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Effect of *CIS* on Activity in *trans* of the Replication Initiator Protein of an IncB Plasmid

J. PRASZKIER, S. MURTHY, AND A. J. PITTARD*

Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Victoria 3010, Australia

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RepA, the replication initiator protein of the IncB plasmid pMU720, acts preferentially in *cis*. The *cis* activity of RepA is thought to be mediated by *CIS*, a 166-bp region of DNA separating the coding region of *repA* from the origin of replication (*ori*) of pMU720. To investigate the *trans* activity of RepA, the *repA* gene, without its cognate *ori*, was cloned on a multicopy plasmid, pSU39. The *ori* on which RepA acts was cloned on pAM34, a plasmid whose replicon is inactive without induction by isopropyl-β-D-thiogalactopyranoside (IPTG). Thus, in the absence of IPTG, replication of the pAM34 derivatives was dependent on activation of the cloned *ori* by RepA produced in *trans* from the pSU39 derivatives. The effect of *CIS*, when present either on the RepA-producing or the *ori* plasmid or both, on the efficiency of replication of the *ori* plasmid in vivo, was determined. The presence of *CIS*, in its native position and orientation, on the RepA-producing plasmid reduced the efficiency of replication of the *ori* plasmid. This inhibitory activity of *CIS* was sequence specific and involved interaction with the C-terminal 20 to 37 amino acids of RepA. By contrast, *CIS* had no effect when present on the *ori* plasmid. Initiation of replication from the *ori* in *trans* was independent of transcription into *CIS*.

Miniplasmid pMU720, a derivative of the large, low-copy-number, conjugative plasmid pMU707, is a member of incompatibility group B (8). Replication of pMU720 requires the synthesis of the RepA protein, which is rate limiting for replication. Analyses of pMU720 and the closely related $IncI_1$ plasmid ColIb-P9 have shown that expression of *repA* is inhibited by a small antisense RNA, RNAI, which binds to its complementary sequence in the leader region of the *repA* mRNA (29, 38). Binding of RNAI to its target in *repA* mRNA prevents formation of a pseudoknot that activates translation of the *repA* mRNA (1–5, 39, 40, 44–46).

Initiation of replication of pMU720 and ColIb-P9 requires the presence in *cis* of two DNA sequences, the origin of replication (*ori*) and *CIS*; the latter separates the RepA coding sequence from *ori* (23, 30, 41). Members of the I complex (IncB, $IncI_1$, $IncI_\gamma$, IncK, and IncZ) as well as IncL/M and IncFII plasmids share this physical arrangement of the three genes required for replication, but the sequences of *repA*, *CIS*, and *ori* are not conserved in all of these plasmids. Thus, RepA, *CIS*, and *ori* of pMU720 show extensive homology to the corresponding sequences of ColIb-P9, R64-11, R144-3 ($IncI_1$), R621a ($IncI_\gamma$), and R387 (IncK) but no significant homology to the sequences of pIE545 (IncZ), R1, and R100 (IncFII) (6, 14, 18, 19, 26, 31, 33). The RepA proteins of pMU720 and the IncL/M plasmid pMU407.1 share ~40% amino acid identity, the *ori* sequences of the two plasmids contain conserved motifs, but their *CIS* regions show no significant similarity to each other (6, 30).

CIS is composed of two domains. The *repA*-proximal domain (5′*CIS*) has strong transcription termination activity (23, 30) and is thought to be involved in the loading of RepA onto *ori* (30). The *repA*-distal domain (3′*CIS*) is a spacer whose function is to place sequences within *ori* at an appropriate distance and on the correct face of the DNA helix vis à vis the *repA*-proximal domain of *CIS* (30). The RepA protein of pMU720 does not appear to recognize a specific sequence(s) in *CIS*, as the *CIS* of pMU720 can be replaced by the *CIS* of both pMU407.1 and pIE545 (30). By contrast, RepA shows sequence specificity for *ori*, in that it activates replication at its cognate *ori* and the *ori* of pMU407.1, which shares conserved sequence motifs with the *ori* of pMU720 but not the *ori* of pIE545 (30).

The Rep protein of the I complex and IncFII plasmids acts in *cis* (12, 18, 21, 23–25); i.e., it preferentially activates the *ori* of the DNA molecule from which its mRNA was transcribed. Studies on the IncFII plasmid R1 have shown that this property of Rep is dependent on the presence of *CIS*, in its native position and orientation (18). In this study, we used a two-plasmid system, in which the *ori* is provided on one plasmid and *repA* is carried on another plasmid, to investigate the *trans* activity of RepA of pMU720 in vivo and the role played by *CIS* in this activity. We find that (i) RepA activity in *trans* is less efficient than activity in *cis*, (ii) the presence of *CIS*, in its native orientation, immediately upstream of *ori* has no effect on the efficiency of replication of the *ori* plasmid, (iii) the presence of *CIS*, in its native orientation, immediately downstream of the *repA* coding sequence of the RepA-producing plasmid reduces the efficiency of replication of the *ori* plasmid, (iv) this inhibitory activity of *CIS* appears to be sequence specific and involves interaction with the C-terminal amino acids of RepA, and (v) transcription, from an upstream promoter, into *CIS* of the *ori* plasmid is not required for initiation of replication in vivo.

MATERIALS AND METHODS

Bacterial strains, plasmids and phages. The following strains of *Escherichia coli* K-12 were used in this study. *E. coli* JM101 [Δ(*lac-proAB*) *supE thi* F′ (*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*ΔM15)] (20) was used for cloning and propagating M13 derivatives. XL1 Blue MRF′ [Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)] (Stratagene) was used to grow M13 derivatives which had undergone mutagenesis as described by Vandeyar et al. (42). JP3438 (*thr-1 leuB6 thi-1 lacY1 gal-351 supE44 tonA21 hsdR4 rpoB364 recA56*) was used for propagating pMU720 derivatives and for all copy number determinations.

Bacteriophage vectors used to clone fragments for DNA sequencing and

* Corresponding author. Mailing address: Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Royal Parade, Victoria 3010, Australia. Phone: 61 3 9344 5679. Fax: 61 3 9347 1540. E-mail: aj.pittard@microbiology.unimelb.edu.au.

