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Role of *CIS* in Replication of an IncB Plasmid

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Replication of the IncB plasmid pMU720 requires the synthesis of the *cis*-acting RepA protein and the presence of two DNA elements, *ori* and *CIS*. *CIS* is the 166-bp sequence separating the RepA coding sequence from *ori*. To investigate how this organization of the pMU720 replicon contributes to the mechanism of initiation of replication, mutations in the sequence and/or the length of *CIS* were introduced into the *CIS* region and their effects on the efficiency of replication of the pMU720 replicon in vivo was determined. The *CIS* region was found to be composed of two domains. The *repA*-proximal domain, which showed strong transcription termination activity, could be replaced by equivalent sequences from I-complex and IncL/M plasmids, whose replicons are organized in the same fashion as pMU720. Replacement by a *trpA* transcription terminator afforded only partial replication activity. The *repA*-distal domain was shown to be a spacer whose role was to position sequence(s) within *ori* on the correct face of the DNA helix vis-à-vis the *repA*-proximal portion of *CIS*. A model for the loading of RepA protein onto *ori* is discussed.

Miniplasmid pMU720, a derivative of a large, low-copy-number, conjugative plasmid, pMU707, belongs to incompatibility group B (8) and is closely related to the other members of the I complex ($IncI_1$, IncIγ, IncK, and IncZ plasmids), with whom it has extensive sequence homology (11, 18, 29, 34). Replication of pMU720 requires the expression of the *repA* gene, which codes for a protein (RepA) that is rate limiting for replication. Extensive studies of pMU720 and the $IncI_1$ plasmid ColIb-P9 established that the expression of the *rep* gene requires the translation and appropriate termination of a leader peptide, which facilitates the formation of an RNA pseudoknot immediately upstream of the Shine-Dalgarno sequence of *repA* (1–3, 17, 35, 45). Formation of this RNA tertiary structure activates the translation of the *repA* mRNA (2, 3, 45). Expression of *repA*, and consequently the copy numbers of pMU720 and ColIb-P9, is regulated by a small, highly structured antisense RNA molecule (RNAI) which is complementary to the leader region of the *repA* mRNA (33, 39). Binding of RNAI to its target in *repA* mRNA inhibits *repA* expression both directly, by sequestering one of the two complementary sequences involved in the formation of the pseudoknot, and indirectly, by inhibiting translation of the leader peptide (1, 4, 40, 41, 45, 46).

Initiation of DNA replication requires the presence of a replication initiator protein and of a *cis*-acting DNA sequence, the origin of replication (*ori*). The binding of the initiator protein to *ori* is the first step in DNA replication. The *ori* of the ColIb-P9 plasmid was proposed to lie within a 172-bp sequence located 152 bp downstream of the coding sequence of *repA* (42). This proposal was based on the finding that the designated sequence is essential for the replication of ColIb-P9 and contains such features common to many origins of replication as a recognition sequence for DnaA protein (DnaA box), AT-rich sequences, and repetitive sequences. The intervening region between *repA* and *ori*, denoted *CIS*, was shown to encode transcription termination signals, which were required for efficient replication of ColIb-P9 (27). Regions of *CIS* lying downstream of these signals appeared to also be required, although no function had been assigned to them (27).

The requirement for *CIS*, in addition to *repA* and *ori*, in DNA replication of ColIb-P9 and the arrangement of these genes vis-à-vis each other resembles the situation described for the IncFII plasmids R1 and R100, which are distantly related to the plasmids of the I complex. The Rep protein of these plasmids is unusual in that it acts preferentially in *cis* i.e., it preferentially activates the origin of replication of the DNA molecule from which its messenger RNA was transcribed (14, 22, 24). This property of Rep is dependent on the presence of *CIS*, in its native position and orientation, and on the transcription-terminating activity of *CIS* (22). The discovery of weak binding sites for Rep in the C terminus of the *repA* coding sequence has led to the suggestion that the newly synthesized Rep is loaded onto the DNA through these sites and then translocates on the DNA until it reaches its primary binding sites in *ori* (22). An alternative hypothesis has been put forward by Maas and Wang, on the basis of studies of the FIC replicon of plasmid P307 (21). They proposed that the *repA* mRNA is not only the messenger RNA for RepA but also a preprimer for initiation of replication and that Rep acts by facilitating the processing of this transcript into the active primer at a site overlapping the *rep* stop codon. Neither of these two conflicting models for the initiation of replication of IncFII and FIC plasmids has been verified.

The Rep protein of I-complex plasmids is also thought to act preferentially in *cis* (27, 28, 30), but little is known about the system that keeps the newly synthesized Rep tethered to its DNA template and then loads it onto *ori*. In this paper we describe mutational analyses of the *CIS* and *ori* sequences of pMU720. We find that (i) the RepA protein shows specificity for *ori* but not for *CIS*, (ii) deletion of the DnaA box reduces the efficiency of replication of pMU720, (iii) deletion of the sequence immediately downstream of the DnaA box produces a plasmid that is unable to replicate, (iv) the *repA*-proximal portion of *CIS* is required for efficient replication but can be replaced by the corresponding sequences from other I-complex plasmids or from IncL/M plasmid, (v) the *repA*-distal portion of *CIS* is a spacer which can be replaced by an unrelated sequence of correct length, (vi) the role of this spacer is to position sequence(s) in *ori* on the correct face of the DNA helix vis-à-vis the *repA*-proximal portion of *CIS*, and (vii) the

