Functional Domains of Yeast Plasmid-Encoded Rep Proteins

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Both of the Saccharomyces cerevisiae 2µm circle-encoded Rep1 and Rep2 proteins are required for efficient distribution of the plasmid to daughter cells during cellular division. In this study two-hybrid and in vitro protein interaction assays demonstrate that the first 129 amino acids of Rep1 are sufficient for self-association and for interaction with Rep2. Deletion of the first 76 amino acids of Rep1 abolished the Rep1-Rep2 interaction but still allowed some self-association, suggesting that different but overlapping domains specify these interactions. Amino- or carboxy-terminally truncated Rep1 fusion proteins were unable to complement defective segregation of a 2µm-based stability vector with rep1 deleted, supporting the idea of the requirement of Rep protein interaction for plasmid segregation but indicating a separate required function for the carboxy-terminal portion of Rep1. The results of in vitro baiting assays suggest that Rep2 contains two nonoverlapping domains, both of which are capable of mediating Rep2 self-association. The amino-terminal domain interacts with Rep1, while the carboxy-terminal domain was shown by Southwestern analysis to have DNA-binding activity. The overlapping Rep1 and Rep2 interaction domains in Rep1, and the ability of Rep2 to interact with Rep1, Rep2, and DNA, suggest a model in which the Rep proteins polymerize along the 2µm circle plasmid stability locus, forming a structure that mediates plasmid segregation. In this model, competition between Rep1 and Rep2 for association with Rep1 determines the formation or disassembly of the segregation complex.

Most strains of the budding yeast Saccharomyces cerevisiae contain an endogenous plasmid, the 2µm circle. This 6,318-bp double-stranded circular DNA plasmid is located in the nucleus at approximately 60 copies per haploid cell and replicates autonomously from, but synchronously with, the chromosomal DNA (for a review, see reference 9). The 2µm circle confers no phenotype or selective advantage on the host yeast; indeed, 2µm plasmid-bearing (2µm) cells grow 1% more slowly than isogenic plasmid-free (2µm) cells (10). Despite this disadvantage, the 2µm plasmid displays a high level of mitotic stability. This stability results from the presence of a plasmid-encoded copy number amplification system and a partition mechanism which together ensure that the rates of plasmid loss in mitosis and meiosis are very low (4, 10, 16, 18). Partitioning of the 2µm plasmid requires two proteins encoded by the plasmid genes REP1 and REP2 and a cis-acting 2µm locus termed STB (16, 18). The role of these three components has been examined in a variety of studies involving mainly deletion or insertion analysis of 2µm-derived plasmids (17, 18, 23). In the absence of any one of these three components, the 2µm plasmid displays a strong maternal bias in inheritance; most plasmids are retained in the mother cell (22). The 2µm circle partition system overcomes this bias by an as yet unknown mechanism.

The Rep1 and Rep2 proteins mediate 2µm plasmid segregation, but their mode of action is unclear. Lack of either Rep1, Rep2, or the STB locus results in the same degree of mitotic instability (18, 27). Experiments designed to reconstitute efficient 2µm segregation by expressing different amounts of Rep1 and Rep2 in the cell have shown that the relative levels of the two proteins are important for their partitioning function (5, 6). In addition to having a role in plasmid partitioning, the Rep proteins repress transcription of the 2µm plasmid FLP gene (25, 27, 31, 35). The FLP gene encodes a site-specific recombinase required for plasmid amplification, and the control of Flp expression by Rep proteins has been proposed as the mechanism by which 2µm plasmid copy number is regulated (8, 37). Rep protein-mediated transcriptional repression, like Rep protein segregation function, requires the concerted action of both Rep1 and Rep2 (25, 31, 35). Taken together, these data suggest that Rep1 and Rep2 may function as part of a complex. Recently, evidence for interaction between Rep1 and Rep2 and for self-association of the two proteins has been obtained both by two-hybrid genetic assays and in vitro protein interaction assays (1, 30, 36). We have used similar approaches here to delineate the regions of Rep1 and Rep2 that are required for these associations. Amino-terminal truncations of Rep1 abolish Rep1-Rep2 interaction and are unable to complement the segregation defect of a 2µm-based stability vector with rep1 deleted, while Rep2 has two separate domains capable of mediating Rep2 self-association, one of which has DNA-binding activity while the other interacts with Rep1.

MATERIALS AND METHODS

Yeast and bacterial strains. Escherichia coli strain DH5α (29) was used for propagating plasmids, strain JF1754 (his5 leu2 met thl) was used for expression of glutathione S-transferase (GST) fusion proteins, and strain BL21(DE3) (Novagen) was used for expression of pET fusion proteins. S. cerevisiae strains used were isogenic [cir-] and [cir+] AS3 (MATα his3A1 ura3-52 leu2-3 leu2-112 trp1-289 ade2::URA3), AS2 (AS3 with an ade2Δ rather than an ade2::URA3 allele), and CTY10/5d [MATα gal4 gal80 his3-200 trp1-901 ade2 ura3-52 leu2-3,112 URA3::lexAop, lacZ (2)].