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Reference or source
pMU720	Miniplasmid derived from pMU707; Gal IncB	8
pMU604	Miniplasmid derived from pMU407.1; Gal IncL/M	11
pMU605	Miniplasmid derived from R64-11; Gal $IncI_1$	11
pMU2200	Miniplasmid derived from pIE545; Gal IncZ	31
pMU2209	Miniplasmid derived from R387; Gal IncK	31
pMU1598	pAM34 carrying nt 1–2170 of pMU720 with a T643C copy up mutation; Ap Cm *lacI*^{q} IncB	30
pMU1599	pAM34 carrying nt 1–2170 of pMU720; Ap Cm *lacI*^{q} IncB	30
pMU1583	pSU39 carrying nt 1–1759 of pMU720 with T642C and T640C mutations; Km IncB; RepA plasmid	This study
pMU1584	pSU39 carrying nt 1–1843 of pMU720 with T642C and T640C mutations; Km IncB; RepA plasmid	This study
pMU1585	pSU39 carrying nt 1–1916 of pMU720 with T642C and T640C mutations; Km IncB; RepA plasmid	This study
pMU1586	pAM34 carrying nt 1916–2170 of pMU720; Ap Cm *lacI*^{q}; *ori* plasmid	This study
pMU1587	pAM34 carrying nt 1840–2170 of pMU720; Ap Cm *lacI*^{q}; *ori* plasmid	This study
pMU1588	pAM34 carrying nt 1759–2170 of pMU720; Ap Cm *lacI*^{q}; *ori* plasmid	This study
pMU1589	pAM34 carrying nt 1825–2160 of pMU604; Ap Cm *lacI*^{q}; *ori* plasmid	This study
pAM34	pMB1 derivative in which the preprimer RNA is expressed from *lacZpo*; *lacI*^{q} Ap Sp	13
pSU39	p15A replicon; Km	7

[a] Abbreviations: Ap, ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Sp, spectinomycin resistance; nt, nucleotides. Mutations introduced into RepA and *ori* plasmids are described in Results.

mutagenesis were M13tg130, M13tg131 (15), and M13tg130BS, a derivative of M13tg130 which contains unique recognition sequences for restriction endonucleases *Sac*II and *Bgl*II in its multiple cloning site. The plasmids used are described in Table 1.

Media, enzymes, and chemicals. The minimal medium used was half-strength buffer 56 (22) supplemented with 0.2% glucose, thiamine (10 μg/ml), and necessary growth factors. Luria-Bertani (LB [35]) agar containing basic fuchsin (3 μg/ml) was used as indicator medium for *E. coli* cells producing chloramphenicol acetyltransferase (CAT). Cells containing high levels of CAT produce dark red colonies on this medium, whereas those containing little or no CAT produce light colonies (32). Enzymes and chemicals of a suitable grade were purchased commercially and not purified further. [^{35}S]dATPαS (>1,000 Ci/mmol) for use in sequencing was obtained from Amersham Corporation. LB-Km-Ap was LB agar supplemented with kanamycin (20 μg/ml) and ampicillin (50 μg/ml). Chloramphenicol was used at 10 μg/ml, isopropylthiogalactoside (IPTG) was used at 1 mM, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 25 μg/ml.

Recombinant DNA techniques. Plasmid and bacteriophage DNA were isolated and manipulated as described by Sambrook et al. (35). DNA was sequenced using a model 373 DNA sequencer and ABI Big Dye Terminator kits (Perkin-Elmer Corporation) or by the method of Sanger et al. (36), modified in that T7 DNA polymerase was used instead of the Klenow fragment and terminated chains were uniformly labeled with [^{35}S]dATPαS. Oligonucleotide-directed in vitro mutagenesis reactions were performed on single-stranded M13 templates by the method of Vandeyar et al. (42). Oligonucleotides were purchased from Bresatec Ltd. or Gibco BRL. DNA sequencing was used to screen for and confirm the presence of mutations.

Construction of *repA* chimeras. The coding sequence of the *repA* gene of the IncL/M plasmid pMU604 (*repA*^{L}) was amplified to contain a unique *Bgl*II site at the 5′ end (at codon 10) and a *Hin*dIII site at the 3′ end, just downstream of the stop codon. The amplified fragment was inserted into the *Bgl*II/*Hin*dIII site of M13tg130BS, and its sequence was determined to ensure that it was free of errors. An error-free clone was used as the template for oligonucleotide-directed mutagenesis, which created unique *Sph*I and *Sal*I sites, at positions corresponding to where these sites occur in the coding sequence of the *repA* gene of the IncB plasmid pMU720. Creation of these two sites did not change the amino acid sequence of RepA^L. RepA^B-RepA^L chimeras were constructed by exchanging corresponding fragments of the two *repA* genes. Thus, RepA^{BLL}-producing plasmid was made by joining the *Bgl*II/*Sph*I fragment of pMU720 to the *Sph*I/*Hin*dIII fragment of pMU604, whereas RepA^{BLB} plasmid was made by joining the *Bgl*I/*Sph*I fragment of pMU720, the *Sph*I/*Sal*I fragment of pMU604 and the *Sal*I/*Hin*dIII fragment of pMU720. RepA^K/RepA^B chimeras were generated by replacing the *Bam*HI/*Sph*I fragment of pMU720 by the *Bam*HI/*Sph*I fragment of the IncK plasmid pMU2209. This resulted in replacement of the IncB *rnaI* gene, the promoter-proximal sequence of *repA* (and thus the target of RNAI), and the sequence coding for the first 99 amino acids of RepA, by the corresponding sequences of the IncK plasmid.

Construction of RepA plasmids. RepA plasmids were derived from pSU39 by insertion of the *repA* gene of pMU720, with or without the *CIS* sequence, into the multiple cloning site. These plasmids do not carry the *ori* of pMU720, so that the RepA protein they produce is freely available to drive the replication of the *ori* plasmid present in *trans*. Expression of *repA* in the RepA plasmids is derepressed due to the presence of promoter down mutations in the *rnaI* gene. Mutations were introduced by replacing the pMU720 sequences by those being tested.

Construction of *ori* plasmids. The *ori* plasmids were derivatives of pMU1599 (30) in which the IncB replicon has been inactivated by deletion or mutation of *repA*. Those plasmids that contain *repA* have had their *Bam*HI/*Sph*I fragment, which carries the *rnaI* gene and the target for RNAI, replaced by corresponding fragment from the IncK replicon. Since RNAI of the IncK plasmid (RNAI^K) does not recognize the target of RNAI^B, this exchange ensured that expression of *repA* on the *ori* plasmid was regulated at the wild-type level, but expression of *repA* from the RepA plasmid present in *trans* remained derepressed. The *ori* plasmids contain the replicon from pAM34 (13), a modified pMB1 replicon in which the essential preprimer RNA is transcribed from the *lacZ* promoter operator. Since these plasmids contain the *lacI*^{q} gene, replication of their pAM34 replicon requires the presence of a *lac* inducer, such as IPTG. Thus, in the absence of IPTG, replication of the *ori* plasmids is dependent on the RepA provided in *trans* by the RepA plasmids. The presence of a CAT reporter gene (derived from pACYC184) allows estimations of the copy numbers of *ori* plasmids to be made.

