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Excess Intracellular Concentration of the pSC101 RepA Protein Interferes with Both Plasmid DNA Replication and Partitioning

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RepA, a plasmid-encoded gene product required for pSC101 replication in *Escherichia coli*, is shown here to inhibit the replication of pSC101 in vivo when overproduced 4- to 20-fold in *trans*. Unlike plasmids whose replication is prevented by mutations in the *repA* gene, plasmids prevented from replicating by overproduction of the RepA protein were lost rapidly from the cell population instead of being partitioned evenly between daughter cells. Removal of the partition (*par*) locus increased the inhibitory effect of excess RepA on replication, while host and plasmid mutations that compensate for the absence of *par*, or overproduction of the *E. coli* DnaA protein, diminished it. A *repA* mutation (*repA46*) that elevates pSC101 copy number almost entirely eliminated the inhibitory effect of RepA at high concentration and stimulated replication when the protein was moderately overproduced. As the RepA protein can exist in both monomer and dimer forms, we suggest that overproduction promotes RepA dimerization, reducing the formation of replication initiation complexes that require the RepA monomer and DnaA; we propose that the *repA46* mutation alters the ability of the mutant protein to dimerize. Our discovery that an elevated intracellular concentration of RepA specifically impedes plasmid partitioning implies that the RepA-containing complexes initiating pSC101 DNA replication participate also in the distribution of plasmids at cell division.

The *repA* gene of plasmid pSC101 encodes a 37-kDa protein essential for plasmid DNA replication (46). While the exact function of the RepA protein in the replication process is unknown, it has been shown to bind to directly repeated sequences within the replication origin (31, 41, 42, 45) as well as to regulate its own expression by interacting with inverted repeats in the *repA* promoter region (30, 47, 49). A number of host-encoded proteins including DnaA, DnaB, DnaC, DnaG, and integration host factor (IHF) (16, 20, 23, 24) are ordinarily required for pSC101 replication, and consensus binding sequences for both the DnaA and IHF proteins are present at the replication origin (20, 46) (Fig. 1). However, the requirement for IHF protein can be circumvented by mutations in the host *topA* gene (8) or by a single-base-pair change in the *repA* gene (*repA7*) (7), both of which stabilize pSC101 plasmids lacking the *par* locus (9, 35), which normally is required for partitioning of plasmids at the time of cell division (34, 44).

While further characterizing the *repA7* mutation, we noted that the mutant produces three times the normal amount of RepA protein, raising the possibility that overproduction of the RepA7 protein is the basis for its ability to stabilize inheritance of *par*-deleted pSC101 derivatives. This finding prompted us to investigate whether overproduction of the wild-type (wt) RepA protein would have similar effects. The results of these investigations indicate that increased intracellular concentrations of wt RepA not only fail to stabilize plasmid inheritance but instead interfere with both pSC101 DNA replication and plasmid partitioning in vivo. Our findings, which suggest a model in which the extent of dimerization of RepA has a crucial role in regulating the formation of origin region DNA-protein complexes that participate in the replication and segregation of pSC101, are described below.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are listed in Table 1. Unless otherwise stated, experiments were performed in PM191 with Luria broth medium containing 20 to 100 μg of ampicillin, kanamycin, or chloramphenicol per ml. Minimal M63 medium (37) containing 1% Casamino Acids, 80 μg each of L-leucine and L-threonine, and 1 μg of thymine per ml was used in [^{3}H]thymidine incorporation experiments.

Cloning of *repA* alleles by PCR. The wt *repA* ($repA_{wt}$) and the *repA7* and *repA46* alleles were cloned by polymerase chain reaction (PCR) amplification using the N-terminal primer

5′-CCCCGGGCGGCCGCCATATGTCTGAATTAGTTGTTTTC-3′
*Nde*I

and the C-terminal primer

5′-CCGGCCCCTAGGGCCGGATCCTTAGATCCTTCCGTATTTAGCCAG-3′.
*Bam*HI

Boldface type shows pSC101-specific sequence. The PCRs (0.1 μg of DNA) consisted of 25 cycles of 2 min at 40°C, 5 min at 50°C, and 1 min at 92°C; these low-stringency annealing conditions were used because of the nonhomologous sequence at the ends of the primers. The products were inserted into the *Nde*I-*Bam*HI-digested plasmid pET3a, yielding pHI729 ($repA_{wt}$), pHI975 (*repA7*), and pHI793 (*repA46*). The sequences of all three cloned *repA* genes were confirmed directly by DNA sequence analysis. Because of a high basal level of RepA expression in JM109(DE3) carrying the T7 RNA polymerase, we recloned the *repA* gene into pET11a, generating pHI780.