Media. Yeast cells were routinely grown at 30°C in liquid or on solid synthetic defined (SD) medium containing 0.67% yeast nitrogen base (without amino
acids) and 2.0% dextrose, supplemented with appropriate amino acids as described by Rose et al. (28). Selection for plasmids in CTY10/5d was maintained by omitting either leucine or histidine or both from the SD medium. Yeast was grown in YEPD rich medium (1% yeast extract, 2% Bacto Peptone, 2% dextrose) for 16 h to induce protein expression. SD medium supplemented only with tryptophan and histidine was used for selective growth conditions for plasmid rate loss experiments, whereas SD medium supplemented with tryptophan, histidine, and adenine was used for nonselective growth. E. coli was grown in 2x YT with 50 μg of ampicillin per ml as described by Sambrook et al. (29). Media were solidified with 2.0% agar. All medium reagents were obtained from Difco Laboratories or Sigma Chemical Co.

**Generation of Rep1 and Rep2 BamHI fragments. BamHI restriction fragments containing Rep1 and Rep2 open reading frames (ORFs) were generated by PCR using the 2μm-based plasmid pMD219B (3) as a template and the following oligonucleotide primers: for Rep1, Rep1-1 (5′GAGTCATGATAAGGCGGAGACTGC3′) and Rep1-2 (5′GGATCCATATGAAATGTGAATAGATGTTCTG 3′). Initiation codons and BamHI sites are underlined. PCR was carried out with Taq DNA polymerase (Boehringer) according to the manufacturer’s instructions except that an additional 10 mM Tris, pH 8.8, was added to the commercial buffer, which significantly improved yields. Amplification was carried out for 30 cycles, each consisting of 1 min at 94°C, 2 min at 50°C, and 4 min at 72°C. PCR products were cloned directly into Smal-digested phagemid vector pTZ18R (Pharmacia) to which 3′ T overhangs had been added by incubation with Taq DNA polymerase and T4 DNA ligase to give pTZ18-Rep1 and pTZ18-Rep2. The BamHI Rep1 fragments were sequenced using a Sequenase 2.0 sequencing kit (U.S. Biochemicals) according to the manufacturer’s instructions.

**Plasmid construction.** Rep1 and Rep2 BamHI fragments were subcloned into the BamHI site in the HJ3 two-hybrid vector pH21-2 (2) to give in-frame fusions with LexA, producing pLEX-Rep1 and pLEX-Rep2, and also to the BamHI site in the LEU2 two-hybrid vector pGAD424 (Clontech) to give in-frame fusions with the Gal4 transcriptional activation domain (Gal4AD), producing pGAD-Rep1 and pGAD-Rep2. pGADRepΔ1–76 and pGADRepΔ1–129 were constructed by ligating an end-filled SpaI/BamHI fragment and an end-filled Smal/BamHI fragment from pTZ18-Rep1, respectively, with pGAD-linearized, end-filled pGAD424. In both constructs, amino-terminally truncated Rep1 is fused in frame to the carboxy terminus of the Gal4AD, pGADRepΔ1–373 was constructed by Stul/BamHI digestion, end filling, and self-ligation of pGAD-Rep1. pGADRepΔ2Δ1–14 was a gift from J. Sherk and is a Mur2 partial digestion product containing the truncated Rep2 ORF inserted at the Clal site in pGAD424. For the expression of GST-Rep fusion proteins in E. coli, the Rep BamHI fragments were end-filled with the Klenow fragment and cloned into Smal-digested pGEX-2T (Pharmacia) in the correct orientation, to give pGEX-REP1 and pGEX-REP2.

**GST-Rep proteins.** GST-Rep1 and GST-Rep2 fusion proteins were expressed in, and extracted from, E. coli transformed with pGEX-REP1 and pGEX-REP2, respectively, as described by Koerner et al. (19). Both GST-Rep fusion proteins were found in the insoluble fraction of E. coli extracts and were further purified by electrophoretic slab gel filtration on a column of polyacrylamide gels in which the insoluble fraction had been electrophoresed (20). Rep1 and Rep2 polyanionic antisera were generated by subcutaneous injection of rabbits with purified GST-Rep fusion proteins monthly and then every 2 weeks until a sufficient titer of anti-Rep antibodies was obtained, as assayed against Western blots of GST-Rep1 and GST-Rep2 fusion proteins and [35S] yeast whole-cell extracts (14). Anti-GST-Rep1 and anti-GST-Rep2 antisera were affinity purified on glutathione-sepharose by the method of Pringle et al. (26) using a Trans-Blot 0.2-μm-pore-size polyvinylidene difluoride membrane (BioRad) for immobilization of gel-purified GST-Rep1 and GST-Rep2 and GEG buffer (0.2 M glycine, 1 mM EGTA, pH 2.5) for antibody elution.

**Western blotting.** Proteins were separated on SDS–10% polyacrylamide gels (20) and transferred to a polyvinylidene difluoride membrane for Western blotting analysis as described by Towbin et al. (32) except that 0.037% SDS was included in the transfer buffer since this was found to be essential for efficient transfer of Rep2. Rabbit anti-Gal4AD antibodies were purchased from Santa Cruz Biotechnology. The secondary antibody for Western blotting was peroxi-dase-labeled goat anti-rabbit immunoglobulin G, which was detected by chemiluminescent reagents (Kirkgaard & Perry Laboratories, Inc.).