Introduction of *ori* and RepA plasmids into *E. coli* cells. *ori* and RepA plasmids were cotransformed into *E. coli* K-12 strain JP3438 by the method of Chung et al. (10). Cells were plated onto medium containing half-strength buffer 56 (22), 0.2% glucose, 0.2% Casamino Acids, thiamine (10 μg/ml), ampicillin, chloramphenicol, and kanamycin, with and without IPTG, and incubated for 72 h at 37°C. Plates were checked after 48 and 72 h of incubation, and the number and size of colonies produced in the presence and absence of IPTG were compared. Single colonies from plates without IPTG were used for copy number estimations.

Measurement of CAT activity. CAT activity of mid-log-phase cultures, grown in minimal medium containing 0.4% glucose, thiamine, leucine, threonine, kanamycin, ampicillin, and chloramphenicol, was assayed as described by Shaw (37). Cells were disrupted by sonication in a Braun Labsonic 2000 Sonicator and cellular debris removed by centrifugation before use in assays. Each assay was performed at least six times. CAT activity was expressed as units per milligram of protein.

Protein assay. The concentration of protein in cleared cell lysates was determined by the method of Bradford (9), using bovine serum albumin as a standard.

Determination of plasmid copy number. The copy number of *ori* plasmids was estimated by comparing CAT activity of cells carrying these plasmids to activity of cells carrying pMU1598. pMU1598 had been shown to have a copy number of ~20 (30). The formula used was (CAT activity of *ori* plasmid)/CAT activity of pMU1598 × 20.

RESULTS

Does the presence of *CIS* affect the ability of *ori* to be activated by RepA provided in *trans*? In pMU720, *CIS* is thought to facilitate the loading of RepA onto the *ori* (30). To determine whether *CIS* also performs this function when RepA is provided in *trans*, a two-plasmid system was developed. RepA was produced from a multicopy (~20 copies per *E. coli* chromosomal equivalent) plasmid pSU39 (7) carrying an intact *repA* gene, so that both the transcription and the translation of *repA* mRNA were driven by its native sequences. Synthesis of RepA was modulated by the introduction of promoter down mutations into the gene for the antisense RNA, RNAI (43). The three mutations used changed the −10 box of the *rnaI* promoter from TATACT to TgTACT (RNAI.1), TgTgCT (RNAI.2), and TgTgCg (RNAI.3), causing progressive decrease in expression of *rnaI* and a corresponding increase in expression of *repA* (43). Expression of *repA* is fully derepressed in the RNAI.3 mutant (43, 45). Since RepA acts preferentially in *cis*, the RepA-producing plasmids were constructed so that they did not carry their cognate, active *ori* sequences, which might trap RepA protein and make it unavailable to activate

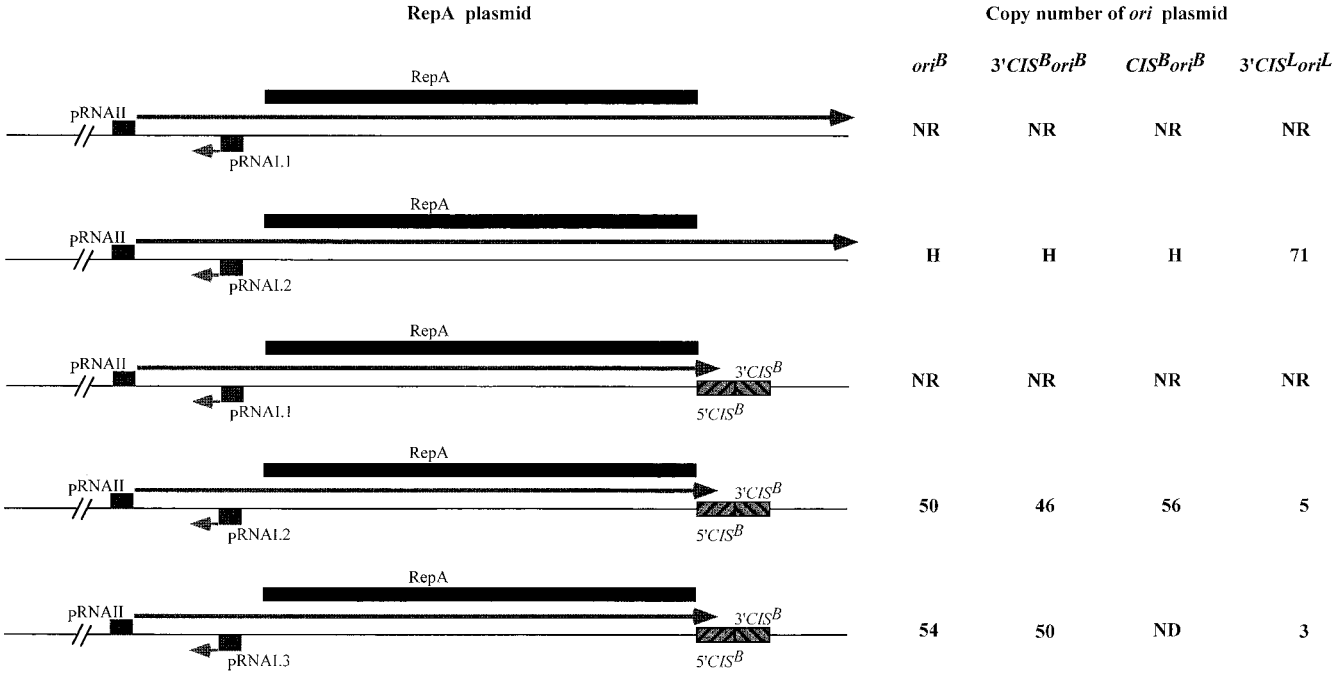

FIG. 1. Effects of mutations in *rnaI*, which increase expression of *repA*, and the presence of *CIS* on the RepA plasmid, on the copy number of ori^B and ori^L plasmids. RNAI, antisense RNA regulating the expression of *repA* of pMU720 replicon; RNAII, *repA* mRNA; RNAI.1, RNAI.2, and RNAI.3 mutations that change the −10 box of the *rnaI* promoter from TATACT to TgTACT, TgTgCT, and TgTgCg, respectively. Copy number was determined by assaying the CAT activity of *E. coli* strain JP3438 carrying the *ori* plasmids, in the absence of IPTG. The values shown are the average of at least six independent determinations. ND, not done; NR, *ori* plasmid unable to replicate without the induction of the pAM34 replicon; H, copy number too high for accurate determination; ⟶, RNA transcript.

replication of the *ori* plasmid in *trans*. The *ori* plasmids were derivatives of pAM34, which carries a fully repressible pMB1 replicon (13). This replicon is inactive in the absence of an inducer but can be switched on by the addition of IPTG. The *ori* sequences were inserted immediately downstream of a transcription terminator, to ensure that transcription from the β-lactamase gene, which is located upstream of the site of insertion, did not read through into *ori*. The *ori* plasmids carry a gene encoding a constitutively expressed CAT, which was used to monitor the plasmid copy number. All tests were carried out by cotransforming *E. coli* K-12 strain JP3438 with the *ori* and RepA plasmids and selecting for both plasmids, in the presence and absence of IPTG. CAT assays were carried out on transformants selected and grown in the absence of IPTG. RepA-*ori* plasmid combinations, which produced colonies only in the presence of IPTG, were scored as unable to activate replication of the *ori* plasmid in *trans*.