Plasmid constructions and mutants generated by site-directed mutagenesis. Plasmids analogous to pZC84 were generated from pSC101 mutant plasmids containing either the *repA7* allele (7, 9), yielding pHI1245, or the *repA46* allele (34), creating pHI1247. An *Xba*I linker (U.S. Biochemical) containing stop codons in all three reading frames was inserted into an *Eco*RI site of pZC84 generated between the fourth and fifth codon of the *repA* gene by using site-directed mutagenesis (32)

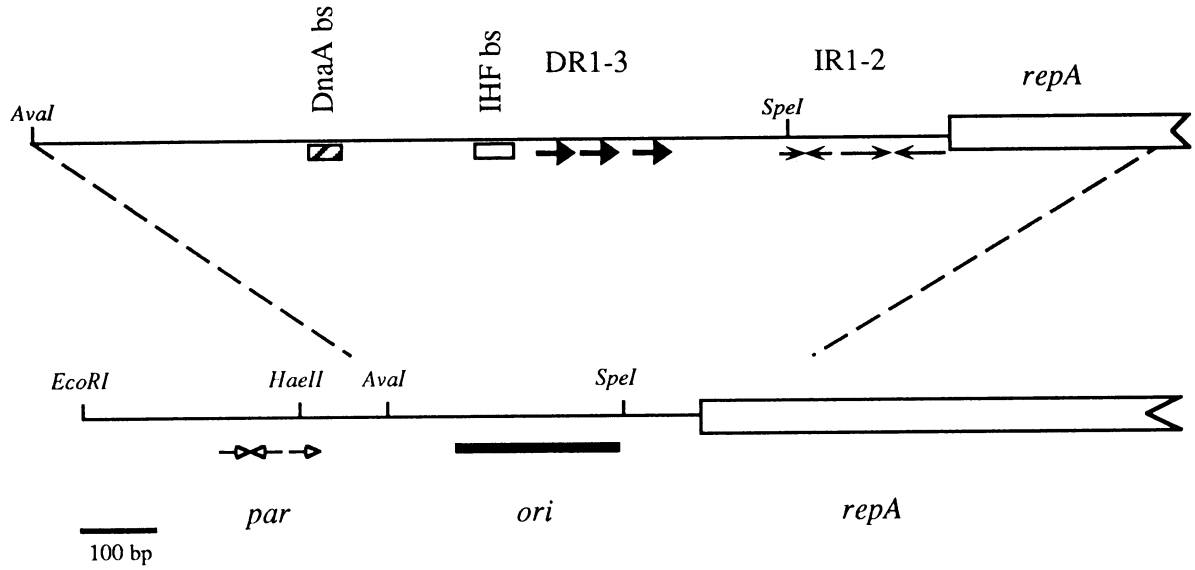

FIG. 1. pSC101 origin and *par* region. The pSC101 origin (ori) comprises a strong DnaA-binding site (DnaA bs) as well as an IHF-binding site (IHF bs). The RepA protein binds to three direct repeats (DR1-3, heavy arrows) within the origin and autoregulates its own synthesis by binding to inverted repeats (IR1-2, thin arrows) outside the origin. The *par* region is located 200 bp away from the origin and consists of one inverted and two direct repeat sequences (open arrowheads). Restriction sites referred to in the text are indicated. The figure is modified from that of Ingmer and Cohen (26).

and the oligonucleotide 5′-GAAAACAACGAATTCA GAC-3′, generating pHI1203. A control plasmid, pHI1190, containing only the promoter region and the first four codons of the *repA* gene, was also created. A pZC20-like plasmid (pHI1312) containing the *repA46* mutation was constructed by deleting the small *Eco*RI-*Bam*HI fragment from pCM128 (44). The $repA_{wt}$ gene and its alleles *repA7* and *repA46* from pHI729, pHI975, and pHI793, respectively, were inserted into the expression vector pGZ119, yielding pHI1281, pHI1282, and pHI1283. Plasmids expressing RepA proteins from the bacteriophage λ_{pRpL} promoters (pHI1298 [$repA_{wt}$], pHI1308 [*repA46*], and pHI1309 [*repA7*]) under control of the *cI857* gene product were created from pHI1281, pHI1282, and pHI1283 by excising the *repA* alleles with *Xba*I-*Cla*I and inserting them into the polylinker of pPL450. A control plasmid (pHI1300) was also made by inserting the *Xba*I-*Cla*I fragment from pGZ119 into pPL450. The *repA* alleles were furthermore excised from pHI729, pHI975, and pHI793 and inserted into a derivative of pMMB66HE containing the *aph* gene, placing the *repA* genes behind a cryptic promoter (pHI745 [$repA_{wt}$], pHI1183 [*repA7*], and pHI914 [*repA46*]). A control plasmid not producing any RepA protein (pHI791) was also constructed.