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TABLE 1. Plasmids

Plasmid	Relevant characteristics[a]	Reference or source
pMU720	Miniplasmid derived from pMU707; Gal IncB	8
pMU604	Miniplasmid derived from pMU407.1; Gal IncL/M	12
pMU605	Miniplasmid derived from R64-11; Gal $IncI_1$	12
pMU1531	Miniplasmid derived from R144-3; Gal Inc $IncI_1$	29
pMU1530	Miniplasmid derived from R621a; Gal Inc IncIγ	29
pMU2200	Miniplasmid derived from pIE545; Gal IncZ	34
pMU2209	Miniplasmid derived from R387; Gal IncK	34
pMU1597	pBR322 derivative carrying a *repA-lacZ* transcriptional fusion containing nt 1–789 of pMU720; Ap IncB	This study
pMU1598	pAM34 carrying nt 1–2170 of pMU720 with a T642C copy up mutation; Ap Cm $lacI^q$ IncB	This study
pMU1599	pAM34 carrying nt 1–2170 of pMU720; Ap Cm $lacI^q$ IncB	This study
pACYC184	p15A replicon; Tc Cm	10
pAM34	pMB1 derivative in which the preprimer RNA is expressed from *lacZpo*; $lacI^q$ Ap Sp	15

[a] Ap, ampicillin resistance; Cm, chloramphenicol resistance; Tc, tetracycline resistance; Sp, spectinomycin resistance. Mutations introduced into plasmids pMU1598 and pMU1599 are described in Results.

length of the CIS spacer can be reduced by one or increased by four helical turns without affecting the efficiency of replication. A model describing the role of *CIS* in loading the RepA protein onto *ori* is presented.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The strains of *Escherichia coli* K-12 used in this study are given below. JM101 [Δ(*lac-proAB*) *supE thi* F′(*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15*)] (23) 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}*Z*ΔM15 Tn*10* (Tet^r)] (Stratagene) was used to grow M13 derivatives which had undergone mutagenesis as described by Vandeyar et al. (43). 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 mutagenesis were M13tg130, M13tg131 (19), and M13tg130S, a derivative of M13tg130 which contains a recognition sequence for restriction endonuclease *Sac*II in its polycloning site. The plasmids used are described in Table 1.

Media, enzymes, and chemicals. The minimal medium used was half-strength buffer 56 (26) supplemented with 0.2% glucose, thiamine (10 μg/ml), and necessary growth factors. 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. Ampicillin was used at a final concentration of 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. (36). DNA was sequenced with a model 373 DNA sequencer and ABI PRISM dye terminator kits (Perkin-Elmer Corporation) or by the method of Sanger et al. (37), 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 with a kit from United States Biochemical Corp. Oligonucleotides were purchased from Bresatec Ltd. or Gibco BRL. DNA sequencing was used to screen for and confirm the presence of mutations.

Construction of plasmids for use in copy number determinations. The plasmids for copy number determinations were derived from pMU1598 (Fig. 1) and pMU1599 by replacement of the *Hin*dIII-*Sac*I and *Sac*I-*Sac*II fragments carrying wild-type *CIS* and *ori*, respectively, by appropriate fragments containing the mutations to be tested. Plasmids pMU1598 and pMU1599 contain both the IncB replicon from pMU720 (nucleotides [nt] 1 to 2170) and the replicon from pAM34 (15). The latter is a modified pMB1 replicon in which the essential preprimer RNA is transcribed from the *lacZ* promoter operator. Since pMU1598 and pMU1599 contain the $lacI^q$ gene, replication of their pAM34 replicons requires the presence of a *lac* inducer, such as IPTG. Thus, in the absence of IPTG, replication of pMU1598 and pMU1599 is dependent on the IncB replicon. As well as allowing the rescue of replication-defective IncB replicons, these plasmids permit determination of relative copy numbers by making use of a chloramphenicol acetyl transferase (CAT) reporter gene (derived from pACYC184), which is expressed constitutively. Plasmid pMU1598 differs from pMU1599 in that it carries the substitution T642C in the *rnaI* promoter, which, by reducing the expression of this gene, increases the synthesis of RepA and hence the copy number of the IncB replicon.

Measurement of CAT activity. The CAT activity of mid-log-phase cultures, grown in minimal medium containing 0.4% glucose, thiamine, leucine, threonine, ampicillin, and chloramphenicol, was assayed as described by Shaw (38). The cells were disrupted by sonication with a Braun Labsonic 2000 sonicator, and cellular debris was removed by centrifugation before the assays were carried out. 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.

Measurement of β-galactosidase activity. The β-galactosidase activity of mid-log-phase cultures was assayed as described by Miller (25). Each sample was done in duplicate, and each assay was performed at least three times.

RESULTS

Characterization of sequences downstream of *repA* that are essential for replication of the IncB plasmid. In order to facilitate the analysis of sequences lying downstream of *repA*, we

