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The yeast 2-μm plasmid Raf protein contributes to plasmid inheritance by stabilizing the Rep1 and Rep2 partitioning proteins

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ABSTRACT

The yeast 2-μm plasmid is a remarkable genetic parasite, managing efficient maintenance at high-copy number with minimal impact on the host. Equal partitioning of the plasmid upon host cell division requires plasmid proteins Rep1 and Rep2 and the plasmid *STB* locus. The Rep proteins and the plasmid-encoded Raf protein also regulate plasmid gene transcription. In this study, protein interaction assays, sequence analyses and mutational approaches were used to identify domains and residues in Rep2 and Raf required for association with Rep1 and Rep2 and to delineate the Rep2 DNA-binding domain. Rep2 and Raf displayed similarities in interactions with Rep1 and Rep2, in having Rep1 promote their *STB* association *in vivo*, and in stabilizing Rep protein levels. Rep2 mutants impaired for self-association were competent for transcriptional repression while those deficient for Rep1 association were not. Surprisingly, Rep2 mutants impaired for either Rep1 interaction or self-association were able to maintain efficient plasmid inheritance provided Raf was present and competent for Rep protein interaction. Our findings provide insight into the Rep protein complexes required for partitioning and transcriptional repression, and suggest that in addition to its transcriptional function, Raf stabilization of Rep partitioning proteins contributes to the remarkable persistence of the 2-μm plasmid.

INTRODUCTION

The 2-μm plasmid of *Saccharomyces cerevisiae* (Figure 1) is the best-studied member of a family of autonomously-replicating circular DNA plasmids found only in the Saccharomycetaceae lineage of budding yeast (1–4). The plasmid is inherited with an efficiency that approaches that of chromosomes, while its presence confers no obvious benefit to the yeast host (5). Retention of the 2-μm plasmid has been attributed to the combined action of mechanisms for plasmid partitioning and copy number control (1–4).

Equal partitioning of the multiple copies of the 2-μm plasmid at cell division is dependent on the plasmid-encoded Rep1 and Rep2 proteins and a repeated sequence at the *STB* partitioning locus (6,7). Deletion of any one of these results in a strong maternal bias in inheritance with few daughter cells receiving copies of the plasmid (8). *In vivo*, Rep1 and Rep2 are both found in association with the *STB* locus (9–11), but the nature of this interaction is unclear. The association of Rep1 with *STB* does not require the presence of other 2-μm proteins, while Rep2 requires the presence of Rep1 for robust association with *STB* (12). Rep1 and Rep2 have been shown to interact directly with each other and to self-associate *in vivo* and *in vitro* (13,14). Rep2 has been shown to bind the *STB* repeated DNA sequence in southwestern assays (15), but neither Rep1 nor Rep2 binds DNA in gel mobility shift assays (16), precluding standard *in vitro* approaches to dissecting the interactions. Amino acid substitutions in Rep1 that impair association with either Rep2 or *STB in vivo* also impair plasmid partitioning, suggesting that the functional 2-μm plasmid partitioning structure includes a complex of Rep1 and Rep2 at *STB* (9). This complex is required for recruitment of host factors, including the Kip1 motor protein (17), the centromere-specific histone H3 variant Cse4 (18), and the cohesin complex (19) to *STB*. These, along with presence of the RSC2 chromatin-remodeling complex (9,20), enable 2-μm plasmid partitioning to be coupled to host chromosome segregation (21).

In addition to their role in plasmid partitioning, Rep1 and Rep2 control plasmid copy number. Amplification of plasmid copy number is triggered by the plasmid-encoded site-specific recombinase Flp, which catalyzes recombination between target sites in a pair of inverted repeats found on the plasmid (Figure 1) (22–24). Flp-mediated recombination during replication converts plasmid copying from

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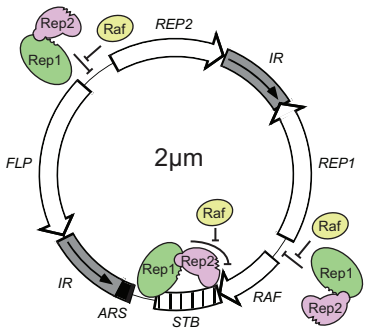

Figure 1. Model for interaction of 2-µm plasmid proteins with plasmid loci. The B form of the 2-µm plasmid is shown with positions of plasmid genes (white arrows), inverted repeat sequences (*IR*; gray boxes with arrows showing orientation), origin of replication (*ARS*; black box) and *STB* (striped box) indicated. Interactions of Rep1, Rep2 and Raf proteins with the 2-µm plasmid are shown. Rep1 and Rep2 associate with *STB* to mediate plasmid partitioning (9,11,16), and repress transcription driven by *STB* and divergent 2-µm plasmid promoters (23,25,26), with Raf acting to relieve this repression (26).

a bi-directional to a rolling circle mode, producing multiple plasmid copies from a single DNA replication initiation event (22–24). Transcription of all four plasmid genes, including the *FLP* gene, and of a 1950-nt transcript of unknown function directed by *STB*, is repressed upon simultaneous expression of Rep1 and Rep2, providing an elegant mechanism whereby further copy number increases are restricted when Rep protein levels in the cell are high (23,25,26). The fourth protein encoded by the 2-µm plasmid, Raf, has been shown to relieve this Rep protein-mediated repression when over-expressed (26), although the mechanism by which this occurs has not been established.

In this study, we have used a combination of *in vivo* assays and bioinformatic analyses to better define the roles of Rep2 and Raf in 2-µm plasmid maintenance. We found that Rep2 and Raf display similarities in their interactions with Rep1 and Rep2, and in requiring Rep1 for robust *STB* association *in vivo*, and that Raf association likely stabilizes Rep protein levels, contributing directly to efficient partitioning of the 2-µm plasmid.

MATERIALS AND METHODS

Strains and media

Saccharomyces cerevisiae yeast strains used in this study are listed in Table 1. Strains lacking the 2-µm plasmid are designated *cir*^{0}and were derived from strains containing the 2-µm plasmid (*cir*$^+$) by expression of a defective Flp recombinase (27). *Escherichia coli* strain DH5α was used for propagation of plasmids and strain BL21(DE3) (Novagen) for fusion protein expression. *Escherichia coli* and yeast were cultured and manipulated according to standard protocols as previously described (15,28).

Yeast were cultured in YPAD (1% yeast extract, 2% Bacto Peptone, 0.003% adenine, 2% glucose) or synthetic defined medium (SD; 0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 0.003% adenine, 0.002% uracil and all required amino acids) at 28°C (28). For induction of galactose-inducible promoters, glucose was replaced with 2% galactose (gal). For selection of plasmids or gene replacements tagged with nutritional genes, SD medium lacking the appropriate bases or amino acids was used. For selection of *kanMX4*-tagged plasmids, YPAD supplemented with 200 µg/l geneticin (G418, GIBCO) was used.

Plasmids

Plasmids and oligonucleotides used as primers for polymerase chain reaction (PCR) or site-directed mutagenesis, or as linkers for addition of restriction sites, are shown in Supplementary Tables S1 and 2, respectively. Phusion Polymerase was used for all PCR-based cloning, as recommended by supplier (Thermo Scientific). All plasmid sequences generated by PCR were confirmed by sequencing.

kanMX4-*tagged 2-µm plasmids.* The 2-µm-based pKan plasmid has been previously described (12) and consists of the entire B form of the 2-µm plasmid. The pKan plasmid is efficiently partitioned in yeast but is defective for Flp-mediated copy number amplification due to disruption of the *FLP* gene by the dominant drug-resistance marker gene *kanMX4*. *E. coli* vector sequences inserted in the inverted repeat downstream of the *REP1* and *REP2* genes replace one of the Flp target sites. Site-directed mutagenesis of *REP2* and *RAF* genes in pKan was carried out using gap repair in yeast to replace the wild-type gene with a PCR product encoding the mutant version. The PCR amplicons containing either the *REP2* or *RAF* ORF and their flanking sequences and containing the designated point mutation(s), were created by assembly PCR and co-transformed into yeast with SphI-digested or StuI-digested pKan plasmids, respectively. Plasmids created by gap repair in yeast were isolated in *E. coli*, sequenced and re-transformed into yeast for subsequent experiments.

One- and two-hybrid assay plasmids. Plasmids for expression in yeast of Rep1 or Rep2 fused to the DNA-binding domain (amino acids 1–87) of the bacterial repressor protein LexA ($LexA_{BD}$; pSH-REP1 and pSH-REP2), to the transcriptional activation domain of Gal4 ($Gal4_{AD}$; pGAD-REP1 and pGAD-REP2) or to an HA epitope-tagged B42 transcriptional activation domain ($B42_{AD}$-HA; pMM3-REP1 and pMM3-REP2) have been previously described (12,15). To create plasmids that would express truncated and mutant versions of Rep1, Rep2 and Raf fused to $LexA_{BD}$ in yeast, relevant portions of the open reading frames (ORFs) were amplified by PCR from 2-µm-based plasmids (pKan plasmids with appropriate wild-type or mutant genes) and the products were inserted in the two-hybrid vector pSH2–1 (29). For expression of proteins as $Gal4_{AD}$ fusions in yeast, EcoRI/SalI fragments encoding the ORFs in pSH-based plasmids were inserted in EcoRI/SalI-digested pGAD424 (Clontech). Full details of constructions are given in the Supplementary Data.

Plasmids for galactose-inducible expression of Rep1, Rep2 and Raf in yeast. A *LEU2*-tagged single-copy (*ARS*/*CEN*) yeast vector (pRSGAL-LEU) and derivatives of pRSGAL-LEU expressing untagged Rep1 or Rep2 under the control of the *GAL1* promoter (pRSGAL-LEU-REP1 and pRSGAL-LEU-REP2) have been previously

Table 1. Yeast strains used in this study

Strain	Genotype	Source
JP48/2b	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1* [cir^+]	W303 (12)
JP49/6b	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1* [cir^0]	W303 (12)
AG8/5	*MATa ade2Δ::URA3 his3–11,-15 leu2–3,-112 trp1–1 ura3–1* [cir^0]	W303, This study
CTMD/3a	*MATa ade2–1 his3 trp1 leu2–3,-112 met URA3::*(lexAop)$_8$-*lacZ* [cir^0]	(12)
JP30/1	*MATa ade2–1 his3–11,15 trp1–1 leu2–3,112 ura3–1 URA3::FLPp-lacZ* [cir^o]	JP49/6b, This study
EP4/MD	*MATa/MATα gal4/GAL4 gal80/GAL80 his3–11,-15/his3–200 trp1–1/trp1–901 ade2–1/ade2 ura3–1/ura3–52 leu2–3,-112/leu2–3,-112 MET/met THR/thr URA3::STB-P-HIS3* [cir^+] *or* [cir^0]	(12)

described (12). A plasmid expressing Raf under the control of the *GAL1* promoter (pRSGAL-LEU-RAF) was generated by BamHI partial/SalI digestion of pGAD-RAF and ligation of the 0.6 kbp *RAF* ORF fragment with BamHI/SalI-digested pRSGAL-LEU. Versions of these plasmids expressing mutant forms of Raf were similarly constructed.

