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The 2μm-Plasmid-Encoded Rep1 and Rep2 Proteins Interact with Each Other and Colocalize to the *Saccharomyces cerevisiae* Nucleus

YONG-TAE AHN,[1] XU-LI WU,[1] SHYAM BISWAL,[1] SOUNDARAPANDIAN VELMURUGAN,[1] FREDERIC C. VOLKERT,[2] AND MAKKUNI JAYARAM[1]*

Department of Microbiology, University of Texas at Austin, Austin, Texas 78712,[1] and Department of Microbiology and Immunology, SUNY Health Science Center, Brooklyn, New York 11203[2]

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The efficient partitioning of the 2μm plasmid of *Saccharomyces cerevisiae* at cell division requires two plasmid-encoded proteins (Rep1p and Rep2p) and a *cis*-acting locus, *REP3* (*STB*). By using protein hybrids containing fusions of the Rep proteins to green fluorescent protein (GFP), we show here that fluorescence from GFP-Rep1p or GFP-Rep2p is almost exclusively localized in the nucleus in a cir$^+$ strain. Nuclear localization of GFP-Rep1p and GFP-Rep2p, though discernible, is less efficient in a cir^0 host. GFP-Rep2p or GFP-Rep1p is able to promote the stability of a 2μm circle-derived plasmid harboring *REP1* or *REP2*, respectively, in a cir^0 background. Under these conditions, fluorescence from GFP-Rep2p or GFP-Rep1p is concentrated within the nucleus, as is the case in cir$^+$ cells. This characteristic nuclear accumulation is not dependent on the expression of the *FLP* or *RAF1* gene of the 2μm circle. Nuclear colocalization of Rep1p and Rep2p is consistent with the hypothesis that the two proteins directly or indirectly interact to form a functional bipartite or high-order protein complex. Immunoprecipitation experiments as well as baiting assays using GST-Rep hybrid proteins suggest a direct interaction between Rep1p and Rep2p which, in principle, may be modulated by other yeast proteins. Furthermore, these assays provide evidence for Rep1p-Rep1p and Rep2p-Rep2p associations as well. The sum of these interactions may be important in controlling the effective cellular concentration of the Rep1p-Rep2p complex.

The 2μm circle, a relatively small circular plasmid (6,318 bp) present in most common strains of *Saccharomyces cerevisiae*, provides a useful model system for benign molecular parasitism (reviewed in reference 1). At its normal density of approximately 60 to 100 molecules per cell, the plasmid apparently confers no selective advantage on the cells harboring it (8) but poses little, if any, growth disadvantage either. How has the plasmid achieved the evolutionary optimization of its steady-state copy number to meet its goal of stable propagation?

Previous genetic analyses have revealed a partitioning system and an amplification system that, in principle, can combine to provide the mechanism for plasmid persistence at a relatively high copy number. Under steady-state conditions, each plasmid molecule is replicated once and only once during a cell cycle with the host replication machinery (32). Following replication, the stability system (11, 14) ensures the efficient partitioning of the plasmids between daughter cells by overcoming the normal segregation bias that favors the mother over the daughter in plasmid retention (17). Two plasmid-encoded proteins, Rep1p and Rep2p, in conjunction with a *cis*-acting locus, *STB* (also called *REP3*), contribute to the stability function (11, 12, 14, 18). Although the results of Hadfield et al. (9) suggest that interaction of Rep1p-Rep2p with *STB* is dependent upon a host factor(s), none have been identified so far. They have observed *STB* binding by urea-solubilized yeast extracts expressing Rep1p and Rep2p or by cir^0 extracts supplemented exogenously with Rep1p and Rep2p.

If the copy number drops below the steady-state value, either because of an occasional failure in partitioning or because of the imposition of artificial experimental situations (24), the amplification system comes into play to rapidly rectify the reduction in copy number. This amplification mechanism overrides the cell cycle restriction on replication and is dependent on the 2μm circle F1p site-specific recombination system. A model for how the recombinational inversion of a bidirectional replication fork can result in multiple rounds of replication from a single initiation event has been proposed (6, 7, 23). Although the act of recombination has been shown to be indispensable in amplification (28), not all of the predictions of the Futcher model have been satisfactorily verified. For example, the replication intermediates predicted by the model have not been directly identified, nor has it been shown (to our knowledge) that amplification fails when the replication origin is placed equidistant from the recombination target sites. The pince-nez forms of the 2μm plasmid molecules (two circular domains linked by a duplex domain) observed by Petes and Williamson (21) in *cdc8* yeast cells at the restrictive temperature support the idea of F1p recombination during plasmid replication (6). However, they are most easily accommodated by recombination occurring within a dimer circle, and not within a monomer circle as proposed by Futcher (6).

How do the partitioning system and the amplification systems communicate and cooperate with each other (and perhaps regulate each other) in the maintenance of the molecular symbiosis between the plasmid and the host genome? Biochemical answers to this fundamental question are sparse. From genetic studies, it appears that, at least up to the normal steady-state value, the plasmid senses its copy number through the levels of one of the Rep proteins (perhaps Rep1p) and that a regulatory complex containing Rep1p and Rep2p negatively controls amplification by turning down expression of the *FLP* gene (20, 22, 25, 27).

Preliminary biochemical characterization of Rep1p shows

* Corresponding author. Phone: (512) 471-0966. Fax: (512) 471-5546. E-mail: jayaram@almach.cc.utexas.edu.

that it is likely associated with the nuclear matrix (31); Rep2p has not been well studied, nor has there been a systematic biochemical analysis of its distribution during subcellular fractionation. There is some cytological evidence to suggest that Rep2p may also be a nuclear protein, and at least a part of it appears to cofractionate with Rep1p during purification of the latter (1). If the two proteins form part of a transcriptional regulator of one or more of the 2μm circle genes, they must associate with each other directly or indirectly at least temporarily during the cell cycle progression of *cir*$^+$ yeast. We have addressed this issue by cytological localization of Rep1p and Rep2p in cir^0 and cir$^+$ yeast strains, by immunoprecipitations of these proteins from mixed extracts of *Escherichia coli* cells expressing them, and by baiting assays using hybrid glutathione *S*-transferase (GST)–Rep proteins. The results and their potential implications in 2μm circle biology are summarized in this report. Additionally, corroboration of these results by in vivo assays with yeast is presented as an appendix.

MATERIALS AND METHODS

Yeast strains. Four yeast strains were used in these assays: FVY2-6B (*leu2-3,112 ura3-52 his3-d1* [cir^0]; Gal$^+$); FVY 889 (*ade2 leu2-3,112 ura3-52 his5-2* [cir^0]; Gal$^+$); FVY93154 (*leu2-3,112 ura3-52 trp1-289 ade2* [cir$^+$]; Gal$^+$); CCY666-1A (*ade2 his3-D200 leu2-3,112 ura3-52* [cir$^+$]; Gal$^+$). The cellular localizations of green fluorescent protein (GFP) fusion proteins shown in Fig. 2 were done in strains FVY2 [cir^0] and FVY93154 [cir$^+$]. Results were unchanged when FVY889 and CCY666-1A were used as the cir^0 host and cir$^+$ host, respectively. Stability of the *ADE2*-containing test plasmids pSTB1 and pSTB2 (see below) was assessed in FVY889 and CCY666-1A.