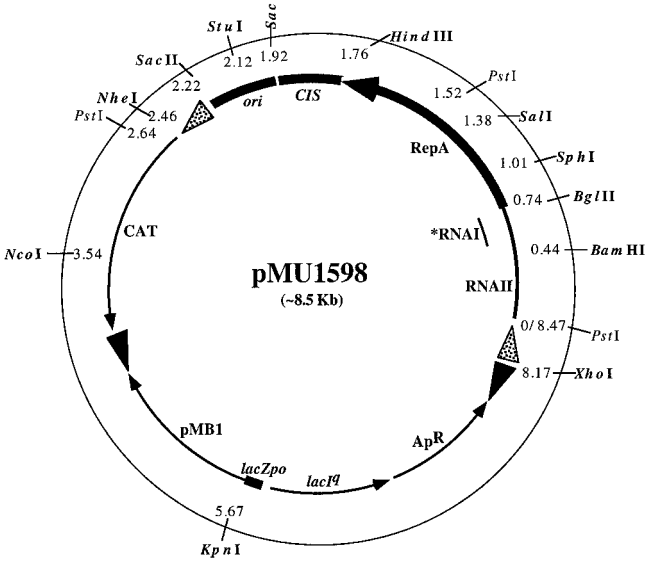

FIG. 1. Schematic representation of the dual-origin plasmid used to construct mutants carrying deletions and substitutions in the *CIS* and *ori* regions of the IncB replicon and to determine their relative copy numbers. Features important for the use of this plasmid and its derivatives are shown. RNAI, antisense RNA regulating the expression of *repA* of the pMU720 replicon; RNAII, *repA*-mRNA; CAT, gene for CAT conferring resistance to chloramphenicol; Ap^R, gene conferring resistance to ampicillin (*bla*). The triangles denote transcription terminators; ▶, T4 gene 32 terminator; ⩥, *pheR* terminator. Unique restriction endonuclease sites are shown in boldface.

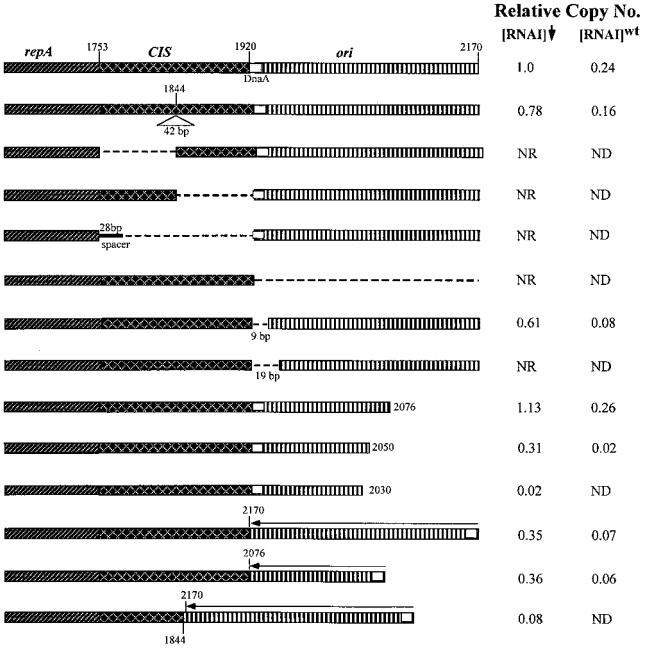

FIG. 2. Effects of deletions in *CIS* and *ori* and of reversing the polarity of *ori* on the relative copy number of the IncB replicons of pMU1598 and pMU1599. The copy number was determined by assaying the CAT activity of *E. coli* JP3438 carrying pMU1598 or its derivatives in the absence of IPTG and is expressed relative to the value obtained for pMU1598. The values shown are the averages of at least six independent determinations. The dashed lines represent deleted sequences, and the open boxes represent the DnaA boxes. The map coordinates shown correspond to those of the published sequence of pMU720 (34). ND, not done; NR, plasmid unable to initiate replication from the IncB replicon.

constructed a dual-origin plasmid, pMU1598 (Fig. 1), which contains both the IncB replicon and a fully repressible pMB1 replicon (15). The pMB1 replicon is inactive in the absence of a *lac* inducer but can be switched on by the addition of IPTG to rescue mutations which inactivate the IncB replicon. The IncB replicon of pMU1598 differs from that of pMU720 in that (i) nt 2171 to 3251 are deleted; (ii) it has a number of unique restriction endonuclease recognition sites, which have been created to facilitate the exchange of DNA fragments; and (iii) its *rnaI* gene carries a mutation (underlined) in the −10 region (RNAI.1; TATACT to TGTACT), which reduces its level of expression and consequently increases the level of expression of *repA* and the plasmid copy number (44). pMU1598 carries a gene encoding constitutively expressed CAT, which can be used as a reporter to assess the plasmid copy number. By using this reporter, it was found that the copy number of pMU1598 was not significantly different from that of pACYC184 (data not shown), which had been reported to be approximately 20 per *E. coli* chromosomal equivalent (10). Creation of the *Bgl*II site at positions 737 to 742 (which changed codons 6 and 7 of *repA*), of a *Hin*dIII site at positions 1759 to 1764 (5 nt downstream of the *repA* stop codon); and of a *Sac*I site at positions 1912 to 1917 (2 nt upstream of the DnaA box) had no significant effect on the copy number (data not shown). Replacing the mutant *rnaI* (RNAI.1) by the wild-type gene, by exchanging the *Bam*HI-*Sph*I fragment of pMU1598 for the *Bam*HI-*Sph*I fragment from pMU720, reduced the copy number of the IncB replicon ~4-fold (Fig. 2), which is in reasonable agreement with data obtained previously (44).

The DNA fragment encompassing nt 1759 to 1843 of pMU720 showed strong transcription termination activity (>98%) when inserted, in the correct orientation, between the promoter and the *lacZ* coding region of the *lacZ* fusion vector pMU1597 (data not shown). This finding is in agreement with those of Mori et al. (27), who found that a 208-bp region of the $IncI_1$ plasmid ColIb-P9, corresponding to nt 1698 to 1905 of pMU720, has strong transcription termination activity and that most of the *rep* transcripts terminated in this region, around map positions 1529 to 1543 (corresponding to positions 1796 to 1810 of pMU720).

Insertion of a 42-bp polylinker at position 1844 of the IncB replicon, which increases the distance between the *repA* coding sequence and *ori* but should not affect termination of *repA* mRNA, had only a slight effect on copy number. However, deletion of the fragment encoding the transcription terminator (nt 1758 to 1843), deletion of the *CIS* region downstream of the terminator (nt 1844 to 1911), deletion of the entire *CIS*, or deletion of *ori* (nt 1918 to 2170) inactivated the IncB replicon (Fig. 2). These data indicate that although the entire *CIS* is necessary for replication, the region downstream of the transcription terminator can be disrupted without significant loss of activity.