A *TRP1*-tagged *ARS/CEN* plasmid expressing *RAF* with an amino-terminal FLAG epitope tag under the control of the *GAL10* promoter (pRSGAL-FLAG-RAF) was created by inserting the *RAF* ORF from BamHI-digested pGAD-RAF into BglII-digested pRSGAL-TRP (12).

A *URA3*-tagged *ARS/CEN* yeast plasmid allowing galactose-inducible expression from the divergent *GAL1/10* promoter (pBM272) (30), and derivatives of this plasmid expressing Rep1 and Rep2 individually (pBM272-REP1 and pBM272-REP2), or simultaneously (pBM272-R1R2) have been previously described (9).

Bacterial expression plasmids. A plasmid (pET32-REP2) for expression of full-length Rep2 as a thioredoxin-, hexahistidine- and S-peptide-tagged (Trx) fusion protein in *E. coli*, and the purification of the fusion proteins from bacterial cell lysates by metal-ion affinity chromatography have been previously described (12,15). Plasmids for expression of Raf and Rep2 truncations as Trx-fusion proteins were created by PCR amplification of the *RAF* ORF and relevant portions of the *REP2* ORF (codons 199–296, and 232–296) with flanking EcoRI and SalI sites and insertion in EcoRI/SalI-digested vector pET32 (Novagen), producing plasmids pET32-RAF, pET32-rep2$_{199–296}$ and pET32-rep2$_{232–296}$.

Two-hybrid assays

Protein–protein association was assayed in a cir^0 yeast strain (CTMD/3a) with eight copies of the LexA operator sequence in the basal promoter region upstream of a *lacZ* reporter gene integrated in the genome at the *URA3* locus (12). The reporter strain was co-transformed with two plasmids expressing the proteins to be assessed for interaction, one (pGAD-based) expressing a Gal4$_{AD}$ fusion protein and the other (pSH-based) expressing a LexA$_{BD}$ fusion protein. Activation of the *lacZ* gene, an indication of interaction of the two hybrid proteins, was monitored using a ß-galactosidase filter assay (29).

Southwestern assays

Southwestern assays were performed as previously described (15). Purified Trx-fusion proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Gels were incubated in western transfer buffer for 30 min to allow renaturation of the proteins, before transfer to nitrocellulose membranes by western blotting. Membranes were incubated twice for 15 min in BBW buffer (3 g/l Ficoll, 3 g/l polyvinylpyrrolidone, 10 mM NaCl, 20 mM Tris [pH 8.0]) with 0.25% milk powder, then incubated for 1 h at RT with 50 ng of a ^{32}P-labeled *STB* duplex oligonucleotide in 1 ml of BBW buffer. The *STB* duplex was created by annealing oligonucleotides *STB6* and *STB7* representing the sequence of a single 63-bp repeat from the 2-μm plasmid *STB* locus with the addition of single-stranded BamHI and BglII restriction site overhangs. The duplex was labeled by incubation with Klenow fragment, 0.08 mM each of unlabeled dGTP, dATP and dTTP, and [α^{32}-P]-dCTP, to a specific activity of 8.0×10^7 cpm/μg. Membranes were then washed twice for 30 min in BBW buffer before exposure to X-ray film overnight at −80°C with an intensifier screen.

2-μm and 2-μm-like plasmid ORF sequences

Sequences of 2-μm and 2-μm-like plasmids and conceptual translations of their *REP2* and *RAF*-positioned ORFs were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Protein sequences used were Rep2 (AAB59343.1) and Raf (AAB59342.1) encoded by the 2-μm plasmid variant (Scp1) found in laboratory strains of *S. cerevisiae*, ORF S (YP_355330.1) of plasmid pSR1 isolated from *Zygosaccharomyces rouxii,* ORF C (NP_040497.1) of plasmid pSB2 from *Zygosaccharomyces bailii*, ORF A (NP_040492.1) and ORF B (NP_040493.1) of plasmid pSM1 from *Zygosaccharomyces fermentati* (since reclassified as *Lachancea fermentati*) (31), ORF C (CAA27592.1) of plasmid pKD1 from *Kluveromyces lactis*, ORF C (CAA39901.1) and ORF B (CAA39903.1) of plasmid pKW1 from *Kluveromyces waltii* (since reclassified as *Lachancea waltii*) (31), and ORF C (CAA71933.1) of plasmid pTD1 from *Torulaspora delbrueckii*. For ORF C of plasmid pSB3, we isolated the plasmid from *Z. rouxii* (ATCC strain 56076) and sequenced the region encoding ORF C, extending the protein from the 157 amino acids originally predicted (XP_001728568.1) (32) to the 213 amino acid sequence used here (Genbank accession number KY549324). The B ORFs of plasmids pSM1 and pKW1 are in the *RAF*-equivalent position in their respective 2-μm-like plasmids. Protein sequences were compared us-

ing ClustalW2 (33), secondary structures predicted using Jpred4 (34) and searches for potential sequence motifs conducted using MEME and GLAM2 (35) accessed through the MEME Suite Web Portal. Protein pI calculations were done through the ExPASy Bioinformatics Resource Portal.

Western blotting analysis

Protein was extracted from yeast cultures using alkali lysis (12) and analyzed by western blotting as previously described (15). Antibodies used were rabbit polyclonal anti-Rep1 and anti-Rep2 (15), rabbit polyclonal anti-LexA (Pierce), mouse monoclonal anti-PGK (Molecular Probes), horseradish peroxidase-conjugated goat anti-rabbit (KPL) and Dylight 549 or 649-conjugated goat anti-mouse IgG (Rockland). Signals were detected by chemiluminescence using a Clarity Western ECL Substrate kit (BioRad) or by fluorescence and imaged using a VersaDoc 4000 MP imaging system with Quantity One software (BioRad) or by autoradiography.

Transcriptional repression assay

To monitor expression directed by the *FLP* promoter, the *lacZ* coding region fused in-frame after the fourth codon of the *FLP* ORF with 306-bp of the *FLP* promoter upstream (pFLPp-lacZ) was integrated at the *URA3* locus in a *cir*^{0}yeast strain creating strain EP8/2-FLPp. EP8/2-FLPp was transformed with pRSGAL-TRP-based plasmids expressing Rep1 and wild-type or mutant Rep2. Strains were cultured in selective medium containing galactose for 24 h before expression of the *FLPp-lacZ* reporter was quantified using a liquid assay for ß-galactosidase activity (28).

Plasmid inheritance assay

Inheritance of *kanMX4*-tagged 2-μm-based pKan plasmids was monitored as previously described (12). Plasmids were used to transform a *cir*^{0} strain of yeast (AG8/5) to G418-resistance. G418-resistant transformants were cultured for 16–24 h (6–8 generations) in selective medium (YPAD+G418) and the fraction of G418-resistant cells in the culture (a measure of partitioning function) was determined by calculating the ratio of colonies on selective (YPAD+G418) versus non-selective (YPAD) medium.

Chromatin immunoprecipitation (ChIP)

A *cir*$^{+}$ yeast strain (JP48/2b) was transformed with pRSGAL-FLAG-RAF and cultured overnight in SD-trp(gal) medium to induce expression of FLAG-tagged Raf, then diluted to a density of ∼2 × 10^{6} cells/ml and cultured in YPA (gal) to a density of ∼1.5 × 10^{7} cells/ml. Cells were formaldehyde-fixed and chromatin immunoprecipitation (ChIP) performed as previously described (12) using rabbit antibodies raised against wild-type Rep1 and Rep2 (15), mouse anti-HA (Roche) and anti-FLAG monoclonal antibodies (Sigma), Protein A Sepharose CL-4B for rabbit antibodies (GE Healthcare) and Protein G-Agarose for mouse antibodies (Roche).

DNA isolated by ChIP was resuspended in 40 μl TE (10 mM TRIS, 0.1 mM ethylenediaminetetraacetic acid, pH 8.0)/50 ml culture, and the enrichment of ∼300-bp 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 30 cycles of PCR. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, imaged on a VersaDoc 4000 MP with Quantity One software and quantified with ImageLab software (BioRad). Yield of PCR products from immunoprecipitated DNA was compared to that from input DNA to give locus immunoprecipitation as a percentage of input DNA.

One-hybrid assays

Protein-DNA association *in vivo* was monitored by one-hybrid assays using yeast strains (EP4/MD *cir*^{0} and EP4/MD *cir*$^{+}$) (12), in which the 295-bp AvaI/HpaI *STB* sequence is integrated in the genome upstream of a *HIS3* reporter gene. Reporter strains were co-transformed with a *TRP1*-tagged pMM2- or pMM3-based plasmid, expressing a B42$_{AD}$-HA fusion protein under the control of a galactose-inducible promoter, and a pRSGAL-LEU-based plasmid, expressing native Rep1, Rep2 or Raf from a galactose-inducible promoter. Transformants were cultured overnight in SD liquid medium lacking leucine and tryptophan (SD-leu-trp) and containing glucose. A series of 5-fold dilutions were prepared from overnight cultures, spotted on solid SD-leu-trp (gal) medium containing galactose and on solid SD-leu-trp (gal) medium lacking histidine and containing a competitive inhibitor of the *HIS3* gene product, 3-aminotriazole (3-AT). Plates were imaged after 5 days of incubation at 28°C.

RESULTS

Raf associates independently with Rep1 and Rep2 *in vivo*

Raf has been reported to co-purify with both Rep1 and Rep2 in tandem-affinity purification from yeast cell extracts (21) but whether Raf could associate independently with either was unclear. To address this and to determine the regions of the Raf protein required for these associations *in vivo*, interactions were assessed using two-hybrid assays, in which two proteins of interest were expressed in yeast, with one fused to the Gal4 transcriptional activation domain and the other to the DNA-binding domain of LexA (Figure 2A). The hybrid proteins were expressed in a reporter strain lacking 2-μm plasmid (*cir*^{0}) to prevent native 2-μm plasmid proteins from bridging or interfering with the interactions being assessed.