Plasmids. The pGFP-Rep plasmids were obtained by joining the 2μm circle *REP1* and *REP2* coding regions to the 3′ end of the GFP coding region via a short in-frame adapter sequence. The manipulations were done in the yeast GFP expression vector pTS408 (an *ARS-CEN* plasmid obtained as a gift from the Botstein laboratory through Clarence Chan), in which the GFP gene is controlled by the yeast *GAL1-10* promoter. The *REP1* and *REP2* gene segments were amplified from 2μm circle templates by PCR with primers such that the products of amplification contained *Bam*HI and *Xba*I restriction sites proximal to the 5′ and 3′ ends of the *REP* reading frames, respectively. After digestion with the two enzymes, the PCR products (approximately 1,200 bp for *REP1* and 900 bp for *REP2*) were ligated into the *Bam*HI-*Xba*I backbone of the pTS408 vector. The resulting plasmids were named pGFP-Rep1 and pGFP-Rep2.

The plasmids for assaying stability, pSTB1 and pSTB2, were derivatives of pYES2 (Invitrogen, Carlsbad, Calif.), which contains the yeast *URA3* marker plus the 2μm circle origin and the *STB* locus. First, the *URA3* in pYES2 was replaced by *LEU2* by removing an *Apa*I-*Nhe*I fragment from it and introducing a roughly 2,200-bp-long *LEU2*-bearing fragment in its place. The yeast *ADE2* gene (approximately 2,500 bp) was then cloned into the *Not*I-*Xho*I interval within the polylinker to obtain the plasmid named pSTB. The addition of the *REP1* and *REP2* genes from the 2μm plasmid into the *Xho*I site of pSTB yielded pSTB1 and pSTB2, respectively. The fragment spanning *REP1* extended from the *Eco*RI site at bp 2407 to the *Xba*I site at bp 703 within the A form of the 2μm circle DNA (10). The *REP2* fragment extended from the *Eco*RV site at position 5542 to the *Xba*I site at position 3949 (A-form coordinates).

In order to test the potential interaction between Rep1p and Rep2p, the pT7Rep and pSRep sets of plasmids were constructed by cloning PCR-amplified *REP1* and *REP2* coding regions into the *Bam*HI sites of pET11a and pET29a vectors (obtained from Novagen, Madison, Wis.). In plasmids pT7Rep1 and pT7Rep2, the arrangement of the *REP* reading frames is such that the expressed proteins would carry the T7 epitope tag at the amino termini. Similarly, Rep proteins expressed from pSRep1 and pSRep2 would carry the S tag at their amino termini.

The plasmids pGST-Rep1 and pGST-Rep2 were constructed for expressing the hybrid proteins used for the GST-baiting assays. In these plasmids, in-frame fusions of *REP1* and *REP2* reading frames with GST were obtained by cloning PCR-amplified fragments into the *Bam*HI site of pGEX2T (Pharmacia Biotech).

Fluorescence microscopy of cells. Single colonies of the host strain containing the appropriate plasmid or plasmids (isolated on selective plates) were grown overnight in 10 ml of medium under selection in raffinose as the sole carbon source. The cells were centrifuged and washed free of the medium in sterile water. Equal aliquots were resuspended in minimal medium containing either raffinose or galactose. After 4 h of growth at 30°C in a shaking incubator, cells were harvested, washed, and prepared for microscopy. For nuclear staining, cells were suspended in DAPI (4,6-diamidino-2-phenylindole) solution (1 μg/ml) and incubated at 30°C for 30 min with gentle shaking. All microscopic examinations were done with a Zeiss AXIOSKOPE or an Olympus BX60 fluorescence microscope. The green fluorescence from GFP (excitation and emission peaks at 395 and 509 nm, respectively) and the blue fluorescence from DAPI complexed with DNA (excitation and emission at 340 to 365 and 450 to 488 nm, respectively) were observed with optical filters recommended by Zeiss or Olympus. Photographs were taken with Kodak T-MAX 400 ASA film. Alternatively, images were captured with a Spot camera from Diagnostic Instruments and printed out with a Sony UP-D8000 color printer.

Plasmid stability assays. Stabilities of pSTB1 or pSTB2 in the cir^0 host harboring pGFP-Rep2p or pGFP-Rep1p, respectively, were assayed as follows. Purified colonies of the plasmid-bearing strains (*LEU2* and *ADE2* markers on pSTB1; *URA3* on pGFP-Rep2p) were maintained on SD Leu$^-$ Ura$^-$-glucose or SD Leu$^-$ Ura$^-$-galactose plates. Single colonies from the glucose and galactose master plates were spread out on yeast extract-peptone-dextrose (YEPD) and YEP-galactose plates and grown for 3 days at 30°C. The colonies were photographed with Kodak Gold (25 ASA) film under bright illumination. The colonies were replica plated on SD Leu$^-$ plates to verify that the red colonies were also Leu$^-$ (to ensure that the *ade2* phenotype was indeed due to plasmid loss).

Protein expression and immunoprecipitations. The Rep1 proteins carrying the T7 or the S tag were expressed in *E. coli* BL21(DE3) by IPTG (isopropyl-β-D-thiogalactopyranoside) induction according to the protocol provided by Novagen. The culture was grown at 37°C prior to induction and shifted to 25°C during induction. All cell manipulations following induction were carried out at 0 to 4°C. The induced cells were collected by centrifugation, washed twice with buffer A (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.10% Nonidet P-40 [NP-40], 2 mM methionine), and resuspended in the same buffer. The cell suspension was treated with lysozyme (100 μg/ml) for 15 min and sonicated by two 15-s pulses at the high-output setting in a Tekmar TM-600 sonicator. The sonicated suspension was spun at 20,000 × *g* for 10 min, and the supernatants were used for the immunological assays. Although sodium dodecyl sulfide (SDS)-polyacrylamide electrophoresis and Western blotting revealed the presence of Rep1p and Rep2p in the supernatants in quantities sufficient for our assays, the major fraction of the proteins were associated with the 20,000 × *g* pellet.

Immunoprecipitations were carried out at 4°C with monoclonal antibody to the T7 epitope according to the method recommended by Novagen. The immunoprecipitates were washed five times with the washing buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 2 mM methionine, 0.2% NP-40) before extraction of proteins into the electrophoresis buffer (15). Assays were done with 20,000 × *g* supernatants corresponding to 2.0 ml of the induced culture. Assays on mixed supernatants corresponded to 4.0 ml of the two cultures together (2.0 plus 2.0 ml).

Baiting assays with hybrid GST-Rep proteins. The GST-Rep hybrid proteins were induced in 100-ml cultures in the presence of 0.3 mM IPTG according to instructions provided by Pharmacia Biotech. Cell pellets were resuspended in 5 ml of 1× phosphate-buffered saline (pH 7.3) containing 0.2% NP-40 and a protease inhibitor cocktail supplied by Boehringer Mannheim. The supernatants used in the baiting assays were obtained as described under the immunoprecipitation protocol. Each baiting mixture contained 1.0 ml of GST-Rep1p (or GST-Rep2p) plus 1.0 ml of the S-Rep2p (or S-Rep1p) supernatant. After incubation for 1 h at 30°C, 50 μl of a 50% slurry of glutathione-agarose beads was added, and samples were gently shaken for 10 min at room temperature. Following centrifugation, the pelleted beads were washed five times with 1× phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), pH 7.3, containing 0.2% NP-40. The adsorbed proteins were dissociated into 50 μl of SDS sample buffer (15) and analyzed by electrophoretic fractionation, Western blotting, and S protein probing.

Adsorption of S-tagged proteins by S protein-agarose beads. The S-tagged Rep proteins present in *E. coli* cell supernatants were trapped by S protein-agarose according to the protocol recommended by Novagen. The beads, collected by low-speed centrifugation, were washed five times with 20 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.1% Triton X-100 before the bound proteins were extracted into the electrophoresis buffer (15).