Generation of antibodies against RepA protein and Western blot (immunoblot) analysis. RepA protein was overexpressed from the T7 promoter on pHI780 in JM109(DE3) and was partially purified from 200 ml of cells by following the protocol described by Sugiura et al. (42) until the $(NH_4)_2SO_4$ precipitation step. After $(NH_4)_2SO_4$ precipitation, 1.2 mg of RepA protein fraction was separated on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel, and the Coomassie-stained RepA band was excised. Rabbit anti-RepA antibodies were prepared by Babco Berkeley Antibody Co. (Richmond, Calif.), using approximately 1 mg of RepA protein per inoculation, and were preabsorbed with an acetone powder made from cells containing the vector (pET11a) as described by Harlow and Lane (22). We routinely used a 1:2,000 dilution of the preabsorbed RepA antibody for Western blot analysis, performed as described by Promega with either an alkaline phosphatase-bound secondary antibody (1:7,500 dilution) and the Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium detection system (Promega) or, when quantitation was desired, the horseradish peroxidase-bound secondary antibody (1:15,000 dilution) and the ECL detection system (Amersham).

Transformation assays, segregation rates, and plasmid copy number. Cells containing either a RepA- or a DnaA-overexpressing plasmid or both of these were transformed with various pSC101 derivatives according to the procedure described by Sambrook et al. (40). The cells were plated on Luria broth plates with selection for all plasmids in the cells. Plasmid stability was determined as described by Meacock and Cohen (34). Plasmid copy number was determined as described by Biek and Cohen (9) with the modification that DNA was quantitated after Southern analysis (13) with ^{32}P-labelled pHI1312 (random priming kit; Pharmacia) as a probe.

Assay for plasmid replication. Replication of the wt pSC101 derivative pSLB204 was determined in the thymine-requiring strain CR34 in the presence of either pHI1298 (*repA* under control of the temperature-sensitive lambda repressor *cI857*) or the control plasmid pHI1300. Overnight cultures were diluted 100-fold into Luria broth medium containing 10 μg of thymine per ml and grown to an optical density at 600 nm of 0.5 to 0.6 with selection for both plasmids. The cells were harvested, washed once with an equal volume of medium, and resuspended in twice the volume of prewarmed (37°C) medium, with selection only for the RepA-overproducing plasmid, where growth was continued. Samples for pulse-labelling (2 ml of culture per sample) were harvested, washed once in 1 volume of preheated minimal medium, and resuspended in 1.5 ml of preheated minimal medium containing 50 μCi of [^{3}H]thymidine (88.8 Ci/mmol; Dupont). Samples were incubated at the growth temperature for 5 min, chased with 400 μg of thymidine, and placed on ice. Plasmid DNA was isolated

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
PM191	*recA thr-1 leuB6 thi-1 lacY1 supE44 rfbD1 fhuA21 mcrA1*	33
MG1655	λ^-	8
SC1127	MG1655 *topA10*	8
CR34	*thr leu thi thyA deoC*	5
JM109(DE3)	T7 polymerase under *lac* promoter control	Promega
Plasmids		
pZC20	4-kb pSC101 (*par*$^+$)	7
pZC119	*Hae*II Δ*par*	9
pZC127	*Eco*RI-*Hae*II Δ*par*	D. Biek
pZC132	*Eco*RI-*Ava*I Δ*par*	9
pCM128	pSC101 (*repA46*)	44
pZC52	pZC20 (*repA7*)	8
pHI1312	pZC20 (*repA46*)	This study
pSLB204	pZC20 with *aph* in *bla*	6
pSLB62	Promoter stabilized Δ*par*	6
pSLB80	Promoter stabilized Δ*par*	6
pET3a	pUC (T7 promoter)	39
pET11a	pUC (T7 promoter-*lac* operator)	15
pHI729	pET3a ($repA_{wt}$)	This study
pHI793	pET3a (*repA46*)	This study
pHI975	pET3a (*repA7*)	This study
pHI780	pET11a ($repA_{wt}$)	This study
pMMB66HE	RSF1010	19
pHI745	RSF1010 ($repA_{wt}$)	This study
pHI914	RSF1010 (*repA46*)	This study
pHI1183	RSF1010 (*repA7*)	This study
pHI791	RSF1010 (control)	This study
pGZ119HE	ColD ($lacI^q$ Ptac)	28
pHI1281	pGZ119HE (*repA46*)	This study
pHI1282	pGZ119HE ($repA_{wt}$)	This study
pHI1283	pGZ119HE (*repA7*)	This study
pPL450	pUC (λ_{pRpL})	N. Dixon
pHI1298	pPL450 ($repA_{wt}$)	This study
pHI1300	pPL450 (control)	This study
pHI1308	pPL450 (*repA46*)	This study
pHI1309	pPL450 (*repA7*)	This study
pZC84	pBR322 (*aph* $repA_{wt}$)	D. Biek
pHI1245	pBR322 (*repA7*)	This study
pHI1247	pBR322 (*repA46*)	This study
pHI1190	pBR322 (control)	This study
pHI1203	pBR322 (*repA* with stop codon)	This study
pFHC871	pACYC184 (*dnaA*$^+$)	2
pACYC184		11
pBR322		10

(25) and separated on a 0.7% agarose gel. The gel was treated with Entensify (Dupont), and after being dried it was subjected to autoradiography. Throughout the experiment, samples were plated on nonselective plates and cells containing the pSC101 derivative were scored by picking onto selective plates.