Full-length Raf (residues 1–181) was seen to associate with both Rep1 and Rep2 but not with itself (Figure 2A). Raf truncations lacking the first 12, 46 or 70 residues retained Rep2 association but no longer interacted with Rep1, while a truncation consisting of residues 1–73 (Raf$_{1–73}$) was sufficient for the association with Rep1 but did not interact with Rep2. Western blotting analysis showed that loss of interaction of the amino-terminally truncated versions of Raf with Rep1 was not due to lack of expression of the relevant fusion proteins (Supplementary Figure S1). For LexA-Raf$_{1–73}$, the level of the fusion protein was lower when co-

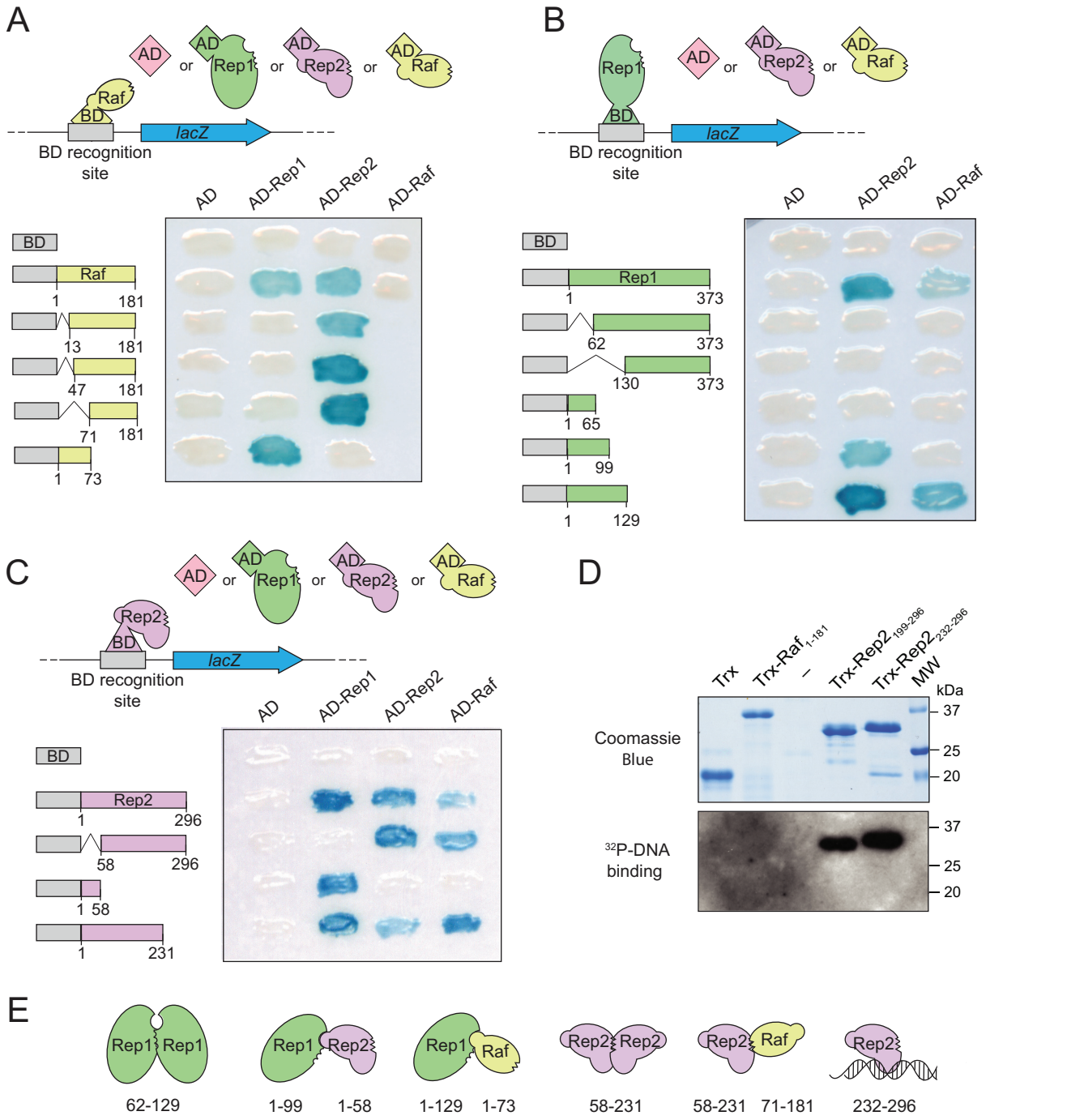

Figure 2. Raf associates with Rep1 and Rep2 independently and displays overlap with Rep2 in the regions required for these associations. (**A**–**C**) A *cir*^{0} two-hybrid reporter strain was co-transformed with two plasmids, one encoding $Gal4_{AD}$ (AD), or $Gal4_{AD}$ fused to Rep1, Rep2 or Raf; and the other encoding $LexA_{BD}$ (BD), or $LexA_{BD}$ fused to full-length or truncated versions of Raf (A), Rep1 (B) or Rep2 (C). Interaction of the two fusion proteins, indicated by activation of expression of the *lacZ* reporter gene, was monitored using a filter assay with the substrate X-gal, which yields a dark-coloured precipitate when cleaved by ß-galactosidase. (**D**) Hexahistidine-tagged thioredoxin (Trx) and Trx-Raf, Trx-$Rep2_{199–296}$ and Trx-$Rep2_{232–296}$ were purified from *Escherichia coli* cell lysates by metal ion affinity chromatography and resolved in duplicate gels by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Proteins were visualized by staining with Coomassie Blue (top) or transferred to a nitrocellulose membrane followed by incubation with a ^{32}P-radiolabeled *STB* 63-bp repeat probe. DNA binding was detected by autoradiography (bottom). (**E**) The regions of Rep1, Rep2 and Raf involved in each of the indicated interactions and for Rep2 with DNA are summarized. Residues shown for Rep1 self-association are based on prior *in vitro* studies (15) and two-hybrid interaction assays (Supplementary Figure S2).

expressed with AD-Rep2 than when co-expressed with AD-Rep1 (Supplementary Figure S1). The level might have been too low to detect interaction with Rep2 in this assay rather than indicating loss of Rep2 association. Alternatively, the reduced level may be a consequence of the truncated Raf protein not being able to interact with Rep2. Taken together, these results suggest that like Rep2, Raf has a small amino-terminal domain sufficient for Rep1 recognition, with a separate non-overlapping carboxy-terminal domain that mediates Rep2 association; however, Raf differs from Rep2 in that it does not display self-association in this assay.

Two-hybrid assays were also used to determine the regions of Rep1 and Rep2 required for association with Raf. Full-length Rep2 and full-length Raf interacted with full-length Rep1 (residues 1–373) and a Rep1 truncation consisting of residues 1 to 129 ($Rep1_{1–129}$), but not Rep1 lacking the amino-terminal 61 ($Rep1_{62–373}$) or 129 ($Rep1_{130–373}$) residues (Figure 2B). Previous studies have shown that Rep1 residues 1–129 are also sufficient for self-association, but, in contrast to association with Raf and Rep2, Rep1 residues 1–61 are dispensable for self-association *in vitro* (15) and *in vivo* (Supplementary Figure S2). Western blotting showed that LexA-$Rep1_{62–373}$ protein levels were lower than that of other Rep1 fusion proteins (Supplementary Figure S1). However, the level was similar to that observed when this Rep1 fusion was co-expressed with AD-Rep1 with which it interacts in a two-hybrid assay (Supplementary Figure S2) (unpublished results), suggesting loss of two-hybrid interaction with Raf and Rep2 was not solely due to the lower steady state level of this truncated Rep1 fusion protein.

A difference between Rep2 and Raf was identified in assessing interaction with more carboxy-terminally truncated versions of Rep1. While neither Raf nor Rep2 interacted with a fusion retaining only the 65 amino-terminal residues of Rep1, the 99 amino-terminal residues of Rep1 were sufficient for Rep2 association but not for Raf interaction (Figure 2B). Western blotting analysis showed that lack of interaction was not due to lack of expression of the fusion proteins (Supplementary Figure S1). The results imply that the regions of Rep1 required for interaction with Rep2 and Raf overlap (1–99 for Rep2 and 1–129 for Raf) and both overlap a region implicated in Rep1 self-association (Rep1 residues 62–129).

Full-length Rep2 (residues 1–296) and Rep2 truncations consisting of residues 1–58 ($Rep2_{1–58}$) or residues 1–231 ($Rep2_{1–231}$), but not residues 58–296 ($Rep2_{58–296}$) were seen to associate with Rep1 in a two-hybrid assay (Figure 2C), showing that, as previously reported, Rep2 residues 1–58 are required and sufficient for association with Rep1 (15). Full-length Rep2 and truncations $Rep2_{1–231}$ and $Rep2_{58–296}$ associated with both full-length Rep2 and full-length Raf in the two-hybrid assay, suggesting that sequences lying between residues 58–231 of Rep2 are required for self-association and for Raf recognition.

We have previously demonstrated that the Rep2 protein binds radiolabeled DNA in a southwestern assay, showing a higher affinity for *STB* repeat DNA over a more GC-rich competitor DNA, and with residues 1–57 being dispensable for this association (15). Here we have used the same approach to show that the C-terminal residues 199–296 and 232–296 of Rep2, expressed in bacteria as hexahistidine-tagged thioredoxin (Trx) fusion proteins (Trx-$Rep2_{199–296}$ and Trx-$Rep2_{231–296}$), also bind *STB* repeat DNA in this assay (Figure 2D) suggesting that the C-terminal 65 amino acids of Rep2 are sufficient for Rep2 DNA-binding activity. Raf expressed as a Trx-fusion protein (Trx-$Raf_{1–181}$) did not bind the DNA probe, suggesting that unlike Rep2, Raf does not recognize DNA, or that, like Rep1, Raf may not be able to associate with DNA under these conditions (15) or in the absence of other proteins (16).

Overall, the results of the two-hybrid and southwestern assays suggest that Raf associates with the same domains of Rep1 and Rep2 as Rep2 (Figure 2E). However, Raf interaction with Rep1 required a larger region of Rep1, Raf self-association was not observed and Raf did not display DNA-binding activity *in vitro* under conditions where Rep2 was able to do so (Figure 2D), suggesting that, while similar, the interactions of Raf are not identical to those of Rep2, and that Raf may lack DNA-binding activity.

Identification of potential sequence motifs in Rep2

As a first step in determining how Rep2 and Raf contribute to 2-μm plasmid inheritance, a bioinformatics approach was taken to identify specific sequence elements in each that might be required for their interactions with other 2-μm plasmid components. Although 2-μm-like plasmids have been found in other species of budding yeast, the plasmids share little identity at the DNA sequence level among themselves or with the *S. cerevisiae* 2-μm plasmid (4,36,37). They do however have a similar size and arrangement of sequence features to 2-μm, and all are predicted to encode a highly conserved version of Flp and a recognizable Rep1 homolog (37). The proteins predicted for the *REP2* and *RAF*-positioned ORFs bear no sequence similarity to Rep2 or Raf (4), but where tested, those in the position of *REP2* have been shown to be required along with the Rep1-encoding ORF for efficient plasmid inheritance (32,38–40), suggesting that the proteins are functional equivalents of 2-μm Rep2. If so, like Rep2, these proteins might be expected to interact with the Rep1 encoded by that plasmid to form a functional partitioning complex (9,20).

To identify a potential Rep1 interaction motif, the sequence of the amino-terminal 57 residues of Rep2 and the sequences predicted for the *REP2*-positioned ORFs on the 2-μm-like plasmids were searched using GLAM2 (35). A potential motif was found within the amino-terminal 33 residues of Rep2 and six of the seven 2-μm-like plasmid proteins (residues 16–31 for Rep2) (Supplementary Figure S3A). Secondary structure analysis (34) predicted an alpha helix within this region for each protein characterized by an aspartic acid residue followed by regularly spaced hydrophobic residues (Dh-x_3-h-x_3-h; where D is aspartic acid, x is any, and h is a hydrophobic residue).