**In vitro protein interaction assay.** Expression of GST and pET fusion proteins was induced with 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 5 h at 25°C. GST fusion proteins were isolated and in vitro protein interaction assays were carried out as described by Ahn et al. (1). Equal amounts of GST fusion proteins were incubated with 50 μl of a 50% slurry of glutathione-agarose beads (Sigma) that had been preequilibrated in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.3) containing 0.2% Nonidet P40 (NP-40) and a protease inhibitor cocktail (1× concentration contains 0.5 μg of leupeptin per ml, 1 μg of aprotinin per ml, and 0.8 μg of pepstatin A per ml). The final volume was increased to 200 μl with buffer A (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.1% NP-40, 2 mM methionine) containing protease inhibitors, 1 mM diithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, and the beads were incubated for 1 h with gentle rocking at room temperature (RT). Bound GST fusion proteins were collected by centrifugation at 500 × g for 3 min. Beads were washed three times with 10 volumes of cold PBS–0.2% NP-40, with rocking for 5 min between washes. Beads were resuspended in 100 μl of PBS–0.2% NP-40–150 μM of bovine serum albumin per ml. pET fusion proteins were prepared by resuspending cell pellets in ice-cold sonication buffer (50 mM NaH2PO4, pH 8.0, 10 mM EDTA, 0.5 mM PMSF, 100 mM NaCl) with 15% glycerol, freezing–thawing (3×) and low-speed centrifugation. The pellet was resuspended in sonication buffer containing 8 M urea. Following sonication at 4°C, the urea-solubilized E. coli extracts were centrifuged at 15,800 × g for 15 min. The pET fusion proteins were purified from the supernatant by affinity chromatography on TALON resin (Clontech) under denaturing conditions according to the manufacturer’s directions. The TALON-purified material was diluted to 10 ng/μl in buffer A containing protease inhibitors, 1 mM PMSF, and 1 M phenylmethylsulfonyl fluoride and dialyzed against three changes of buffer A at 4°C. The purified pET fusion protein extracts were centrifuged at 15,800 × g for 10 min at 4°C to remove any particulate matter.

The equivalent of 500 ng of renatured pET fusion protein was added to the bead-bound GST fusion proteins, and the volume was increased to 500 μl with buffer A. Samples were rocked for 1 h at RT, followed by centrifugation at 500 × g for 5 min. Beads were washed five times with an equal volume of PBS–0.2% NP-40 by rocking them for 5 min, followed by centrifugation at 500 × g for 3 min. The beads with any bound proteins were then resuspended in 50 μl of 2× gel loading buffer and one-fifth of each sample was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on a 12% polyacrylamide gel and Western blot analysis. The presence of pET fusion proteins was detected by incubation with chemiluminescent reagents directed against the S-tag present on the TALON-purified proteins.

**Southwestern assay.** Southwestern blotting was performed as described by Mello et al. (21). Western blots of SDS-PAGE-separated TALON-purified pET fusion proteins were incubated with radiolabeled probes generated by using the Klenow fragment; unlabeled dGTP, dATP, dTTP, and [α-32P]CTP to label either a 375-bp EcoRI/BamHI fragment from the E. coli plasmid pBR322 or a 312-bp EcoRI/BamHI fragment containing the 2μm STB-proximal locus [the 2μm STB AvoI/HpaI fragment had been end filled and subcloned at the EcoRV site in the pBlueScript II SK(+)] (Strategene) polynucleotide region, where it is flanked by these two sites]. Specific activities of the probes were 1.4 × 106 for the pBR322 probe and 7.5 × 105 cpm/μg probe for the STB-proximal probe. Duplicate Western
blots were preincubated in BBW buffer (3 g of Ficoll per liter, 3 g of polyvinylpyrrolidone per liter, 10 mM NaCl, 20 mM Tris [pH 8.0], 0.25% milk powder) and then placed in 1 ml of this buffer in a sealed bag for a 1-h incubation at RT with 5 µg of unlabeled sonicated salmon sperm DNA and 50 ng of either radiolabeled probe. The membranes were then washed four times for 30 min in the BBW buffer before exposure to X-ray film for 3.5 h at −70°C with an intensifier screen.

**RESULTS**

**Rep protein interactions in vivo.** Genetic data have suggested that Rep1 and Rep2 function as part of a complex to regulate *FLP* gene expression and to mediate 2μm plasmid segregation (5, 6, 35). A two-hybrid genetic assay has previously shown that these two proteins interact in vivo (1, 36). We have used a similar approach here to investigate the ability of truncated versions of Rep1 and Rep2, expressed as in-frame fusions with the Gal4AD, to interact with either Rep1 or Rep2 expressed as in-frame fusion proteins with the bacterial DNA-binding protein LexA. Plasmids were cotransformed into the yeast two-hybrid host, where transcription of a lacZ reporter gene (detected by measuring β-galactosidase activity) requires interaction of the two fusion proteins to recruit RNA polymerase to the LexA binding site upstream of the reporter gene (2).