As shown in Fig. 1, RepA plasmids carrying the RNAI.1 mutation were unable to support the replication of the *ori* plasmids in *trans*. Introduction of the RNAI.2 and RNAI.3 mutation into the *rnaI* gene of the RepA plasmids resulted in an efficient activation of the *ori* present in *trans*. This suggests that the failure of the RNAI.1 mutants to activate replication of the *ori* plasmid was caused by their inability to produce enough RepA. The presence of 3′*CIS* or the intact *CIS* of the IncB replicon (CIS^B), in its native position and orientation on the ori^B plasmid, had no effect on its efficiency of replication. However, the presence of CIS^B in its native position and orientation on the RepA plasmid resulted in a decrease in the copy number of the *ori* plasmid in *trans*. Unfortunately, it was impossible to measure the extent of this effect with ori^B plasmids, because estimations of their copy number in the presence of the RepA plasmid that was not carrying CIS^B were not reproducible, ranging from ~10 to ~70 per chromosomal equivalent. Analysis on agarose gel of plasmid DNA extracted from cells cotransformed with the ori^B plasmid and the RepA plasmid that was not carrying CIS^B revealed that the ratio of *ori* plasmid DNA to RepA plasmid DNA varied from colony to colony but did not appear to be less than 1. The cotransformants produced extremely small colonies, in both the presence and absence of IPTG, and exhibited segregational instability, as evidenced by the production of very small, dark red colonies showing extensive sectoring, on LB-Km-Ap plates containing basic fuchsin, an indicator for CAT. The sectored areas of these colonies were lighter colored and faster growing than the bulk of the colony. Upon restreaking on LB-Km-Ap-basic fuchsin plates, the cells from the red portion of the sectored colonies produced sectored colonies again, whereas cells from the light-colored sectors failed to grow. The latter cells did grow on LB-Km-basic fuchsin plates (i.e., in the absence of selection for the ori^B plasmid), producing light-colored colonies. The simplest interpretation of these data is that the copy number of the ori^B plasmid in the presence of RepA-producing plasmid that does not carry *CIS* is too high to be sustained, resulting in selection of faster-growing cells that have lost the ori^B plasmid. In the IncB replicon, replacement of the *ori* by the corresponding sequence from the IncL/M replicon (ori^L) reduced the copy number of the replicon (30), indicating that RepA interacts less efficiently with this heterologous *ori* than with its cognate *ori*. Therefore, we examined the copy number of the ori^L plasmid in the presence of RepA-producing plasmid that does not carry *CIS*, to determine whether it was low enough to be sustainable. The copy number was found to be ~70 chromosome equivalents/cell, and the estimations were reproducible; thus, the effect of *CIS* could be measured. The copy number of the ori^L plasmid was reduced more than 10-fold when the RepA-producing plasmid carried CIS^B. These data are consistent with the notion that the segregational in-

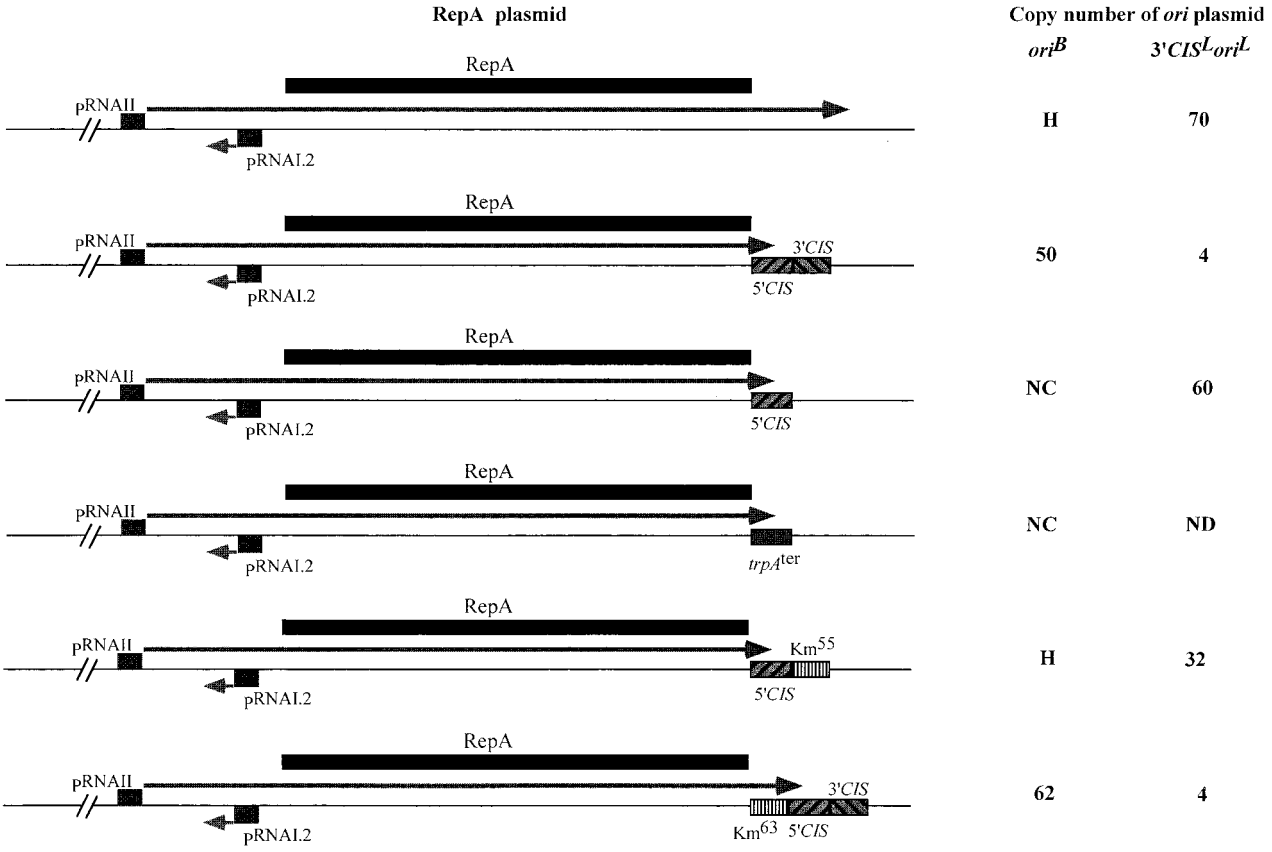

FIG. 2. Effects of deletions, insertions, and substitutions in *CIS* of the RepA-producing plasmid on the copy number of ori^B and ori^L plasmids. Copy number was determined by assaying the CAT activity of *E. coli* strain JP3438 carrying the *ori* plasmids, in the absence of IPTG. The values shown are the average of at least six independent determinations. $trpA^{ter}$, 28-bp *trpA* transcription terminator; Km^{55} and Km^{63}, 55- and 63-bp DNA fragments amplified from the coding sequence of aminoglycoside 3′-phosphotransferase; NC, no transformants detected, regardless of whether the pAM34 replicon was induced or not; ND, not done; H, copy number too high for accurate determination; ⟶, RNA transcript.

stability of the ori^B plasmid in the presence of RepA plasmid lacking CIS^B is due to its frequency of replication being too high to be sustained.