A second motif identified in Rep2 and the 2-μm-like plasmid proteins encoded by *REP2*-positioned ORFs was located in a more carboxy-terminal region (residues 170–191 for Rep2) within the region required for association with Raf and Rep2 self-association (Supplementary Figure S3B). Secondary structure algorithms (34) predicted mainly alpha-helical conformations in this region of Rep2 and the 2-μm-like plasmid proteins, with the positioning

of hydrophobic residues suggesting the potential for forming amphipathic helices. The identified motif spans a region containing one of these predicted helices (residues 174–187 in Rep2) with the most notable feature being the regularly spaced hydrophobic residues. The hydrophobic face of the alpha helix in this central portion of Rep2 might represent an element involved in Rep2 self-association and/or association of Rep2 with Raf.

Several potential motifs characterized by similar positioning of basic residues, but differing slightly in extent, were also identified nearer the carboxy terminus of Rep2 (residues 231–251) and the 2-μm-like plasmid *REP2*-positioned ORF proteins (Supplementary Figure S4A, data not shown) in the region of Rep2 identified as sufficient for binding DNA in the southwestern assay (Figure 2D), suggesting this motif may contribute to Rep2 DNA-binding activity.

Amino acid substitutions in Rep2 that impair association with Rep1 or with Rep2 and Raf

To test whether the motifs identified in Rep2 by GLAM2 searches might represent functionally significant elements, residues deemed to be characteristic of these regions were altered. Aspartic acid 22 in Rep2, representing the most conserved site in the amino terminal motif, was replaced with asparagine to create $Rep2_{D22N}$, 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. A pair of leucines at positions 185 and 186, representing hydrophobic residues in the predicted amphipathic helix identified by the second motif, were replaced with alanines to create $Rep2_{AA}$; because alanine is less hydrophobic than leucine, it was anticipated that these substitutions would weaken associations mediated by hydrophobicity, but not significantly perturb structure. A third mutant, $Rep2_{NAA}$ containing all three amino acid substitutions (D22N, L185A and L186A) was also generated.

The ability of the mutant versions of Rep2 to associate with wild-type Rep1, Rep2 and Raf was tested *in vivo* in a cir^0 strain using two-hybrid assays (Figure 3A). The $Rep2_{D22N}$ mutant was unable to associate with Rep1, but was not impaired for association with Rep2 or Raf, while $Rep2_{AA}$ was unable to associate with Rep2 or Raf, but not impaired for association with Rep1. The $Rep2_{NAA}$ mutant did not interact with any of the proteins. Western blotting analysis with antibodies recognizing the LexA DNA-binding domain (BD), to which the Rep2 proteins had been fused to perform the two-hybrid assay, showed that none of the amino acid changes significantly reduced BD-Rep2 protein levels, although effects were seen on levels of some of the co-expressed AD fusion proteins. For example, levels of the wild-type Rep2 and Raf AD-fusions were reduced when co-expressed with the BD-$Rep2_{AA}$ mutant, which could be a consequence of loss of interaction with the mutant protein (Figure 3C). Consistent with the hypotheses presented above, these 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 and association with Raf.

Identification of amino acid substitutions in Raf that impair association with Rep1 or with Rep2

A search for potential motifs in Raf was hampered by a lack of sequences for comparison. Variants of the 2-μm plasmid have been identified (41), including one in a closely-related yeast species, *Saccharomyces eubayanus* (3), but differences within their *RAF* coding regions are few, with most representing conservative amino acid changes. Of the two 2-μm-like plasmids that have a *RAF*-positioned ORF, pKW1 and pSM1, the predicted proteins show some similarity with each other (25% identity), but no similarity to Raf. Disruption of pKW1 ORF B did not affect plasmid maintenance (6,38), precluding any inferences about the function of these proteins.

For the Raf domain identified as sufficient for Rep1 interaction in the two-hybrid assay, MEME and GLAM2 searches of the sequences of 2-μm Raf and of the proteins encoded by the *RAF*-positioned ORFs in pSM1 and pKW1 did not return any potential shared motifs, and for the Rep2-associating domain, identified only low-scoring potential motifs. As an alternative approach, we conducted a screen for mutant versions of Raf that no longer interacted with Rep1 in a two-hybrid assay but retained Rep2 association, and identified a phenylalanine-to-serine substitution at residue 18 (Raf_{F18S}) that specifically impaired Rep1 association (Figure 3B). This residue lies within the most amino terminal α-helix predicted for Raf, and like the first predicted α-helix in Rep2, has an aspartic acid residue near its amino-terminal end; however, the positioning of hydrophobic residues in the helix does not match the motif identified by the GLAM2 search in the first alpha helix of Rep2.

To identify a mutant version of Raf selectively impaired for Rep2 association, we targeted selected pairs of hydrophobic residues positioned in predicted amphipathic helices in the Rep2-interacting domain of Raf that were also conserved in the Raf proteins encoded by variants of the 2-μm plasmid. Substitutions of leucines 115 and 116 or of leucines 130 and 131 with alanines did not selectively impair Raf interaction with Rep2 in a two-hybrid assay (Supplementary Figure S5), whereas Raf with tyrosine 95 and leucine 96 switched to alanines (Raf_{AA}) retained Rep1 association, albeit at a reduced level, but failed to interact with Rep2 (Figure 3B). Western blotting analysis showed the steady-state level of the Raf_{AA} mutant fusion protein was reduced relative to that of the wild-type Raf (Figure 3D). A lower steady-state level for this fusion protein likely accounts for the weaker signal observed in the Rep1 two-hybrid interaction, but in comparison to the level of other Raf fusion proteins (Supplementary Figure S1), was unlikely to be sufficiently reduced to account for lack of two-hybrid interaction with Rep2.

$Rep2_{D22N}$, but not $Rep2_{AA}$, is impaired for transcriptional repression

Having identified amino acid substitutions in Rep2 and Raf that selectively impaired their interactions, we wanted to assess the effect of loss of each association on their known functions. Co-expression of Rep1 and Rep2 has been shown to repress transcription of 2-μm plasmid-encoded genes, with over-expressed Raf able to alleviate this repression

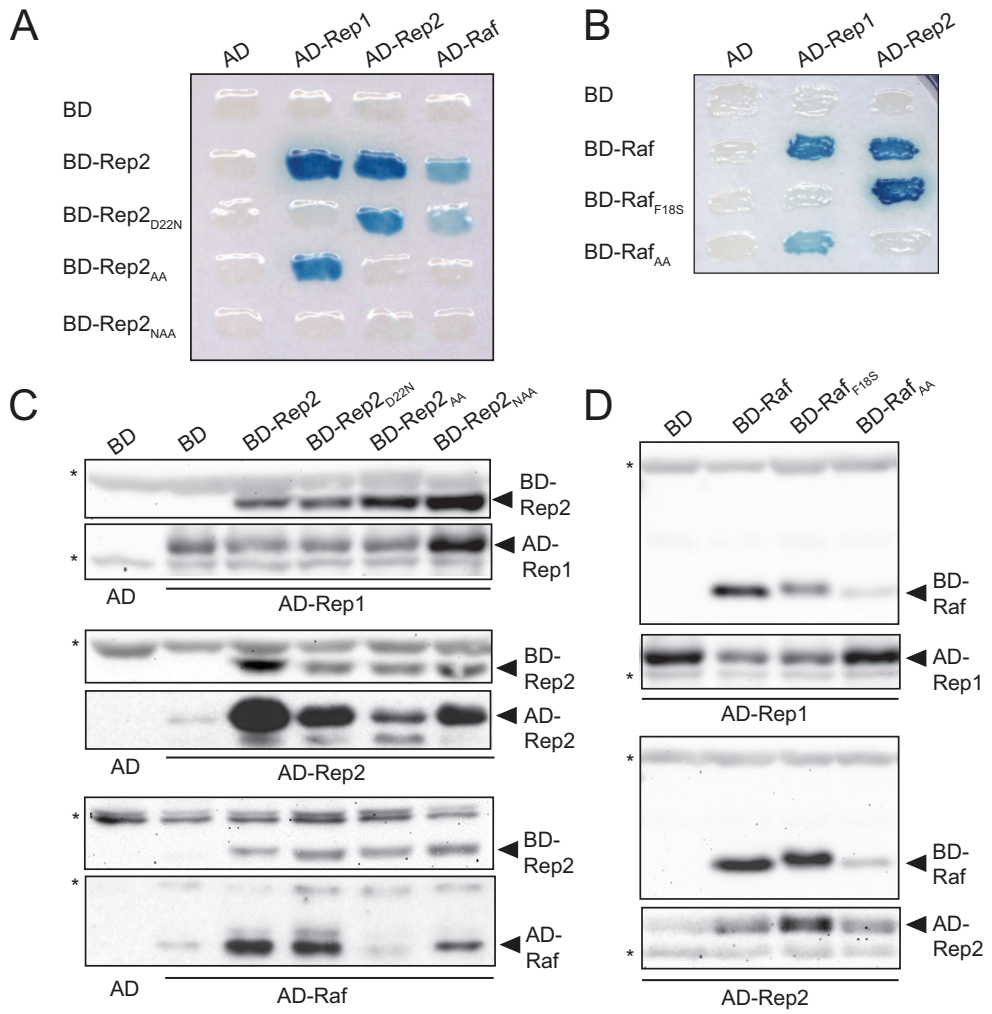

Figure 3. Identification of amino acid substitutions in Rep2 and Raf that selectively impair interaction with Rep1 or Rep2. A *cir*^{0} two-hybrid reporter strain was co-transformed with two plasmids, one expressing $Gal4_{AD}$ (AD) or $Gal4_{AD}$ fused to Rep1, Rep2 or Raf and the other expressing $LexA_{BD}$ (BD) or $LexA_{BD}$ fused to wild-type or mutant Rep2 (**A** and **C**) or Raf (**B** and **D**). (A and B) The two-hybrid assay for protein–protein interaction was performed as described in the legend for Figure 2. (C and D) Total protein was extracted from the co-transformants shown in (A) and (B) respectively, and equal amounts from each analyzed by western blotting. BD-fusions were detected with anti-LexA with the exception of BD-Rep2 expressed with AD-Raf where they were detected with anti-Rep2. Blots were stripped and reprobed for AD fusions with anti-Gal_{AD} antibody with the exception of AD-Rep1 fusions where duplicate blots were probed with anti-Rep1. Asterisks denote non-target host proteins detected by the antibodies.