Deletion of the 9-bp DnaA box (nt 1920 to 1928) reduced the copy number by only 1.6-fold when *repA* expression was elevated by the RNAI.1 mutation, although it had a more profound effect (3-fold reduction) in the presence of wild-type *rnaI*. Thus, the DnaA box, while not essential, greatly increases efficiency of replication, and overproduction of RepA can partly compensate for the loss of this sequence. Deletion of an additional 10 bp (nt 1920 to 1938) inactivated the IncB replicon. Removal of 94 bp from the 3′ end of the IncB replicon had no effect on copy number. However, removal of 120 bp reduced the copy number 3- and 12-fold in the plasmids carrying the mutant and wild-type *rnaI*, respectively. Deletion of an additional 20 bp reduced the copy number of the RNAI.1 mutant 50-fold and inactivated the replicon carrying wild-type *rnaI* (Fig. 2). These data indicate that the minimal *ori* of pMU720 lies within the region encompassing nt 1929 to 2076. However, the possibility that the two ends of *ori* code for redundant functions cannot be excluded.

To determine whether the orientation of *ori* with respect to *CIS* and *repA* is important for replication of the IncB plasmid, the polarities of the DNA fragments carrying *ori* were reversed. Reversing the orientation of *ori* reduced the copy number approximately three- and fourfold in the plasmids carrying the mutant and wild-type *rnaI*, respectively (Fig. 2). Interestingly, the *ori*-proximal portion of *CIS* (*3′CIS*; nt 1844 to 1917) could be deleted from the RNAI.1 mutant carrying the inverted *ori*, although the copy number of this derivative was reduced ~13-fold. This is in contrast to the absolute requirement for the *ori*-proximal portion of *CIS* shown by the plasmid carrying *ori* in its native orientation (Fig. 2).

RepA does not have base-specific interactions with *CIS*. The RepA protein of the IncB plasmid shows ~95% sequence identity with the RepA proteins of the closely related I-complex plasmids R144-3 ($IncI_1$), R64-11 ($IncI_1$), R621a (IncIγ), and R387 (IncK) but has no significant homology with the RepA protein of the IncZ plasmid pIE545 (34). The *CIS* and *ori* sequences of these I-complex plasmids show a level of homology with each other similar to that exhibited by their RepA proteins (34).

Replacement of the *CIS ori* sequences of the IncB replicon of pMU1598 (CIS^B ori^B) by the corresponding sequences from R144-3, R64-11, R621A, or R387 had no significant effect on the copy number (data not shown). However, the *CIS ori* sequence of the IncZ plasmid could not replace the function of CIS^B ori^B (Fig. 3). This was due to the mismatch between

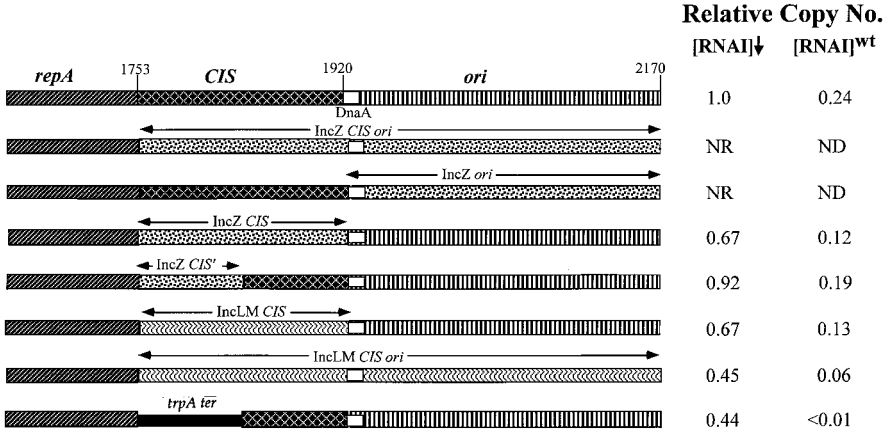

FIG. 3. Effects of substitutions of *CIS* and *ori* on the relative copy numbers of the IncB replicons of pMU1598 and pMU1599. The copy number was determined by assaying the CAT activity of *E. coli* JP3438 carrying pMU1598 or its derivatives in the absence of IPTG and is expressed relative to the value obtained for pMU1598. The values shown are the averages of at least six independent determinations. *trpA ter* is a 28-bp *trpA* transcription terminator flanked by linker sequences so that the overall length of *5′CIS* is maintained. ND, not done; NR, plasmid unable to initiate replication from the IncB replicon.

RepA and *ori*, as the IncZ *CIS* (CIS^Z) could replace CIS^B, with only a slight impairment of function (Fig. 3). Similarly, the *CIS* sequence from the IncL/M plasmid pMU407.1 (CIS^L), whose replicon has been shown to be distantly related to that of the IncB plasmid (5), could replace CIS^B, albeit with partial loss of function (Fig. 3). The sequences of CIS^Z and CIS^L show little homology either with each other or with CIS^B, suggesting that the function of *CIS* does not involve its specific recognition by RepA. Both of these *CIS* sequences, as well as those from R144-3, R64-11, R621A, and R387, showed strong transcription termination activity within their *repA*-proximal halves (*5′CIS*; data not shown). However, the function of *5′CIS* could not be performed efficiently by a *trpA* transcription terminator (Fig. 3). This was particularly evident in the plasmid carrying the wild-type *rnaI*, whose ability to replicate was so severely affected that it formed very small colonies, and only after prolonged incubation.