Does 3′*CIS* play a role in reducing the *trans* activity of RepA? In the intact IncB replicon, where RepA activates the *ori* present in *cis*, the 3′*CIS* acts as a spacer for appropriately positioning 5′*CIS* and *ori* with respect to each other and can be replaced by an unrelated sequence of the same length without any loss in activity (30). Since this role should be superfluous when *CIS* is carried on a RepA-producing plasmid that has no cognate *ori*, we determined whether the presence of $3'CIS^B$ had any effect on the ability of RepA to activate *ori* in *trans*. Deletion of $3'CIS^B$ from the RepA-producing plasmid resulted in the loss of the inhibitory activity seen with the intact CIS^B (Fig. 2). Moreover, replacement of 3′*CIS* by the same spacer fragment (Km^{55}) that was its fully functional substitute in *cis* (30) did not restore the inhibitory activity of *CIS*. Insertion of a 63-bp spacer (Km^{63}) between *repA* coding sequence and the intact *CIS* had no effect on the *trans* activity of RepA (Fig. 2). By contrast, this insertion resulted in a three- to sixfold reduction in the copy number of the intact IncB replicon (30). Insertion of $5'CIS^B$ or the *trpA* transcription terminator, immediately downstream of the *repA* coding sequence of the RepA-producing plasmid, appeared to result in the runaway replication of the ori^B plasmid, as no transformants could be detected even on the induction of the pAM34 replicon. Since both *trpA* terminator and $5'CIS^B$ produced the same effect, it is most likely due to prevention of read-through transcription from *repA* into the p15A replicon of the RepA plasmid. If, as seems likely, such read-through has a deleterious effect on the replication of the RepA plasmid, then obviation of this effect by efficient termination of *repA* transcripts would be expected to increase or stabilize the copy number of this plasmid and thus lead to an increase in the level of RepA available to the *ori* plasmid. Since the frequency of replication of the ori^B plasmid in the presence of RepA plasmid that lacks CIS^B was already so high as to produce tiny, segregationally unstable colonies, even a small increase in this frequency might be expected to lead to runaway replication.

Characterization of sequences involved in reducing the *trans* activity of RepA. In the IncB replicon, *CIS* (CIS^B) could be replaced by the corresponding sequences from pMU407.1 (CIS^L) and pIE545 (CIS^Z), with only slight loss of function (30). However, replacement of CIS^B in the RepA-producing plasmid, by either CIS^L or CIS^Z, resulted in almost complete loss of inhibition of the *trans* activity of RepA (Fig. 3), suggesting that this effect is a result of sequence-specific interactions between RepA and *CIS*. To investigate this interaction further, the *repA* gene on the RepA-producing plasmid was replaced by a chimeric gene, in which the promoter and promoter-proximal portion of the coding sequence came from pMU720 and the remainder of the coding sequence was from pMU407.1. The resulting protein, $RepA^{BLL}$, is composed of the N-terminal 99 amino acids of $RepA^B$ and the C-terminal 247 amino acids of $RepA^L$. $RepA^{BLL}$ resembles $RepA^L$ in that it can activate ori^L present in *cis* but fails to activate replica-

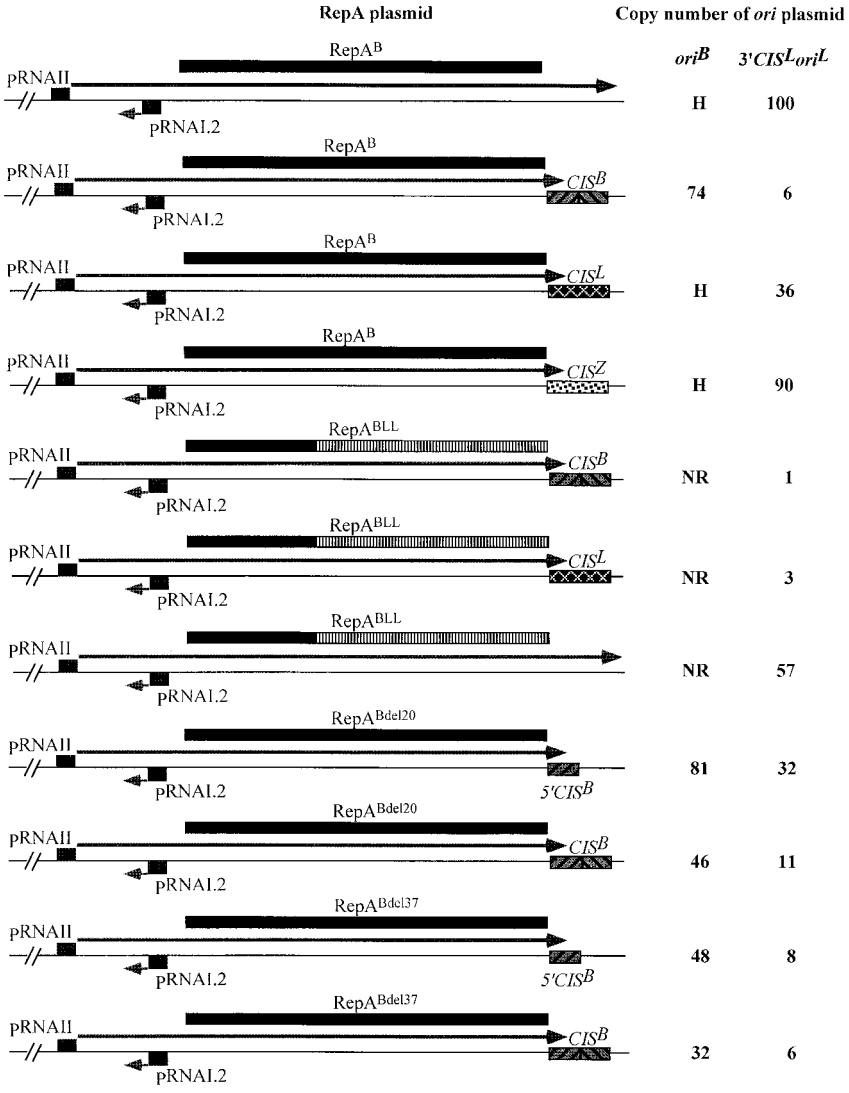

FIG. 3. Effects of heterologous and homologous RepA-*CIS* combinations and C-terminal deletions in RepA on the copy number of ori^B and ori^L plasmids. Copy number was determined by assaying the CAT activity of *E. coli* strain JP3438 carrying the *ori* plasmids, in the absence of IPTG. The values shown are the average of at least six independent determinations. NR, *ori* plasmid unable to replicate without the induction of the pAM34 replicon; H, copy number too high to be determined accurately; ⟶, RNA transcript.