RESULTS

The current working hypothesis on the action of Rep1p and Rep2p, based largely on genetic experiments (with some biochemical support), is presented in the model shown in Fig. 1 (modified from 25). According to the model, Rep1p and Rep2p must interact with each other in order to mediate stable plasmid partitioning or to function as a transcriptional regulator of at least a subset of the 2μm plasmid genes. The model does not address whether the two proteins interact directly to form a bipartite complex or do so indirectly through the mediation of a host factor(s) to form a high-order complex. Regardless of this detail, it makes two key predictions. First, Rep1p and Rep2p must colocalize within the cell, at least transiently during their functional phase within the cell cycle. Second, they must harbor peptide domains that directly establish a dimer interface or bring them into association via interaction with host factors. Cytological, genetic, immunological,

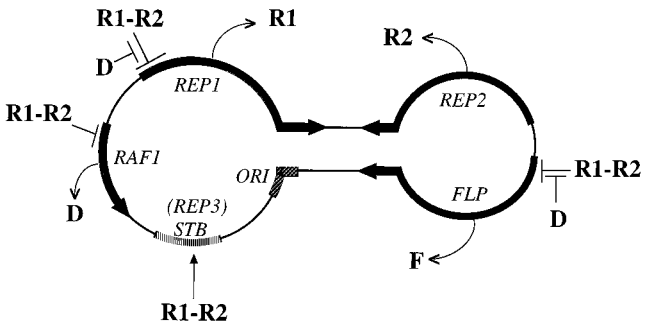

FIG. 1. A model for gene regulation in the 2μm plasmid by a bipartite regulator. The 2μm plasmid is shown in its standard representation with the parallel lines indicating the 599-bp inverted repeats. The products of the *REP1* and *REP2* genes (R1 and R2, respectively) are proposed to form a bipartite regulator that represses transcription from the *REP1*, *FLP*, and *RAF1* promoters. The R1-R2 complex is believed to interact with the *cis*-acting *STB* (or *REP3*) locus to promote plasmid partitioning at cell division. The product of the *RAF1* gene (D) may antagonize the R1-R2 repression. The plasmid replication origin is denoted by *ORI*. The product of the *FLP* gene (F) is a site-specific recombinase that is required for copy number compensation by an amplification mechanism (6, 28). The diagram, adapted from the work of Som et al. (25), summarizes contributions from several laboratories (2, 11, 14, 20, 22, 25).

and biochemical experiments that test these predictions are described below.

Cellular localization of Rep1p and Rep2p with GFP hybrids. Given that the 2μm plasmid exists primarily in the nucleus (with perhaps only occasional excursions outside of it), the logical site of action of the proposed Rep1p-Rep2p complex is the nucleus. In Fig. 2A to D, we display the distribution of hybrid proteins obtained by in-frame fusion of Rep1p or Rep2p to the carboxy terminus of GFP in a cir^+ yeast strain. The fusion proteins were induced by shifting cells from raffinose to galactose to turn on the *GAL10* promoter that controls their expression. The cells were examined at 4 h following transfer to galactose medium. The left panels (A and C) display the green fluorescence from the fusion proteins. In this host, over 90% of the cells exhibited exclusive nuclear fluorescence from GFP-Rep1p (A) or from GFP-Rep2p (B). The right panels (B and D) depict the correspondence between the green fluorescence from GFP-Rep proteins and the blue fluorescence from DAPI-stained nuclei.

The nuclear localization of Rep1p and Rep2p was less efficient in a cir^0 host strain (Fig. 2E to J). Although fluorescence from GFP-Rep1p was localized primarily to the nucleus in almost all cells (E and F), there was a dull background fluorescence of the cytoplasm (E). Rarely (in approximately 10% of the cells), Rep1p accumulation could not be associated with the nucleus with confidence. An example of such cells is shown in Fig. 2I. The fluorescence profile of GFP-Rep2p was somewhat different from that of GFP-Rep1p. In 60 to 70% of the cells, fluorescence was confined to the nucleus (G and H) and was more compact than that from GFP-Rep1p. In the remaining 30 to 40% of the cir^0 cells, fluorescence from GFP-Rep2p appeared to be clumped within the cell, with no specific organelle or subcellular association detectable under the resolution of the microscopy (Fig. 2J).

It should be pointed out that there was no detectable fluorescence in raffinose-grown cells harboring the GFP expression vector or the GFP-Rep expression vectors (results not shown). Thus, transcription of the fusion proteins was fairly tightly controlled under these experimental conditions. Furthermore, in galactose-induced cells expressing GFP alone, the cells showed global fluorescence with no indication of compartmentation (data not shown). We conclude that the subcellular confinement of the hybrid proteins is a function of the Rep component of the hybrid and not of the GFP component.

The nearly exclusive nuclear colocalization of Rep1p and Rep2p in cir^+ yeast is consistent with the notion that their association is a prerequisite for their functionality.

GFP-Rep2p and GFP-Rep1p are functional in stable plasmid partitioning. Do the nuclear enrichment and the sharply focused nuclear fluorescence of GFP-Rep1p or of GFP-Rep2p in a cir^+ background reflect the concentration of the two proteins at a subnuclear site that is their functional location? If so, can the GFP-Rep proteins substitute for the native Rep proteins in mediating plasmid stability in a cir^0 host strain?

The stability assays were done with pSTB1 and pSTB2, the two related plasmids that contain the 2μm circle replication origin, the *STB* locus, the yeast *ADE2* gene, and a second yeast marker (*LEU2*). Whereas pSTB1 harbored the 2μm circle *REP1* gene, pSTB2 harbored the *REP2* gene. In these plasmids, expression from the *REP1* and *REP2* genes was controlled by their native promoters. In a cir^0 *ade2* strain, pSTB1 or pSTB2 was lost rapidly under nonselective growth, giving rise to *ade2* cells at a very high frequency (data not shown). They were easily identified by the red color of the Ade^- colonies. A completely red colony indicated that it was clonally derived from an *ade2* cell that seeded it. The number of red sectors within a red-white colony reflected the frequency of plasmid loss during its growth, and the size of a red sector reflected the time of a plasmid loss event with respect to the age of the colony. In the cir^+ strain, the loss rate was reduced significantly as a result of complementation by the *REP1* or *REP2* gene function provided by the resident 2μm circle (data not shown).

The stability of pSTB1 was then assayed in a cir^0 host that harbored pGFP-Rep2p. Since the latter plasmid contained a centromere, it was not lost at any appreciable rate even under nonselective growth. The cells picked from selective glucose plates and spread on YEPD plates gave rise to colonies that were almost exclusively red (Fig. 3A). Thus, when GFP-Rep2p was not expressed, pSTB1 was as unstable as it was in a cir^0 host. In contrast, cells transferred from selective galactose plates to YEP-galactose plates showed a large reduction of red colonies in the population (Fig. 3B). Thus, expression of GFP-Rep2p from the *GAL* promoter ameliorated the loss of pSTB1. Intermediate levels of stability were observed when cells from selective galactose plates were transferred to YEPD plates (Fig. 3C) or when those from selective glucose plates were transferred to YEP-galactose plates (Fig. 3D). Note, however, that shifting cells from the preinduced state of GFP-Rep2p to the noninducible state (selective galactose to YEPD [Fig. 3C]) was more effective in stabilizing pSTB1 than the reverse mode of transfer (selective glucose to YEP-galactose [Fig. 3D]). When the assay was repeated in the same cir^0 parent strain harboring pGFP-Rep1p instead of pGFP-Rep2p, pSTB1 was equally unstable during growth in glucose or galactose, as indicated by the preponderance of red colonies (results not shown). A similar set of experiments using pSTB2 and pGFP-Rep1p (Fig. 4) gave comparable results for transfer of cells from selective glucose medium to YEPD (compare Fig. 4A to 3A) or from selective galactose medium to YEPD or YEP-galactose (compare Fig. 4B and C to Fig. 3B and C, respectively). Note, though, that cells transplanted from selective glucose plates to YEP-galactose plates did not show significant improvement in pSTB2 stability over those transferred to YEPD plates (compare Fig. 4D to A). Perhaps the rate of plasmid loss under this situation outpaces the buildup of the functional levels of GFP-Rep1p.

