plasmid prophages of bacteriophages and mammalian viral plasmids. A thorough understanding of 2µm plasmid maintenance will therefore not only contribute to the understanding of yeast plasmid biology, but may also provide insight into the maintenance strategies of plasmids found in a diversity of cell types.

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APPENDIX

This appendix contains the results of two genome-wide screens, summarized in table form.

Table 8. Strains showing decreased expression of the *STB-P(O)-lacZ* reporter

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
1	O1	dark	normal	v.pale blue	nib	normal	present	YCR077C	PAT1
	O4	dark	normal	pale	nib	normal	present	YGR241C	YAP1802
2	A10	pale	pale	pale	absent	absent	present, small	YMR064W	AEP1
	A14	pale	spot	v.pale blue	absent	absent	present, small	YMR066W	SOV1
	C1	normal	normal	pale	nib	normal	present	YER111C	SWI4
	J7	normal	normal	v.pale blue	absent	small	present, small	YLR038C	COX12
	J18	normal	normal	pale	absent	absent	present, small	YMR097C	MTG1
	N17	normal	normal	v.pale blue	absent	absent	present, small	YNL252C	MRPL17
	P21	pale	normal	pale	smooth	absent	present, small	YPL132W	COX11
3	B6	normal	normal	pale	absent	absent	present, small	YJL003W	COX16
	N1	normal	normal	pale	absent	absent	present	YJR144W	MGM101
	N24	normal	dark	v.pale blue	nib	absent	present	YJR054W	KCH1
4	C1	normal	normal	v.pale blue	nib	absent	present	YDR461W	MFA1
	D12	normal	normal	v.pale blue	v.smooth	absent	present	YDR512C	EMI1
	D20	dark	normal	v.pale blue	nib	absent	present	YFL001W	DEG1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
4	G20	normal	dark	v.pale blue	absent	absent	present, small	YKR085C	MRPL20
	L22	normal	dark	pale	v.smooth	absent	present, small	YNL315C	ATP11
	P7	normal	normal	white	v.smooth	large	present	YNL107W	YAF9
	P8	absent	normal	white	v.smooth, large	absent	present	YOR302W	YOR302W
	P22	normal	normal	v.pale blue	absent	absent	present, small	YPL078C	ATP4
5	H21	normal	pale	pale	absent	med/small	present, small	YBL045C	COR1
	G11	pale	dark	v.pale blue	absent	med/small	present, small	YNL284C	MRPL10
	G13	normal	dark	v.pale blue	absent	med/small	present, small	YNL315C	ATP11
	P13	normal	dark	v.pale blue	nib	normal	present	YBL089W	AVT5
	P15	pale	dark	white	absent	absent	present, small	YBL090W	MRP21
6	D18	normal	normal	pale	nib	normal	present	YDL021W	GPM2
	F16	normal	normal	white	v.smooth	medium	present, small	YDL032W	YDL032W
	F18	normal	normal	white	v.smooth	medium	present, small	YDL033C	SLM3
7	A16	normal	normal	v.pale blue	v.smooth	absent	present, small	YDL198C	YHM1
	A23	normal	normal	v.pale blue	v.smooth	small	present, small	YDL107W	MSS2

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
7	D15	normal	dark	pale	v.smooth	absent	present	YDR069C	DOA4
	P4	normal	normal	white	absent	absent	present, small	YDR231C	COX20
8	H20	normal	normal	pale	nib	absent	present	YEL045C	YEL045C
	K2	normal	normal	v.pale blue	nib	normal	present	YDR401W	YDR401W
	O19	normal	normal	white	nib, small	small	present	YDR335W	MSN5
9	A1	dark	normal	v.pale blue	absent	normal	present	YDR523C	SPS1
	H12	normal	normal	v.pale blue	nib	med/large	present	YGL139W	FLC3
	G19	normal	normal	white	nib	absent	present	(YER055C)	(HIS1)
	I3	normal	dark	white	absent	small	present, small	YER058W	PET117
	L4	normal	normal	v.pale blue	nib	normal	present	YGL159W	YGL159W
	P21	pale	present	white	nib	normal	present	YGL096W	TOS8
10	A2	normal	dark	pale	nib	normal	present	YGR027C	RPS25A
	A3	normal	normal	pale	nib	med/normal	present	YGL195W	GCN1
	D2	normal	dark	pale	absent	small	present, small	YHL038C	CBP2
	E12	normal	normal	v.pale blue	smooth	normal	present	YGR056W	RSC1
	I5	normal	normal	v.pale blue	v.smooth	normal	present	YGL244W	RTF1
	P10	pale	absent	white	absent	absent	present, small	YHR038W	RRF1
11	A13	normal	normal	white	absent	med/sm	present, small	YHR051W	COX6

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
11	A18	normal	normal	pale	absent	absent	present, small	YHR147C	MRPL6
	E10	normal	normal	white	smooth	absent	present	YHR167W	THP2
	G1	normal	normal	white	nib	normal	present	YHR081W	LRP1
	M7	normal	normal	white	absent	med/sm	present, small	YHR120W	MSH1
12	B9	dark	normal	pale	v.smooth, small	normal	present	YJL124C	LSM1
	C8	normal	normal	white	absent	colony	present, small	YJL209W	CBP1
	G20	normal	normal	pale	nib, large	med/normal	present	YJL179W	PFD1
	H13	normal	normal	v.pale blue	nib	normal	present	YJL084C	ALY2
	I15	normal	normal	v.pale blue	nib, small*	normal	present	YIL153W	RRD1
	O1	normal	normal	v.pale blue	nib	normal	present	YIR005W	IST3
13	A5	normal	dark	white	nib, large	absent	present, small	YKL003C	MRP17
	B4	normal	normal	v.pale blue	nib, medium	absent	present	YLL002W	RTT109
	E17	normal	normal	white	nib	absent	present	YKL032C	IXR1
	K12	normal	dark	pale	nib	absent	present	YKL160W	ELF1
14	E15	normal	normal	v.pale blue	nib	absent	present	YLR061W	RPL22A
	G3	normal	normal	pale	v.smooth	absent	present, small	YLR067C	PET309
	I4	normal	normal	v.pale blue	nib	normal	present	YLR181C	VTA1
	I6	normal	pale	pale	v.smooth	absent	present, small	YLR182W	SWI6

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
14	I15	normal	normal	v.pale blue	nib	normal	present	YLR085C	ARP6
	J4	normal	normal	v.pale blue	nib	absent	present	YLR373C	VID22
	K24	normal	normal	white	smooth	colony	present, small	YLR203C	MSS51
	L20	normal	normal	pale	v.smooth	med/normal	present	YLR393W	ATP10
15	B21	normal	normal	pale	v.smooth	absent	present, small	YMR151W	YIM2
	F6	normal	normal	pale	absent	absent	present, small	YMR257C	PET111
	G8	normal	normal	v.pale blue	nib, small	small	present	YMR234W	RNH1
	M7	normal	normal	white	smooth	med/normal	present	YML062C	MFT1
16	B23	dark	normal	pale	nib	absent	present	YNL136W	EAF7
	L13	normal	absent	v.pale blue	absent	absent	present, small	YNL081C	SWS2
	L14	normal	normal	pale	v.smooth	small	present	YNR020C	ATP23
	N22	normal	pale	v.pale blue	absent	absent	present, small	YNR036C	MRPS12
	P8	normal	pale	v.pale blue	absent	absent	present, small	YNR041C	COQ2
17	A20	normal	normal	v.pale blue	nib	normal	present	YOR106W	VAM3
	B11	normal	normal	pale	absent	absent	present	YOR198C	BFR1
	B13	normal	dark	pale	absent	absent	present, small	YOR199W	YOR199W

