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Molecular Analysis of RNAI Control of *repB* Translation in IncB Plasmids

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The translation of RepA, the replication initiation protein of the IncB plasmid pMU720, requires that its mRNA (RNAII) folds to form a pseudoknot immediately upstream of the *repA* Shine-Dalgarno sequence. The formation of this pseudoknot is dependent in turn on the translation and correct termination of a leader peptide, RepB. A small countertranscript RNA, RNAI, controls the replication of pMU720 by interacting with RNAII to negatively regulate the expression of *repA* both directly, by sequestering the proximal bases required for pseudoknot formation, and indirectly, by inhibiting the translation of *repB*. Inhibition of the translation of *repB* by RNAI was found to depend on the close proximity of the RNAI-RNAII complex to the translational initiation region of *repB*, indicating that the primary mechanism of RNAI control involves steric hindrance. Disruption of RNAI control of *repB* had only a small effect on the copy number of the IncB plasmid, indicating that inhibition of the expression of *repA* by RNAI is achieved predominantly by inhibition of pseudoknot formation rather than by inhibition of *repB* translation.

The IncB miniplasmid pMU720 is a low-copy-number plasmid with approximately two to four copies per cell. The basic replicon consists of a 3.25-kb DNA fragment which contains the genetic information required for autonomous replication and copy number control (Fig. 1). The replication frequency of pMU720 is dependent on the *repA* gene, the product of which is thought to be necessary for replication initiation (18, 19). Expression of *repA* is negatively regulated at the posttranscriptional level by a small countertranscript RNA, RNAI, which is transcribed from the opposite strand to the RepA mRNA (RNAII) and is therefore complementary to it. Mutational and computer analyses of the folding of RNAII indicate that the translational initiation region (TIR) of *repA* is sequestered within a secondary structure, designated stem-loop III (SLIII), which prevents de novo translation by sterically inhibiting ribosome loading (20, 29). It has been established for both the IncB plasmid pMU720 (20, 29) and its close relative, the $IncI_1$ plasmid ColIb-P9 (1, 2), that for *rep* to be expressed, SLIII must be disrupted by the prior translation and correct termination of a small leader peptide, enabling the generation of a pseudoknot structure. As first demonstrated with ColIb-P9, the formation of the pseudoknot is essential for the translation of *rep* and involves pairing between complementary sequences in the Rep mRNA. The proximal pseudoknot sequence lies in the loop of stem-loop I (SLI), a large structure which is complementary to RNAI, and the distal pseudoknot sequence lies adjacent to the Shine-Dalgarno (SD) sequence of *repA* (Fig. 1). The pseudoknot, which must be in close proximity to the *repA* SD sequence, has been shown to act as an enhancer of the translation of *repA*. Its mode of action is unknown but may involve a direct pseudoknot-ribosome interaction (20, 29).

RNAI negatively regulates the translation of *repA*, primarily by pairing with SLI to form a structure which sequesters the proximal bases required for the formation of the pseudoknot. This notion is supported by the finding that the initial site of RNAI-RNAII interaction in pMU720 involves three of the four proximal bases critical for pseudoknot formation (24). Apart from its primary role in the inhibition of pseudoknot formation, pairing between RNAI and RNAII also inhibits the translation of the leader peptide RepB. The mechanism by which RNAI regulates *repB* is unknown. However, since RNAI transcription was shown to initiate at a base adjacent to the putative *repB* SD sequence (18, 24), it was postulated that the binding of the 5′ single-stranded tail of RNAI to its complementary region in RNAII would produce an RNA duplex adjacent to the *repB* SD sequence and that this structure would sterically inhibit ribosome binding (20, 25). Recently, it was demonstrated that an RNAI lacking the 5′ tail is capable of efficient regulation of Rep expression in pMU720 (25) and in the FII plasmid R1 (28). However, the effect of this RNAI species on the expression of the leader peptide had not been determined.