RESULTS

RepA protein inhibits pSC101 plasmid DNA replication. To investigate the effects of intracellular RepA concentration on the propagation of pSC101, we constructed a series of plasmids unrelated to pSC101 that express a range of intracellular levels of RepA protein (Fig. 2 and Table 2). We found that 10-fold overexpression of the pSC101 RepA protein from the ColD replicon (pHI1282) under control of the uninduced *tac* promoter affected transformation by both a wt pSC101 derivative

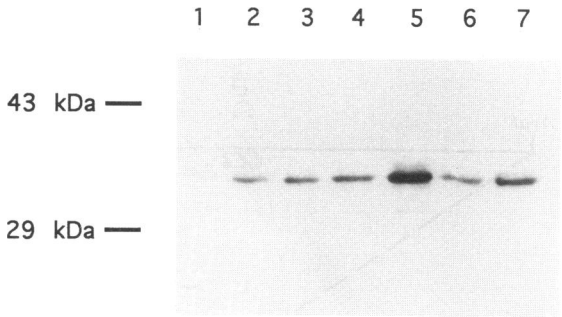

FIG. 2. Western blot analysis of plasmids that overproduce RepA. Proteins from approximately equal amounts of cells containing the indicated plasmids were separated on an SDS–10% polyacrylamide gel, reacted with antibody against RepA, and visualized with the ECL system (Amersham). Lanes: 1, no plasmid; 2, pZC20; 3, pZC84; 4, pHI745; 5, pHI1282; 6, pHI1298 at 30°C; 7, pHI1298 after 30 min at 37°C (fivefold dilution). The positions of the molecular mass markers (ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa) are indicated on the left.

(pZC20) and plasmids mutated in the *par* locus (pZC119, pZC127, and pZC132), whereas a 4-fold overexpression of RepA from a cryptic adventitious promoter (pHI745) only reduced transformation by pSC101 replicons containing substantial *par* locus deletions (pZC127 and pZC132) (Table 3). When RepA was expressed from its own autoregulated promoter on a high-copy-number plasmid (pZC84), only transformation by the pSC101 derivative containing the most extensive *par* deletion (pZC132) was severely inhibited by a fourfold increase in RepA protein concentration. The slight difference in transformation by *par*-mutated derivatives in strains containing pZC84 or pHI745 is probably due to titration of RepA protein by RepA binding sites present in the promoter region of the *repA* gene on pZC84 not found in pHI745. These results show that the more extensive the disruption of the *par* locus is, the more severe is the effect of increased RepA protein concentration. Insertion of a stop codon at the 5′ end of the *repA* gene of pZC84 (pHI1203) restored the ability of *par*-deleted plasmids to be established, indicating that the observed inhibition of transformation is mediated by the RepA protein itself. Consistent with this conclusion were results showing that the corresponding control plasmids lacking the *repA* gene (pHI1190 and pHI791) had no effect on the establishment of any of the pSC101 derivatives (Table 3).

Factors that previously were found to stabilize the inheritance of *par*-deleted plasmids, i.e., increased overall plasmid

TABLE 2. Relative RepA production

Plasmid	Replicon	Promoter	Ribosome binding site	Amt of RepA protein (fold)[a]
pZC20	pSC101	*repA*	*repA*	1
pZC84	pBR322	*repA*	*repA*	4
pHI745	RSF1010	Cryptic	T7 gene 10	4
pHI1282	ColD	*tac*	T7 gene 10	10
pHI1298	pUC	λ_{pRpL}	T7 gene 10	2 (20)[b]

[a] The amount of RepA protein was determined by Western blot analysis (see Materials and Methods) and after densitometric tracing normalized to the optical cell density measured at 600 nm. The results are normalized to the value obtained for the wt pSC101 plasmid pZC20 and are representative of >5 experiments of the type shown in Fig. 2.

[b] The value in parentheses was obtained after 30 min of growth at 37°C to induce the λ_{pRpL} promoters.