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
17	B15	normal	normal	pale	absent	absent	present, small	YOR200W	YOR200W
	B17	normal	normal	pale	absent	absent	present, small	YOR201C	MRM1
	E6	normal	normal	pale	nib	normal	present	YOR123C	LEO1
	K9	normal	normal	pale	absent	absent	present, small	YOR065W	CYT1
18	C21	normal	normal	v.pale blue	smooth	medium	present	YOL012C	HTZ1
	D4	normal	normal	pale	absent	absent	present, small	YPL078C	ATP4
	D5	normal	normal	pale	absent	absent	present, small	YPL173W	MRPL40
	D7	normal	normal	pale	absent	colony	present, small	YPL172C	COX10
	E19	normal	absent	v.pale blue	absent	med/small	present, small	YOL023W	IFM1
	K3	dark	dark	pale	v.smooth	absent	present, small	YOL051W	GAL11
	K18	normal	normal	pale	absent	small	present, small	YPL215W	CBP3
19	B9	normal	normal	v.pale blue	absent	med/small	present, small	YOR054c	VHS3
	B16	normal	normal	white	v.smooth	colony	present, small	YGR222W	PET54
	B21	normal	normal	pale	nib	absent	present	YIL134W	FLX1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
19	F14	normal	dark	v.pale blue	absent	med/small	present, small	YPL132W	COX11
	F20	normal	normal	v.pale blue	absent	colony	present, small	YML129C	COX14
	H2	normal	dark	pale	nib	normal	present	YMR125W	STO1
	I12	normal	normal	v.pale blue	nib, small*	normal	present	YPR159W	KRE6
	K24	normal	normal	white	smooth	absent	present	(YPR177C)	(YPR177C)
	N20	normal	dark	pale	absent	small	present, small	YCR046C	IMG1
	O4	normal	normal	pale	v.smooth	small	present	YPR191W	QCR2
	O16	normal	normal	v.pale blue	nib	normal	present	YPR197C	YPR197C
	P2	normal	absent	pale	absent	small	present, small	YNL073W	MSK1
	P6	normal	normal	white	smooth	normal	present	YFL001W	DEG1
	P18	dark	normal	white	nib	med/small	present	YJR055W	HIT1
20	A13	normal	pale	pale	absent	small	present, small	YAL039C	CYC3
	B15	normal	normal	pale	absent	med/small	present, small	YKL170W	MRPL38
	C17	normal	dark	pale	absent	colony	present, small	YBL045C	COR1
	D13	normal	dark	pale	absent	small	present, small	YLR067C	PET309

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
20	D20	dark	dark	pale	absent	med/normal	present, small	YPL172C	COX10
	E14	normal	normal	pale	absent	med/small	present, small	YER058W	PET117
	E17	pale	normal	pale	absent	colony	present, small	YBR037C	SCO1
	F3	dark	pale	pale	nib	absent	present	YLR182W	SWI6
	G1	normal	normal	v.pale blue	v.smooth, large	nib, medium	present, small	YBR120C	CBP6
	G15	normal	dark	white	absent	colony	present, small	YBR251W	MRPS5
	J13	normal	absent	pale	absent	med/small	present, small	YMR256C	COX7
	J15	normal	normal	pale	absent	colony	present, small	YMR257C	PET111
	K5	normal	normal	pale	absent	small	present, small	YDL107W	MSS2
	M4	normal	normal	v.pale blue	smooth	med/normal	present, small	YHR116W	COX23
	M6	normal	dark	v.pale blue	absent	med/small	present, small	YHR120W	MSH1
	P11	normal	normal	pale	absent	normal	present, small	YOR199W	YOR199W
	P17	normal	normal	pale	absent	colony	present, small	YOR205C	GEP3

Table 9. Strains showing increased expression of the *STB-P(O)-lacZ* reporter and absence of 2µm hybridization signal

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
1	A16	absent	absent	dark blue	nib	small	present	YCR099C	YCR099C
	N12	pale	absent	dark blue	nib, small	normal	present	YNR051C	BRE5
	N17	absent	absent	dark blue	nib	normal	present	YKR069W	MET1
	P19	pale	absent	dark blue	nib	small	present	YKR082W	NUP133
2	C11	pale	pale	dark blue	nib	small	present	YER116C	SLX8
3	J16	absent	absent	dark blue	nib	absent	present	YJR026W	YJR026W
	L20	pale	absent	dark blue	nib	absent	present	YJR040W	GEF1
4	C11	absent	absent	dark blue	nib	large	present	YGR028W	MSP1
	G11	absent	absent	dark blue	nib, small*	absent	present	YJL136C	RPS21B
5	F3	absent	absent	dark blue	nib	normal	present	YBL024W	NCL1
	J11	absent	absent	dark blue	absent	normal	present	YBL052C	SAS3
	P22	pale	absent	dark blue	nib	normal	present	YBR078W	ECM33
6	A3	absent	absent	blue	v.smooth	v.small	present	YBR081C	SPT7
	A24	absent	absent	dark blue	nib	normal	present	YBR184W	YBR184W
	D2	absent	absent	dark blue	nib	medium	present	YDL013W	SLX5
	N18	absent	absent	dark blue	nib	normal	present	YDL080C	THI3
	O6	absent	absent	dark blue	nib	normal	present	YBR259W	YBR259W
	P16	absent	absent	dark blue	nib	normal	present	YDL091C	UBX3
7	C17	absent	absent	dark blue	v.smooth	small	present	YDL116W	NUP84
	D6	absent	absent	dark blue	nib	normal	present	YDR161W	TCI1
8	E23	absent	absent	dark blue	nib	normal	present	YDR277C	MTH1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
9	B23	absent	pale	dark blue	absent	absent	present	YGL012W	ERG4
	J9	absent	absent	dark blue	nib	normal	present	YGL054C	ERV14
10	G14	absent	absent	dark blue	nib	normal	present	YGR069W	YGR069W
	K17	absent	absent	dark blue	nib	normal	present	YGL262W	YGL262W
	N16	absent	absent	dark blue	nib	normal	present	YHR029C	YHI9
11	L9	pale	absent	dark blue	nib	normal	present	YIL064W	EFM4
12	C2	pale	absent	dark blue	nib	normal	present	YJL212C	OPT1
	K8	pale	pale	dark blue	nib	normal	present	YJL161W	FMP33
13	C14	pale	pale	dark blue	nib	absent	present	YKL113C	RAD27
	D9	absent	absent	dark blue	nib	normal	present	YKL207W	EMC3
	G15	absent	absent	dark blue	nib, small	normal	present	YKL043W	PHD1
	J5	absent	absent	dark blue	nib	absent	present	YKR020W	VPS51
	N20	absent	absent	dark blue	smooth	normal	present	YLR015W	BRE2
15	J3	absent	absent	dark blue	absent	absent	present	YMR185W	RTP1
	J10	pale, v.small	absent	dark blue	nib	normal	present	YMR283C	RIT1
16	C13	absent	absent	dark blue	nib	normal	present	YNL321W	VNX1
	H14	absent	absent	dark blue	nib	normal	present	YNL046W	YNL046W
	J10	absent	absent	dark blue	absent	absent	present, small	YNR006W	VPS27
17	A10	absent	absent	dark blue	nib	normal	present	YOR101W	RAS1
	A11	absent	absent	dark blue	nib	normal	present	YOR006C	TSR3
	C10	absent	absent	dark blue	nib	normal	present	YOR113W	AZF1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
17	G9	absent	absent	dark blue	nib	normal	present	YOR041C	YOR041C
	J4	absent	absent	dark blue	nib	normal	present	YOR337W	TEA1
	O15	absent	absent	dark blue	nib, small	normal	present	YOR092W	ECM3
	O16	absent	absent	dark blue	nib	normal	present	YOR188W	MSB1
18	O13	absent	absent	dark blue	nib	normal	present	YOL080C	REX4
19	E20	absent	absent	dark blue	v.smooth, small	absent	present	YPR139C	LOA1
	L10	absent	absent	dark blue	v.smooth	normal	present	YJR040W	GEF1
	L15	absent	absent	dark blue	nib	normal	present	YMR119W	ASI1
20	D8	absent	absent	dark blue	absent	med/small	present, small	YPL271W	ATP15
	E2	absent	absent	dark blue	absent	med/normal	present, small	YER014W	HEM14
	G8	absent	absent	dark blue	nib	normal	present	YGL038C	OCH1
	I5	pale	absent	dark blue	nib	med/normal	present	YDL013W	SLX5
	J6	absent	absent	dark blue	absent	med/normal	present, small	YGL223C	COG1
	K16	absent	absent	dark blue	nib	normal	present	YHL035C	VMR1
	I18	absent	absent	dark blue	nib	normal	present	YGR069W	YGR069W

Table 10. Strains showing increased expression of the *STB-P(O)-lacZ* reporter and presence of 2µm hybridization signal

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
1	C16	pale	normal	blue	smooth	normal	present	YDR363W-A	SEM1
	D11	normal	absent	blue	nib	normal	present	YGR270W	YTA7
	E15	normal	pale	blue sector	nib	normal	present	YBR296C	PHO89
	E22	pale	absent	dark blue	absent	absent	absent	YER188W	YER188W
	F22	dark	absent	dark blue	absent	small	absent	YLR432W	IMD3
	H19	pale	absent	dark blue	absent	absent	absent	YIL009C-A	EST3
	K8	pale	absent	dark blue	absent	absent	present, small	YGR219W	YGR219W
	K10	pale	normal	dark sector	absent	absent	present, small	YGR220C	MRPL9
	L17	normal	absent	dark blue	nib	normal	present	YIR038C	GTT1
	L18	normal	normal	dark blue	v.smooth	absent	present, small	YMR060C	TOM37
	N9	normal	absent	dark blue	nib	absent	present	YKR035W-A	DID2
2	A9	normal	pale	blue sector	absent	absent	present, small	YER103W	SSA4
	A23	pale	pale	dark blue	nib	absent	present, small	YER110C	KAP123
	B1	dark	normal	blue sector	nib	normal	present	YBR232C	YBR232C
	C2	normal	dark	blue sector	absent	normal	present, small	YMR072W	ABF2