The results of the two-hybrid assay are shown in Fig. 1. None of the Rep fusion proteins or their truncated derivatives activated the lacZ reporter gene when they were singly expressed in the yeast two-hybrid host (data not shown), demonstrating that neither Rep1 nor Rep2 has an intrinsic transcriptional activation function in this assay. In accordance with the results of earlier studies (1, 36), expression in the reporter strain of both Gal4AD-Rep1 and LexA-Rep1, or both Gal4AD-Rep2 and LexA-Rep1, resulted in activation of the lacZ reporter gene, indicating a Rep1-Rep2 interaction. β-Galactosidase production was also observed when the plasmids expressing Gal4AD-Rep2 and LexA-Rep2 were cotransformed into the yeast two-hybrid host, although at a lower level than that observed for the Rep1-Rep2 fusion proteins. Although Rep1 self-association has previously been observed using a two-hybrid assay (1, 36), no association was detected in our assay. This may reflect interference with the interaction by the LexA moiety fused to the amino terminus of the Rep1 protein or differences between the two-hybrid assay systems used in the two studies. Neither of the pGAD-REP plasmids expressing Rep1 proteins with amino-terminal deletions induced β-galactosidase expression when they were coexpressed with LexA-Rep2, suggesting that loss of Rep1 amino-terminal sequences abolishes interaction with Rep2. In contrast, when LexA-Rep2 was coexpressed with the version of Rep1 with a carboxy-terminal deletion, expression of the reporter gene was significantly enhanced. These results suggest that the first 129 amino acids of Rep1 are sufficient for interaction with Rep2 and that Rep1 carboxy-terminal sequences may influence this association.

In the Rep2 two-hybrid analysis, deletion of either the first 14 amino acids of Rep2 or all but the first 58 amino acids did not significantly reduce the level of expression of β-galactosidase when these fusion proteins were coexpressed with LexA-Rep1, while removal of the first 57 amino acids of Rep2 led to loss of expression of the reporter gene. Taken together, these data suggest that the amino-terminal 57 amino acids of Rep2 are required for Rep1 interaction and that residues 15 to 58 are sufficient for this association. All Rep2 truncations gave lower β-galactosidase levels than full-length Rep2 when they were coexpressed as Gal4AD fusions with LexA-Rep2. However, the level of activation was significantly higher than when LexA-Rep2 was expressed in the absence of a Gal4AD fusion protein, suggesting that none of the truncations completely abolished the ability of Rep2 to interact with the LexA-Rep2 fusion protein.
Rep fusion protein expression. Lack of β-galactosidase expression in the two-hybrid assay may be due to a lack of interaction between the Rep1 and Rep2 portions of the two fusions, or it may be due to a lack of expression or lower abundance of the Rep deletion fusion proteins. To verify expression of the Rep fusion proteins, whole-cell protein extracts from the two-hybrid cotransformants were isolated and analyzed by SDS-PAGE and Western blotting using affinity-purified anti-Rep1 (a) or anti-Rep2 (b) antibodies. Protein was from strain AS2 [cir0] or [cir+] and the two-hybrid host CTY10/5d [cir+] cotransformed with either pLEX-REP2 expressing the LexA-Rep2 fusion protein and pGAD424 containing either full-length or truncated versions of the REP1 ORF, allowing them to be expressed as Gal4AD-Rep1 fusion proteins (a), or pLEX-REP1 and pGAD424 containing either full-length or truncated versions of the REP2 ORF (b). Protein from CTY10/5d transformed with either pLEX-REP1 (a) or pLEX-REP2 (b) (lanes 7) was also included. The positions of native Rep1 and Rep2, as well as those of Rep fusion proteins, are indicated by arrowheads. Autoradiographs were scanned using Molecular Analyst (Bio-Rad) software and prepared for figures using Adobe Photoshop. WT, wild type.
proteins and probably represent products of premature transla-
tional termination or proteolysis.

The anti-Rep2 antibody Western blot (Fig. 2b) shows a band
migrating with a molecular mass of 35 kDa in all protein
extracts made from [cir−] yeast (lanes 2 to 7) but not from the
[cir+] yeast strain (lane 1). The size of this band is consistent
with the predicted molecular mass of native Rep2 protein, 33
kDa (15). The anti-Rep2 antibody also detected a band of
approximately 57 kDa and, more variably, two lower-molecu-
lar-mass bands in all protein extracts. These proteins must be
non-2μm related since they were present in extracts derived
from both [cir−] and [cir+] yeasts. A novel Rep2-antigenic band
with a mobility of approximately 43 kDa was observed in the
extract derived from yeast containing the pLEX-Rep2 plas-
mid. In addition, novel bands with mobilities of approxima-
tely 53, 52, and 45 kDa were detected by both anti-Rep2 and
anti-Gal4AD antibodies in extracts derived from yeast con-
taining the plasmids pGAD-Rep2, pGADrep2Δ1–14, and
pGADrep2Δ1–76, respectively. These molecular masses are
consistent with the sizes expected for these Gal4AD-Rep fusion
proteins. No novel anti-Rep2 antigenic band was observed in
extracts derived from yeast containing the pGADrep2Δ59–296
plasmid. The anti-Gal4AD antibody did detect low levels of a
protein with an apparent mobility of 31 kDa in these extracts,
close to the expected size for this fusion protein, suggesting
that the Rep2 truncation eliminated the epitopes recognized
by the polyclonal anti-Rep2 antibodies. Despite the low steady-
state level of the Gal4AD-Rep2Δ59–296 fusion protein, the
level was sufficient to activate the reporter gene when it was
coenzymed with LexA-Rep1. In contrast, the Gal4AD-
Rep2Δ1–57 fusion protein, which was expressed at a higher
level than the carboxy-terminally truncated Rep2 fusion pro-
did not activate the reporter gene when it was coex-
pressed with LexA-Rep1 and neither did the higher levels of