(23,25,26). To determine whether Rep2 association with Rep1 or Rep2 self-association were required for this repression, expression of a *FLPp-lacZ* reporter, consisting of the *FLP* gene promoter and the first four codons of *FLP* fused in-frame to the *lacZ* gene, was monitored in *cir*^{0} yeast (Figure 4A). As expected, when Rep1 was co-expressed with wild-type Rep2 in the absence of Raf, *lacZ* expression directed by the *FLP* promoter was decreased compared to that observed when Rep1 was expressed without Rep2 or when Raf was co-expressed with Rep1 and Rep2. Expression of $Rep2_{AA}$ with Rep1 also decreased *FLPp-lacZ* expression although to a slightly lesser extent than wild-type Rep2, implying that $Rep2_{AA}$ is able to mediate transcriptional repression, and therefore that Rep2 self-association is dispensable for repressor function. In contrast, when Rep1 was expressed with $Rep2_{D22N}$, *FLPp-lacZ* expression was similar to that observed in the absence of Rep2, suggesting that $Rep2_{D22N}$ is impaired in its ability to mediate transcriptional repression, and consequently that the association of Rep1 and Rep2 may contribute to repressor function. $Rep2_{NAA}$ also failed to repress *FLPp-lacZ* expression (data not shown).

Raf_{F18S} retains anti-repressor function but Raf_{AA} does not

The anti-repressor activity of the mutant versions of Raf was also assessed. Raf_{F18S} was as competent as wild-type Raf in alleviating the repression of the *FLP* promoter conferred by co-expression of wild-type Rep1 and Rep2, suggesting Raf does not need to interact with Rep1 to perform this role. In contrast, expression of the Raf_{AA} mutant did not significantly increase *FLPp-lacZ* expression

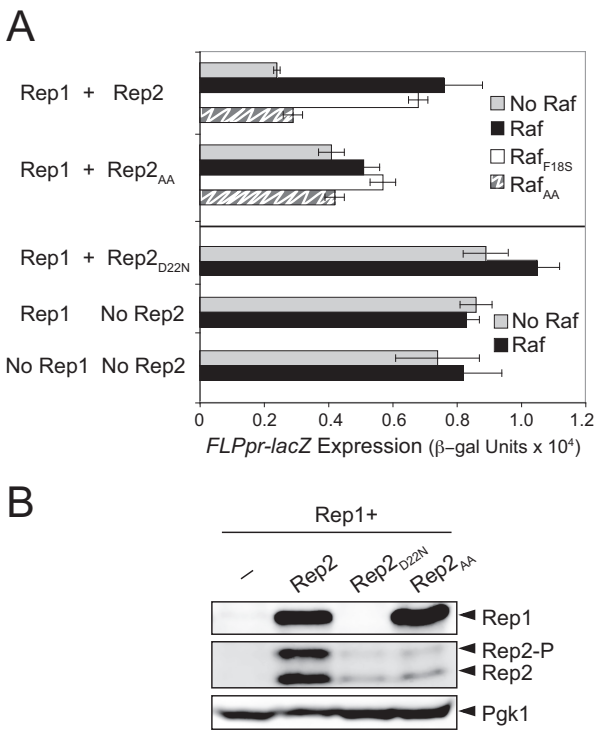

Figure 4. Rep2 transcriptional repressor activity is retained by $Rep2_{AA}$ but not by $Rep2_{D22N}$, with Raf_{F18S} but not Raf_{AA} able to alleviate this repression. The indicated Rep1, Rep2 and Raf proteins were expressed under the control of galactose-inducible promoters from *ARS/CEN* pRSGAL-based plasmids in a *cir*^{0} strain containing a *FLP* promoter-*lacZ* fusion reporter gene (*FLPp-lacZ*) integrated in the genome. (**A**) Expression of the *FLPp-lacZ* reporter was monitored by assaying for ß-galactosidase activity after 24 h of growth in selective medium containing galactose. Results represent the average (±s.d.) from analyzing four independent transformants. (**B**) Total protein extracted from the strains in (A) expressing Rep1, and either no Rep2 or the indicated form of Rep2, was analyzed by western blotting with antibodies specific for Rep1, Rep2 and Pgk1. Rep2-P is a hyperphosphorylated form of Rep2.

above the level observed in the absence of Raf. Lack of anti-repressor function might reflect a lower steady-state level for the Raf_{AA} mutant protein, or could indicate that Raf needs to be able to interact with Rep2 to counteract repression mediated by the combined presence of Rep1 and Rep2. Consistent with the latter, wild-type Raf and the Raf_{F18S} were both equally and significantly compromised for alleviating repression conferred by Rep1 when it was co-expressed with $Rep2_{AA}$, the Rep2 mutant impaired for Raf interaction and self-association. In this case, *FLPp-lacZ* expression was slightly increased above the level detected when no Raf was expressed, which may represent some interaction of the Raf protein with the $Rep2_{AA}$ mutant not detected in the two-hybrid assay, or an effect of Raf that is not dependent on its interaction with the Rep proteins. Unsurprisingly, the Raf_{AA} mutant (impaired for Rep2 interaction) that was unable to alleviate repression conferred by wild-type Rep1 and Rep2 was also incapable of doing so in the presence of wild-type Rep1 and the $Rep2_{AA}$ mutant (impaired for Rep2 and Raf association). The mutant versions of the Raf protein were not tested for their anti-repressor activity in cells co-expressing Rep1 with the $Rep2_{D22N}$ mutant because no repression of *FLPp-lacZ* expression was detected even in the absence of Raf expression.

Rep1 protein levels are reduced in the absence of Rep2 and in presence of $Rep2_{D22N}$

The loss of repressor function observed for the $Rep2_{D22N}$ mutant might reflect a requirement for interaction between Rep1 and Rep2 to form the repressor complex, but might also be expected if Rep1 or Rep2 protein levels were significantly reduced by loss of the interaction between the two proteins. We have previously shown that the presence of Rep2 protects the Rep1 protein from degradation (12). Western blotting of protein extracted from the yeast co-expressing Rep1 with wild-type or mutant versions of Rep2 analyzed in the *FLPp-lacZ* reporter assay (Figure 4A) showed that Rep1 protein levels were indeed drastically reduced both in the absence of Rep2 and when Rep2 carried the D22N substitution, but were not affected by the two alanine substitutions in $Rep2_{AA}$ (Figure 4B). Rep2 protein levels were also reduced when Rep2 carried the D22N substitution; however, the decrease was similar to that observed for the $Rep2_{AA}$ mutant, which retained repressor function, making it more likely that the loss of repression for $Rep2_{D22N}$ is due to the reduction in Rep1 protein levels. The changes in Rep1 and Rep2 protein levels observed when Rep2 carried the D22N or AA substitution suggest that the association between Rep1 and Rep2 protects both proteins from degradation, and that Rep2 self-association may contribute to Rep2 protein stability.

$Rep2_{D22N}$ and $Rep2_{AA}$ are unable to mediate plasmid inheritance in the absence of Raf

In addition to repressing transcription, Rep1 and Rep2 act together to ensure equal partitioning of 2-μm plasmid copies at cell division (6,7,9,42). To determine whether association of Rep2 with Rep1 or Rep2 self-association is required for plasmid partitioning, mutations that led to loss of these associations were introduced into the *REP2* gene in the *kanMX4*-tagged 2-μm plasmid, pKan (12). The *kanMX4* cassette in pKan disrupts the *FLP* ORF, preventing Flp-mediated plasmid amplification from compensating for and potentially obscuring plasmid missegregation events, and confers resistance to the aminoglycoside antibiotic G418, thereby providing a phenotype to the otherwise cryptic 2-μm plasmid. Use of a marker gene conferring drug-resistance rather than nutritional prototrophy removes the growth advantage conferred by higher plasmid copy number, an advantage observed even when transformants are cultured in medium containing the nutritional supplement (12) (Pinder,J., unpublished observations). We have previously demonstrated that the pKan plasmid is 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 (12).

The pKan plasmids encoding either $Rep2_{D22N}$ or $Rep2_{AA}$ with wild-type Rep1 and Raf were inherited with similar efficiency to the parental version encoding wild-type Rep2 (Figure 5). The copy number for the $Rep2_{AA}$-encoding plas-

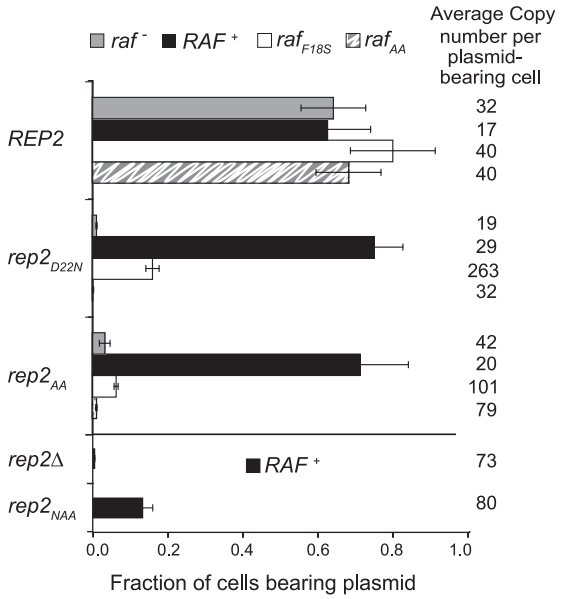

Figure 5. Raf is required for partitioning competence of $Rep2_{D22N}$ and $Rep2_{AA}$. A *cir*^{0} yeast strain was transformed with *kanMX4*-tagged, amplification-incompetent (*flp*$^{-}$) 2-μm plasmids (pKan-based) carrying a wild-type *REP1* gene and the indicated version of the *REP2* and *RAF* genes. Due to the absence of a functional *FLP* gene, plasmid missegregation events cannot be corrected by Flp-mediated copy number amplification, making efficient maintenance of the pKan-based plasmids dependent on Rep protein partitioning function. Transformants were cultured overnight (six to eight generations) in selective medium, and the fraction of plasmid-bearing cells determined by a plating assay. Results represent the average (±s.d.) of five independent transformants for each plasmid. Plasmid copy number in each culture was determined by polymerase chain reaction (PCR) using total DNA extracted from the transformants as template and quantifying the ratio of product obtained with primers specific for a plasmid relative to a chromosomal locus. This value was then corrected for the fraction of cells in the population containing plasmid to obtain the average plasmid copy number per plasmid-bearing cell (See Supplementary Figure S6 for details).

mid was similar to that of the parental version while that encoding $Rep2_{D22N}$ was increased to the level observed for native 2-μm in this strain background (Figure 5 and Supplementary Figure S6). The results suggest that both mutant Rep2 proteins could supply the associations required for establishing a functional partitioning complex at *STB*. This finding was surprising given that amino acid substitutions in Rep1 that lead to loss of Rep2 association have previously been shown to eliminate Rep protein-mediated plasmid partitioning (9), and that Rep2 self-association might also have been expected to contribute to the organization of the Rep1–Rep2-*STB* partitioning complex. The pKan plasmid encoding the $Rep2_{NAA}$ mutant was severely compromised for inheritance, suggesting loss of Rep2–Rep1 interaction combined with loss of Rep2–Raf interaction and Rep2 self-association leads to loss of partitioning function. The copy number in cells containing the $Rep2_{NAA}$-encoding plasmid was also significantly increased above the level of the partitioning-competent plasmids, consistent with the expected effect of ineffective partitioning combined with ongoing selection for the marker gene on the plasmid. A similar increase in copy number was observed for the pKan plasmid from which the *REP2* coding region had been deleted (pKanΔrep2) although this was retained even less efficiently that the plasmid encoding $Rep2_{NAA}$. This difference in retention could indicate that $Rep2_{NAA}$ might still interact with its partner proteins at some low level, or that Rep2 can contribute to 2-μm plasmid inheritance in a way that does not require these associations.