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
2	E1	pale	normal	dark blue	nib	normal	present, small	YER122C	GLO3
	E5	normal	absent	dark blue	nib	normal	present	YER124C	DSE1
	H1	pale	normal	dark blue	absent	absent	present	YHR191C	CTF8
	H3	pale	normal	dark blue	nib	absent	present	YHR193C	EGD2
	H5	spot	normal	dark blue	nib	normal	present	YLL030C	RRT7
	H11	pale	normal	dark blue	smooth, small	absent	present	YLL049W	LDB18
	J8	pale	absent	dark blue	nib	nib	present	YML131W	YML131W
	K5	normal	normal	blue	nib	normal	present	YER163C	GCG1
	O15	normal	absent	dark blue	nib	normal	present	YMR054W	STV1
	O19	pale	pale	dark sector	nib	normal	present	YMR056C	AAC1
3	B2	normal	normal	blue	nib	absent	present	(YJL001W)	(PRE3)
	N18	normal	normal	blue sector	nib	absent	present	YJR051W	OSM1
	N20	normal	normal	blue sector	nib	absent	present	YJR052W	RAD7
	O2	normal	normal	blue sector	nib	absent	present	YOR364W	YOR364W
	P12	normal	pale	dark blue	nib	absent	present	YJR060W	CBF1
4	C4	pale	normal	blue sector	nib	absent	present	YFL014W	HSP12
	F14	normal	normal	blue	nib, small	absent	present	YFL013C	IES1
	F15	normal	normal	blue	v.smooth	absent	present	YCR047C	BUD23
	J22	pale	normal	blue sector	nib	absent	present	YIR044C	YIR044C
	M10	pale	normal	blue sector	nib	absent	present	YOL153C	YOL153C

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
4	O22	pale	normal	dark blue	absent	absent	present, small	YPR067W	ISA2
5	D18	normal	absent	dark blue	nib	normal	present	YBR006W	UGA2
	D20	normal	absent	dark blue	nib	normal	present	YBR007C	DSF2
	D21	normal	absent	blue	smooth	medium	present, small	YBL021C	HAP3
	D23	normal	absent	dark blue	absent	absent	absent	YBL022C	PIM1
	E2	pale	normal	blue sector	nib	med/large	present	YAL044C	GCV3
	G1	normal	normal	blue sector	nib	normal	present	YNL109W	YNL109W
	H16	normal	absent	dark blue	nib	normal	present	YBR027C	YBR027C
	H23	normal	normal	blue	nib	normal	present	YBL046W	PSY4
	J2	normal	absent	dark blue	nib	normal	present	YBR032W	YBR032W
	J21	normal	absent	dark blue	absent	absent	absent	YBL057C	PTH2
	M22	normal	pale	dark blue	nib	normal	present	YAR027W	UIP3
	N2	normal	normal	dark sector	nib	normal	present	YBR056W	YBR056W
	N14	normal	absent	dark blue	nib	normal	present	YBR062C	YBR062C
	N21	normal	normal	blue sector	nib	normal	present	YBL081W	YBL081W
6	C9	pale	normal	blue sector	nib	normal	present	YBR094W	PBY1
	E23	normal	normal	blue sector	nib	normal	presesnt	YBR113W	YBR113W
	G22	pale	normal	blue sector	nib	normal	present	YBR219C	YBR219C

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
6	H11	pale	absent	dark blue	absent	absent	absent	(YCL043C)	(PDI1)
	H12	normal	normal	dark blue	absent	absent	present	YDL042C	SIR2
7	C21	normal	present	dark blue	nib	normal	present	YDL118W	YDL118W
	F12	dark	dark	dark sector	nib	small	present, small	YDR176W	NGG1
	G4	normal	normal	blue sector	nib	normal	present	YDL229W	SSB1
	G24	normal	normal	blue	nib	normal	present	YDL239C	ADY3
	M2	normal	normal	dark sector	nib	normal	present	YDR022C	ATG31
	M15	pale	pale	dark blue	nib	normal	present	YDL174C	DLD1
	M23	dark	normal	blue sector	nib	normal	present	YDL178W	DLD2
	N9	pale	absent	blue	smooth	small	present	YDR127W	ARO1
	P24	normal	normal	dark blue	nib	normal	present	YDR241W	BUD26
8	A18	normal	dark	blue sector	nib	normal	present	YDR348C	PAL1
	A19	normal	normal	dark sector	nib	normal	present	YDR251W	PAM1
	B19	normal	dark	dark blue	nib	absent	present, small	YDR448W	ADA2
	B20	normal	absent	dark sector	nib	normal	present	YEL010W	YEL010W
	C21	normal	dark	dark blue	smooth	absent	present	YDR264C	AKR1
	C24	normal	normal	blue sector	nib	normal	present	YDR363W	ESC2
	D3	normal	pale	dark blue	nib	normal	present	YDR452W	PPN1
	D9	normal	normal	blue sector	nib	normal	present	YDR455C	YDR455C
	D11	normal	normal	blue sector	nib	medium	present	YDR456W	NHX1
	D23	pale	pale	blue	smooth	med/large	present	YDR462W	MRPL28

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
8	E5	normal	dark	dark blue	absent	medium	present, small	YDR268W	MSW1
	E13	pale	absent	dark blue	nib	normal	present	YDR272W	GLO2
	E14	normal	absent	dark blue	nib	absent	absent	YDR371W	CTS2
	E19	normal	absent	dark blue	nib	normal	present	YDR275W	BSC2
	F4	normal	normal	blue sector	nib	normal	present	YEL025C	YEL025C
	F5	normal	dark	blue sector	nib	normal	present	YDR465C	RMT2
	F10	normal	normal	blue sector	nib	normal	present	YEL028W	YEL028W
	F17	normal	pale	blue sector	nib	med/normal	present	YDR471W	RPL27B
	G12	normal	normal	blue	nib, small	med/normal	present	YDR382W	RPP2B
	I10	normal	normal	blue	v.smooth	med/normal	present, small	YDR393W	SHE9
	J3	normal	normal	blue	nib	normal	present	YDR488C	PAC11
	K15	normal	normal	dark blue	nib	normal	present, small	YDR309C	GIC2
	P5	normal	normal	blue sector	nib	normal	present	YDR525W	API2
9	A15	pale	normal	blue sector	nib	normal	present	YER020W	GPA2
	A23	normal	normal	blue sector	nib	normal	present	YER024W	YAT2
	B7	normal	normal	dark blue	nib	normal	present	YGL004C	RPN14
	C10	normal	normal	blue sector	nib	normal	present	YFL015C	YFL015C
	E10	normal	normal	dark blue	absent	absent	present	YFL026W	STE2
	F1	pale	normal	dark blue	v.smooth	small	present	YGL025C	PGD1
	F12	normal	normal	dark blue	nib	normal	present	YGL127C	SOH1
	F13	normal	dark	dark blue	nib	absent	present	YGL031C	RPL24A