FIG. 3. In vitro baiting assay demonstrating Rep protein interaction. Rep proteins and deletion derivatives were expressed as pET fusions;
shown are results with full-length Rep1, Rep1Δ1–76, and Rep1Δ130–373 (top panels) and full-length Rep2, Rep2Δ1–57, and Rep2Δ59–296
(bottom panels). The pET-Rep fusion proteins were incubated with GST-Rep1, GST-Rep2, or GST bound to glutathione-agarose beads. The input
(1/10 of that added to the reaction mixtures) and bound pET fusion proteins were analyzed by SDS-PAGE and Western blotting and detected with
an S protein probe and chemiluminescence. The positions of the pET-Rep fusion proteins are indicated by filled arrowheads. A caret indicates the
position of GST-Rep2, which cross-reacts with the S protein probe. The autoradiograph was scanned using Ofoto (Light Source) software and
prepared for figures using Adobe Photoshop. WT, wild type.
studies showing Rep1 self-association in vivo (1, 36) and suggests that the region comprising amino acids 77 to 129 is required for this interaction.

The baiting assay with the two truncated pET-Rep2 fusion proteins showed that both parts of the Rep2 protein were capable of independently mediating interaction with either GST-Rep1 or GST-Rep2, although the pET-Rep2Δ59–296 protein was brought down much less efficiently by the GST-Rep2 beads than was the amino-terminally truncated Rep2 fusion. The converse was observed for the interaction with the GST-Rep1 beads; the carboxy-terminally truncated Rep2 was more efficiently retained than the pET-Rep2Δ59–57 fusion. The two-hybrid assay results support an interaction between Rep1 and the amino-terminal portion of Rep2 but did not provide evidence for the in vitro association observed between Rep1 and the carboxy-terminal domain of Rep2.

Rep2 DNA-binding activity. Although the Rep proteins are hypothesized to interact with the 2μm STB locus to mediate plasmid segregation, direct association of either of the Rep proteins with DNA has yet to be demonstrated. Transient association of the Rep proteins with STB DNA has been shown by a biosensor assay but requires the presence of other unidentified host proteins (13). Biochemical studies of Rep1 and Rep2 have been complicated by the inherent insolubility of the native proteins when they were isolated from yeast (13, 38) or when they were expressed as fusion proteins in E. coli (M. J. Dobson, unpublished results). To circumvent these insolubility problems, a Southwestern assay was used to determine whether either Rep1 or Rep2 might have intrinsic DNA-binding activity (Fig. 4). Rep1 and the carboxy-terminally truncated Rep2 both demonstrated DNA-binding activity in this assay. Rep2 and amino-terminally truncated Rep2 both bound a DNA fragment containing the repeat region of STB and with lower efficiency bound a DNA fragment derived from the E. coli plasmid pBR322.

**Rep1 segregation function.** The results of the two-hybrid and baiting assays provide evidence for in vivo association of Rep1 and Rep2. To determine the connection between this association and 2μm plasmid segregation, we decided to test the ability of the truncated forms of Rep1 to mediate plasmid segregation. To assay plasmid segregation, we used a 2μm-based stability vector, pAS4 (A. Sengupta, unpublished data). In addition to containing all 2μm sequences, the vector carries an ADE2 gene, which allows selection for the plasmid in an ade2 host strain. If the REP1 and REP2 genes on the vector are wild type, the plasmid segregates in a [cir] assay system, the stability vector with rep1 deleted supplies Rep2 while the Gal4AD-Rep1 fusion proteins to determine whether the fusion proteins could complement the rep1 deletion in the stability vector. pGAD424 is itself a 2μm-based vector but contains only the cis-acting STB locus; its normal mitotic stability relies on Rep1 and Rep2 being supplied in trans from the 2μm plasmids in the [cir+] two-hybrid host. In our [cir+] assay system, the stability vector with rep1 deleted supplies Rep2 while the Gal4AD-Rep1 fusion protein is the only source of Rep1. If the Gal4AD-Rep1 fusion protein is able to supply Rep1 function, most cells grown under conditions selecting for the presence of both plasmids should be able to form colonies on medium lacking adenine and leucine since both contain the STB locus and will be efficiently partitioned at each cell division. Conversely, if the Gal4-Rep1 fusion protein does not restore Rep1 function, a significant proportion of the cells grown in selective medium will not contain both plasmids, reflecting the inability of the plasmids to be segregated to the daughter cells. Similarly, the rate of loss of the ADE2 stability vector with rep1 deleted from cells containing the LEU2 Gal4AD-Rep1 fusion protein-expressing plasmid should be high if the Rep1 fusion protein is
nonfunctional but low if Rep1 function is restored. The percentage of Leu<sup>+</sup> cells which also contain the ADE2 plasmid was determined for the cotransformants, both during growth selecting for the presence of both plasmids and after a period of growth in which selection for the ADE2 plasmid had been relaxed. The results are shown in Table 1. A comparison of yeast cotransformed with pAS4-D<sub>rep1</sub> and pGAD-REP1 versus those cotransformed with pAS4-D<sub>rep1</sub> and the vector pGAD424 indicates that the full-length Rep1-Gal4 AD fusion protein is able to restore mitotic stability to the version of pAS4 with rep1 deleted. The rate of loss of the pAS4-D<sub>rep1</sub> plasmid during nonselective growth was also reduced by expression of the full-length Rep1-Gal4 AD fusion protein. In contrast, none of the truncated Rep1-Gal4 AD fusion proteins was able to increase the mitotic stability of pAS4-D<sub>rep1</sub> beyond that observed with the pGAD424 cotransformant. The results show that deletion of the amino-terminal 76 amino acids or the carboxy-terminal 244 amino acids of Rep1 destroys its ability to mediate plasmid segregation.