The RepA proteins of the IncB and IncL/M replicons show 40% sequence identity (5). The *ori* sequences of these two replicons show 65% identity within the first 83 bp but have much less homology in the remainder of the sequence. Despite these limits in sequence identity, the RepA protein of the IncB plasmid is able to activate ori^L, albeit less efficiently than its own *ori* (Fig. 3).

Role of *3′CIS*. The finding that *3′CIS* (nt 1844 to 1917) could be separated from *5′CIS* (the *repA*-proximal portion of *CIS*; nt 1759 to 1843) with little loss of replication activity and that it could be deleted from the RNAI.1 mutant, provided the polarity of the *ori* region was reversed, raised the possibility that *3′CIS* was a spacer, whose sole role was to keep sequence(s) within *ori* at the appropriate position with respect to *repA* and/or *5′CIS*. To test this possibility, the entire *CIS* (nt 1759 to 1917) and *3′CIS* were replaced by sequences of equivalent lengths, which were amplified from the coding region of the aminoglycoside 3′-phosphotransferase of Tn*903*. Replacement of *3′CIS* by the spacer fragment (spacer 1) had no significant effect on the efficiency of replication of the IncB replicon of the dual-origin plasmid, even in the presence of the wild-type *rnaI* gene (Fig. 4). Surprisingly, replacement of the entire *CIS* by a spacer fragment (spacer 2) did not inactivate the IncB replicon, although its ability to replicate was severely reduced (Fig. 4). Once again, the increase in synthesis of RepA, caused by the reduction in the expression of *rnaI*, compensated in part for the loss of *CIS*, so that the copy number was reduced only fourfold. In contrast, the copy number of the plasmid carrying wild-type *rnaI* was reduced to the point that its transformants exhibited severe slowing in growth rate, producing very small colonies even after prolonged incubation, and their CAT activity could not be measured. Insertion of spacer fragment 2 between the promoter and the *lacZ* of pMU1597 had no significant effect on *repA-lacZ* expression, confirming that this fragment does not contain a transcription terminator. These data suggest that simply separating the RepA coding region from *ori* may be sufficient to permit replication to occur when *repA* expression is elevated. However, the spacer sequence must be of sufficient length, as replacing CIS by a 28-bp linker produced a plasmid that was unable to replicate (Fig. 2).

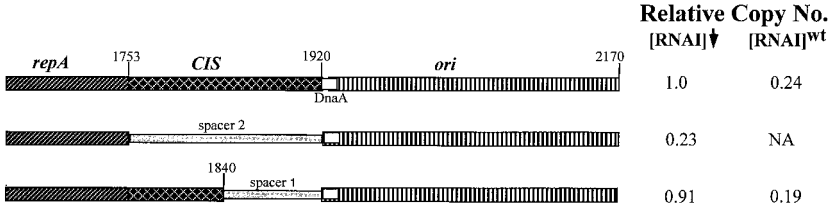

FIG. 4. Effect of replacement of the entire *CIS* and *3′CIS* by spacer fragments of equivalent length, amplified from the coding sequence of aminoglycoside 3′-phosphotransferase, on the relative copy number of the IncB replicon of pMU1598 and pMU1599. The copy number was determined by assaying the CAT activity of *E. coli* JP3438 carrying pMU1598 or its derivatives in the absence of IPTG and is expressed relative to the value obtained for pMU1598. The values shown are the averages of at least six independent determinations. NA, not able to be assayed.

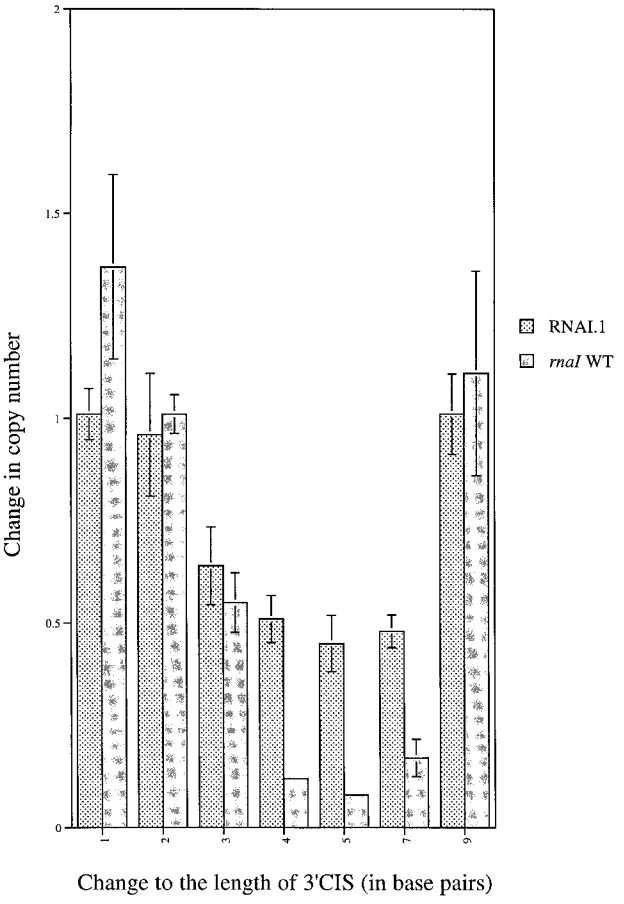

FIG. 5. Effect of increasing the length of *3′CIS*, by adding 1 to 9 nt at map position 1912, on the copy number of the IncB replicon of pMU1598 and pMU1599. The copy number was determined by assaying the CAT activity of *E. coli* JP3438 carrying pMU1598, pMU1599, or their derivatives in the absence of IPTG and is expressed relative to the value obtained for the parental plasmid (i.e., either pMU1598 or pMU1599, as appropriate). The values shown are the averages (± standard deviations) of at least six independent determinations. WT, wild type.