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
9	F23	pale	normal	dark sector	nib	absent	present	YGL036W	MTC2
	G24	normal	absent	dark blue	nib	absent	present	YFL043C	YFL043C
	H1	normal	normal	dark blue	nib	normal	present	YGL037C	PNC1
	H13	normal	pale	dark blue	nib	med/normal	present	YGL043W	DST1
	H22	pale	pale	dark blue	nib	normal	present	YGL144C	ROG1
	I1	normal	normal	blue sector	nib	normal	present	YER057C	HMF1
	I5	pale	normal	dark blue	nib	normal	present	YER059W	PCL6
	J2	normal	normal	blue	nib	normal	present	YGL146C	RRT6
	J13	normal	normal	dark blue	nib	absent	present	YGL056C	SDS23
	J16	normal	normal	blue	nib	normal	present	YGL153W	PEX14
	K1	normal	normal	blue	nib	normal	present	YER067W	RGI1
	K9	normal	normal	blue	smooth	absent	present	YER069W	ARG5,6
	K11	normal	dark	dark blue	absent	absent	present, small	YER070W	RNR1
	L16	pale	normal	blue, small	nib	normal	present	YGL165C	YGL165C
	L22	pale	normal	dark blue	nib	absent	present	YGL168W	HUR1
	P1	normal	present	blue	nib	normal	present	YGL086W	MAD1
10	C9	normal	pale	blue	smooth	med/normal	present	YGL211W	NCS6
	D1	normal	pale	dark sector	v.smooth	normal	present	YGR135W	PRE9
	F8	pale	normal	dark blue	nib	normal	present	YHL023C	NPR3
	F15	normal	normal	blue sector	nib	normal	present	YGR154C	GTO1
	H15	normal	pale	blue sector	nib	absent	present	YGR166W	TRS65
	J1	normal	dark	blue sector	absent	small	present, small	YGR171C	MSM1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
10	L24	normal	normal	blue sector	nib	small	present	YHR021C	RPS27B
	M8	normal	dark	blue sector	absent	small	present, small	YGR102C	GTF1
	M9	pale	pale	dark blue	nib	normal	present	YGR007W	ECT1
11	D6	pale	dark	dark blue	nib	normal	present	YIL015C-A	YIL015C-A
	E21	pale	normal	blue	nib	absent	present	YHR079C	IRE1
	G4	normal	normal	blue	nib	normal	present	YHR176W	FMO1
	L2	normal	normal	blue	nib	normal	present	YIL060W	YIL060W
	M9	normal	normal	dark sector	nib	normal	present	YHR121W	LSM12
	P18	pale	normal	dark blue	nib	normal	present	YIL094C	LYS12
12	B5	normal	normal	dark sector	nib	normal	presesnt	YJL126W	NIT2
	B14	normal	normal	blue sector	nib	med/small	present	YJR073C	OPI3
	C2	pale	absent	dark blue	nib	normal	present	YJL212C	OPT1
	F1	normal	normal	dark blue	absent	absent	present, small	YJL102W	MEF2
	G12	normal	normal	blue	smooth	normal	present	YJL183W	MNN11
	H21	normal	normal	blue sector	nib	normal	present	YJL080C	SCP160
	I4	normal	normal	blue	v.smooth	absent	present, small	YJL176C	SWI3
	K8	pale	pale	dark blue	nib	normal	present	YJL161W	FMP33
	K20	normal	absent	blue	nib	normal	present	YJL155C	FBP26
	K22	normal	normal	blue sector	nib	normal	present	YJL154C	VPS35
	L3	normal	pale	blue sector	nib	normal	present	YJL064W	YJL064W

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
12	M10	normal	normal	blue	v.smooth	medium	present	YJL148W	RPA34
	M12	normal	normal	blue sector	nib	normal	present	YJL147C	MRX5
13	D6	normal	normal	blue	nib	normal	present	YLL015W	BPT1
	D21	dark	normal	dark blue	nib, small	absent	present	YKL213C	DOA1
	E6	normal	normal	blue	nib	med/normal	present	YKL121W	DGR2
	G6	pale	normal	blue sector	nib	normal	present	YKL133C	YKL133C
	G8	pale	pale	dark blue	absent	absent	present, small	YKL134C	OCT1
	J6	normal	normal	blue	nib	normal	present	YLL051C	FRE6
	J12	pale	normal	dark blue	nib	normal	present	YLL054C	YLL054C
	K14	normal	normal	blue sector	nib	normal	present	YKL161C	KDX1
	L2	normal	pale	blue sector	nib	normal	present	YLL061W	MMP1
	M18	normal	normal	blue sector	nib	normal	present	YKL175W	ZRT3
	N13	normal	normal	blue sector	nib	normal	present	YKR048C	NAP1
	N20	spot	absent	dark blue	nib	normal	present	YLR015W	BRE2
	O20	normal	pale	blue sector	nib	absent	present	YKL188C	PXA2
14	C13	normal	normal	blue sector	nib	absent	present	YLR048W	RPS0B
	E4	normal	normal	blue sector	nib	normal	present	YLR149C	YLR149C
	F3	normal	normal	blue sector	nib	normal	present	YLR253W	MCP2
	I3	normal	pale	dark blue	smooth	absent	present	YLR079W	SIC1
	J9	pale	normal	dark blue	nib	normal	present	YLR280C	YLR280C
	K13	pale	normal	blue sector	nib	normal	present	YLR096W	KIN2
	L10	pale	absent	dark blue	nib	med/normal	present	YLR388W	RPS29A
	L14	pale	normal	blue sector	nib	normal	present	YLR390W	ECM19

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
14	N5	pale	absent	dark blue	nib	absent	present	YLR303W	MET17
	O13	pale	normal	blue	nib	normal	present	YLR120C	YPS1
	O17	normal	normal	blue sector	nib, small	normal	present	YLR309C	IMH1
15	B7	normal	normal	blue	nib	absent	present	YMR143W	RPS16A
	E2	normal	normal	blue sector	nib	normal	present	YML020W	YML020W
	E14	normal	normal	blue sector	v.smooth	normal	present	YML014W	TRM9
	F4	pale	normal	blue sector	absent	absent	present, small	YMR256C	COX7
	I3	normal	normal	dark blue	absent	normal	present, small	YML088W	UFO1
	I21	normal	normal	blue sector	nib	normal	present	YML079W	YML079W
	L3	normal	normal	blue sector	nib	normal	present	YMR196W	YMR196W
	M12	normal	normal	blue sector	nib	normal	present	YMR031W-A	YMR031W-A
16	A16	normal	pale	dark blue	smooth	absent	present	YNL236W	SIN4
	A18	normal	pale	dark blue	nib	normal	present	YNL235C	YNL235C
	D17	pale	normal	dark blue	nib	normal	present	YNL127W	FAR11
	D22	normal	normal	blue, small	nib	normal	present	YNL025C	SSN8
	E3	pale	normal	dark blue	nib	medium	present	YNL314W	DAL82
	E6	pale	normal	blue sector	nib	normal	present	YNL217W	YNL217W

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
16	H2	normal	normal	blue sector	nib	normal	present	YNL040W	YNL040W
	I23	normal	normal	blue sector	nib	normal	present	YNL280C	ERG24
	J14	pale	normal	blue	nib	normal	present	YNR008W	LRO1
	J18	normal	normal	blue	nib	absent	present	YNR010W	CSE2
	K7	normal	normal	blue sector	nib	normal	present	YNL276C	YNL276C
	L16	normal	pale	blue sector	nib	absent	present	YNR021W	YNR021W
	N8	pale	absent	blue	nib	normal	present	YNR029C	YNR029C
	N13	normal	normal	blue, small	smooth	v.small	present	YNL069C	RPL16B
	N14	normal	normal	blue sector	nib	normal	present	YNR032W	PPG1
17	A19	normal	normal	blue	nib	normal	present	YOR010C	TIR2
	B10	normal	normal	dark blue	nib	small	present	YOR293W	RPS10A
	E2	normal	normal	blue sector	nib	normal	present	YOR121C	YOR121C
	F10	normal	pale	dark blue	nib	normal	present	YOR316C	COT1
	J13	normal	normal	blue	nib, small	normal	present	YOR247W	SRL1
	J20	normal	normal	blue sector	nib	normal	present	YOR345C	YOR345C
	O24	pale	normal	blue sector	nib	normal	present	YOR192C	THI72
	P1	pale	normal	blue sector	nib	normal	present	YOR277C	YOR277C
	P19	pale	normal	blue	nib	normal	present	YOR286W	RDL2
18	C10	normal	dark	blue	nib, small	medium	present	YPL267W	ACM1
	D18	normal	normal	blue sector	nib	normal	present	YPL071C	YPL071C
	E7	normal	normal	blue sector	nib	normal	present	YOL017W	ESC8
	E9	normal	normal	blue sector	nib	med/small	present	YOL018C	TLG2