The similarity between the interactions of Rep2 and Raf (Figure 2) raised the possibility that Raf, although unable to compensate for absence of the Rep2 protein in plasmid partitioning (6,43), might be able to compensate for the loss of specific Rep2 interactions. To test this hypothesis, plasmid inheritance assays were undertaken using pKan plasmids in which the *RAF* gene was mutated either to encode Raf_{F18S} or Raf_{AA}, or to prevent Raf expression by replacing the third codon in the *RAF* gene with a stop codon (*raf*$^{-}$). As expected from earlier studies (6), the absence of Raf had no effect on inheritance of the pKan plasmid expressing wild-type Rep2 (Figure 5). However, for pKan plasmids expressing either $Rep2_{D22N}$ or $Rep2_{AA}$, the absence of Raf drastically reduced the fraction of plasmid-bearing cells to the level observed for the pKan plasmid lacking the *REP2* gene. This suggests that when Rep2 is impaired for either Rep1 interaction or self-association, Raf is able to play a compensatory role in 2-μm plasmid inheritance.

Replacement of the *RAF* gene in pKan with either of the mutant alleles, *raf*$_{F18S}$ or *raf*$_{AA}$, did not impair plasmid inheritance when the plasmid encoded a wild-type *REP2* gene. Indeed, a small but significant increase in the fraction of plasmid-bearing cells was observed for the plasmid with the *raf*$_{F18S}$ allele. The copy number per plasmid-bearing cell for this plasmid was higher than that of the wild-type *RAF* parental plasmid. However, plasmids with the *raf*$^{-}$ or *raf*$_{AA}$mutant alleles also had a similar increase in copy number and the percentage of cells in the population containing these plasmids was not significantly increased, suggesting Raf_{F18S} function rather than just the increased copy number might be contributing to the higher fraction of cells bearing plasmid.

As was found for the pKan plasmid lacking a functional *RAF* gene (*raf*$^{-}$), the presence of either *raf*$_{F18S}$ or *raf*$_{AA}$, in a plasmid encoding Rep_{D22N} or $Rep2_{AA}$, significantly impaired plasmid inheritance, indicating Raf interactions with both Rep1 and Rep2 are required for Raf to fully compensate for loss of Rep2 interactions. For those with the *raf*$_{AA}$ allele, the defect was of similar severity to that of pKan plasmids expressing either mutant version of Rep2 in the absence of Raf or that of a pKan plasmid that lacked a *REP2* gene. The mutant Raf_{AA} protein might be present at too low level to compensate for loss of Rep2 functions, or the amino acid changes may make it unable to substitute for the lost Rep2 functions. The *raf*$_{F18S}$ allele combined with the *rep*$_{D22N}$ allele did increase the fraction of plasmid-containing cells above the level observed for the *rep*$_{D22N}$ allele in the absence of Raf. This improved inheritance may due to the extremely elevated copy number per plasmid-bearing cell which could increase the chance of plasmids being delivered to daughter cells by random diffusion rather than by active partitioning. Raf_{F18S} might also at least partially be able to compensate for loss of Rep2–Rep1 interaction without itself interacting with Rep1, presumably by

being competent for Rep2 interaction. Consistent with this, when the raf_{F18S} allele was combined with the $rep2_{AA}$ allele, which should impair Rep2 interaction with Raf, the fraction of plasmid-bearing cells did not increase to the same extent despite plasmid levels per plasmid-bearing cell being elevated.

Raf expression leads to an increase in Rep1 and Rep2 protein levels

Presence of Rep2 has been previously been shown protect Rep1 from degradation (12) (Figure 4B). As Raf and Rep2 associate with the same domains in Rep1 and Rep2 (Figure 2), we hypothesized that Raf might be compensating for the loss of specific Rep2 associations by protecting the Rep proteins from degradation. To assess a potential chaperoning role for Raf, various combinations of Rep1, Rep2 and Raf were expressed from galactose-inducible promoters on *ARS/CEN* plasmids in a cir^0 yeast strain to prevent plasmid copy number variation and Rep1–Rep2–Raf-mediated transcriptional regulation of plasmid protein genes from confounding observations regarding protein stability.

When Rep1 was co-expressed with Rep2 (Figure 6, lane 3), Rep1 protein levels were increased above the level observed in the absence of Rep2 (lane 2), consistent with previous results (12) (Figure 4B). Rep2 protein levels were also seen to increase slightly in the presence of Rep1 (Figure 6, lane 8 compared to lane 7). Expression of Raf increased Rep1 and Rep2 protein levels, both in the absence (lanes 4 and 9 compared to lanes 2 and 7) and presence (lanes 5 and 10 compared to lanes 3 and 8) of the other Rep protein. The Raf-dependent increase in Rep1 protein levels (lane 4) was greater than that produced by co-expression of Rep1 with Rep2 (lane 3), while total Rep2 protein levels in the presence of Raf without Rep1 (lane 9) were similar to those observed in the presence of Rep1 without Raf (lane 8). Simultaneous expression of Rep1, Rep2 and Raf (lanes 5, 10 and 11) led to the largest increase in protein levels for both Rep1 and Rep2. Raf expression also increased Rep1 and Rep2 levels when Rep2 was impaired in its ability to interact with either Rep1 ($Rep2_{D22N}$) or Rep2 and Raf ($Rep2_{AA}$). The increased level of the $Rep2_{D22N}$ mutant likely reflects direct stabilization by Raf association, whereas for the $Rep2_{AA}$ mutant that was unable to associate with Raf in a two-hybrid assay (Figure 3A), stabilization may be indirect, for example, a result of Raf protecting Rep1, making more Rep1 available to associate with and stabilize $Rep2_{AA}$.

In addition to changes in Rep2 protein levels, Raf expression also shifted the relative abundance of a Rep2 species from a 37-kDa form, previously shown to be a hyperphosphorylated Rep2 species (12), toward a 35-kDa form (Figure 6, lanes 9 and 10 compared to lanes 7 and 8). These shifts were also seen for the mutant versions of Rep2, suggesting that the presence of Raf limits Rep2 phosphorylation either directly by associating with Rep2 or indirectly, as would be the case for $Rep2_{AA}$, potentially by competing with Rep2 for interaction with the unidentified kinase that phosphorylates Rep2.

Raf associates with *STB in vivo* in the presence of Rep1

The ability of Raf to sustain Rep1 and Rep2 protein levels undoubtedly contributes to plasmid partitioning but Raf might also play a more direct role, potentially by providing some aspects of Rep2 function at the *STB* locus. Although Raf did not display *STB* DNA-binding activity in the *in vitro* southwestern assay (Figure 2D), it is possible that Raf associates with the *STB* locus *in vivo*, perhaps requiring the assistance of host proteins to do so. To examine Raf association with *STB in vivo*, an amino-terminally FLAG epitope-tagged Raf protein was expressed from an *ARS/CEN* plasmid in yeast containing native 2-μm plasmid. *STB* DNA was enriched by ChIP with antibodies specific for Rep1, Rep2 and FLAG-tagged Raf, as were the two 2-μm plasmid gene promoter regions (*FLP/REP2p* and *REP1/RAFp*) (Figure 7A). These enrichments were not seen upon immunoprecipitation with an unrelated antibody (anti-HA), and a genomic locus (the *TRP1* gene) was not enriched in any of the immunoprecipitates. These results demonstrate that *in vivo*, Rep1, Rep2 and Raf are present at the *STB* partitioning locus, and are also present at the 2-μm plasmid gene promoters, as expected from their role as transcriptional regulators of these genes.

Rep2 association with *STB in vivo* has previously been shown to be largely dependent on the presence of Rep1 (12). To determine whether Raf requires the presence of other 2-μm plasmid proteins to associate with the *STB* locus, a one-hybrid assay was performed in which Raf was expressed fused to an HA-tagged B42 transcriptional activation domain ($B42_{AD}$-HA) in a cir^0 strain containing *STB* upstream of a *HIS3* reporter gene integrated in the genome. Strains were also transformed with an *ARS/CEN* plasmid that expressed no Rep proteins, or expressed Rep1 or Rep2. Activation of the reporter gene by the $B42_{AD}$-HA-Raf fusion protein was only detected in the presence of Rep1 (Figure 7B), suggesting that Raf, like Rep2, is dependent on Rep1 for efficient association with *STB in vivo*.

As both Rep2 and Raf interact with the same region of the Rep1 protein, and both require Rep1 for robust association with *STB*, it seemed likely that they might compete for *STB* association. To test this, a one-hybrid assay was performed in which $B42_{AD}$-HA-tagged Rep1 (AD-Rep1) or Rep2 (AD-Rep2) was expressed in a cir^+ *STB-HIS3* reporter strain in either the presence or absence of Raf over-expression (Figure 8). Activation of the *STB-HIS3* reporter by AD-Rep2 was decreased upon Raf over-expression, suggesting that, as predicted, Raf competes with Rep2 for *STB* occupancy. Unexpectedly, activation of the *STB-HIS3* reporter by AD-Rep1 was slightly improved in the presence of Raf. This suggests that Raf may lead to an increase in the level of the AD-Rep1 fusion protein as it does for Rep1 (Figure 6), although it is also possible that Raf stabilizes the association between AD-Rep1 and *STB*.

DISCUSSION

Maintenance of the yeast 2-μm plasmid is dependent on equal partitioning of plasmid copies during host cell division, amplification of plasmid copy number and strict transcriptional regulation of the plasmid-encoded genes required for these activities. In this study we have investigated

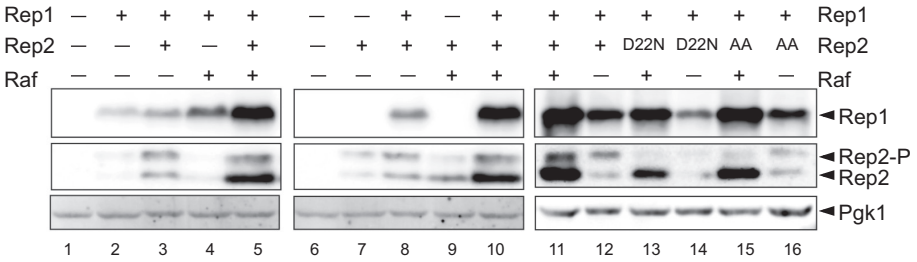

Figure 6. Raf increases Rep1 and Rep2 protein levels. A *cir*^{0} yeast strain was transformed with two *ARS*/*CEN* galactose-inducible expression plasmids: one (pBM272-based) expressing either no protein, or expressing Rep1 and Rep2 variants individually or simultaneously; and the other expressing no protein (pRSGAL-LEU), or expressing Raf (pRSGAL-LEU-RAF). Transformants were cultured overnight in selective medium containing galactose to induce protein expression. Total protein was analyzed by western blotting with antibodies specific to Rep1, Rep2 and Pgk1. Rep2-P is a hyperphosphorylated form of Rep2. For each cell extract, presence (+) or absence (−) of the wild-type version of each protein, or presence of a mutant version of Rep2 (D22N or AA) is indicated above the lane.

two of the 2-μm plasmid-encoded proteins: Rep2, which, with the plasmid Rep1 protein, has roles in both partitioning and transcriptional repression; and Raf, for which the only known function prior to this study was in alleviation of Rep protein-mediated repression of plasmid gene expression. We have identified similarities between Rep2 and Raf in their associations with Rep1, Rep2 and the plasmid *STB* partitioning locus that provide insight into how these proteins contribute to 2-μm plasmid partitioning and transcriptional regulation (Figure 9).