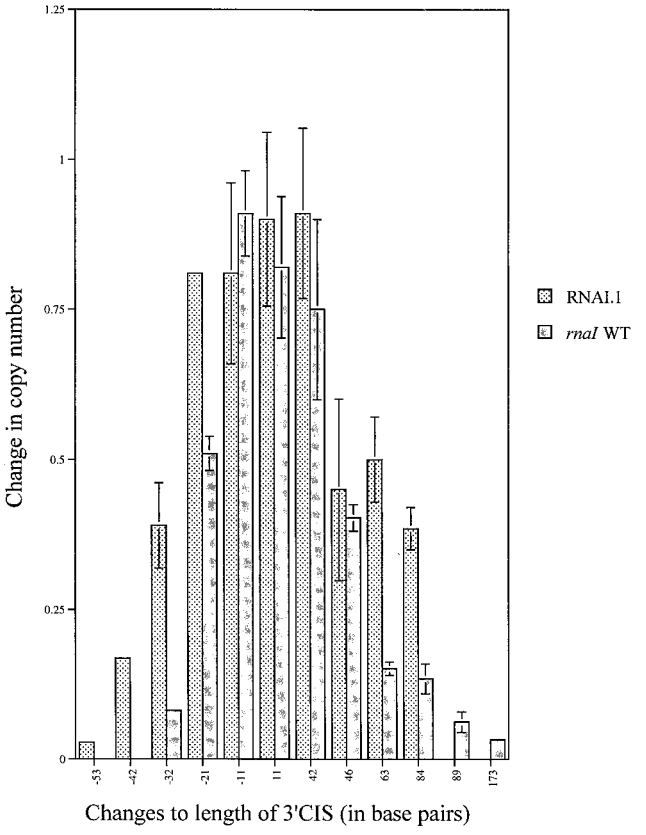

FIG. 6. Effect of increasing or decreasing the length of *3′CIS* by full turns of the helix (i.e., ~10.5 bp) on the copy number of the IncB replicon of pMU1598 and pMU1599. The length of *3′CIS* was altered by replacing nt 1844 to 1911 by spacers of appropriate length, except for the +42 mutant, which was constructed by insertion of a 42-bp polylinker at map position 1844. The copy number was determined by assaying the CAT activity of *E. coli* JP3438 carrying pMU1598, pMU1599, or their derivatives in the absence of IPTG and is expressed relative to the value obtained for the parental plasmid (i.e., either pMU1598 or pMU1599, as appropriate). The values shown are the averages (± standard deviations) of at least six independent determinations. WT, wild type.

Effect of moving *ori* sequences along the face of the DNA helix. To determine whether it was important for sequences within *ori* to be at a particular position on the face of the DNA helix, the length of *3′CIS* was increased by 1 to 9 bp, in small increments, and the effects of these changes on the copy number of the IncB plasmid were measured. Because data (presented in this paper) indicated that increased expression of *repA* can compensate for decreased efficiency of RepA-*ori* interactions, these experiments were carried out on both pMU1598 and its derivative carrying the wild-type *rnaI* gene. The changes in the copy number, relative to those of the two parental plasmids, are presented in Fig. 5. These data show that increasing the length of *3′CIS* by 1 bp resulted in a slight but significant increase in the copy number of the plasmid carrying the wild-type *rnaI* gene but had no effect on pMU1598. Increasing the length of *3′CIS* by 2 or 9 bp had no significant effect, but adding 3, 4, 5, or 7 bp reduced the copy number, with the plasmid carrying the wild-type *rnaI* gene being much more severely affected than pMU1598. The greatest reduction in copy number was seen with plasmids whose *3′CIS* was increased by 4 to 7 bp, which moved sequences within *ori* approximately half a helical turn relative to sequences within *repA* and *5′CIS*.

What are the limits to the length of *3′CIS* for efficient plasmid replication? To determine the permissible limits for the spacing between the RepA coding sequence or *5′CIS* and *ori*, the effects on the copy number of decreasing and increasing the length of *3′CIS* by multiples of DNA helical turns (10.5 bp) were measured. As seen from the data in Fig. 6, shortening *3′CIS* by 32 bp, which meant that it was only 44 bp long, reduced the copy number of the plasmid carrying wild-type *rnaI* ~12-fold, and *CIS* could not be shortened further without the total loss of the ability to replicate. The plasmid carrying the RNAI.1 mutation could still replicate when *3′CIS* was shortened by 42 and 53 bp, but its copy number was reduced ~6- and 30-fold, respectively. Shortening *3′CIS* by 21 bp reduced the copy number of the plasmid carrying wild-type *rnaI* twofold, but deleting 11 bp or inserting 11 or 42 bp had little effect. Insertions of 63 bp or more caused progressive decreases in the copy number. These data show that efficient replication of the IncB replicon requires that *ori* be separated from *5′CIS* by no less than 55 and no more than 118 bp.