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
18	F5	normal	normal	dark blue	v.smooth	medium	present	YPL161C	BEM4
	H15	normal	normal	blue sector	nib	normal	present, small	YPL144W	POC4
	H22	pale	dark	dark blue	absent	absent	present	YPL045W	VPS16
	I23	normal	absent	dark blue	nib	medium	present	YOL049W	GSH2
	L5	normal	normal	blue sector	nib	small	present	YPL125W	KAP120
	O6	normal	normal	dark blue	nib	normal	present	YPL197C	YPL197C
19	B22	normal	normal	dark blue	smooth	v. small	present, small	YIR026C	YVH1
	C24	normal	normal	blue	nib	normal	present	YPR129W	SCD6
	D8	normal	normal	blue	absent	normal	present, small	YER122C	GLO3
	D9	normal	normal	blue	smooth	absent	present	YBR081C	SPT7
	G21	normal	normal	dark blue	nib	normal	present	YPR052C	NHP6A
	H5	pale	normal	blue	absent	med/normal	present, small	YDL063C	SYO1
	J17	normal	normal	blue sector	nib	normal	present	YGR025W	YGR025W
	J20	pale	normal	blue, small	absent	absent	present, small	YJR144W	MGM101
	K9	normal	normal	dark blue	nib	normal	present	YPR070W	MED1
20	A4	normal	normal	dark blue	absent	normal	present, small	YDR283C	GCN2
	B8	normal	pale	blue	absent	normal	present, small	YOR304C-A	BIL1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
20	C3	pale	absent	dark blue	v.smooth	medium	present, small	YBL021C	HAP3
	C4	pale	pale	dark blue	nib	normal	present	YDR440W	DOT1
	C5	pale	pale	dark blue	absent	v.small	present, small	YBL022C	PIM1
	C18	dark	pale	blue	absent	med/normal	present, small	YEL029C	BUD16
	C20	pale	absent	dark blue	absent	absent	absent	YEL036C	ANP1
	C21	pale	pale	dark blue	nib	med/normal	present	YBL072C	RPS8A
	D10	pale	pale	dark blue	smooth, large	v.small	present	YPL234C	VMA11
	F1	normal	normal	blue sector	absent	absent	present	YLR148W	PEP3
	F10	normal	normal	blue	absent	v.small	present, small	YPL078C	ATP4
	F15	normal	normal	blue sector	absent	absent	present	YLR240W	VPS34
	F20	pale	normal	blue sector	absent	absent	present, small	YPL029W	SUV3
	G7	normal	normal	blue sector	absent	absent	present, small	YBR163W	EXO5
	G16	normal	normal	blue sector	nib, medium	med/normal	present	YGL105W	ARC1
	J12	normal	normal	blue sector	absent	normal	present, small	YDL063C	SYO1
	J16	pale	pale	blue sector	absent	small	present, small	YGL218W	YGL218W
	J23	normal	normal	blue sector	absent	med/normal	present, small	YMR287C	DSS1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
20	K22	pale	normal	dark blue	absent	med/small	present, small	YHR051W	COX6
	L1	normal	normal	blue sector	absent	absent	present, small	YMR293C	HER2
	L3	pale	pale	dark blue	nib	normal	present	YNL302C	RPS19B
	L5	pale	pale	dark blue	v.smooth	absent	present	YNL248C	RPA49
	L9	pale	normal	dark blue	v.smooth	normal	present	YNL236W	SIN4
	M14	normal	normal	blue sector	nib	med/normal	present	YIL128W	MET18
	M20	normal	normal	blue	v.smooth	absent	present	YJL188C	YJL188C
	O5	normal	absent	dark blue	nib	med/small	present	YDR140W	MTQ2
	O8	pale	absent	dark blue	v.smooth	absent	present	YJL075C	APQ13
	O13	normal	normal	blue	absent	absent	present, small	YDR175C	RSM24
	O24	normal	normal	dark blue	absent	large	present	YKL054C	DEF1
	P21	normal	normal	dark blue	nib	med/normal	present	YOR221C	MCT1
	P23	normal	normal	blue	absent	normal	present, small	YOR241W	MET7

Table 11. Strains that appear as small colonies upon overexpression of Rep1 and Rep2.

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
1	A10	dark	dark	pale blue	nib, small*	medium	present	YCR094W	CDC50
	B7	absent	normal	pale blue	nib, small	normal	present	YGR256W	GND2
	I17	normal	normal	pale blue	nib, small	normal	present	YCR045C	RRT12
	P6	pale	normal	pale blue	nib, small*	normal	present	YNR060W	FRE4
	P14	normal	normal	pale blue	nib, small*	normal	present	YNR064C	YNR064C
2	B7	normal	normal	pale blue	nib, small	normal	present	YEL011W	GLC3
	L10	normal	normal	pale blue	nib, small*	med/small	present	YMR105C	PGM2
	L12	normal	normal	pale blue	nib, small	normal	present	YMR106C	YKU80
	L13	absesnt	normal	pale blue	nib, small*	norm/med	present	YMR311C	GLC8
3	E17	normal	dark	pale blue	v.smooth, small	small	present, small	YHR026W	PPA1
	L8	normal	normal	pale blue	nib, medium	absent	present	YJR034W	PET191
	L13	absent	pale	pale blue	nib, small	normal	present	YJR133W	XPT1
	P2	absent	normal	absent	nib, small*	normal	present	YJR055W	HIT1
4	G11	absent	absent	dark blue	nib, small*	absent	present	YJL136C	RPS21B
	H10	normal	normal	pale blue	nib, small*	normal	present	YGR258C	RAD2
	H13	normal	normal	pale blue	nib, small*	abesnt	present	YCR073C	SSK22
	I11	normal	normal	pale blue	nib, small*	normal	present	(YJL174W)	(KRE9)
	I12	normal	normal	pale blue	nib, small*	normal	present	YLR442C	SIR3
	I13	normal	normal	pale blue	nib, small*	med/sm	present	YJL175W	YJL175W
	J11	normal	normal	pale blue	nib, small*	normal	present	YFR011C	MIC19

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
4	J12	normal	normal	pale blue	nib, small*	absent	present	YIL092W	YIL092W
5	G18	normal	normal	pale blue	nib, small	normal	present	YAL027W	SAW1
	K8	normal	normal	pale blue	nib, small*	normal	present	YAL008W	FUN14
	L13	absent	normal	absent	nib, small*	normal	present	YBL065W	YBL065W
6	I10	normal	normal	pale blue	nib, small*	normal	present	YBR225W	YBR225W
7	N7	normal	normal	pale blue	smooth, small	norm/med	present	YDR126W	SWF1
	O3	normal	normal	pale blue	nib, small	normal	present	YDL180W	YDL180W
8	A7	normal	dark	pale blue	smooth, small*	norm/med	present	YDR245W	MNN10
	F16	absent	normal	blue	nib, small	normal	present	YEL031W	SPF1
	G9	normal	normal	pale blue	nib, small*	normal	present	YDR282C	YDR282C
	G12	normal	normal	blue	nib, small*	norm/med	present	YDR382W	RPP2B
	H9	normal	normal	pale blue	nib, small*	normal	present	YDR479C	PEX29
	H19	normal	normal	pale blue	nib, small	small	present	YDR484W	VPS52
	H22	normal	normal	pale blue	nib, small*	med/sm	present	YEL046C	GLY1
	L13	absent	normal	pale blue	nib, small*	normal	present	YDR505C	PSP1
	L14	normal	normal	pale blue	nib, small*	normal	present	YEL065W	SIT1
	M12	absent	absent	absent	nib, small*	med/small	present	YDR418W	RPL12B
	N12	pale	absent	dark blue	nib, small	absent	absent	YNR051C	BRE5
	O14	normal	normal	pale blue	nib, small*	normal	present	YDR431W	YDR431W