tion from ori^B. The copy number of the replicon carrying the $repA^{BLL}$ gene is sevenfold lower than that of the one with $repA^L$-gene (data not shown). The activity of $RepA^{BLL}$ in *trans* was reduced 57-fold by CIS^B and 19-fold by CIS^L (Fig. 3), suggesting that this protein, unlike $RepA^B$, is able to recognize both the *CIS* sequences.

To determine whether the sequence, or part of the sequence, recognizing *CIS* lies within the C-terminal portion of RepA, deletion derivatives missing the last 20 ($RepA^{Bdel20}$) and the last 37 ($RepA^{Bdel37}$) amino acids were tested. IncB replicons carrying these mutant *repA* genes are unable to replicate (I. W. Wilson, J. Praszkier, and A. J. Pittard, unpublished data) unless expression of *repA* is increased by the introduction of the RNAI.1 mutation. However, the efficiency of replication of these *repA*/*rnaI* mutants is severely affected, as evidenced by the 11-fold ($repA^{Bdel20}$) and 59-fold ($repA^{Bdel37}$) reduction in their copy number (data not shown). Comparison of the *trans* activity of $RepA^{Bdel20}$ produced from the plasmid carrying $5'CIS^B$ with one produced from the plasmid carrying an intact CIS^B showed that the inhibitory effect of CIS^B (3-fold with $3'CIS^L$ ori^L) was much lower than that seen with the intact $RepA^B$ (17-fold with $3'CIS^L$ ori^L) (Fig. 3). Deletion of further 17 amino acids from this protein ($RepA^{Bdel37}$) resulted in almost complete loss of the inhibitory effect of CIS^B (Fig. 3; compare $RepA^{Bdel37}$ $5'CIS^B$ with $RepA^{Bdel37}$ CIS^B).

Does the presence of the *repA* gene on the *ori* plasmid increase its ability to respond to RepA produced in *trans*? It has been suggested that the mRNA for the Rep protein of the IncFIC replicon of plasmid P307, which is closely related to the replicon of pMU720, is required not only for the synthesis of the initiator protein but also to prime initiation of DNA synthesis (16). It was postulated that the *repA* mRNA is processed at the junction of the RepA coding sequence and *CIS-ori*, producing a replication primer that is used by the polymerase for initiation of leading-strand synthesis (16). However, as there was no transcription directed towards the *ori* region of the plasmids used in the assays described above, synthesis of such a primer is not essential for activation of *ori* in *trans*. To

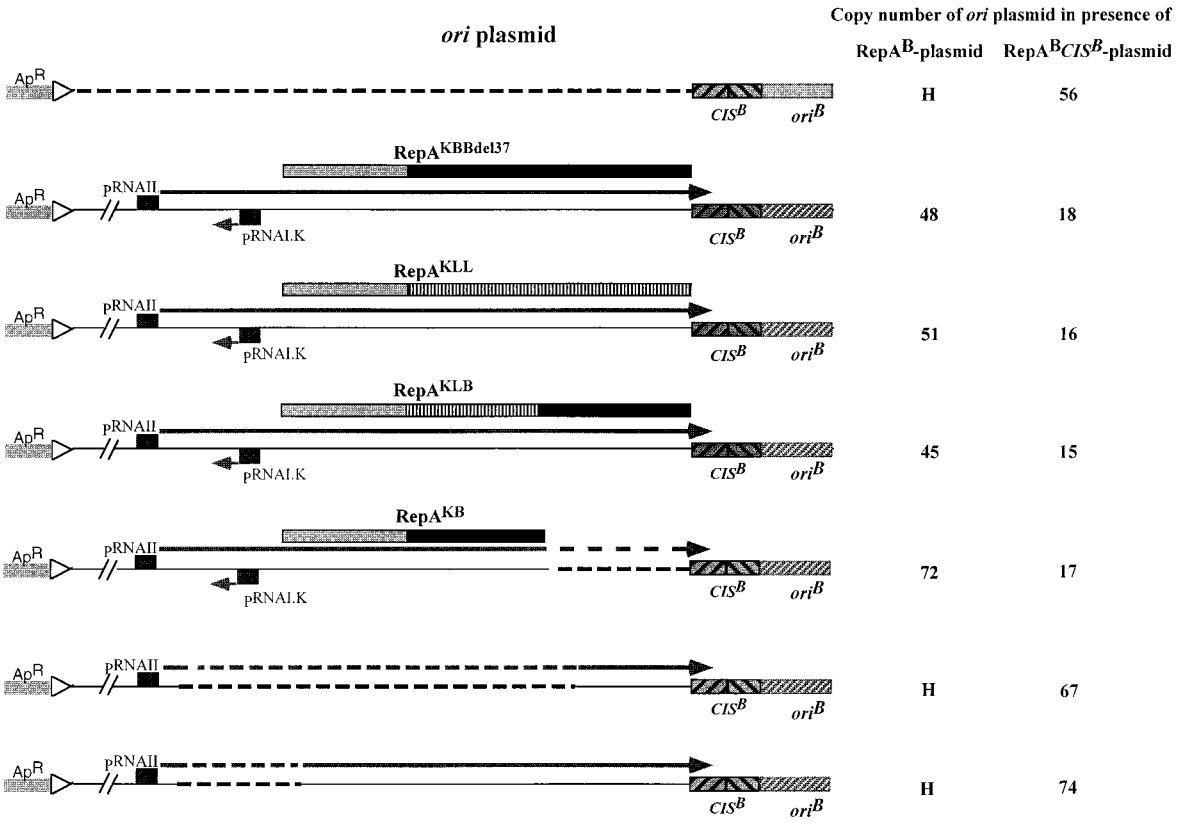

FIG. 4. Effects of deletions and substitutions in its *repA* coding sequence, and the presence of *repA* transcription and translation initiation signals, on the ability of the *ori* plasmid to be activated for replication by RepA provided in *trans*. Copy numbers were determined by assaying the CAT activity of *E. coli* strain JP3438 carrying the *ori* plasmids, in the absence of IPTG. The values shown are the average of at least six independent determinations. H, copy number too high to be determined accurately. Ap^R, β-lactamase gene; ▷, transcription terminator; ⟶, RNA transcript; – – – –, region deleted; RNAI.K, replacement of the sequence encoding the IncB RNAI and its target by the corresponding sequence from the IncK replicon.