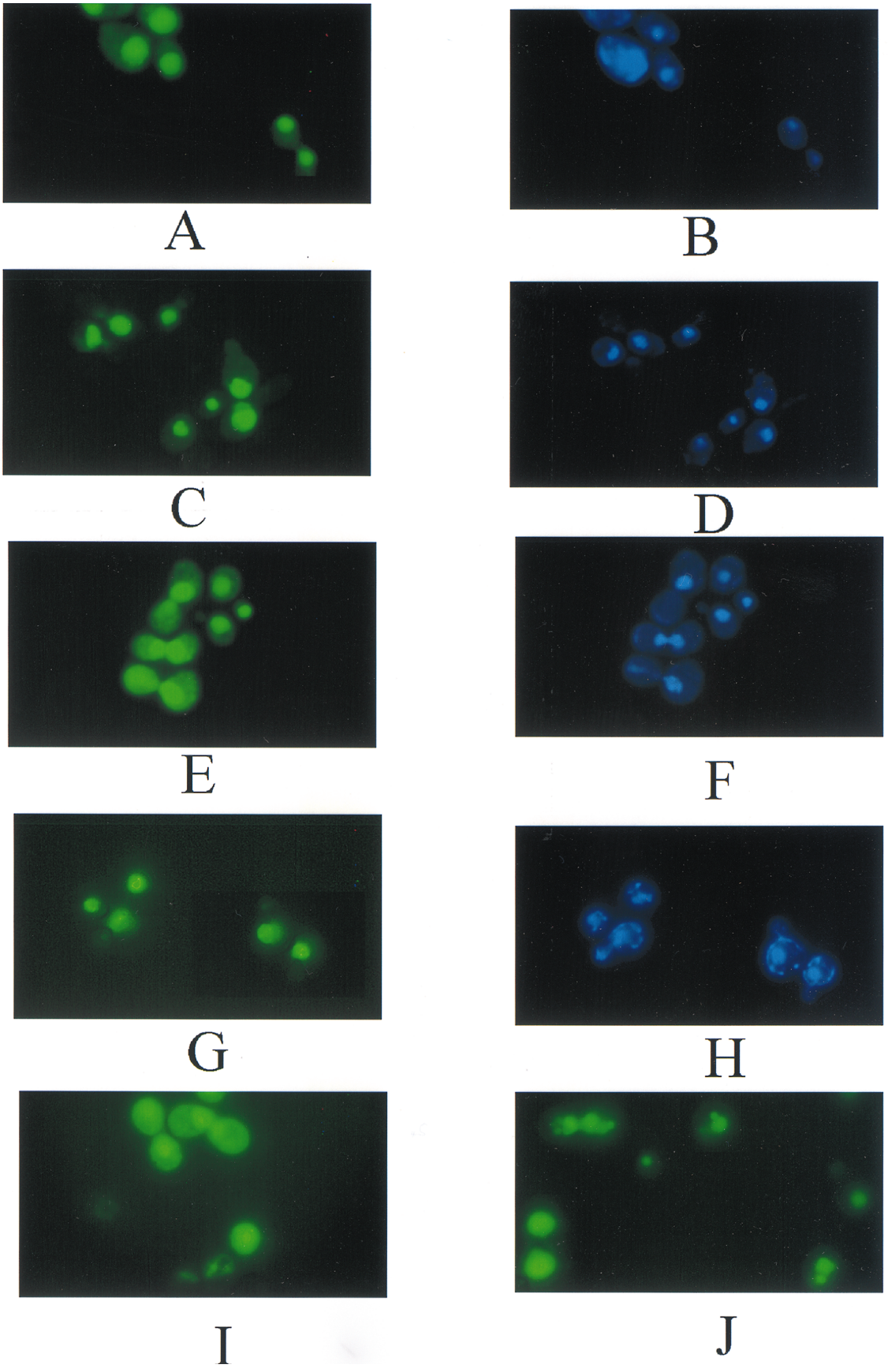
A
B
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FIG. 2. Fluorescence localization from GFP-Rep1p and GFP-Rep2p in cir^+ and cir^0 cells. The cells were prepared for microscopy after 4 h of induction of the GFP-Rep hybrid proteins (see Materials and Methods). (A, C, E, and G) Green fluorescence from GFP-Rep1p (A and C) and GFP-Rep2p (E and G). (B, D, F, and H) Correspondence between the green fluorescence from GFP-Rep proteins and the blue nuclear fluorescence from DAPI treatment. (I and J) Representative cells in which the green fluorescence from Rep1p (I) and Rep2p (J) could not be associated confidently with the nucleus.

We also examined the cellular localization of GFP-Rep1p and GFP-Rep2p in the cir^0::Rep2p and cir^0::Rep1p backgrounds, respectively. Cells grown selectively for both pGFP-Rep1p and pSTB2 (Fig. 5A and B) or pGFP-Rep2p and pSTB1 (Fig. 5C and D) were galactose induced for 4 h and prepared for fluorescence microscopy. In over 90% of the cells, the hybrid proteins were resident exclusively in the nucleus. Furthermore, the sharpness of the fluorescent spots was akin to that seen in the cir^+ host (compare Fig. 5A and C to Fig. 2A and C).

Based upon the sum of the stability assays, we conclude that GFP-Rep1p and GFP-Rep2p must retain at least partial functionality in plasmid partitioning. Furthermore, the 2μm plasmid functions present on pSTB1 or pSTB2 are sufficient for the exclusive, sharply focused homing of Rep2p or Rep1p, respectively, to the nucleus. Neither the F1p recombinase nor the *RAF* gene product is required for their nuclear targeting.

Rep1p and Rep2p can be coimmunoprecipitated from bacterial extracts containing them. As alluded to above, the interaction between Rep1p and Rep2p has been hypothesized to be an important regulatory step in the physiology of the 2μm plasmid. However, there is no evidence for this proposed protein-protein association. To test whether Rep1p and Rep2p can interact with each other without the mediation of other yeast proteins, we performed immunoprecipitations of mixed extracts of *E. coli* cells expressing them.