**DISCUSSION**

We have used two-hybrid and in vitro baiting assays to determine the regions of the yeast 2<sup>μm</sup> plasmid-encoded proteins Rep1 and Rep2 that mediate the interactions of these proteins and a Southwestern assay to identify Rep2 DNA-binding activity. These interactions are summarized in Fig. 5. A plasmid segregation assay was used to demonstrate that amino-terminal deletions of Rep1 that abolish Rep1-Rep2 interaction and a carboxy-terminal deletion that does not impair this interaction both result in loss of Rep protein-mediated plasmid partitioning.

The predicted amino acid sequence of the Rep proteins reveals little about how they mediate plasmid partitioning. Proteins with functions analogous to those of Rep1 and Rep2 are encoded by other 2<sup>μm</sup>-like plasmids in several closely related yeasts, *Zygosaccharomyces rouxii*, *Zygosaccharomyces bisporus*, and *Kluyveromyces marxianus* (33). Despite the lack of any apparent DNA sequence homology between these yeast plasmids, all share remarkably similar structures; they are similar in size and have a large perfect inverted repeat which divides the plasmid into two unique regions encoding at least three ORFs and a locus required in cis for mitotic stability (33). The encoded proteins include a site-specific recombinase which, like 2<sup>μm</sup> Flp, promotes amplification of the plasmids by catalyzing recombination between the inverted repeats and two proteins which, like Rep1 and Rep2, are required in trans for

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**TABLE 1. Plasmid segregation assay**

<table>
<thead>
<tr>
<th>LEU2 plasmid</th>
<th>% of Leu&lt;sup&gt;+&lt;/sup&gt; cells retaining the ADE2 pAS4-rep1 plasmid after growth in medium lacking adenine and leucine</th>
<th>Rate of loss of the ADE2 pAS4-rep1 plasmid from Leu&lt;sup&gt;+&lt;/sup&gt; cells during growth in adenine-containing medium (%/generation&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGAD424</td>
<td>66.7 (± 14.7)</td>
<td>13.3 (± 8.0)</td>
</tr>
<tr>
<td>pGAD-REP1</td>
<td>96.0 (± 7.7)</td>
<td>0.4 (± 1.4)</td>
</tr>
<tr>
<td>pGAD-rep1Δ1–76</td>
<td>61.7 (± 12.3)</td>
<td>13.8 (± 6.2)</td>
</tr>
<tr>
<td>pGAD-rep1Δ1–129</td>
<td>72.1 (± 5.6)</td>
<td>13.3 (± 4.8)</td>
</tr>
<tr>
<td>pGAD-rep1Δ130–373</td>
<td>44.9 (± 12.2)</td>
<td>15.9 (± 5.1)</td>
</tr>
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</table>

<sup>a</sup> The ability of Gal4AD-Rep1 fusion proteins to complement a REP1 deletion was tested by measuring the mitotic stability of the 2<sup>μm</sup>-based ADE2 plasmid pAS4-Δrep1 when it was cotransformed in an ade2 leu2 [cir<sup>0</sup>] yeast strain, AS3, with either the LEU2 pGAD424 vector or derivatives of this plasmid expressing Rep1 fusion proteins. Data are averages ± standard deviations from assays of a minimum of four independent yeast transformants. See Materials and Methods for details.

<sup>b</sup> Numbers of generations in adenine-containing medium ranged from 10 to 12 for all cultures.