determine whether transcription of *repA* mRNA, upstream of *ori*, enhances its ability to be activated by RepA produced in *trans*, IncB replicons encoding mutant RepA proteins unable to initiate replication from the *ori* present in *cis* were used as *ori* plasmids. To prevent RNAI (a *trans*-acting molecule) produced by these replicons from repressing the synthesis of RepA from the RepA-producing plasmid, the DNA fragment encoding their *rnaI* gene and the promoter-proximal part of their *repA* gene was replaced by the corresponding fragment from the IncK plasmid, pMU2209. $RNAI^K$ does not interact with the *repA* mRNA of the IncB plasmid pMU720 (31). Exchange of the DNA fragment carrying *rnaI* resulted also in replacement of the N-terminal 99 amino acids of $RepA^B$ by the corresponding sequence from $RepA^K$. However, these two proteins are so similar that only two amino acids are changed by this exchange (30).

All three *repA* genes used in these experiments differed in their 3′ coding sequences. The first gene had the sequence from pMU720, but its termination codon was present 112 bp upstream of the wild-type position ($repA^{Bdel37}$). The second gene had the sequence from pMU604 ($repA^{KLL}$), and the third gene contained the wild-type sequence from pMU720 ($repA^{KLB}$). Despite these differences, all three *ori* plasmids replicated with very similar efficiencies, resulting in copy numbers of 45 to 51 and 15 to 18 in the absence and presence, respectively, of *CIS* on the RepA-producing plasmid (Fig. 4). These copy numbers are significantly lower than those seen when the only pMU720 sequences present on the *ori* plasmid are *CIS* and *ori* and there is no transcription reading into *CIS* (Fig. 4, compare lines 2 to 4 with line 1). Furthermore, deletion of the coding sequence for the last 121 amino acids of RepA ($RepA^{KB}$) from the *repA* gene of the *ori* plasmid had no significant effect on its copy number. However, deletion of a sequence encompassing the translation initiation region of *repA* increased the copy number of the *ori* plasmid to levels similar to those seen with *ori* alone, regardless of whether all or only part of the coding sequence of RepA was present on the *ori* plasmid (Fig. 4). These data suggest that transcriptional activity immediately upstream of *CIS-ori* has no effect on the efficiency of replication of the *ori* plasmid, but that synthesis of replication-defective RepA from the *ori* plasmid reduces the copy number of this plasmid. However, the possibility that removal of translational activity had a polar effect on the level of transcription reading into *CIS*, which contributed to the observed increase in the copy number of the *ori* plasmids, cannot be discounted.

The finding that synthesis of replication-defective RepA from the *ori* plasmid reduces its copy number raised the possibility that these proteins are able to bind to *ori* and block initiation of replication by the replication-proficient RepA produced in *trans*. Since these proteins are produced in *cis*, they should be loaded onto *ori* more efficiently than the protein produced from the RepA plasmid. However, comparison of an *ori* plasmid carrying $repA^{KBBdel37}$ to one carrying $repA^{KBB}$ showed that their copy numbers were not significantly different from each other (data not shown). The latter plasmid is able to

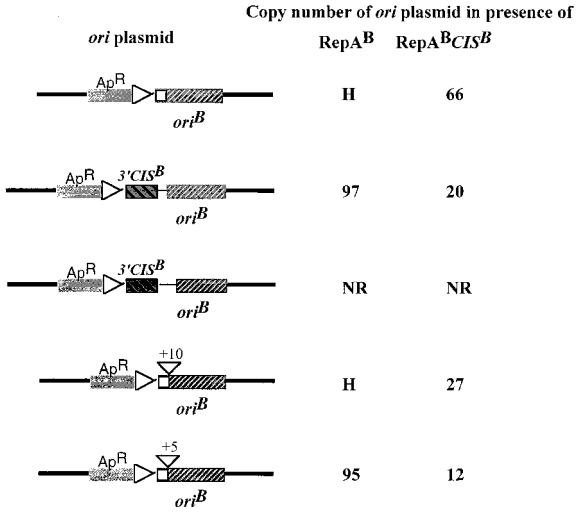

FIG. 5. Effects of mutations in and immediately downstream of the DnaA box on the ability of the *ori* plasmid to be activated for replication by RepA provided in *trans*. Copy numbers were determined by assaying the CAT activity of *E. coli* strain JP3438 carrying the *ori* plasmids, in the absence of IPTG. The values shown are the average of at least six independent determinations. NR, *ori* plasmid unable to replicate without the induction of the pAM34 replicon; H, copy number too high to be determined accurately; the thin line, region deleted; □, DnaA box; ▽, 5- or 10-bp insertion; ▷, transcription terminator.

replicate in the absence of RepA in *trans*, with a copy number of 7, which is comparable to that of the wild-type pMU720 replicon. This copy number increased ~10-fold in presence of a RepA-producing plasmid and ~4-fold in presence of one in which CIS^B is present immediately downstream of the *repA* coding sequence. A plasmid which was unable to replicate in the absence of a RepA in *trans* because its $3'CIS^B$ was deleted reached copy number not significantly different from those of the *ori* plasmids carrying $repA^{KBBdel37}$ and $repA^{KBB}$, in the presence of RepA-producing plasmids (data not shown). Deletion of the 3′*CIS* is thought to reduce the efficiency of loading of RepA onto *ori* present in *cis* (30). Thus, *ori* plasmids that produce RepA replicate with a lower copy number than ones that do not, irrespective of whether the protein is replication proficient or not. Moreover, all of these plasmids replicate with similar copy numbers, suggesting that the same mechanism is operating in all of them to lower their frequency of initiation of replication.

Effects of mutations involving the DnaA box on the copy number of the *ori* plasmid. The *ori* of pMU720 contains the sequence 5′ TTATCCACA 3′, which is a consensus sequence for a binding site of the DnaA protein (DnaA box). Although this sequence is not essential for replication of pMU720, its deletion lowers the copy number of the wild-type plasmid 3-fold and the copy number of the RNAI.1 mutant 1.6-fold (30). Deletion of the DnaA box from the *ori* plasmid reduced its copy number 3.3-fold (Fig. 5). As found with *ori* present in *cis*, deletion of additional 10 bp inactivated the *ori*. Insertion of 10 bp immediately downstream of the DnaA box reduced the copy number of the *ori* plasmid 2.4-fold, whereas insertion of 5 bp reduced it 5.5-fold (Fig. 5). These data show that the DnaA box is required for efficient activation of *ori* in *trans* and that its effectiveness is largely lost when it is moved by as little as one turn of the DNA helix. Moving the box by half a helical turn had a greater effect than moving it by a full turn, indicating that it must be positioned on the correct face of the helix relative to other sequences within *ori*.