In the experiments depicted in Fig. 6, Rep2p harbored an amino-terminal T7 epitope tag and could be immunoprecipitated as well as probed in Western blots with T7-specific antibody (see Materials and Methods). Rep1p harbored an amino-terminal S tag and could not be directly immunoprecipitated in our assays but could be probed with the S-specific probe. The top and bottom panels of Fig. 6 represent the outcomes of probing electrophoretically fractionated extracts (lanes 3 and 5; also marked E) or immunoprecipitates (lanes 4 and 6 to 8; also marked I) with the T7 antibody and S probe, respectively. The presence of Rep2p or Rep1p in the extracts obtained from cells expressing either protein was easily detected by probing with the antibody or S protein probes (lane 3, top, and lane 5, bottom). There was no cross-reactivity between the T7 antibody and the S tag on Rep1p (lane 5, top), or between the S probe and the T7 epitope on Rep2p (lane 3, bottom). Furthermore, the T7 antibody immunoprecipitated Rep2p from the 20,000 × *g* supernatant of an extract containing this protein (lane 4, top) but did not immunoprecipitate Rep1p from an equivalent Rep1p-containing supernatant (lane 6, bottom). The most significant results are those shown in lanes 7 and 8. From a mixture of the Rep1p and Rep2p supernatants, the T7

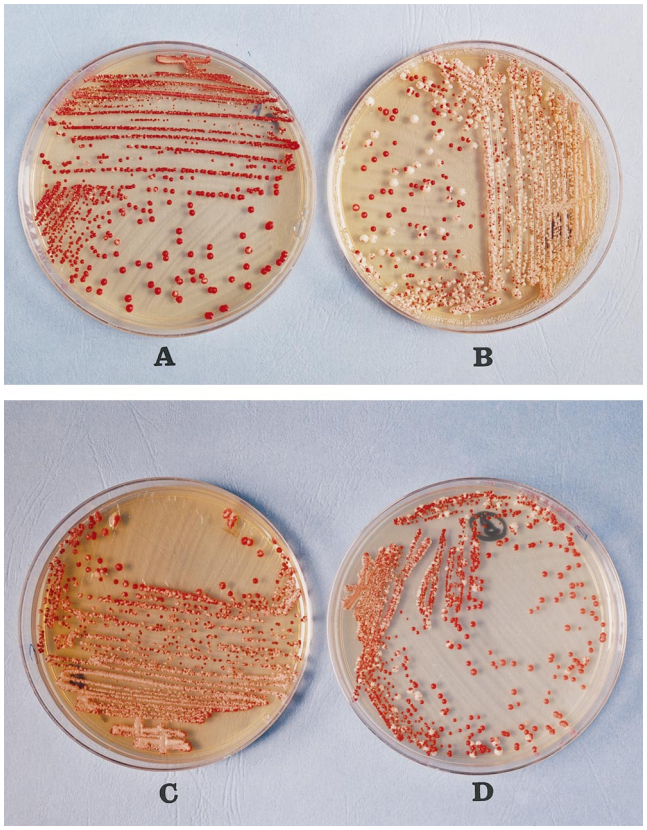

FIG. 3. Stability of pSTB1 in a cir^0 host under conditions that repress or induce GFP-Rep2p expression. Cells were transferred from selective plates containing glucose or galactose as the sole carbon source to YEPD or YEP-galactose plates and grown for 3 days at 30°C before being photographed. (A and D) Cells transferred from selective glucose plates to YEPD (A) and YEP-galactose (D) plates. (B and C) Cells transferred from selective galactose plates to YEP-galactose (B) and YEPD (C) plates.

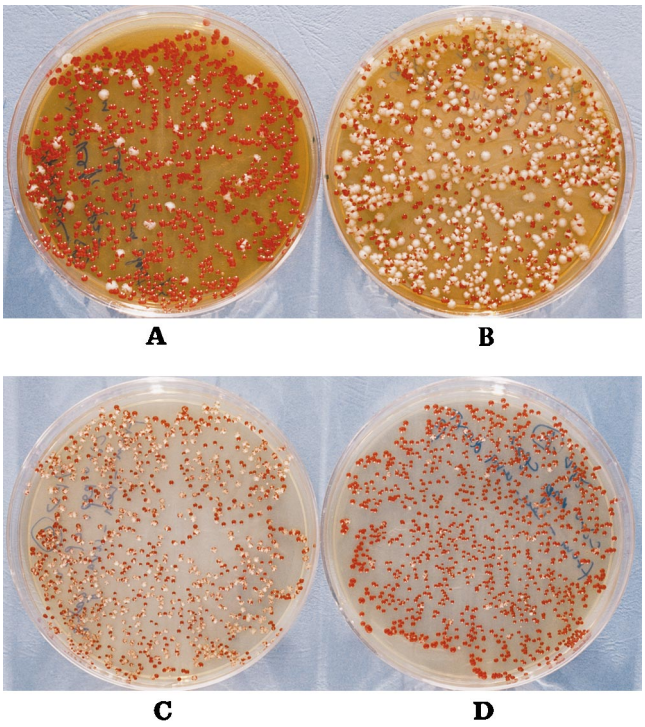

FIG. 4. Stability of pSTB2 in a cir^0 host during induction or repression of GFP-Rep1p. Experiments and panel designations are as described for Fig. 3.

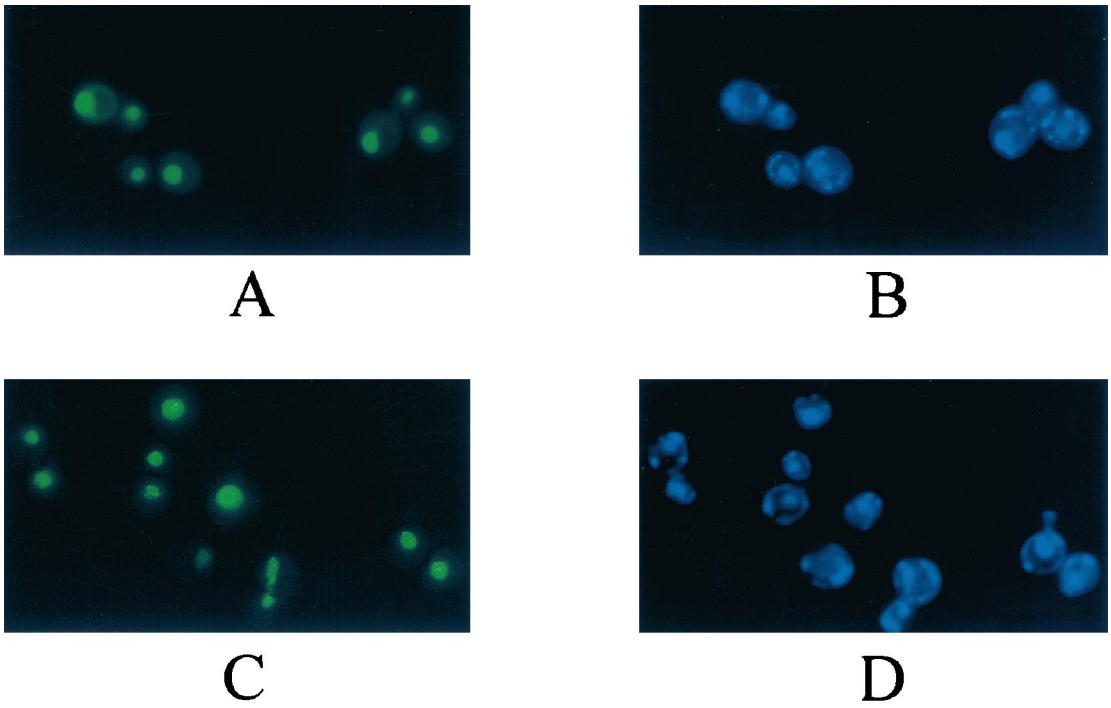

FIG. 5. Fluorescence from GFP-Rep1p and GFP-Rep2p in cir^0 cells harboring pSTB2 and pSTB1, respectively. Cells harboring pSTB2 and pGFP-Rep1p (A and B) or those harboring pSTB1 and GFP-Rep2p (C and D) were grown overnight in selective medium containing raffinose, washed, and transferred to selective medium containing galactose to induce GFP-Rep1p or GFP-Rep2p expression. Cells were prepared for microscopy after 4 h of induction.

antibody immunoprecipitated not only Rep2p (lane 7, top) but also Rep1p (lane 7, bottom). By contrast, no Rep1p could be immunoprecipitated from a mixture of the Rep1p supernatant and the supernatant from cells containing the T7 vector without the cloned *REP2* gene (lane 8, bottom). A reciprocal set of assays using T7-tagged Rep1p and S-tagged Rep2p yielded similar results (Fig. 7).