In this paper, we investigate the regulation of the expression of the leader peptide RepB by RNAI and determine the effect of disruption of this control on IncB plasmid replication. We show that the inhibition of *repB* translation by RNAI depends on the close proximity of the RNAI-RNAII complex to the TIR of *repB*, indicating that the primary mechanism of this control involves steric hindrance. We demonstrate that although inhibition of the translation of *repB* relies on the 5′ tail of RNAI, a tailless RNAI species is able to exert significant control on the expression of *repB*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The strains of *Escherichia coli* K-12 used in this study are as follows. Strain JM101 [Δ(*lac-proAB*) *supE thi* F′ (*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*)] (15) was used for cloning and propagating M13 derivatives. Strain SDM [*hsdR17 mcrAB recA1 supE44* Tet^r Δ(*lac-proAB*) F′ (*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*)] was used to grow M13 derivatives which had undergone mutagenesis as described by Vandeyar et al. (27). Strain JP3923 (*thr-1 leuB6 thi-1 lacZ*Δ *M15 lacY1 gal-351 supE44 tonA21 hsdR4 gyrA379 rpsL743 recA56 srl-1300*::Tn*10 aroL513*) was used for all β-galactosidase assays with translational and transcriptional *lacZ* fusions. Strain JM8042 (Δ*lacU169 tyrR366 recA56*) was used for all

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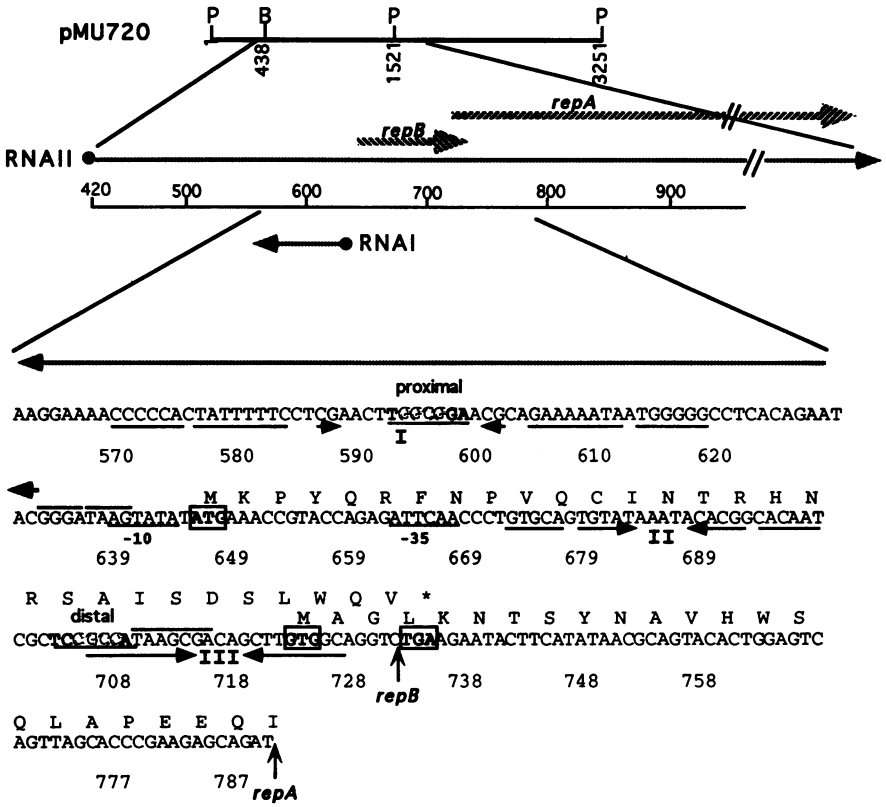

FIG. 1. Replication control region of pMU720 (18). RNAI and RNAII transcripts and the coding regions of the two *rep* genes (hatched arrows) are indicated. The nucleotide sequence between base positions 561 and 789, together with the amino acid sequence of *repB* and the amino-terminal end of *repA*, is shown. The initiation and termination codons are boxed, and the putative SD sequences of the two genes are overlined. The promoter region of RNAI and the putative SLI, SLII, and SLIII structures are indicated. The complementary proximal and distal pseudoknot sequences are underlined and shown in boldface type with the bases indispensable for pseudoknot formation in outline type. The vertical arrow indicates the 3′ end of the pMU720 fragment inserted into the fusion vectors. P, *Pst*I; B, *Bam*HI.

β-galactosidase assays to determine relative plasmid copy numbers (33).

Bacteriophage vectors used to clone fragments for DNA sequencing and mutagenesis were M13tg130 and M13tg131 (12). The plasmids used are described in Table 1.

Media, enzymes, and chemicals. The minimal medium used was half-strength buffer 56 (17) supplemented with 0.2% glucose, thiamine (10 μg/ml), and the necessary growth factors. Enzymes and chemicals of a suitable grade were purchased commercially and not purified further. [α-^{35}S]dATPαS (1,000 to 15,000 Ci/mmol) for use in sequencing was obtained from NEN Research Products. Ampicillin was used at a final concentration of 50 μg/ml, trimethoprim 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 DNAs were isolated and manipulated as described by Sambrook et al. (22). The method used for DNA sequencing was as described by Sanger et al. (23), except that T7 DNA polymerase was used instead of the Klenow fragment and terminated chains were uniformly labelled with [α-^{35}S]dATPαS.