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
8	O19	normal, small	normal	white	nib, small	small	present	YDR335W	MSN5
	P10	normal	normal	pale blue	nib, small*	present	present	YER007W	PAC2
	P11	normal	normal	pale blue	nib, small*	present	present	YDR528W	HLR1
9	D21	absent	normal	absent	nib, small	normal	present	YGL023C	PIB2
	J12	absent	normal	dark blue	nib, small*	normal	present	YGL151W	NUT1
	K19	normal	normal	pale blue	smooth, small	present	present	YER074W	RPS24A
	M2	normal	normal	pale blue	nib, small	absent	present	YFR010W	UBP6
10	E10	normal	normal	pale blue	nib, small	norm/med	present	YGR055W	MUP1
	G9	normal	normal	pale blue	nib, small*	medium	present	YGL234W	ADE5,7
	H22	normal	normal	pale blue	nib, small	norm/med	present	YHL005C	YHL005C
	I13	normal	normal	pale blue	nib, small*	absent	present	YGL248W	PDE1
	I14	absent	normal	dark blue	nib, small*	present	present	YGR081C	SLX9
	K13	normal	normal	pale blue	nib, small*	normal	present	YGL260W	YGL260W
	L13	normal	normal	pale blue	nib, small*	normal	present	YGR189C	CRH1
11	J14	normal	normal	pale blue	nib, small*	normal	present	YIL054W	YIL054W
12	B9	dark	normal	pale	v.smooth, small	normal	present	YJL124C	LSM1
	C18	dark	normal	pale blue	smooth, small	small	present	YJL204C	RCY1
	D6	normal	normal	pale blue	nib, small	normal	present	YJR082C	EAF6
	E16	normal	normal	pale blue	nib, small	normal	present	YJL193W	YJL193W
	H17	normal	normal	pale blue	nib, small*	normal	present	YJL082W	IML2
	I15	normal	normal	v.pale blue	nib, small*	normal	present	YIL153W	RRD1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
12	M18	normal	normal	pale blue	nib, small	normal	present	YJL144W	YJL144W
13	G15	absent	absent	dark blue	nib, small	normal	present	YKL043W	PHD1
	H6	normal	normal	pale blue	nib, small	absent	present	YLL039C	UBI4
	H15	normal	normal	pale blue	nib, small*	absent	present	YKR013W	PRY2
	H17	normal	normal	pale blue	nib, small	norm/med	present	YKR014C	YPT52
	I18	normal	normal	pale blue	nib, small*	absent	present	YKL151C	YKL151C
	P8	normal	normal	pale blue	smooth, small*	norm/med	present	YLR021W	IRC25
14	H18	normal	normal	pale blue	nib, small*	normal	present	YLR368W	MDM30
	N14	dark	pale	pale blue	nib, small	absent	present	YLR402W	YLR402W
	O17	normal	normal	pale blue	nib, small	normal	present	YLR122C	YLR122C
15	F10	normal	normal	pale blue	nib, small	normal	present	YMR259C	TRM732
	G8	normal	normal	v.pale blue	nib, small	small	present	YMR234W	RNH1
	K12	normal	normal	pale blue	nib, small*	normal	present	YMR019W	STB4
	L13	normal	normal	pale blue	nib, small*	normal	present	YMR201C	RAD14
16	A12	absent	normal	absent	v.smooth, small	normal	present	YNL238W	KEX2
17	C3	normal	dark	pale blue	nib, small	norm/med	present	YOR014W	RTS1
	J11	normal	normal	pale blue	smooth, small*	normal	present	YOR246C	ENV9
	J13	normal	normal	blue	nib, small*	normal	present	YOR247W	SRL1
	M15	normal	normal	pale blue	nib, small*	small	present	YOR080W	DIA2
	O15	absent	absent	dark blue	nib, small	normal	present	YOR092W	ECM3
18	B16	absent	present	absent	nib, small*	absent	present	YPL084W	BRO1
	C10	normal	dark	blue	nib, small	medium	present	YPL267W	ACM1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
18	G14	normal	dark	pale blue	nib, small	normal	present	YPL241C	CIN2
	G18	dark, small	normal	absent	nib, small	absent	present	YPL239W	YAR1
	G21	normal	normal	pale blue	nib, small	norm/sm	present	YOL036W	YOL036W
	H14	normal	normal	pale blue	nib, small*	small	present	YPL049C	DIG1
	H20	normal	normal	pale blue	nib, small	normal	present	YPL046C	ELC1
	H21	normal	normal	pale blue	nib, small	normal	present	YPL141C	FRK1
	I13	normal	normal	pale blue	nib, small*	normal	present	YOL044W	PEX15
	J13	normal	normal	pale blue	nib, small*	normal	present	YPL133C	RDS2
	K13	normal	normal	pale blue	nib, small	normal	present	YOL055C	THI20
	M7	pale	normal	pale blue	nib, small*	medium	present	YOL064C	MET22
19	Ill	normal	normal	pale blue	nib, small*	normal	present	YPR059C	YPR059C
	I12	normal	normal	v.pale blue	nib, small*	normal	present	YPR159W	KRE6
20	G12	normal	normal	pale blue	nib, small	small	present	YGL076C	RPL7A
	M18	absent	normal	absent	nib, small	absent	present	YJL189W	RPL39

Table 12. Strains with colonies that appear larger and/or smoother upon Rep1 and Rep2 overexpression.

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
1	A20	normal	normal	pale blue	smooth	present	present	YCR101C	YCR101C
	C16	pale	normal	blue	smooth	normal	present	YDR363W-A	SEM1
	D20	normal	normal	pale	v.smooth	absent	present, small	YLL018C-A	COX19
	F17	normal, small	normal	absent	v.smooth	absent	present	YGR285C	ZUO1
	G20	normal	normal	pale blue	smooth	normal	present	YFR040W	SAP155
	H15	normal	normal	pale blue	v.smooth	absent	present	YHR039C-B	VMA10
	K23	normal	dark	pale blue	smooth	med/small	present	YCR063W	BUD31
	L18	normal	normal	dark blue	v.smooth	absent	present, small	YMR060C	TOM37
	M4	absent	normal	pale blue	smooth	medium	present	YGR229C	SMI1
2	C11	absent	absent	dark blue	smooth	medium	present	YER116C	SLX8
	F1	normal	normal	pale blue	v.smooth	v.small	present, small	YHL025W	SNF6
	F11	normal	normal	pale blue	smooth	absent	present	YHR064C	SSZ1
	F12	normal	pale	pale blue	v.smooth	medium	present, small	YML110C	COQ5
	F16	dark	normal	pale blue	v.smooth	medium	present	YML112W	CTK3
	G13	normal	normal	pale blue	smooth	norm/med	present	YER141W	COX15
	G19	normal	normal	pale blue	v.smooth	medium	present	YER145C	FTR1

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
2	H11	pale	normal	dark blue	v.smooth, small	absent	present	YLL049W	LDB18
	H12	dark	dark	pale blue	v.smooth	normal	present	YML121W	GTR1
	K1	pale	pale	pale blue	v.smooth	normal	present	YER161C	SPT2
	K12	normal	normal	pale blue	smooth	small	present	YOL108C	INO4
	L15	absent	normal	blue	smooth	absent	present	YMR312W	ELP6
	N15	normal	normal	pale blue	v.smooth	med/small	present	YNL250W	RAD50
	N24	normal	normal	pale blue	smooth	normal	present	YMR123W	PKR1
	P4	absent	normal	absent	v.smooth	norm/med	present	YMR125W	STO1
	P21	pale	normal	pale	smooth	absent	present, small	YPL132W	COX11
3	C16	dark	normal	pale blue	smooth	absent	present	YDR074W	TPS2
	D1	normal	normal	pale blue	v.smooth	absent	present	YCL058C	FYV5
	E1	normal	normal	pale blue	v.smooth	absent	present	YHR004C	NEM1
	E9	normal	normal	pale blue	smooth	absent	present	YHR008C	SOD2
	E17	normal	dark	pale blue	v.smooth, small	absent	present, small	YHR026W	PPA1
	G8	absent	normal	absent	v.smooth	absent	present	YLR358C	YLR358C
	J17	normal	dark	pale blue	v.smooth	absent	present	YJR118C	ILM1
	J21	dark	normal	pale blue	v.smooth	absent	present	YJR120W	YJR120W
	J23	pale	normal	pale blue	v.smooth	absent	present	YJR121W	ATP2
4	D12	normal	normal	v.pale blue	v.smooth	absent	present	YDR512C	EMI1
	E3	normal	normal	pale blue	v.smooth, large	absent	present	YGR063C	SPT4
	E11	normal	normal	pale blue	smooth	absent	present	YGR092W	DBF2

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
4	F15	normal	normal, small	blue	v.smooth	absent	present	YCR047C	BUD23
	F19	dark	normal	pale blue	smooth	absent	present	YCR053W	THR4
	H2	dark	dark	pale blue	smooth	absent	present	YGR252W	GCN5
	J14	normal	normal	pale blue	v.smooth	absent	present	YIR023W	DAL81
	J19	normal	normal	pale blue	v.smooth	absent	present, small	YNL052W	COX5A
	K14	normal	normal	pale blue	v.smooth	absent	present	YNR052C	POP2
	L22	normal	dark	pale	v.smooth	absent	present, small	YNL315C	ATP11
	M7	absent	normal	absent	v.smooth	absent	present	YKL204W	EAP1
	M14	normal	normal	pale blue	v.smooth	absent	present	YPL268W	PLC1
	N16	normal	normal	pale blue	v.smooth	absent	present	YOR270C	VPH1
	P7	normal	normal	white	v.smooth	large	present	YNL107W	YAF9
	P8	absent	normal	white	v.smooth, large	absent	present	YOR302W	YOR302W
5	A5	normal	normal	pale blue	v.smooth	small	present	YDR417C	YDR417C
	D21	normal	absent	blue	smooth	medium	present, small	YBL021C	HAP3
	F22	normal	normal	pale blue	smooth	medium	present	YBR018C	GAL7
	F24	normal	normal	pale blue	v.smooth	normal	present	YBR019C	GAL10
	H4	normal	normal	pale blue	smooth	normal	present	YBR021W	FUR4
	I6	normal	normal	pale blue	smooth	medium	present	YAL021C	CCR4
	N19	absent	dark	absent	v.smooth	med/normal	present	YBL080C	PET112