Thus, coimmunoprecipitation results show that Rep1p and Rep2p can interact with each other in the absence of any other yeast protein(s). In addition, we have also demonstrated in vivo interactions between these proteins in yeast by using the dihybrid analysis (see Appendix).

Rep1p and Rep2p exhibit self-association. In order to probe self-association in the Rep proteins, we carried out immunoprecipitations in supernatants containing a mixture of T7-Rep1p plus S-Rep1p or T7-Rep2p plus S-Rep2p. As in experiments shown in Fig. 6 and 7, the immunoprecipitations were carried out with the T7 antibody, and coprecipitation of the S-tagged proteins was assayed with the S protein probe (Fig. 8). The S-tagged Rep1p or Rep2p could be quantitatively recovered from the respective supernatants by using S protein-agarose and revealed by the S probe (lanes 2 and 3). Identically processed supernatants containing the T7-tagged Rep proteins did not show the Rep1p or Rep2p bands in response to S probing (lanes 4 and 5). The results of the immunoprecipitations of the mixed supernatants are shown in lanes 6 and 7. Approximately 10% of S-Rep1p present in the T7-Rep1p–S-Rep1p supernatant was coimmunoprecipitated with T7-Rep1p (lane 6); similarly, about 20 to 25% of S-Rep2p from T7-Rep2p–S-Rep2p was coimmunoprecipitated with T7-Rep2p (lane 7). In these assays, nearly all of T7-Rep1p and T7-Rep2p was recovered in the immunoprecipitates from the corresponding mixed supernatants (as revealed by T7 probing [data not shown]).

The results from immunoprecipitations were verified by a GST-Rep hybrid baiting assay. In this analysis, GST-Rep1p or GST-Rep2p was used to trap S-tagged Rep1p and Rep2p from supernatants of *E. coli* extracts containing them (details are given under Materials and Methods). The results of the baiting assays are displayed in Fig. 9. The electrophoretically fractionated samples were probed with S protein to reveal Rep1p or Rep2p harboring this tag. The amounts of S-Rep1p and S-Rep2p present in the supernatants used for baiting are shown in lanes 2 and 3. When GST-Rep1p was used as the bait, S-Rep1p or S-Rep2p could be pulled down from the respective supernatants (lanes 4 and 5). Similar results were obtained in the reciprocal experiment with GST-Rep2p as the bait (lanes 6 and 7). Control assays with GST alone failed to trap either S-Rep1p (lane 8) or S-Rep2p (lane 9). There was no cross-reactivity between the S probe with GST-Rep1p (lane 10) and that with GST-Rep2p (lane 11). Under the stringency of the assay conditions, GST-Rep1p trapped approximately 12% of the S-Rep1p (lane 4) and 70% of the S-Rep2p (lane 5) present in the respective supernatants. The corresponding values with GST-Rep2p were approximately 60% for S-Rep1p (lane 6) and 15% for Rep2p (lane 7).

The inferences drawn from the immunoprecipitation tests are corroborated well qualitatively and roughly quantitatively by the results of the GST-Rep hybrid baiting assays. At this time, we do not know whether the apparently weaker self-association between the Rep proteins (relative to the cross-interaction) is real or is a function of the available interaction surfaces in the molecular forms of GST-Rep or T7-Rep present in the assays. When overexpressed in *E. coli*, a large fraction of Rep1p or Rep2p was in aggregate form and was associated with the insoluble cell fraction. It is not clear whether the aggregation reflects the native self-association of these proteins or is an artifact of overexpression. An interest-

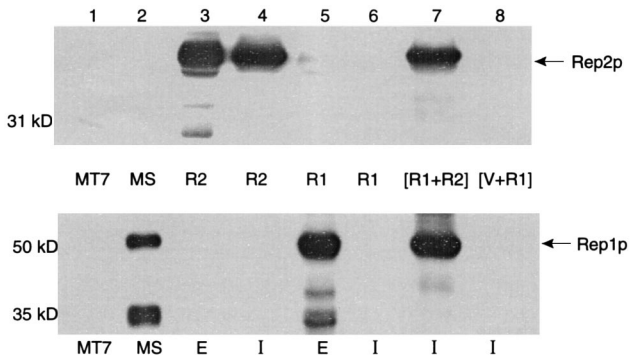

FIG. 6. Coimmunoprecipitation of Rep1p with epitope-tagged Rep2p from *E. coli* extracts expressing them. Rep1p harbored the S tag, while Rep2p harbored the T7 tag. Immunoprecipitations were done with a monoclonal antibody to the T7 epitope. Following electrophoretic fractionation of the *E. coli* extracts or the immunoprecipitates in SDS–12% polyacrylamide gels, proteins were transferred to polyvinylidene difluoride membranes and probed with the T7 probe (top) or with the S probe (bottom). Lanes 1 and 2 display the T7-specific (MT7) and S-specific (MS) protein markers supplied by Novagen, respectively. Lanes marked E (3 and 5) refer to extracts; those marked I refer to immunoprecipitates (4 and 6 to 8). The E lanes represent cell pellets corresponding to 50 μl of liquid cultures extracted into the loading buffer by being heated at 90°C for 3 min. The I lanes represent the 20,000 × *g* supernatants from 2.0-ml cultures (or 2.0- plus 2.0-ml cultures in the case of mixed extracts) immunoprecipitated with the T7 antibody. All immunoprecipitations were done at 4°C. Supernatant mixtures were incubated at 25°C (lanes 7 and 8) for 1 h prior to immunoprecipitation. Lane 8 represents immunoprecipitation of the Rep1p supernatant mixed with that from cells containing the expression vector without Rep2p. R1 and R2 refer to cell extracts or supernatants containing Rep1p and Rep2p, respectively. V refers to the cell supernatant from the mock-induced cells containing neither Rep1p nor Rep2p. In this figure and in Fig. 7, the higher sensitivity of the T7 probe relative to the S protein probe (as determined by the relative band intensities observed for standard amounts of T7- or S-tagged Rep1p or Rep2p) was compensated for by enhancing the panels probed with the S protein probe with an image analysis program supplied by Bio-Rad.

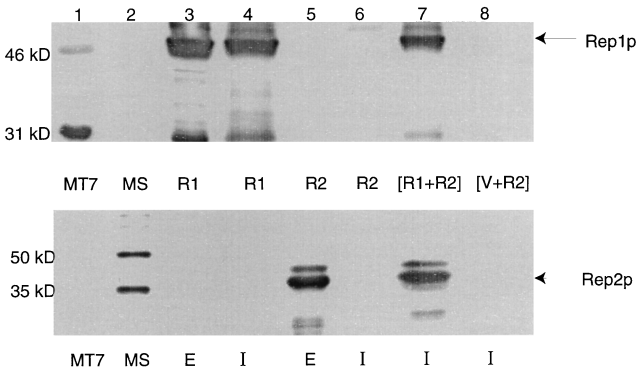

FIG. 7. Coimmunoprecipitation of Rep2p with epitope-tagged Rep1p from *E. coli* extracts. The experiment was exactly the reciprocal of that shown in Fig. 6. Rep1p was tagged with the T7 epitope, and Rep2p was tagged with the S tag. The lane designations are the same as for Fig. 6. An unknown band just above Rep2p (lanes 5 and 7 of the bottom panel) that cross-reacted with the S probe was occasionally seen in other assays as well. Interpretations of results are not affected by this band.

ing feature of the predicted secondary structure of Rep1p is that it includes a carboxy-terminal α-helical region with the potential to form extended coiled-coil structures (31). It should be pointed out that coimmunoprecipitation of T7-Rep2p and S-Rep2p was more efficient when the proteins were coexpressed than when they were expressed separately and then mixed (data not shown). Although we are ignorant of the molecular details of the Rep protein interactions, their propensity to self-associate as revealed in this study could be important in fine-tuning the partitioning of Rep1p and Rep2p into [Rep1p-Rep2p] as a function of their individual concentrations. Results of the dihybrid interaction assays in yeast that test Rep1p-Rep1p and Rep2p-Rep2p interactions (see Appendix) are consistent with those of the in vitro assays.