Functional differences between Rep2 and Raf

Despite the similarities identified in this study between Rep2 and Raf with respect to their interaction with 2-μm plasmid components (Figures 2 and 7), Rep2 and Raf have distinct functions. Raf does not confer partitioning function in the absence of Rep2 (6) (McQuaid,M.E., unpublished data). Additionally, unlike Rep2, Raf does not repress 2-μm plasmid gene expression when co-expressed with Rep1, and instead antagonizes the repression mediated by co-expression of Rep2 with Rep1 (23,25,26).

The functional differences between Rep2 and Raf may stem from differences in the way in which they associate with Rep1 or with DNA. Raf association with Rep1 required a more extensive amino-terminal region of Rep1 than Rep2 association (Figure 2B). A potential Rep1-interaction motif identified in Rep2 was not found in Raf although a residue in the most amino-terminal α-helix of both was required for Rep1 recognition. Rep2 and Raf both seem to depend on Rep1 for robust association with the *STB* locus *in vivo* (12) (Figure 7B), but the results of southwestern assays suggest that Rep2 may interact more directly with *STB* DNA than Raf (Figure 2D). In this study, the DNA-binding activity of Rep2 was mapped to the C-terminal 65 residues. We also found that Raf was unable to bind DNA under these conditions, suggesting either that Raf is unable to bind DNA directly or that Raf interaction with DNA differs from that of Rep2.

A striking feature of Rep2 is its highly basic carboxy-terminal region ($Rep2_{232–296}$ pI = 11.9). Raf, which is a smaller protein, lacks this basic region and has an acidic carboxy terminus ($Raf_{117–181}$ pI = 4.4). These differences suggest that despite other similarities between Rep2 and Raf, the presence or absence of a carboxy-terminal DNA-binding domain may be a key feature that distinguishes the two. A potential basic residue motif signature was identified in this terminal region of Rep2 and in the carboxy-terminal region of proteins encoded by *REP2*-positioned ORFs of 2-μm-like plasmids from other closely related species of budding yeast (Supplementary Figure S4A). For the proteins encoded by the pTD1 and pKW1 plasmids, the region containing the basic motif also contained an AT-hook motif (44) (residues 222–234 and residues 264–276, respectively, Supplementary Figure S4B).

AT-hooks are small DNA-binding modules with a preference for binding AT-rich DNA (45) and have been identified in a large number of proteins, including high-mobility group non-histone proteins, transcription and chromatin-remodeling factors, the yeast DNA replication origin recognition factor Orc2 and the yeast centromere-binding protein Mif2 (44). The residues flanking the core GRP (glycine-arginine-proline) of the AT-hook motif in the pTD1 and pKW1 proteins suggest these are low-affinity DNA binding Type II AT-hooks (44). Although no match to an AT-hook consensus was found for the proteins encoded by the other *REP2*-positioned ORFs all, with the exception of ORF C for the *K. lactis* plasmid pKD1, are predicted to have a highly basic C-terminal region (pIs of 10.0–11.9 for the terminal 65 residues), suggesting they may also bind DNA.

Rep1–Rep2 interaction is required for 2-μm plasmid gene repression

Despite the Rep2-equivalent partitioning proteins of 2-μm-like plasmids sharing no sequence similarity with Rep2, comparison to these proteins allowed us to identify residues in Rep2 required for association with Rep1 and for self-association. Our results with mutant Rep2 proteins impaired for these interactions suggest that Rep1–Rep2 association is required for the transcriptional repression function of the Rep proteins, while Rep2 self-association is dispensable for this repression (Figure 4A). These results, taken together with the finding that Rep2 interaction with Rep1 competes with Rep1 self-association (14), support a model in which a Rep1–Rep2 dimer, rather than a multimeric Rep protein complex, in association with gene promoters or *STB* DNA, is sufficient to mediate transcriptional repression (Figure 9A). The DNA-binding activity of Rep2 may

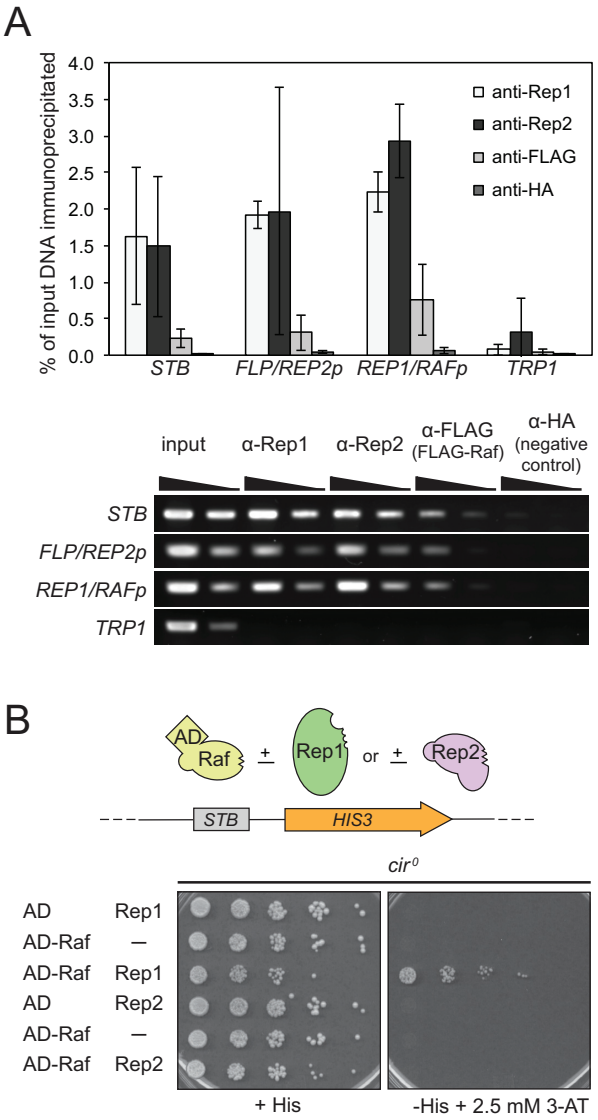

Figure 7. Raf associates with *STB* and 2-μm plasmid gene promoters, and association with *STB* is dependent on Rep1. (**A**) A *cir*$^+$ yeast strain was transformed with an *ARS*/*CEN* plasmid expressing FLAG-tagged Raf under the control of a galactose-inducible promoter. Transformed yeast were cultured in medium containing galactose and chromatin was immunoprecipitated with antibodies specific for native Rep1 and Rep2, anti-FLAG (FLAG-Raf) and, as a negative control, anti-HA. The precipitated DNA was analyzed by semi-quantitative PCR with primers specific for the *STB* locus, for the divergent *FLP*/*REP2* and *REP1*/*RAF* promoter regions (*FLP*/*REP2p* and *REP1*/*RAFp*), and, as a negative control, a chromosomal locus (*TRP1*). The bar graph indicates ChIP efficiency as the percent of input DNA immunoprecipitated; results are average (±s.d.) from triplicate assays. Ethidium bromide-stained agarose gels of PCR products from a representative assay are shown below the graph. Template DNA amplified in 'input' PCR reactions is 10% of that amplified in 'ChIP' PCR reactions. Products obtained from neat and 1:4 dilutions of each template are shown. (**B**) A *cir*^{0} yeast strain with *STB* integrated in the chromosome upstream of a *HIS3* reporter gene was co-transformed with two galactose-inducible expression plasmids: one expressing B42$_{AD}$-HA (AD) or B42$_{AD}$-HA-Raf (AD-Raf), and the second, expressing either no protein (−), or expressing Rep1 or Rep2. Five-fold serial dilutions of co-transformants were spotted onto solid media that selected for the presence of the two plasmids, with galactose to induce Rep protein expression, and either containing histidine (+His), or lacking histidine (−His) and containing 3-aminotriazole (3-AT). Growth on the −His + 3-AT medium indicates recruitment of the AD fusion protein to *STB*.

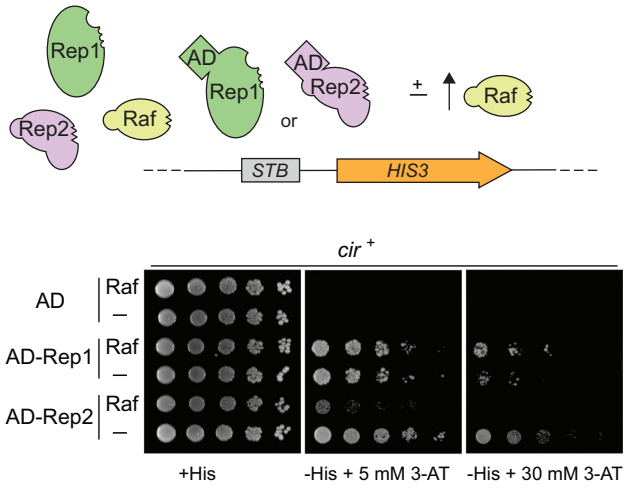

Figure 8. Overexpression of *RAF* impairs Rep2 association with *STB*. A *cir*$^+$ yeast strain with *STB* integrated in the genome upstream of a *HIS3* reporter gene was co-transformed with two galactose-inducible expression plasmids: one that expressed either B42$_{AD}$-HA (AD), or Rep1 or Rep2 fused to B42$_{AD}$-HA (AD-Rep1 or AD-Rep2); and the second expressing no protein (−) or expressing Raf. Recruitment of AD fusion proteins to *STB* was monitored as described in the legend of Figure 7.

be required for the Rep1–Rep2 dimer to be more tightly or appropriately associated with target sites on the 2-μm plasmid than Rep1-bridged DNA association alone would allow. The ability of Raf to compete with Rep2 for Rep1-mediated *STB* DNA association *in vivo* (Figure 8), combined with a putative lack of DNA-binding capacity for Raf (Figure 2D), may allow Raf to effectively disrupt Rep protein-mediated repression of 2-μm plasmid transcription. Raf interaction with Rep2 may also contribute to Raf-mediated anti-repression, potentially serving to destabilize Rep2 association with DNA.