DISCUSSION

RepA protein of IncB and $IncI_1$ plasmids is *cis* acting, preferentially activating the origin of replication on the DNA molecule from which its mRNA was transcribed (23–25). We have constructed a two-plasmid system for studying the activity of RepA, when it is present in *trans* to the *ori* on which it acts. Using this system, we show that in the absence of a cognate *ori* in *cis*, RepA does activate an *ori* present in *trans*. However, this activation requires a much higher level of *repA* expression than activation of *ori* in *cis*, confirming that some aspect of the interaction between RepA and the *ori* in *trans* is inefficient. The level of *repA* expression necessary to initiate replication from an *ori* in *trans* is unsustainable when the *ori* is present in *cis*, as IncB replicons carrying the RNAI.2 mutation cannot be recovered, presumably due to their runaway replication (data not shown).

The presence of *CIS*, in its native position and orientation, on the RepA-producing plasmid reduced the copy number of the *ori* plasmid. This effect required the presence of 3′*CIS* and involved sequence-specific interaction between *CIS* and the C terminus of RepA. Thus, it is unlike the interaction between RepA and *CIS* that is proposed to be the first step in the loading of this protein onto the *ori* in *cis* (30). These data suggest that *CIS* has an additional role, which involves the trapping and/or inactivation of the initiator protein. The current model for the control of frequency of replication initiation of IncB and other I-complex plasmids involves translational regulation of the synthesis of RepA. For this system to work satisfactorily, it is necessary for RepA to be synthesized de novo and to be modified during its first round of initiation, so that it is not available for initiation of subsequent rounds of DNA synthesis. The observed interaction between RepA and *CIS* might ensure that the initiator protein does not participate in more than one round of replication. The finding that this interaction is sequence specific solves the apparent anomaly posed by the observation that the 3′*CIS* acts as a nonspecific spacer for loading of RepA in *cis* (30) yet its sequence is very highly conserved in the I-complex plasmids (14, 31). Studies on the replication initiator protein of the IncFII plasmid NR1 also showed that the presence in *cis* of the *CIS-ori* region results in an apparent "titration" of the protein (12). Because this effect was lost when the sequence encoding 3′*CIS ori* was deleted, it was suggested that the "titration" of Rep was the consequence of its binding to *ori*. Our data show that, at least in the case of RepA of the IncB plasmid and the chimera composed of N-terminal sequence of IncB and C-terminal sequence of IncL/M proteins, the presence of *CIS* alone is sufficient to reduce the amount of RepA available for activation of *ori* in *trans*.

The only sequence required in *trans* to the RepA-producing plasmid was *ori*, supporting the notion that in the intact replicon *CIS* is involved in loading of RepA onto the *ori*, but not in the initiation of replication. The efficiency of initiation of replication was not influenced by the presence or absence of transcription into the region upstream of *ori*, indicating that such transcripts are not involved in priming of DNA synthesis. Initiation of replication of IncFII plasmids was also shown to be independent of transcription into the *ori* region (12, 27).

Synthesis of RepA protein in *cis* to *ori* reduced the copy number of the *ori* plasmid, regardless of whether the protein was competent for replication or not. The finding that all constructs tested, including one missing the 3′*CIS* and thus predicted to be impaired in the loading of RepA onto *ori* in *cis*, showed similar reduced levels of activation by RepA provided in *trans* indicates that the mechanism responsible for this phenomenon is unlikely to involve competition between *cis*- and

trans-produced RepA for binding sites in *ori*. This effect was seen even with proteins carrying C-terminal deletions, showing that its cause is different to the interaction that reduced the availability of protein from the RepA-producing plasmid. It has been suggested that loading of RepA in *cis* might involve bending of DNA to bring *ori* and the RepA positioned on 5′*CIS* close together (30). If that is the case, then it is likely that in this closed-configuration *ori* is less easily accessible to RepA produced in *trans*.

All I-complex, IncFII, and FIC plasmids examined to date encode a DnaA box at the 5′ end of *ori* (14, 26, 31, 34). Although this box is not part of minimal *ori*, its deletion or inactivation reduces the efficiency of replication of IncB and IncFII plasmids (28, 30). Deletion of the DnaA box reduced the copy number of the *ori* plasmid, showing that the efficiency of activation of *ori* in *trans* is also dependent on this sequence. Analysis of binding between RepA and *ori* of the IncB plasmid in vitro showed that the protein binds immediately downstream of the DnaA box (T. Betteridge, J. Praszkier, J. Yang, and A. J. Pittard, unpublished data). Therefore, the finding that moving the DnaA box by one turn of the helix reduced the copy number of the *ori* plasmid, and that moving the box by half a helical turn reduced it even more, suggests cooperative interaction between DnaA and RepA. Binding in vitro of DnaA protein to the DnaA box located at the 5′ end of the *ori* of the IncFII plasmid R1 required the presence of RepA, which is indicative of cooperative interaction between the two proteins (17).

The use of a repressible replicon as delivery vector for *ori* allowed us to distinguish between RepA-*ori* combinations that resulted in lack of replication of the *ori* plasmid and those that led to too high a level of replication. Thus, dependence of cotransformation of *E. coli* cells by *ori* and RepA plasmids on induction of the repressible replicon was a clear indication of failure of replication by the *ori* plasmid. On the other hand, the inability to detect cotransformants in both the presence and absence of the inducer was taken as evidence of high level of uncontrolled replication by the *ori* plasmid. This view was supported by the finding that RepA-*ori* combinations in which the *ori* plasmids replicated with high copy number formed small colonies, both in the presence and in the absence of the inducer. Replacement of the ori^B by ori^L, which has been shown to reduce the copy number of the IncB replicon (30), led to an increase in the size of the colonies and a reduction in the copy number of the *ori* plasmid. Similarly, although cotransformation of *E. coli* cells by ori^B and RepA-producing plasmids carrying 5′*CIS* failed, replacement of ori^B by ori^L or deletion of the C-terminal amino acids of RepA led to high efficiency of transformation. Analysis of these transformants showed that the *ori* plasmid was replicating with high efficiency, reaching a copy number of ~50 to 60 per chromosomal equivalent (Fig. 2 and 3). In the intact IncB replicon, upward fluctuations in copy number result in increased gene dosage of *rnaI*. The consequent increase in the concentration of the antisense RNA, RNAI, leads to inhibition of synthesis of RepA, and thus a decrease in frequency of initiation of replication. This feedback loop is missing in our two-plasmid system, because expression of *repA* from the RepA-producing plasmid is not sensitive to fluctuations in the copy number of the *ori* plasmid. Consequently, efficient activation of *ori* in *trans* may lead to runaway replication of the *ori* plasmid.

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