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**FIG. 5.** Summary of in vivo and in vitro Rep protein interactions. Rep fusion proteins and their truncated derivatives, tested for interaction with Rep1, Rep2, or DNA by two-hybrid, baiting, and Southwestern assays, are shown as rectangles. Gray rectangles indicate interacting fusions, while white rectangles indicate no interaction observed. The degree of interaction is indicated as strongest (+++) to weakest (+). A minus indicates no interaction.
mitotic stability of the plasmids. The Rep1-equivalent trans-
acting stability factors encoded by the other 2μm-like plasmids
share a limited degree of amino acid sequence similarity with
2μm Rep1, whereas the Rep2-like factors are too dissimilar to
be aligned (24). The first 129 amino acids of 2μm Rep1, which
we have shown to be essential and sufficient for Rep1 self-
association and for interaction with Rep2, include a domain
which is conserved between Rep1 and the Rep1-like ORFs
from the other 2μm-like plasmids (6). The proteins share
about 44% amino acid similarity across a 60-amino-acid stretch
(residues 30 to 89) (24). The sequence conservation in this
amino-terminal domain may reflect its dual functions of me-
diating Rep1 and Rep2 interaction. However, given the lack of
sequence conservation between the Rep2 analogues and their
inability to complement the segregation defect of rep2 deriva-
tives of 2μm plasmids other than their own, we might not
expect the Rep1-Rep2 interaction domain to be as highly con-
served as the Rep1 self-association domain (24). In our in vitro
association assay, deletion of the first 76 amino acids of Rep1
reduced but did not abolish Rep1 self-association, while data
from Velmurugan et al. (36) demonstrated that loss of the first
100 amino acids of Rep1 abolished interaction with both Rep1
and Rep2. Taken together, these data imply that residues crit-
ical for Rep1 self-association lie between residues 76 and 100.
Almost completely contained within this region is the most
significant block of identity shared by the different Rep1 pro-
teins, a region spanning residues 72 to 85. We suggest that this
conserved block is at least part of the Rep1 self-association
domain. Our data do not allow us to precisely delineate the
Rep1 domain responsible for Rep2 interaction, other than by
indicating that residues amino terminal to residue 77 and those
between positions 77 and 129 must be required.

The inability of the amino-terminally truncated Rep1 fusion
proteins analyzed in our study to interact with Rep2 or to
provide Rep1 plasmid segregation function indicates that the
carboxy-terminal two-thirds of the protein is insufficient for
these functions. Interestingly, deletion of this carboxy-terminal
portion of Rep1 appeared to enhance both Rep1 self-associa-
tion and interaction with Rep2, suggesting that this region may
play an inhibitory role in Rep protein interactions. Despite the
ability of carboxy-terminally truncated Rep1 to associate with
both Rep1 and Rep2, the truncated protein was unable to
promote segregation of a rep1 – 2μm-based plasmid, indicat-
ing that this domain is required for the plasmid segregation
function of Rep1. Examination of the amino acid sequence of S.
cerevisiae Rep1 has revealed that the carboxy-terminal 40% of
the protein has about 60% similarity to two coiled-coil pro-
teins: myosin heavy chain and the intermediate filament pro-
tein vimentin (38). This carboxy-terminal domain of Rep1 also
shows some degree of conservation between Rep1 analogues
from other 2μm-like plasmids, although to a lesser extent than
that observed for the amino-terminal domain; only 37% simi-
larity is present over an 80-amino-acid stretch (24). Subcellular
fractionation experiments have shown that S. cerevisiae Rep1
copurifies with a nuclear karyoskeletal fraction (38). These
observations have led to the suggestion that Rep1 intercalates
in the nuclear matrix or lamina by means of this carboxy-
terminal domain to provide dispersed attachment sites for the
2μm plasmid, thereby ensuring its efficient partitioning when
the nucleus divides (38). If Rep1 is normally tethered at these
sites through this domain, the Rep1 two-hybrid fusion proteins
may be restricted in their ability to participate in the two-
hybrid assay. Deletion of this domain may release the seques-
tered proteins and may account for the enhanced two-hybrid
interaction of carboxy-terminally truncated Rep1 with Rep2.

Protein interaction assays with truncated versions of Rep2
showed that the amino-terminal 58 amino acids were sufficient
for promoting interaction with Rep1 and, to a lesser extent,
self-association but that the carboxy-terminal 239 amino acids
promoted self-association. The in vitro baiting assay also indi-
cated that this carboxy-terminal protein of Rep2 might interact
with Rep1. These nonoverlapping interaction domains raise
the possibility that the Rep proteins can form a multimeric
complex in vivo and may explain their inherent insolubility
when they are purified from yeast. Further deletion derivatives
of both Rep1 and Rep2 are needed to precisely delineate which
protein domains determine the ability of the Rep pro-
teins to interact with and to mediate 2μm plasmid segregation.