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
5	N20	normal	normal	pale blue	smooth	normal	present	YBR065C	ECM2
6	A3	absent	absent	blue	v.smooth	v.small	present	YBR081C	SPT7
	D5	normal	normal	pale blue	v.smooth	normal	present	YCL016C	DCC1
	F16	normal	normal	white	v.smooth	medium	present, small	YDL032W	YDL032W
	F18	normal	normal	white	v.smooth	medium	present, small	YDL033C	SLM3
	G13	normal	normal	pale blue	v.smooth	norm/med	present, small	YBR120C	CBP6
	G14	normal	normal	pale blue	v.smooth	normal	present	YBR215W	HPC2
	I22	normal	normal	pale blue	v.smooth	normal	present	YBR231C	SWC5
7	A16	normal	normal	v.pale blue	v.smooth	absent	present, small	YDL198C	GGC1
	A23	normal	normal	v.pale blue	v.smooth	small	present, small	YDL107W	MSS2
	A24	normal	absent	pale blue	v.smooth	normal	present, small	YDL202W	MRPL11
	C17	absent	absent	dark blue	v.smooth	small	present	YDL116W	NUP84
	D15	normal	dark	pale	v.smooth	absent	present	YDR069C	DOA4
	F6	normal	normal	pale blue	v.smooth	normal	present, small	YDR173C	ARGR3
	J4	normal	normal	pale blue	v.smooth	absent	present	YDR195W	REF2
	K16	dark	dark	pale blue	v.smooth	absent	present, small	YDR017C	KCS1
	L4	normal	normal	pale blue	v.smooth	absent	present	YDR207C	UME6

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
7	L11	normal	normal	pale blue	smooth	med/sm	present	YDR116C	MRPL1
	M12	absent	absent	pale blue	v.smooth	med/sm	present	YDR027C	LUV1
	M18	absent	present	pale blue	v.smooth	normal	present	YDR030C	RAD28
	N9	pale	absent	blue	smooth	small	present	YDR127W	ARO1
	P11	absent	normal	absent	smooth	absent	present	YDR140W	MTQ2
8	A7	normal	dark	pale blue	v.smooth, small	normal	present	YDR245W	MNN10
	B13	normal	normal	pale blue	v.smooth	medium	present	YDR443C	SRB9
	C21	normal	dark	dark blue	smooth	absent	present	YDR264C	AKR1
	D23	pale	pale	blue	smooth	med/large	present	YDR462W	MRPL28
	G23	absent	normal	blue	smooth	normal	present	YDR289C	RTT103
	H18	absent	normal	absent	v.smooth	v.small	present	YEL044W	IES6
	I10	normal	normal	blue	v.smooth	norm/med	present, small	YDR393W	SHE9
	J8	normal	dark	pale blue	v.smooth	colony	present, small	YEL051W	VMA8
	L2	normal	normal	pale blue	smooth, large	normal	present	YEL059W	HHY1
	N3	normal	normal	pale blue	v.smooth	small	present	YDR512C	EMI1
	P19	absent	absent	absent	v.smooth, large	small	present	YDR532C	KRE28
9	C16	normal	normal	pale blue	v.smooth	medium	present	YFL018C	LPD1
	D12	absent	dark	absent	v.smooth	absent	present, small	YGL115W	SNF4
	D23	absent	present	pale blue	v.smooth	absent	present	YGL024W	YGL024W

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
9	E17	absent	absent	absent	v.smooth, small	colony	present, small	(YER044C)	(ERG28)
	F1	pale	normal	dark blue	v.smooth	small	present	YGL025C	PGD1
	H11	normal	normal	pale blue	smooth	normal	present	YGL042C	YGL042C
	I11	normal	dark	pale blue	smooth	small	present	YER061C	CEM1
	J17	normal	normal	pale blue	v.smooth	absent	present, small	YGL058W	RAD6
	K7	normal	normal	pale blue	v.smooth	present	present	YER068C-A	
	K9	normal	normal	blue	smooth	absent	present	YER069W	ARG5,6
	L9	normal	normal	pale blue	v.smooth	absent	present	YGL066W	SGF73
	N8	normal	absent	pale blue	v.smooth	norm/med	present	YGL173C	KEM1
10	A7	absent	absent	pale blue	smooth	normal, nib	present	YGL197W	MDS3
	A20	present	absent	pale blue	smooth	small	present	YGR036C	CAX4
	C9	normal	pale	blue	smooth	med/normal	present	YGL211W	NCS6
	D1	normal	normal	pale blue	v.smooth	normal	present	YGR135W	PRE9
	E12	normal	normal	v.pale blue	smooth	normal	present	YGR056W	RSC1
	F10	normal	normal	pale blue	smooth	normal	present	YHL022C	SPO11
	F21	normal	normal	pale blue	smooth	absent	present	YGR157W	CHO2
	G15	normal	normal	pale blue	smooth	absent	present, small	YGL237C	HAP2
	H1	normal	normal	pale blue	smooth	medium	present	YGR159C	NSR1
	H3	normal	normal	pale blue	v.smooth	small	present	YGR160W	YGR160W

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
10	H13	normal	normal	pale blue	v.smooth	absent	present	YGR165W	MRPS35
	H23	normal	normal	pale blue	smooth	normal	present	YGR170W	PSD2
	I5	normal	normal	v.pale blue	v.smooth	normal	present	YGL244W	RTF1
	I9	normal	normal	pale blue	v.smooth, large	small	present	YGL246C	RAI1
	L2	normal	normal	pale blue	v.smooth	small	present	YHR010W	RPL27A
	L18	normal	normal	pale blue	v.smooth	absent	present	YHR018C	ARG4
	M12	normal	normal	pale blue	v.smooth	absent	present, small	YGR104C	SRB5
	M14	normal	normal	pale blue	v.smooth, large	v. small	present, small	YGR105W	VMA21
	O11	absent	normal	pale blue	v.smooth	colony	present	YGR020C	VMA7
11	D17	normal	normal	pale blue	v.smooth	absent	present	YIL020C	HIS6
	E10	normal	normal	white	smooth	absent	present	YHR167W	THP2
	I15	normal	normal	pale blue	smooth	small	present	YHR100C	GEP4
	J10	normal	normal	pale blue	v.smooth	small	present	YIL052C	RPL34B
12	A1	dark	dark	pale blue	v.smooth	normal	present	YIL098C	FMC1
	B3	normal	normal	pale blue	smooth	absent	present	YJL127C	SPT10
	B9	dark	normal	pale	v.smooth, small	normal	present	YJL124C	LSM1
	G2	normal	absent	blue	v.smooth, small	v. small	present, small	YJL188C	BUD19
	G12	normal	normal	pale blue	smooth	normal	present	YJL183W	MNN11
	I4	normal	normal	blue	v.smooth	absent	present, small	YJL176C	SWI3
	J15	normal	normal	pale blue	v.smooth	absent	present	YJL071W	ARG2

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
12	K5	normal	normal	pale blue	smooth	normal	present	YIL160C	POT1
	M10	normal	normal	blue	v.smooth	medium	present	YJL148W	RPA34
13	A13	normal	normal	pale blue	v.smooth	medium	present	YKL006W	RPL14A
	A21	normal	normal	pale blue	smooth	normal	present	YKL010C	UFD4
	G3	normal	normal	pale blue	smooth	norm/med	present	YKL037W	AIM26
	G14	normal	normal	pale blue	v.smooth	medium	present, small	YKL137W	CMC1
	N20	absent	absent	dark blue	smooth	normal	present	YLR015W	BRE2
	O14	normal	normal	pale blue	smooth	normal	present	YKL185W	ASH1
	P16	normal	normal	pale blue	smooth	absent	present	YLR025W	SNF7
14	B23	normal	normal	pale blue	smooth, large	absent	present, small	YLR239C	LIP2
	D4	normal	normal	pale blue	v.smooth	absent	present	YLR337C	VRP1
	D9	normal	normal	pale blue	v.smooth	absent	present	YLR244C	MAP1
	F21	normal	normal	pale blue	smooth	normal	present	YLR262C	YPT6
	G3	normal	normal, small	pale	v.smooth	absent	present, small	YLR067C	PET309
	I3	normal	pale	dark blue	smooth	absent	present	YLR079W	SIC1
	I6	normal	pale	pale	v.smooth	absent	present, small	YLR182W	SWI6
	K1	normal	normal	pale blue	smooth	normal	present	YLR090W	XDJ1
	K24	normal	normal	white	v.smooth	colony	present, small	YLR203C	MSS51
	L20	normal	normal	pale	v.smooth	norm/med	present	YLR393W	ATP10