Roles of Rep2 interaction with Rep1 and Rep2 self-association in 2-μm plasmid partitioning

In this study, mutant Rep2 proteins impaired for association with Rep1 (Rep2$_{D22N}$) or Rep2 (Rep2$_{AA}$) were still proficient for plasmid partitioning, provided that Raf was present (Figures 5 and 9B). These results suggest that direct interaction of Rep2 with Rep1 or Rep2 self-association may not be essential for establishing a functional partitioning complex, and that one of these interactions can compensate for the lack of the other. The ability of Raf to sustain efficient plasmid inheritance when Rep2 was impaired for association with itself or Rep1 could indicate that Raf interaction with Rep1 and Rep2 can substitute for loss of one of these normal Rep2 associations, allowing the mutant Rep2 to fulfill other roles in partitioning that do not require this specific association. Alternatively, Raf may compensate for the impaired Rep protein interactions by associating with and stabilizing the mutant Rep2 protein at a level sufficient to mediate partitioning despite sub-optimal interactions. Our data show that impairing Rep1–Rep2 or Rep2–Rep2 associations, or loss of Raf expression, all lead to decreases in Rep protein levels (Figures 4 and 8). In the absence of Raf, if Rep2 self-association or interaction with

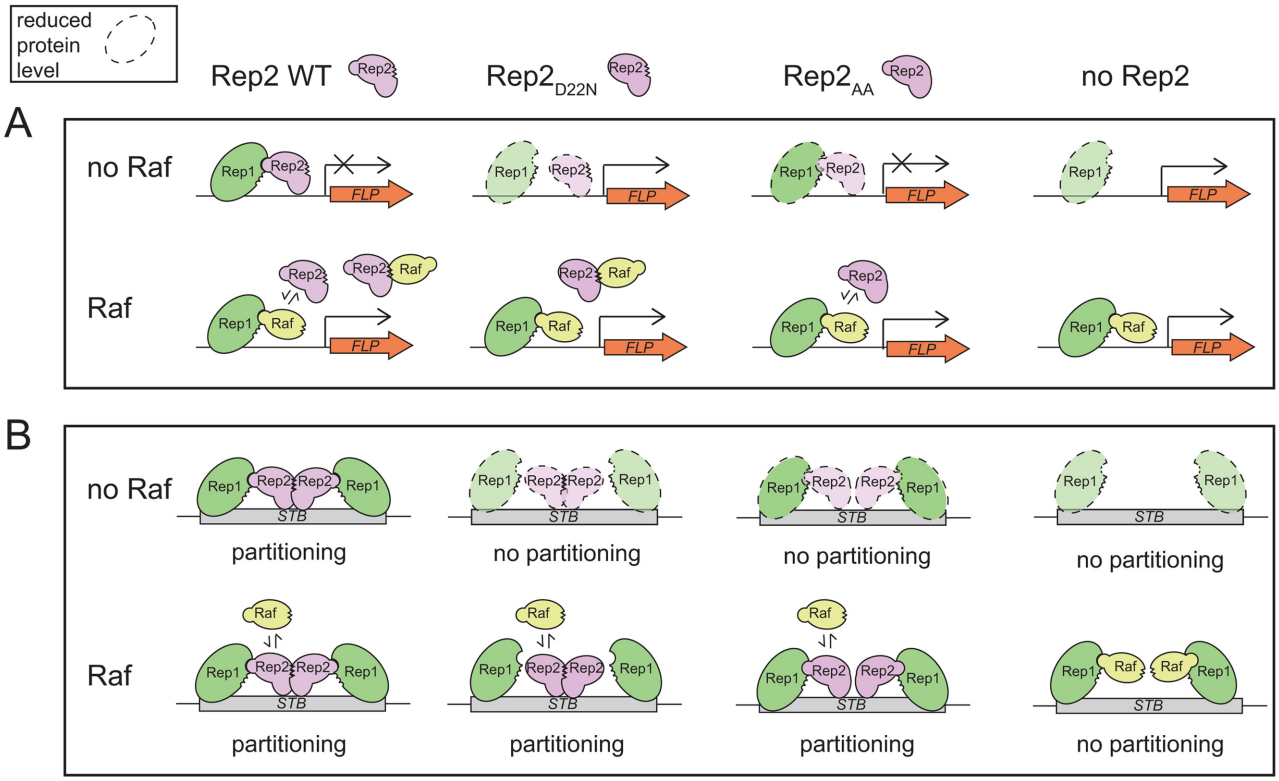

Figure 9. Models for the effect of Raf and loss of specific Rep2 associations on Rep protein complexes at the 2-μm plasmid *FLP* gene promoter and *STB* partitioning locus. (**A**) In the absence of Raf (top), *FLP* gene transcription is repressed by co-expression of wild-type Rep1 with wild-type Rep2 or with $Rep2_{AA}$, suggesting that a Rep1–Rep2 dimer associating with the *FLP* promoter is sufficient for repression. Expression of Rep1 in the absence of Rep2, or with $Rep2_{D22N}$ does not repress *FLP* transcription, consistent with a complex of Rep1 with Rep2 being required for repression, although low Rep1 protein levels in the absence of Rep2 association preclude determining whether Rep1 alone would be sufficient to block transcription. The presence of Raf (bottom) relieves Rep protein-mediated *FLP* repression, likely by competing with Rep2 for association with Rep1, and stabilizes Rep protein levels. (**B**) Wild-type Rep1 and Rep2 proteins, in association with the *STB* locus, are sufficient to mediate plasmid partitioning in both the absence (top) and presence (bottom) of Raf. Interaction is shown between the Rep2 subunits of the Rep1–Rep2 heterodimer at left but may not be essential for establishing the functional partitioning complex at *STB*, as indicated by Rep2 mutants lacking this interaction. When Rep2 association with Rep1 or Rep2 is impaired (as is the case for the $Rep2_{D22N}$ and $Rep2_{AA}$ mutants, respectively), partitioning function is impaired unless Raf is present. Raf may compensate for these impaired associations by stabilizing Rep protein levels. Raf might also contribute more directly to the partitioning complex by replacing Rep2 at a subset of sites in the repeated *STB* sequence; however, Raf is not sufficient to provide partitioning function in the absence of Rep2, despite stabilizing Rep1 protein levels. The potential role of Rep1 self-association in Rep-mediated transcriptional repression and partitioning is not shown in these models and remains to be determined. Proteins present at levels lower than those observed when Rep2 is wild-type are shown with a dashed line with more significantly reduced levels indicated by lighter shading.

Rep1 is impaired, Rep protein levels may be reduced to the point where they are no longer sufficient to form the partitioning complex at *STB* (Figure 9B).

Role of Raf in 2-μm maintenance

The origin of the 2-μm plasmid *RAF* gene remains uncertain. Only two of the seven 2-μm-like plasmids encode a fourth protein in addition to the Flp recombinase required for copy number amplification and the two proteins required for equal partitioning of the plasmid copies at cell division, one of which is related to 2-μm Rep1. None of the proteins encoded by the 2-μm-like plasmids resembles Raf. Prior studies have not shown impaired 2-μm plasmid maintenance resulting from disruption of *RAF*, raising the question of why the 2-μm plasmid retains a *RAF* gene. One explanation has been that Raf alleviation of Rep protein-mediated repression of the *FLP* gene allows more rapid induction of plasmid amplification in response to a drop in plasmid copy number (23,25,26). Our results demonstrating functional overlap between Raf and the Rep2 associations provide a probable basis for Raf anti-repressor function and suggest an additional potential selective advantage for retention of the *RAF* gene. Our evidence shows a strong correlation between Raf expression and stabilization of Rep1 and Rep2 protein levels. At low plasmid copy number, Raf might be critical for sustaining sufficient Rep protein at *STB* to ensure equal partitioning of the plasmid copies at cell division. At low Rep protein levels, Raf competition with Rep2 for Rep1 interaction may be more effective at the plasmid gene promoters than at the *STB* locus if, for example, at the latter, self-association of Rep2 molecules bound at adjacent *STB* repeats provides a protection from Raf competition to the Rep1–Rep2 interaction that is not present at the individual Rep1–Rep2 dimers at gene promoters. Raf could thereby provide a distinct advantage at low copy number by combatting formation of repressive Rep1–Rep2 complexes at the promoters until sufficient Flp recombinase has been

expressed to return plasmid copy number to normal levels, without compromising formation of the Rep protein partitioning complex at *STB*.

The arrangement of the 2-μm plasmid genes might also contribute to the advantage of having the *RAF* gene. The *REP1* and *RAF* genes share a common promoter region, whereas *REP2* gene expression is co-regulated with that of the *FLP* gene (Figure 1) (Dobson,M., unpublished observations). This poses a problem if plasmid copy number is already optimal, a situation in which Rep2 is still needed for partitioning whereas further expression of *FLP* would be detrimental. Raf stabilization of Rep2 may explain how this issue has been accommodated. Repression of *RAF* gene transcription has previously been shown to require a higher level of the Rep proteins than that required to repress the *FLP* gene (26). This differential repression would allow the Raf protein to be expressed and be available to increase the steady-state level of Rep2 protein at plasmid copy numbers sufficient to limit transcription of the *REP2* gene.

Another possible explanation for the retention of the 2-μm *RAF* gene might be a role for Raf in chaperoning the Rep proteins in environmental situations or during other cellular events such as meiosis not assessed in standard laboratory culture conditions. We have observed a significant drop in Rep2 levels relative to Rep1 levels as cells enter late stationary phase when the *REP1* and *REP2* genes were present at one copy per cell and expressed under the control of their own promoters in the absence of other 2-μm plasmid sequences (Pinder,J., unpublished observations). Whether this drop represents a difference in gene expression or protein stability remains to be determined. If the latter, Raf stabilization of Rep proteins might help maintain the 2-μm plasmid during cellular quiescence, a role that would not have been detected in prior lab-based assessments.

In addition to stabilizing Rep protein levels, the possibility remains that Raf may also be able to partially substitute for some aspects of Rep2 function in partitioning, either directly, by potentially facilitating interactions between Rep1 and Rep2, or indirectly, by antagonizing Rep1 self-association (an aspect of Rep1 protein function not addressed in this study) or by positioning the Rep proteins at *STB* to establish the functional Rep protein/*STB* partitioning complex. Sequence analysis of variants of the 2-μm plasmid found in industrial strains of yeast showed a correlation between variations in the *RAF* gene and sequence diversity at the *STB* locus and in the *REP1* gene, suggesting Raf might play a more active role in plasmid partitioning (41).

In summary, this study has identified the Rep2 domains required for interaction with Raf, Rep1 and DNA, and shown functional overlap between Rep2 and Raf in stabilizing Rep protein levels. These findings suggest models for the 2-μm protein complexes required for plasmid partitioning and transcriptional regulation (Figure 9). Our analyses have also identified features common to the Rep2 and Rep2-equivalent partitioning proteins encoded by the 2-μm and 2 μm-like plasmids, thereby contributing to our understanding of how this family of plasmids manages to persist in their budding yeast hosts.

ACCESSION NUMBER

Genbank accession number: KY549324.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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