DISCUSSION

The 2μm circle plasmid is a selfish DNA that has evolved a dual strategy, that of efficient plasmid partitioning and of copy number amplification, for its maintenance as a benign parasite genome in yeast. It is not known whether the partitioning system is an active or a passive one: whether there is a directed segregation of replicated plasmid molecules into the daughter cells or whether the replicas are simply freed from a subcellular replication anchor so as to allow free diffusion. Any deviation from the copy number resulting from the passive distribution mechanism can, in principle, be corrected by the amplification mechanism. Genetic experiments (11, 14, 20, 22, 25) have given rise to a model in which the components of the stability system provide a measure of the plasmid copy number and contribute to the transcriptional regulation of the amplification system (Fig. 1). Experiments described in this paper address one central aspect of the model: direct or indirect interaction between the *REP1* and *REP2* gene products, the two plasmid-encoded *trans*-acting components of the stability system.

We reasoned that if Rep1p and Rep2p act in association, they must colocalize, albeit temporarily, at the site of their action within the cell, very likely the nucleus. Hybrids between GFP and the Rep proteins have allowed us to test this notion visually with minimal perturbations to the live yeast cells. Fusions between GFP and cellular proteins have been previously utilized to reveal the location and dynamics of these proteins in bacteria, yeast, and higher cells (3, 5, 16, 30). The nearly exclusive concentration of GFP-Rep1p or GFP-Rep2p in the nucleus in cir^+ cells, contrasted with their less efficient nuclear localization in cir^0 cells, is consistent with the interpretation that efficient nuclear transport of either protein is facilitated by the other, perhaps by mutual association. Our findings are in general agreement with the inferences drawn from earlier immunofluorescence analysis of Rep1p and Rep2p (summarized in reference 1). Although our results do not substantiate the claim that Rep2p is found in the nucleus only when coexpressed with Rep1p, we do find that coexpression significantly enhances nuclear accumulation of Rep2p. We have shown in this study that the *FLP* and *RAF* gene functions are dispensable without affecting the effective targeting of GFP-Rep2p or GFP-Rep1p to the nucleus. We cannot rule out, though, a possible involvement of *cis*-acting loci such as *STB* or the plasmid replication origin in localizing the Rep proteins to the nucleus. Experiments that address this issue are currently being done. Preliminary results indicate that quantitative nuclear restriction of Rep1p is Rep2 dependent but independent of *STB*; however, either Rep1p or *STB* appears to suffice for efficient nuclear deposition of Rep2p (31a). Thus, both protein-protein and protein-DNA interactions may contribute to the subcellular compartmentation of the Rep proteins. Functional analysis indicates that GFP-Rep2p and GFP-Rep1p can substitute for Rep2p and Rep1p, respectively, with reasonable efficiency in conferring stability on appropriate 2μm circle-derived test plasmids. Overall, the cytological and genetic assays can be accommodated by the hypothesis that Rep1p-Rep2p association may provide a mechanism for the effective, simultaneous delivery of the two proteins to the nuclear locale where the plasmid stability function is expressed.

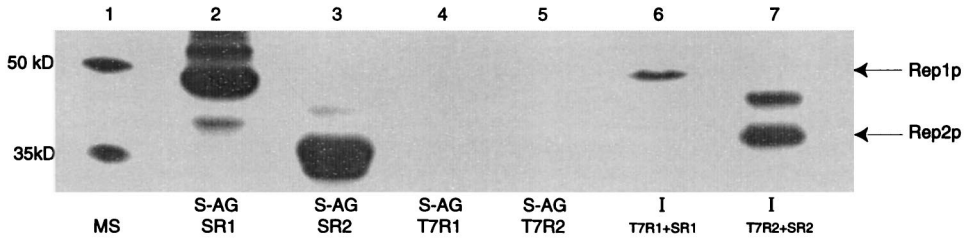

FIG. 8. Coimmunoprecipitation of S-tagged Rep1p with T7-tagged Rep1p and of S-tagged Rep2p with T7-tagged Rep2p. The samples, fractionated by electrophoresis, were probed with the S probe. Rep1p and Rep2p are denoted by R1 and R2 with the appropriate prefix, T7 or S, indicating the tag harbored by them. I refers to samples immunoprecipitated by the T7 antibody; S-AG stands for samples treated with S protein-agarose. MS stands for the molecular mass standards. The origin of the band above Rep2p in lane 7 is unknown. A band of similar mobility was also seen in some other assays (see legend to Fig. 7).

Our localization of Rep1p to the nucleus agrees with the earlier finding that the protein copurifies with the nuclear cytoskeletal fraction (31). The similarity (approximately 60%) of the carboxy-terminal portion of Rep1p to two coiled-coil proteins, myosin heavy chain and the intermediate filament protein vimentin, has spawned the speculation that Rep1p may play a role in anchoring the 2μm plasmid to the nuclear matrix during replication and partitioning. There is a limited amount of amino acid similarity between Rep1p and its homologs encoded by 2μm circle-like plasmids found in *Zygosaccharomyces* and *Kluyveromyces* yeasts (19, 26, 29). The *Saccharomyces* Rep2p and its counterparts from the other yeasts are much less similar and do not permit meaningful alignments. Nevertheless, the striking similarity among the yeast plasmids in their structural and functional organization (despite the divergence at the nucleotide and amino acid level) suggests that they utilize similar mechanisms for stability and copy number control.

Immunoprecipitation experiments and trapping assays with GST-Rep fusion proteins confirm the interaction between Rep1p and Rep2p suspected from the cytological observations. Since the assays are done with the proteins contained in *E. coli* extracts, we conclude that the interaction is not absolutely dependent on the presence of other yeast factors. The minimal interpretation of our results is that Rep1p interacts directly with Rep2p. This interpretation would fit in nicely with the idea that each protein promotes the import of the other from the cytoplasm to the nucleus. We cannot, however, rule out the mediation of an adapter moiety that is conserved in yeast and in *E. coli*. We also do not know at this time whether the extent or strength (or both) of Rep1p-Rep2p association can be modified by the presence of other yeast proteins in a functionally relevant manner (9). Nevertheless, the self-association of Rep1p and Rep2p (detected by immunoprecipitation and by using GST fusion proteins as bait) suggests that the effective intracellular Rep1p-Rep2p concentration at any particular time could be the result of multiple binding equilibria.