To determine whether the requirement for preserving the helical phasing of *3′CIS* still held when its length was increased by 42 and 84 bp, an additional 4 bp was inserted into these sequences. Introduction of these 4-bp insertions reduced the copy number ~2-fold, showing that moving the *ori* sequences

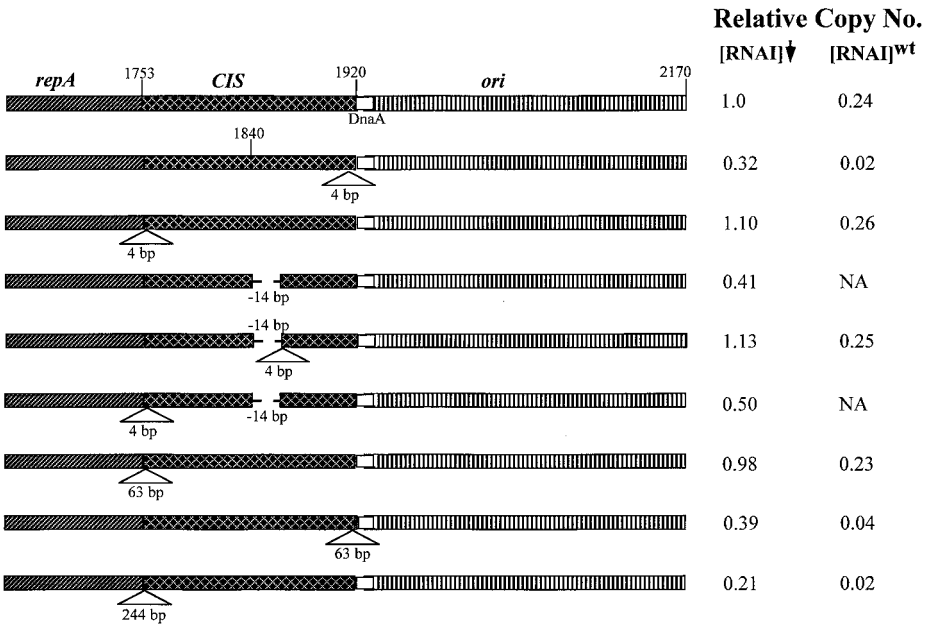

FIG. 7. Effect of deleting and inserting sequences immediately downstream of the *repA* stop codon, at the boundary between *5′CIS* and *3′CIS*, or at the end of *3′CIS* on the relative copy numbers of the IncB replicons of pMU1598 and pMU1599. The copy number was determined by assaying the CAT activity of *E. coli* JP3438 carrying pMU1598 or its derivatives in the absence of IPTG and is expressed relative to the value obtained for pMU1598. The values shown are the averages of at least six independent determinations. NA, not able to be assayed.

approximately half a helical turn relative to sequences within *repA* and *5′CIS* decreases the efficiency of replication even when the spacing between *ori* and *5′CIS* has been doubled.

Spacing between *5′CIS* and *ori* is critical for efficient replication. To determine whether the effects observed when the length of *3′CIS* was altered were due to changes in the spacing between *ori* and *5′CIS* or between *ori* and the end of the RepA coding sequence, insertions and deletions were introduced in *3′CIS* or immediately downstream of the *repA* stop codon. The effects of these mutations on the copy numbers of plasmids expressing wild-type or reduced levels of RNAI were determined, and they are presented in Fig. 7. The data show that insertion of 4 bp immediately downstream of the *repA* stop codon had no effect on the copy number, which is in stark contrast to the severe reduction in copy number seen when the 4 bp is inserted into *3′CIS*. Similarly, the reduction in copy number caused by deleting 14 bp from *3′CIS* was completely reversed by the introduction of 4 bp into *3′CIS* but not by the introduction of 4 bp between the RepA coding sequence and *5′CIS*. These data show that it is the spacing between *ori* and *5′CIS* that is crucial for efficient replication and that the helical phasing of *3′CIS* must be preserved. The introduction of 63 bp immediately downstream of the *repA* stop codon had no effect on the copy number, but insertion of 244 bp decreased the copy number ~2.6- and 6-fold in the plasmid carrying the mutant and wild type *rnaI*, respectively. Thus, the spacing between the RepA coding sequence and *CIS* and/or between the RepA coding sequence and *ori* is also important, as only relatively short insertions (longer than 63 but shorter than 244 bp) are tolerated.

DISCUSSION

We have constructed an IncB plasmid carrying a ColE1-type replicon that is inactive in the absence of a *lac* inducer but whose induction permits the rescue of replication-defective mutants of the IncB replicon. This plasmid also carries a constitutively expressed CAT gene, so that its CAT activity is directly proportional to its copy number. This dual-origin plasmid was used to construct mutants carrying deletions, insertions, and substitutions within the *CIS* and *ori* regions of the IncB replicon and to accurately measure the effects of these mutations on the efficiency of replication of this replicon.

The minimal origin of replication of pMU720 was mapped to a sequence lying immediately downstream of the DnaA box. This sequence has base-specific interactions with RepA, as indicated by the finding that the RepA protein of pMU720 showed a preference for its own *ori*. The RepA protein of pMU720 was able to activate the *ori* of an IncL/M plasmid, pMU407.1, but not that of an IncZ plasmid. Examination of these two *ori* sequences revealed that the first contained a motif (5′A/TANCNGCAAA/T3′) which is also present in the *ori* of pMU720. This motif, which was not found in the *ori* of the IncZ plasmid, was repeated four and two times in the *ori* of the IncB and IncL/M plasmids, respectively. It is thus tempting to speculate that this motif represents the binding site of RepA. In both pMU720 and pMU407.1 a copy of this motif is present 2 bp downstream of the DnaA box, and preliminary data indicate that in vitro the RepA protein of pMU720 binds immediately downstream of this box (7). Although the DnaA box is not essential, its deletion reduced the copy number of the IncB replicon threefold. Increased expression of RepA compensated in part for the loss of the DnaA box. The minimal *ori* of IncFII plasmids also lies immediately downstream of a DnaA box (31). Moreover, an R1 plasmid whose DnaA box has been inactivated by mutations so that it no longer binds the DnaA protein in vitro replicates very inefficiently unless its RepA protein is overexpressed (32). Similarly, the *E. coli* chromosome of a *dnaA* null mutant, whose replication was placed under the direction of an integrated R1 plasmid, was underreplicated unless the synthesis of RepA was increased (6).