Most models of 2μm plasmid segregation predict that Rep1
and/or Rep2 will interact with the DNA repeats of the plasmid
STB locus. Direct association of either of the Rep proteins with
any 2μm DNA sequence has not previously been demon-
strated. Evidence for such an association in vivo comes from
observations of alterations in the DNase sensitivity of the STB
region when Rep1 is not expressed in the cell and in that of the
FLP promoter region, if either Rep1 or Rep2 is absent, com-
pared to the pattern observed in [cir+] cells (34). These al-
terations might also occur if the Rep proteins interact with host
proteins that bind to the DNA rather than binding directly to
the DNA themselves. Hadfield et al. (13) reported an unchar-
acterized yeast host protein that interacts with the 2μm segre-
gation locus, STB, in vitro. The binding activity was observed
only in urea-solubilized yeast extracts isolated from cells ex-
pressing both Rep1 and Rep2 or in [cir+] extracts to which
exogenous Rep1 and Rep2 had been added. This result sugg-
ests that the host protein may need to interact with the Rep1-
Rep2 complex in order to bind STB or that Rep1 and Rep2
may need the host factor to promote formation of a complex
that binds STB. The requirement for urea solubilization of the
host factor may indicate that this protein(s) is normally asso-
ciated with a subcellular structure (13). Our Southwestern
assay demonstrated that Rep2 can preferentially bind STB DNA,
but efficient binding of Rep2 to other AT-rich repeated DNA
sequences has also been observed (M. J. Dobson, unpub-
lished results). It is possible that additional sequence specificity
is conferred when Rep2 is associated with Rep1 or other host
proteins. Recently, Velmurugan et al. (36) isolated a potential
candidate for a host protein involved in STB binding when they
identified the SHFI gene on the basis of its ability to activate
expression of a reporter gene lying downstream of the STB
repeats in a one-hybrid screen.

On the basis of the data presented here and in previous
studies of the 2μm plasmid, we propose a model for plasmid
partitioning in which there is competition between the Rep
proteins for heterodimerization versus self-association. In this
model, the Rep1-Rep2 complex is the functional unit for seg-
regation, with Rep2 mediating polymerization of a multimeric
Rep protein complex along the STB locus, both through its
eDNA-binding activity and its potential ability to interact simul-
taneously with both Rep1 and Rep2. Homodimerization of
Rep1 may block the formation or allow the disassembly of this partitioning complex in synchrony with detachment of chromosomal kinetochores from the spindle apparatus during mitosis. The overlap in Rep1 of the regions required for Rep2 interaction and for self-association suggests that these two interactions may be mutually exclusive. Support for this comes from the results of in vitro assays similar to those reported here in which simultaneous addition of Rep1 and Rep2 fusion proteins to GST-Rep1 beads reduced the amounts of bound protein for both compared to when either was added alone, whereas no reduction was observed when they were both added to GST-Rep2 beads (30). In our baiting assays, deletion of the carboxy-terminal portion of Rep1 consistently led to an increase in the amount of self-association of Rep1 while the effect on Rep2 interaction was generally less pronounced. These results suggest that the carboxy-terminal region of Rep1 may inhibit the ability of the amino-terminal domain to interact with either Rep1 or Rep2. In vivo, it is possible that host proteins interacting with the carboxy-terminal domain of Rep1 influence the balance between hetero- and homodimerization and hence between formation and disassembly of the partitioning complex.

There is some experimental support for this model. First, insufficient expression of Rep1 relative to normal Rep2 levels should disrupt segregation of 2μm-based plasmids. Cashmore et al. (5) have shown that a single chromosomally integrated copy of the \textit{REP2} gene expressed from its own promoter was able to fully complement the segregation defect of a multicopy \textit{rep2}\textsuperscript{−} plasmid. In contrast, one integrated copy of the \textit{REP1} gene was not sufficient to stabilize a multicopy \textit{rep1}\textsuperscript{−} plasmid. When the number of integrated copies of the \textit{REP1} gene was increased, the stability of the \textit{rep1}\textsuperscript{−} plasmid increased. These results suggest that Rep1 expression must reach a certain level for efficient plasmid segregation to be established. Further support for this model comes from the experiments of Hadfield et al. (13), in which an observed in vitro \textit{STB}\textsuperscript{−} binding activity was found to be dependent on the relative levels of expression of Rep1 and Rep2, insufficient Rep1 relative to Rep2 resulting in reduced binding.

Second, if the only requirement for segregation is to maintain a sufficient supply of the Rep1-Rep2 heterodimer, overexpressing Rep1, without altering Rep2 levels, should have no effect on plasmid segregation. Support for this possibility comes from experiments in which an efficient heterologous promoter was used to direct expression of the \textit{REP1} ORF on a 2μm-based vector in a \textit{[cir\textsuperscript{−}] cell} (6). The plasmid displayed high mitotic stability, suggesting that segregation was unperturbed. Concomitant overexpression of Rep1 and Rep2 might have an effect on the cell if the excess Rep1-Rep2 complex can polymerize on other chromosomal DNA sequences, forming a functioning partitioning complex, or is otherwise able to interact with and titrate host proteins, preventing them from performing their normal functions. These host proteins might be required for mediating chromosome segregation. Reynolds et al. (27) reported a reduction in the growth rate of \textit{[cir\textsuperscript{+}] cells} when Rep1 and Rep2 were simultaneously overexpressed under the control of the \textit{GAL1-10} promoter. This reduced growth was not observed when either of the Rep proteins was overexpressed alone, suggesting that the Rep1-Rep2 complex was interfering with some normal cellular process. We are currently continuing our studies to further elucidate the nature of the Rep1-Rep2 complex and to determine the host proteins with which it interacts to ensure the efficient segregation of the 2μm plasmid when the nucleus divides.

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