TABLE 3. Effect of RepA-expressing plasmids on transformation by pSC101 derivatives

Incoming plasmid	No. of transformants obtained with resident plasmid (relevant allele)[a]						
	pGZ119	pHI1282 ($repA_{wt}$)	pHI791	pHI745 ($repA_{wt}$)	pHI1190	pZC84 ($repA_{wt}$)	pHI1203
pZC20	1.3×10^5	6.0×10^3	10^5	10^5	4×10^5	10^5	10^5
pZC119	1.3×10^5	$<10^2$	8×10^4	3×10^4	4×10^5	4×10^5	3×10^5
pZC127	1.0×10^5	$<10^2$	7×10^4	30[b]	2×10^5	3×10^3	3×10^5
pZC132	0.7×10^5	$<10^2$	5×10^4	<25	2×10^5	<25	2×10^5
pACYC184	ND[c]	ND	ND	ND	5×10^5	3×10^5	4×10^5
pBR322	0.7×10^5	1.5×10^5	10^5	10^5	ND	ND	ND

[a] The number of transformants per microgram of DNA was determined by transforming 200 μl of competent PM191 cells containing one of the indicated plasmids with 40 ng of each of the pSC101 derivatives or with one of the control plasmids pBR322 or pACYC184. In some strain backgrounds, transformants containing both the RepA-overproducing plasmid and a *par*-deleted pSC101 derivative were observed. However, transformability did not correlate with a specific genotype, and the copy number of the *par* mutant derivatives was drastically reduced compared with the copy number in cells not overexpressing RepA (data not shown).
[b] Small colonies, which did not grow on selective media when restreaked.
[c] ND, not determined.

superhelicity as a consequence of a host mutation in the *topA* gene (SC1127) (8, 35), superhelicity generated by a strong promoter reading away from the pSC101 replication origin (pSLB62 and pSLB80) (6), and mutation of the *repA* gene (*repA7*) (9), also mimicked *par* in reducing the effects of excess RepA (Table 4). Overproduction of the *E. coli* DnaA protein, which binds to pSC101 in vitro at origin region sites that overlap loci occupied by RepA (41), similarly reduced the inhibitory effects of excess RepA on pSC101 derivatives containing smaller deletions in the *par* locus (pZC119 and pZC127) but failed to allow transformation of a *par*-deleted plasmid (pZC132). The inheritance of *par*-deleted plasmids was furthermore stabilized by overproduction of DnaA in cells producing a normal amount of the RepA protein: after 100 generations of cell growth, 95% of the cells retained the *par*-deleted pSC101 derivative pZC132 in the presence of the DnaA-producing plasmid pFHC871 (2) whereas only 2% contained pZC132 when the vector alone was present.

To determine the basis for the observed inhibition of transformation by RepA overproduction, we used a tandem arrangement of the λ_{pRpL} promoters (pPL450; N. Dixon), both of which are regulated by the temperature-sensitive λ repressor, to control expression of RepA. The resulting plasmid construct, pHI1298, produces twice the wt amount of RepA at 30°C but shows induction to 20 times the wt RepA level at 37°C (Table 2). We examined directly the effect of this extent of RepA excess on the replication of wt pSC101 DNA by monitoring the rate of incorporation of [^{3}H]thymidine into pSLB204, a pSC101 derivative carrying the native replication origin and *par* locus (6) in the presence of pHI1298. Our results (Fig. 3A) show that induction of excess RepA leads to a prompt and dramatic cessation of the incorporation of [^{3}H]thymidine into pSLB204 while not affecting replication of the concurrently present ColE1-type pHI1298 plasmid carrying the overexpressing *repA* gene. No inhibition of replication occurred following the temperature shift in cells containing the same ColE1-derived replicon lacking the pSC101 *repA* gene (pHI1300) (Fig. 3B).

A mutation in the *repA* gene that alters plasmid copy number control reduces inhibition of replication. Certain mutations within the *repA* gene lead to an increase in pSC101 copy number (1, 27, 34, 48). We speculated that this effect might result from the inability of the mutated RepA proteins to exercise the negative control of replication we observed for the wt RepA protein. As seen in Fig. 3C, replication of pSLB204 was unaffected by even a 20-fold excess of one such mutant protein (34) over the normal concentration of wt RepA, whereas replication of the test plasmid was inhibited by a similar concentration of either the wt RepA protein (Fig. 3A) or another mutant protein, RepA7, that did not affect the propagation of pSC101 when overproduced in a less excessive amount (Fig. 3D and pHI1183 in Table 4). Sequencing of the copy number mutant allele showed that it contains a single-base-pair transition in codon 46, changing CGG to TGG (Arg to Trp), leading us to designate this copy number mutation *repA46*.