Site-directed mutagenesis. In vitro mutagenesis was performed with the commercially available United States Biochemical Corp. kit. Oligonucleotides used for generating insertions or deletions contained at least eight bases of complementarity flanking the site of the insertion or deletion, and only five nucleotides were inserted for any single mutagenesis reaction. Oligonucleotides were synthesized by using the Gene Assembler Plus (Pharmacia LKB Biotechnology). DNA sequencing was used to screen for and confirm the presence of mutations.

Construction of the *lacZ* fusion plasmids. The construction of these plasmids has been described previously (20). The plasmid pMU1550 is pMU525 (18) carrying nucleotides (nt) 1 to 789 of pMU720, which fuses codon 23 of *repA* with codon 8 of *lacZ*. pMU1578 is pMU525 carrying nt 1 to 730 of pMU720, fusing codon 29 of *repB* with codon 8 of *lacZ*. β-Galactosidase expression in pMU1550 and pMU1578 is therefore dependent on transcription from the RNAII promoter and translational initiation from *repA* and *repB*, respectively. The transcriptional *lacZ* fusion vector pMU1590 was constructed from pMU2385 (20) by the insertion of nt 1 to 730 of pMU720, so that β-galactosidase expression is dependent on transcription from the RNAII promoter and translational initiation from *galK*.

pBR322 derivatives. The construction of pMU617 and pMU662 has been described previously (18). pMU617, which is pBR322 carrying nt 438 to 718 of pMU720 (Fig. 1), expresses RNAI (but not RNAII) from its own promoter and is used to deliver extra copies of RNAI. pMU662 is pBR322 carrying the first 637 nt of pMU720 (Fig. 1) and therefore expresses the leader region of RNAII including SLI, which is the target for RNAI, but does not express RNAI. This plasmid is used to titrate out RNAI molecules synthesized by other plasmids. The

TABLE 1. Plasmids

Plasmid	Relevant characteristics[a]	Source or reference
pBR322	pMB1 derivative; Ap Tc	26
pAM34	pBR322 derivative in which the preprimer RNA is expressed from *lacZpo*; *lacI*^{q} Ap Sp	9
pMU720	Miniplasmid; Gal IncB	3
pMU617	pBR322 carrying nt 438 to 718 of pMU720; Ap IncB	18
pMU662	pBR322 carrying nt 1 to 637 of pMU720	18
pMU525	Low-copy-number translational fusion vector; *lac'ZYA'* Tp IncW	18
pMU575	Low-copy-number transcriptional fusion vector; *galK'-lac'ZYA* Tp IncW	34
pMU2385	Low-copy-number transcriptional fusion vector derived from pMU575; *galK'-lac'Z* Tp IncW	20
pMU1550	*repA-lacZ* translational fusion carrying nt 1 to 789 of pMU720; Tp IncW IncB	20
pMU1578	*repB-lacZ* translational fusion carrying nt 1 to 730 of pMU720; Tp IncW IncB	20
pMU1590	*repB-lacZ* transcriptional fusion carrying nt 1 to 730 of pMU720; Tp IncW IncB	This study
pMU2371	*repA-lacZ* translational fusion carrying nt 1 to 789 of pMU720 with RNAI.3 mutation; Tp IncW IncB	This study
pMU3435	*repA-lacZ* transcriptional fusion carrying nt 1 to 789 of pMU720 with RNAI.3 and RepBS mutations; Tp IncW IncB	This study
pMU4525	pBR322 carrying nt 553 to 789 of pMU720 with the RNAI-9 mutation; Ap IncB	This study
pMU4526	pBR322 carrying nt 553 to 789 of pMU720 with the RNAI-7 mutation; Ap IncB	This study
pMU4527	pBR322 carrying nt 553 to 789 of pMU720 with the RNAI-2 mutation; Ap IncB	This study
pMU4528	pBR322 carrying nt 553 to 789 of pMU720 with the RepBS mutation; Ap IncB	This study
pMU4529	pBR322 carrying nt 553 to 789 of pMU720 with the RNAI+5 mutation; Ap IncB	This study
pMU4530	pBR322 carrying nt 553 to 789 of pMU720 with the RNAI+9 mutation; Ap IncB	This study
pMU4531	pBR322 carrying nt 553 to 789 of pMU720 with the RNAI+15 mutation; Ap IncB	This study
pMU4532	pBR322 carrying nt 553 to 789 of pMU720 with the RNAI+20 mutation; Ap IncB	This study
pMU4406	pAM34 with the nt 1-3251 of pMU720 plus flanking sequences from the Gal operon and *tyrP'-lacZ*; *lacI*^{q} Ap IncB	This study
pMU4533	pMU4365 with nt 553 to 789 of pMU720; *lacI*^{q} Ap IncB	This study
pMU4534	pMU4365 with nt 553 to 789 of pMU4528 insert; *lacI*^{q} Ap IncB	This study
pMU4535	pMU4533 with nt 1 to 789 of pMU720 from pMU2371 insert; *lacI*^{q} Ap IncB	This study
pMU4536	pMU4533 with nt 1 to 789 of pMU720 from pMU3435 insert; *lacI*^{q} Ap IncB	This study