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
14	O6	normal	normal	pale blue	v.smooth	small	present	YLR218C	COA4
	N16	absent	pale	pale blue	smooth	absent	present	YLR403W	SFP1
15	B21	normal	normal	pale	v.smooth	absent	present, small	YMR151W	YIM2
	B23	pale	normal	pale blue	v.smooth	colony	present, small	YMR150C	IMP1
	B24	absent	normal	pale blue	smooth	medium	present	YMR242C	RPL20A
	E14	normal	normal	dark blue	v.smooth	normal	present	YML014W	TRM9
	G2	normal	normal	pale blue	v.smooth	small	present	YML010W-A	YML010W-A
	H2	absent	normal	absent	v.smooth	absent	present, small	YMR267W	PPA2
	I1	normal	normal	pale blue	smooth	normal	present	YMR002W	MIX17
	I15	normal	normal	pale blue	smooth	normal	present	YML082W	YML082W
	J15	normal	normal	pale blue	smooth	norm/med	present	YMR191W	SPG5
	L15	absent	normal	absent	smooth	colony	present	YMR202W	ERG2
	M5	normal	normal	pale blue	v.smooth	small	present	YML063W	RPS1B
	M7	normal	normal	white	smooth	norm/med	present	YML062C	MFT1
	P19	pale	pale	pale blue	smooth	absent	present, small	YMR228W	MTF1
16	A12	absent	normal	absent	smooth, small	normal	present	YNL238W	KEX2
	A16	normal	pale	dark blue	smooth	absent	present	YNL236W	SIN4
	D14	normal	normal	pale blue	smooth	absent	present	YNL021W	HDA1
	E10	normal	normal	pale blue	v.smooth	norm/med	present	YNL215W	IES2

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
16	G20	normal	normal	pale blue	smooth	medium	present	YNL198C	YNL198C
	I8	normal	normal	pale blue	smooth	norm/med	present	YNL192W	CHS1
	K21	absent	normal	absent	smooth	absent	present	YNL269W	BSC4
	L14	normal	normal	pale	v.smooth	small	present	YNR020C	ATP23
	M4	normal	pale	pale blue	v.smooth	med/small	present	YNL171C	YNL171C
	N13	normal	normal	blue	smooth	colony	present	YNL069C	RPL16B
	O15	absent	absent	absent	v.smooth	absent	present	YNL248C	RPA49
17	B4	absent	normal, small	absent	v.smooth, small	absent	present, small	YOR290C	SNF2
	E11	normal	normal	pale blue	v.smooth	norm/med	present	YOR030W	DFG16
	E20	absent	normal	pale blue	v.smooth	absent	present	YOR130C	ARG11
	E23	normal	normal	pale blue	smooth	absent	present	YOR036W	PEP12
	F9	dark	normal	pale blue	smooth	small	present	YOR221C	MCT1
	H16	absent	normal	absent	v.smooth	absent	present	YOR331C	YOR331C
	K19	normal	normal	pale blue	v.smooth	absent	present	YOR070C	GYP1
	M11	absent	pale	absent	v.smooth	v.small	present	YOR078W	BUD21
18	C21	normal	normal	v.pale blue	smooth	medium	present	YOL012C	HTA3
	E24	normal	normal	pale blue	v.smooth	med/small	present	YPL248C	GAL4
	F5	normal	normal	dark blue	v.smooth	medium	present	YPL161C	BEM4
	F13	normal	normal	pale blue	smooth	normal	present	YPL157W	TGS1
	I15	normal	normal	pale blue	v.smooth	normal	present	YOL045W	PSK2
	K1	normal	absent	pale blue	v.smooth, large	normal	present	YOL050C	YOL050C
	K3	dark	dark	pale	v.smooth	absent	present, small	YOL051W	GAL11

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
18	K19	normal	normal	pale blue	v.smooth	absent	present	YOL058W	ARG1
	O5	normal	normal	pale blue	v.smooth	v.small	present	YOL076W	MDM20
	M23	normal	pale	pale blue	smooth, small	small	present	YOL072W	THP1
	O24	normal	normal	pale blue	v.smooth	small	present	YPL188W	POS5
	P11	normal	normal	pale blue	smooth	norm/med	present	YPL098C	MGR2
19	B8	normal	normal	pale blue	v.smooth	normal	present	YCR044C	PER1
	B16	normal	normal	white	v.smooth	v.small	present, small	YGR222W	PET54
	B20	normal	normal	pale blue	smooth	med/small	present	YGR285C	ZUO1
	B22	normal	normal	dark blue	smooth	v.small	present, small	YIR026C	YVH1
	D9	normal	normal	blue	smooth	absent	present	YBR081C	SPT7
	E10	normal	normal	pale blue	v.smooth	normal	present, small	YPR134W	MSS18
	E12	normal	normal	pale blue	v.smooth	normal	present	YPR135W	CTF4
	E13	normal	normal	pale blue	v.smooth	absent	present, small	YPR036W	VMA13
	E20	absent	absent	dark blue	v.smooth, small	absent	present	YPR139C	LOA1
	F6	normal	dark	pale blue	smooth	small	present	YHR064C	PDR13
	G12	normal	normal	pale blue	smooth, small	normal	present	YPR147C	YPR147C
	H1	normal	normal	pale blue	v.smooth	medium	present	YDR290W	YDR290W
	H4	normal	normal	pale blue	v.smooth	normal	present	YGR188C	BUB1
	J7	normal	dark	pale blue	v.smooth	normal	present, small	YGL218W	YGL218W

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
19	J14	normal	dark	pale blue	v.smooth	med/small	present	YJR118C	ILM1
	J16	normal	normal	pale blue	v.smooth	med/small	present, small	YJR121W	ATP2
	J23	absent	normal	dark blue	v.smooth	absent	present	YBR279W	PAF1
	K24	normal	normal	white	smooth	absent	present	(YPR177C)	(YPR177C)
	L7	normal	normal	pale blue	smooth	med/small	present, small	YJL006C	CTK2
	L10	absent	absent	dark blue	v.smooth	normal	present	YJR040W	GEF1
	N24	normal	normal	pale blue	v.smooth, large	absent	present, small	YCR084C	TUP1
	O4	normal	normal	pale	v.smooth	small	present	YPR191W	QCR2
	P6	normal	normal	white	smooth	normal	present	YFL001W	DEG1
	P16	normal	normal	pale blue	v.smooth, large	absent	present	YGR272C	YGR272C
	P22	normal	normal	pale blue	smooth	small	present, small	YNL315C	ATP11
20	A1	normal	normal	pale blue	v.smooth	small	present	YDR417C	YDR417C
	B2	normal	absent	pale blue	v.smooth	v.small	present, small	YOR290C	SNF2
	B3	normal	normal	pale blue	v.smooth	v.small	present, small	YKL080W	VMA5
	B6	normal	normal	pale blue	v.smooth	absent	present	YOR302W	YOR302W
	B20	normal	normal	pale blue	v.smooth	med/small	present, small	YOR358W	HAP5

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
20	C3	normal	absent	dark blue	v.smooth	medium	present, small	YBL021C	HAP3
	C19	normal	normal	pale blue	v.smooth	small	present	YBL058W	SHP1
	D4	normal	normal	pale blue	smooth	med/small	present	YOL072W	THP1
	D6	normal	normal	pale blue	v.smooth, small	v.small	present, small	YOL051W	GAL11
	D7	normal	normal	pale blue	v.smooth	medium	present, small	YLL041C	SDH2
	D10	pale	pale, small	dark blue	v.smooth, large	v.small	present	YPL234C	VMA11
	D16	normal	normal	pale blue	v.smooth	absent	present	YPL188W	POS5
	E1	normal	normal	pale blue	v.smooth	normal	present	YBL080C	PET112
	F6	normal	normal	pale blue	v.smooth	absent	present	YPL106C	SSE1
	F11	normal	normal	pale blue	smooth	small	present	YLR226W	BUR2
	F19	normal	normal	pale blue	v.smooth	absent	present	YLR244C	MAP1
	G1	normal	normal	v.pale blue	v.smooth	medium	present, small	YBR120C	CBP6
	I14	normal	normal	pale blue	v.smooth	absent	present	YGL246C	RAI1
	I21	absent	normal, small	dark blue	v.smooth	absent	present, small	YDL067C	COX9
	J1	normal	dark, small	pale blue	v.smooth, large	small	present, small	YMR151W	YIM2
	L5	pale, small	pale	dark blue	v.smooth	absent	present	YNL248C	RPA49

Plate	Position	Southerns		*STB(O)-lacZ*	Rep overexpression	growth controls		ORF name	
		mated	non-mated			galactose	dextrose	systematic	standard
20	L9	pale, small	normal	dark blue	v.smooth	normal	present	YNL236W	SIN4
	M4	normal	normal	v.pale blue	smooth	med/normal	present, small	YHR116W	COX23
	M20	normal	normal	blue	v.smooth	absent	present	YJL188C	YJL188C
	O8	pale	absent	dark blue	v.smooth	absent	present	YJL075C	YJL075C