TABLE 4. Effect of RepA expression on transformation by pSC101 derivatives under plasmid-stabilizing conditions

Incoming plasmid	No. of transformants obtained with host strain with relevant genotype and resident plasmid(s) (relevant allele[s])[a]								
	MG1655/ pHI791	MG1655/ pHI745 ($repA_{wt}$)	SC1127 *topA10*/ pHI791	SC1127 *topA10*/ pHI745 ($repA_{wt}$)	PM191/ pHI791	PM191/ pHI745 ($repA_{wt}$)	PM191/ pHI1183[b] (*repA7*)	PM191/pHI745/ pACYC184 ($repA_{wt}$)	PM191/pHI745/ pFHC871 ($repA_{wt}$ *dnaA*$^+$)
pZC20	3.0×10^5	1.5×10^5	4.0×10^4	5.0×10^4	2×10^6	4×10^5	4×10^5	5×10^4	4×10^4
pZC119	2.0×10^5	1.3×10^5	4.0×10^4	2.5×10^4	8×10^5	4×10^4	3×10^5	0.6×10^4	10^4
pZC127	2.2×10^5	$<7 \times 10^2$	4.7×10^4	3.0×10^4	7×10^5	$<7 \times 10^2$	3×10^5	<25	10^3
pZC132	1.3×10^5	$<7 \times 10^2$	3.4×10^4	1.8×10^4	6×10^5	$<7 \times 10^2$	3×10^5	<25	<25
pSLB62	ND[c]	ND	ND	ND	2×10^5	6×10^4	ND	ND	ND
pSLB80	ND	ND	ND	ND	1×10^4	5×10^3	ND	ND	ND
pBR322	7.5×10^5	5×10^5	1.3×10^5	8.0×10^4	5×10^6	2×10^6	3×10^6	5×10^4	2×10^4

[a] The number of transformants per microgram of DNA was determined by transforming 200 μl of cells with 40 ng of pSC101 plasmid DNA. The data shown were obtained in a single experiment but are representative of results obtained from more than five separate experiments.
[b] Western blot analysis showed that pHI1183 and pHI745 produce approximately equal amounts of RepA protein (data not shown).
[c] ND, not determined.