The *CIS* of pMU720 can be separated into two domains,

which have different functions and can be moved apart from each other. The 3′ domain is a spacer whose sole function is to place a sequence(s) within *ori* at an appropriate distance and helical phasing, relative to sequences in *5′CIS*. *5′CIS* possesses strong transcription termination activity, but it is unlikely that terminating the *repA* mRNA is its only function. Replacing *5′CIS* by a fragment of equal length, carrying the *trpA* transcription terminator, led to a severe reduction in replication activity. Since transcription termination in this mutant is predicted to occur at the same point, relative to the *repA* stop codon and *ori*, as the predominant termination site mapped in ColIb-P9 (27), it is unlikely that the replication defect of this mutant is due to an inappropriate site of termination. However, it is possible that the system for the loading of RepA onto *ori* requires that termination of transcription of *repA* be Rho dependent, as has been suggested for plasmid R1 (22). The proposed reason for this requirement was that Rho-dependent termination is likely to take longer to complete, allowing the ribosome translating *repA* mRNA time to reach the stop codon before this transcript is released from the template (22). Transcription termination in ColIb-P9 was shown to be only partly Rho dependent (27), and it is unclear whether this dependence was important for efficient replication, because the ColIb-P9 derivative used in the study was a mutant whose copy number was elevated ninefold due to increased expression of *repA* (4). In pMU720, the replication activity of the mutant carrying the *trpA* terminator could be largely restored by increasing the synthesis of RepA, suggesting that under these conditions the native system for loading RepA onto *ori* may be bypassed. However, it should be pointed out that replication initiation in the mutants expressing *repA* at elevated levels requires the presence of an intervening sequence between *ori* and the *repA* coding sequence, indicating that their RepA proteins are still acting in *cis*.

The *5′CIS* sequences of the IncZ and IncL/M plasmids were found to be adequate replacements for the *5′CIS* of the IncB plasmid, despite the lack of any discernible homology between them, indicating that they contain functional features lacking in the *trpA* terminator. The IncZ replicon is a naturally occurring chimera in which the sequences upstream of the *repA* coding sequence are highly homologous to those of the IncB and other I-complex plasmids, whereas the *repA* coding sequence, *CIS*, and *ori* are highly homologous with those of the IncFII plasmids R1 and R100 (34). The RepA protein of the IncL/M replicon shows 40% identity to RepA of pMU720 (5), the *ori* sequences of these two plasmids show strong homology at their 5′ ends, and the RepA of pMU720 is able to initiate replication at the *ori* of the IncL/M replicon. Thus, it is reasonable to assume that the IncL/M, IncFII, and I-complex plasmids use very similar mechanisms to load their Rep proteins onto *ori*.

A recent model postulates that replication of the IncFIC replicon of P307 initiates at the stop codon of *repA* and involves the processing of the *repA* mRNA at that site to produce an RNA primer for leading-strand synthesis (21). The IncFIC replicon of P307 is a naturally occurring chimera which is the mirror image of the IncZ replicon in that its sequences lying upstream of the *repA* coding sequence are highly homologous to those of the IncFII plasmids R1 and R100, whereas the *repA* coding sequence, *CIS*, and *ori* are highly homologous with those of the IncB plasmid (20, 21, 34). Given these similarities between their sequences, it is reasonable to assume that the IncFIC replicons of P307 and pMU720 use the same strategy for initiation of replication. However, it is difficult to envisage how our data, showing the necessity for maintaining correct distance and native helical phasing between *5′CIS* and *ori*, can be reconciled with the model put forward by Maas and Wang (21).

There are two lines of evidence suggesting that the mechanism for loading Rep onto *ori* is unlikely to involve loading onto secondary binding sites in the C terminus of the *rep* coding sequence followed by translocation to *ori*. Firstly, the requirement for preserving the helical phasing between *5′CIS* and *ori* suggests that the newly synthesized RepA finds its initial target within *5′CIS*. Secondly, the secondary binding sites detected in R1 are not conserved in the IncZ replicon, despite the strong homology between the RepA proteins and the *CIS* and the *ori* sequences of these two plasmids. If the secondary binding sites were an integral part of the replication machinery of these plasmids, there would have been a strong selective pressure for their retention. It is noteworthy that the sequence lying immediately downstream of the DnaA box, which is thought to be the primary binding site for RepA (16), has been conserved.

The requirement that the spacing between the *repA* coding sequence and *5′CIS* be kept relatively short is consistent with the notion that the nascent protein has to interact with nucleotide sequences or with the RNA polymerase transcribing its messenger RNA in order to be efficiently loaded onto *ori*. If RepA is recognizing a specific nucleotide motif, then this motif must be present in the *5′CIS* of all the plasmids tested in this work. Although some potential motifs can be found in *5′CIS*, they are relatively short and not well conserved, and most importantly, the helical phasing between these sequences and *ori* is not always maintained. For these reasons we favor the notion that the first step in the loading of RepA onto *ori* involves an interaction between the nascent RepA and the RNA polymerase transcribing its messenger RNA. RepA is then translocated onto *ori* by a process which does not involve passive sliding along the DNA until its primary binding site in *ori* is encountered but may involve bending of the DNA. Although at present there is no information regarding DNA bending near the *ori* of I-complex plasmids, static DNA bending was found to occur within the *CIS* of the IncFII plasmid R100 (13). This model for the loading of RepA onto *ori* accommodates the data presented in this paper and explains the importance of transcription terminating at the appropriate position in *5′CIS*, as well as the *cis* action of RepA.

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