Currently available data in literature are consistent with the idea that the Rep1p-Rep2p complex provides a sensitive measure of the plasmid copy number, the levels of this complex exquisitely controlling the turn-on or turn-off of the amplification system as well as the execution of the partitioning function. If the expression or turnover of Rep1p is finely controlled as a function of plasmid copy number, one would expect that a certain threshold level of Rep1p is required in order to build up the critical concentration of Rep1p-Rep2p. Consistent with this idea, Cashmore et al. (2) find that the *REP1* function cannot be complemented in *trans* from one copy of the chromosomally integrated *REP1* gene controlled by its own promoter. In contrast, the *REP2* function can be complemented by a single-copy *REP2* integrant. Overexpression of Rep1p, on the other hand, does not impede plasmid partitioning. Inducible expression of *REP1* from the *GAL10* promoter results in inducible expression of the stability system, glucose and galactose, causing low and high plasmid stabilities, respectively (12). The importance of the relative amounts of Rep1p and Rep2p in stable plasmid partitioning is also indicated by the results of Dobson et al. (4). Under the normal physiology of the yeast cell, abnormally high levels of Rep1p (and consequently of Rep1p-Rep2p) would be undesirable. The amplification system would then be insensitive to small decreases in plasmid copy number. Thus, autoregulation of *REP1* can provide a

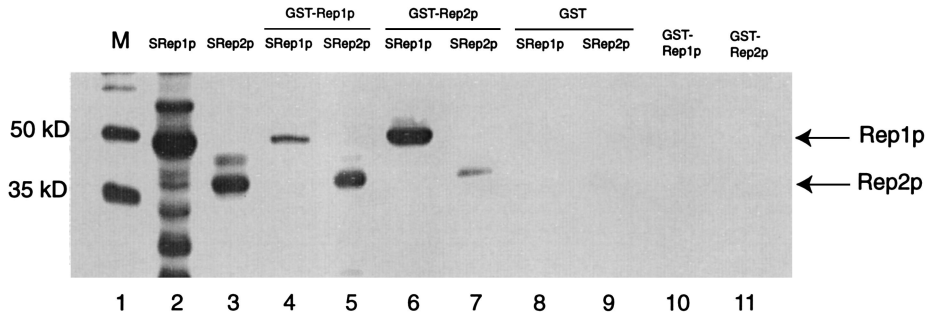

FIG. 9. Baiting assays for Rep1p and Rep2p using GST-Rep1p and GST-Rep2p hybrid proteins. The assays were carried out as described under Materials and Methods. Proteins were fractionated in SDS–12% polyacrylamide gels and probed with the S protein probe. The lane marked M displays molecular mass standards. The controls in lanes 2 and 3 represent supernatants from extracts containing S-Rep1p or S-Rep2p, respectively, that were not mixed with GST-Rep supernatants. These S-tagged proteins were pulled down with S protein-agarose beads prior to fractionation. The GST-Rep hybrids used as the baits and the corresponding baited S-Rep1p or S-Rep2p supernatant are indicated above lanes 4 to 7. In the reactions represented in lanes 8 and 9, the baiting was performed with GST and not with the GST-Rep hybrids. GST-Rep1p and GST-Rep2p in lanes 10 and 11 were adsorbed onto glutathione-agarose beads before fractionation.

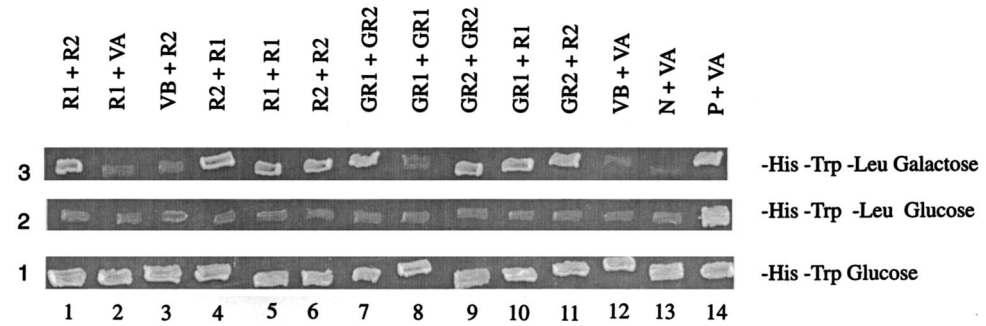

FIG. A1. In vivo baiting assays using Rep1p and Rep2p or their GFP fusion derivatives. Abbreviations: R1, Rep1p; R2, Rep2p; GR1, GFP-Rep1p; GR2, GFP-Rep2p; VB, vector containing LexA without Rep fusion; VA, vector containing the transcriptional activation domain without Rep fusion. For a given binary protein combination, the protein at the left of the plus sign is the bait (fused to LexA) and the protein at the right is the suspected prey (fused to the activation domain). The positive control (P) provided by the Brent laboratory contained a direct fusion of LexA to the *GAL4* activation domain. The negative control (N; also provided by the Brent laboratory) was a transcriptionally inert protein, *Drosophila* bicoid, fused to the LexA DNA binding domain.

mechanism for maintaining a level of Rep1p that is sufficient to ensure plasmid partitioning without impeding plasmid amplification. It is likely that the *RAF1* gene of the 2μm circle also contributes to the plasmid regulatory circuit. The Rep1p-Rep2p complex appears to repress *RAF1* expression, although less effectively than *FLP* expression (20). The Raf1p, on the other hand, acts as an antagonist of Rep1p-Rep2p, promoting transcription of *FLP*.

The 2μm plasmid is a remarkable genome. Its circular geometry, structural compactness, and functional parsimony represent an evolutionary model for the high-copy maintenance of an extrachromosomal selfish DNA element. The 2μm circle paradigm almost certainly holds for the other circular plasmids found in *Zygosaccharomyces* and *Kluyveromyces* yeasts. We suspect that at least some of the elements of this paradigm have global implications in benign molecular parasitism.

APPENDIX

In this appendix, we present results of the in vivo dihybrid assays for Rep protein interactions in yeast that substantiate the results from the in vitro assays. These experiments were performed by S. Velmurugan, and a more detailed analysis will be presented elsewhere (27a). Strains and plasmids were kindly provided by the Roger Brent laboratory (5a). The bait protein, a hybrid between LexA and Rep, was expressed constitutively from the yeast *ADH* promoter harbored by a *HIS3* plasmid. The prey, a fusion between Rep and a transcriptional activation domain, was expressed from the inducible *GAL1* promoter contained within a *TRP1* plasmid. The reporter cassette was a chromosomally integrated copy of the yeast *LEU2* gene (rows 2 and 3, Fig. A1) placed under the control of the LexA operator DNA serving as the *UAS* (upstream activating sequence). The simultaneous presence of the plasmids expressing the bait and prey was verified by the growth of the tester strain in SD His^- Trp^- medium (row 1, Fig. A1). A growing patch in row 3 (*LEU2* reporter; SD His^- Trp^- Leu^- galactose) was indicative of positive protein-protein interaction. Note that, whereas the LexA hybrid would be constitutively expressed, the fusion protein containing the transcriptional activator would be induced only in the presence of galactose. As glucose would repress expression of the latter, row 2 should not support growth. Since the positive control used here, a direct fusion of the activation domain to LexA, was constitutively made from the *ADH* promoter, its growth in rows 2 and 3 (column 14) was expected. The following conclusions can be drawn from the cumulative results shown in Fig. A1. Rep1p interacts with Rep2p and with itself; Rep2p also interacts with itself. GFP-Rep1p can interact with Rep1p, Rep2p, or GFP-Rep2p. Similarly, GFP-Rep2p can interact with Rep1p, Rep2p, GFP-Rep1p, or GFP-Rep2p. No interaction was detected by this assay between GFP-Rep1p and GFP-Rep1p (for explanations, see the legend to Fig. A1). The outcomes of these in vivo assays in yeast complement and extend the in vitro results obtained with the Rep proteins expressed in *E. coli*.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

M. J. Dobson and colleagues at Dalhousie University, Halifax, Canada, have also identified interactions between the Rep1 and Rep2 proteins of the 2μm plasmid (personal communication).

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