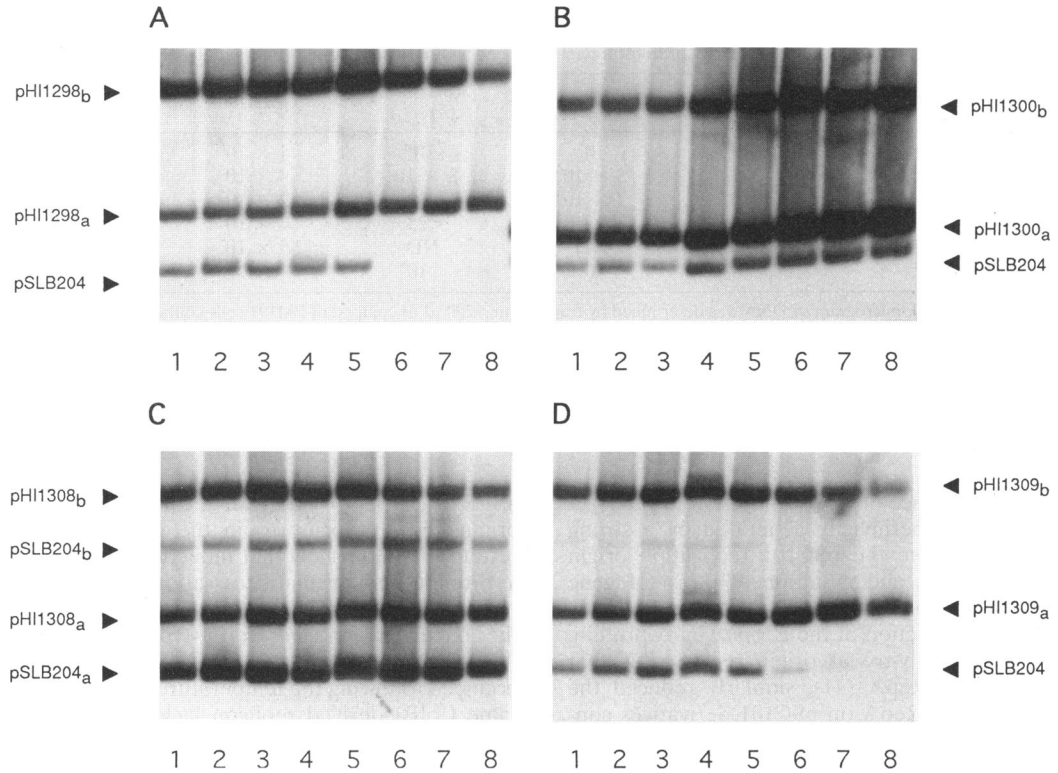

FIG. 3. Replication of pSLB204 in the presence of RepA overproduction. Cells containing the pSC101 derivative pSLB204 and the RepA overproducers pHI1298 (*repA*$_{wt}$) (A), pHI1308 (*repA46*) (C), or pHI1309 (*repA7*) (D) or the control plasmid pHI1300 (B) were pulse-labelled with [^{3}H]thymidine. Plasmid DNA was separated on a 0.7% agarose gel and subjected to autoradiography. Samples in lanes 1 through 4 were isolated from cells growing exponentially at 30°C, and those in lanes 5 through 8 were from cells that had been incubated 3, 15, 25, or 45 min at 37°C. The subscript letters a and b refer to the two different forms of each plasmid present in the undigested sample. Western blot analysis showed that pHI1298, pHI1308, and pHI1309 all produced the same amount of RepA protein at 37°C. At the end of the experiment, more than 95% of the cells still contained pSLB204.

While a 20-fold excess of the RepA46 protein failed to inhibit pSC101 replication, overproduction of the RepA46 protein from the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *tac* promoter (pHI1281), which yields a 300-fold excess of the gene product after induction, reduced the copy number of pHI1312 to 70% of the elevated copy number seen when the *repA46* gene is expressed from its own promoter (Table 5). During the course of these studies, we observed that overexpression of RepA46 protein under control of the uninduced *tac* promoter resulted in an almost threefold increase in pHI1312 copy number. These observations, which are considered further in the Discussion, indicate that (i) the *repA46* mutation enables the mutant protein to be tolerated by pSC101 in vastly greater excess than the wt RepA protein and (ii) the normal steady-state concentration of the RepA46 protein is suboptimal given its ability to exist at a high concentration with little or no observable inhibition of plasmid replication.

RepA protein overproduction affects segregation of a nonreplicating pSC101 plasmid. Cessation of pSC101 DNA replication subsequent to a mutation that causes the loss of RepA activity yields plasmid-free cells after the intracellular pool of nonreplicating plasmids has been diluted by partitioning among several generations of daughter cells, so long as the plasmid carries a functional *par* locus (44). In contrast, while investigating the effects of RepA overproduction on wt pSC101 replicons, we noted that plasmid loss occurred soon after the induction of excess *repA* gene expression, suggesting that excess RepA leads to defective plasmid partitioning (Fig. 4). Cells free of the *par*$^+$ pSC101 derivative pSLB204 were observed less than one generation after induction of wt RepA overproduction from pHI1298, and further gradual loss of the plasmid was observed upon prolonged incubation at nonpermissive conditions. At 30°C, both pCM301 and pSLB204 in the presence of pHI1298 had approximately the same copy number as the wt pSC101 plasmid pZC20 (data not shown).

TABLE 5. Copy number of pHI1312 and relative concentration of RepA46 protein

Additional plasmid	Relative copy no. of pHI1312[a]	Relative concn (fold) of RepA46[b]
pGZ119HE	1.0 ± 0.4	1 (4)
pHI1281	2.7 ± 0.3	4 (15)
pHI1281 + IPTG[c]	0.7 ± 0.1	80 (300)

[a] The relative copy number of pHI1312, a pSC101 derivative containing the *repA46* allele, was determined in the presence of the RepA46-overproducing plasmid pHI1281 and normalized to the value obtained in the presence of the vector pGZ119HE. The data (means ± standard deviations) represent the average of at least four determinations.

[b] The total amount of RepA46 protein was determined by Western blot analysis in cells containing pHI1312 and the RepA46-overproducing plasmid pHI1281, and the values obtained were normalized to those found for pHI1312 in the presence of pGZ119HE. The RepA46 protein concentration was also normalized to the amount of RepA protein produced by the wt plasmid pZC20 (values in parentheses).

[c] Cells were treated with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 90 min.

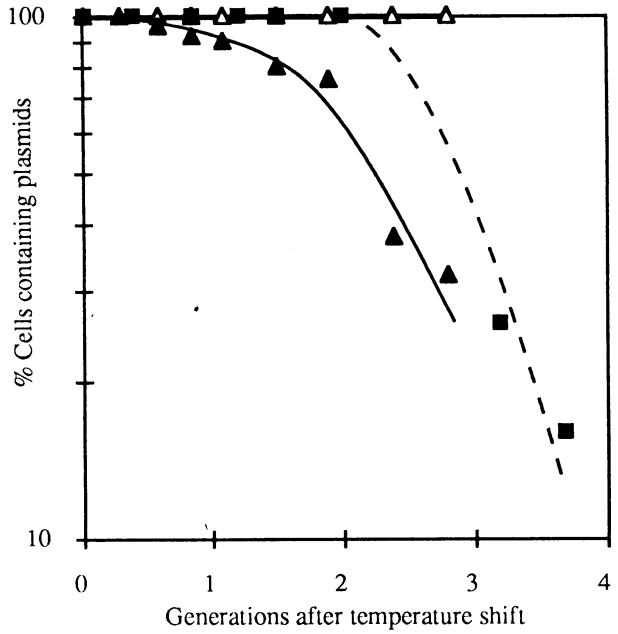

FIG. 4. Stability of pSC101 after replication inhibition. The accumulation of plasmid-free cells was monitored after a shift to 42°C at time zero for cells carrying pCM301 [*repA*(Ts), shaded squares] and a shift to 37°C at time zero for cells carrying pSLB204 and either pHI1298 ($repA_{wt}$, shaded triangles) or pHI1300 (control, open triangles). Viable cell counts showed an exponential increase following both the temperature shifts (data not shown).

DISCUSSION

It has long been known that the RepA protein encoded by pSC101 is required for replication of this plasmid (1, 29, 46, 50). The results reported here indicate that RepA also can inhibit pSC101 DNA replication and plasmid partitioning when present in excess. While interference with cotransformation and replication by excess amounts of other plasmid-encoded replication proteins has been observed previously for P1, Rts1, and the Pi protein of plasmid R6K (12, 17, 43), no effects of replication protein overproduction on plasmid partitioning have been reported.

While RepA has been reported to exist almost entirely in dimer form in vitro (42), the ratio of dimers to monomers in vivo under normal physiological conditions is not known. Gel shift assays using linear fragments of pSC101 DNA as a target indicate that it is the monomeric form of RepA that binds to directly repeated sequences (iterons) at the replication origin of pSC101 (31). We suggest that an excessive intracellular concentration of RepA promotes its dimerization and consequently reduces the availability of the RepA monomers, which could participate with DnaA in formation of the complexes that (i) initiate plasmid DNA replication and (ii) subsequently participate in the distribution of plasmids to daughter cells at division. As RepA dimers can bind efficiently to the *repA* gene operator-promoter (31, 42) autoregulating production of the protein, elevation of the intracellular concentration of RepA ordinarily is self-correcting and RepA is maintained at a level that allows plasmid replication and controls copy number. However, overproduction of RepA from an adventitious promoter that is not regulated by the intracellular concentration of RepA should lead, according to the model proposed here, to an increase in RepA dimers, which reportedly do not participate in the protein-DNA complexes that initiate replication (31). The *par* locus and mutations or conditions that compensate for the absence of *par* (i.e., the *repA7* and *topA* mutations, strong transcription near the origin, and DnaA overproduction) could potentially facilitate RepA binding to the origin. Evidence that at least the *par* locus can affect formation of origin region DNA-protein complexes (26) is consistent with this notion.

We suggest that the *repA46* mutation exerts its effects by decreasing dimerization of the gene product and consequently allowing replication to occur in the presence of excess RepA46 protein. Even at a 300-fold excess of the protein, only slight inhibition of pSC101 replication was detected. When RepA46 was expressed under control of the *repA* gene promoter, the intracellular concentration of the protein was increased about fourfold over the concentration found for the autoregulated wt RepA protein (Table 5). Our finding that a further fourfold increase in the already-elevated level of RepA46 stimulated pSC101 DNA replication implies that the normal steady-state concentration of the mutant protein is suboptimal.

Sequence analysis of the *repA46* gene shows that the mutant protein contains an arginine-to-tryptophan substitution at amino acid 46. The mutation flanks a previously identified leucine zipper motif in the RepA protein covering amino acids 12 to 33 (21). As leucine zipper motifs have been implicated in the ability of proteins to dimerize (38), we speculate that the *repA46* mutation may affect the function of the adjacent leucine zipper domain.

It has been suggested that bacteriophage P1 plasmids coupled together at their ParS sites by the P1-encoded ParB protein are separated and partitioned to daughter cells at division (3, 4, 14). If pSC101 plasmids bound to RepA monomers undergo analogous intermolecular coupling following their replication, as has been proposed by Miller and Cohen (36), factors that facilitate the interaction of the RepA monomers with the origin might be expected to facilitate both replication and partitioning. Conversely, a shift toward dimerization of unbound RepA as a result of its overproduction should interfere with both functions. We found that every tested condition that stabilizes the inheritance of *par*-deleted plasmids also antagonizes the RepA-mediated inhibition of pSC101 DNA replication. Moreover, when the wt RepA protein was greatly overproduced, cells free of a nonreplicating pSC101 plasmid containing the *par* locus appeared after less than one generation time, indicating directly that overproduction of RepA interferes with partitioning; this segregation pattern contrasted with the normal partitioning of nonreplicating plasmids observed when initiation of replication was prevented by a temperature-sensitive mutation in RepA (44) (Fig. 4). Interestingly, an excess of the ParB protein, which appears not to be involved in plasmid replication per se, leads to P1 plasmid instability (18) analogous to what we have observed for pSC101 during RepA overproduction.

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