[a] Abbreviations: Ap, ampicillin resistance; Sp, spectinomycin resistance; Tc, tetracycline resistance; Tp, trimethoprim resistance; Gal, ability to promote fermentation of galactose. Mutations introduced into the *repA-lacZ* and *repB-lacZ* fusion plasmids and pMU4535 derivatives are described in Results.

fragments containing the RNAI genes used to construct the pBR322 derivatives pMU4525 to pMU4532 were created by using PCR. The forward primer used in these reactions corresponded to nt 553 to 571 of pMU720 and contained an *Eco*RI linker at its 5′ end. The reverse primer corresponded to nt 789 to 773 of pMU720 and contained a *Bgl*II linker at the 5′ end. The fragments generated by PCR were cloned into M13 vectors, and their sequences were checked for the presence of misincorporated nucleotides. Clones carrying error-free inserts were used as the source of DNA fragments for the construction of the pBR322 derivatives. The derivatives were made by inserting *Eco*RI-*Bgl*II fragments into *Eco*RI- and *Bam*HI-cleaved pBR322. None of the pBR322 derivatives carries *lacZ*.

Construction of plasmids for use in copy number determinations. The chimeric plasmid pMU4535 contains both the IncB replicon and the replicon from pAM34 (Fig. 2). The latter is a modified pMB1 replicon in which the essential preprimer RNA is transcribed from the *lacZ* promoter operator. Since pMU4535 also contains the *lacI*^{q} gene, replication of the pAM34 replicon is dependent on the presence of IPTG. Thus, in the absence of IPTG, replication of pMU4535 is reliant on the IncB replicon. As well as allowing the rescue of mutations deficient in IncB plasmid replication, this plasmid permits determination of relative copy numbers by making use of a *lacZ* reporter gene which in a *tyrR* strain (JP8042) is expressed constitutively from the *tyrP* promoter. The pMU4535 derivatives were created so that the genes for RNAI and RNAII no longer overlap, which allows RNAI and RNAII to be manipulated and analyzed independently of one another. pMU4535 derivatives were obtained in two steps from pMU4365 (Fig. 2). Fragments containing the different "active" RNAI genes which control the replication of the plasmid were created by PCR. The forward primer used corresponded to nt 553 to 571 of pMU720 and contained a *Xba*I linker at its 5′ end. The reverse primer corresponded to nt 789 to 773 of pMU720 and also contained an *Xba*I linker at the 5′ end. The fragments generated by PCR were cloned into M13 vectors, and their sequences were checked. Clones carrying error-free inserts were used for cloning into pMU4365. The *Xba*I fragments containing the RNAI gene were inserted into *Nhe*I-cleaved pMU4365, and the orientation was checked by sequencing; only clones containing the RNAI gene transcribing in the same direction as RNAII were used for further clonings. The pMU4535 derivatives were then obtained by exchanging the 789-bp *Eco*RI-*Bgl*II fragment containing an "inactive" RNAI gene from pMU1550 derivatives with the wild-type *Eco*RI-*Bgl*II region from pMU4533.

Measurement of β-galactosidase activity. β-Galactosidase activity of mid-log-phase cultures was assayed as described by Miller (16). Each sample was done in duplicate, and each assay was performed at least three times.

Prediction of RNA secondary structures. The computer programs of Zuker and his colleagues (10, 11, 35) were used to predict RNA secondary structures.

RESULTS

Mapping of the *repB* SD sequence. Since the hypothesis that we wish to test is that binding of RNAI to RNAII sterically hinders access of ribosomes to the ribosome binding site of *repB*, it is important to unambiguously identify the SD sequence for this gene. In the region upstream of the *repB* start codon there are two potential sequences showing some resemblance to the consensus SD sequence TAAGGAGG (21). The

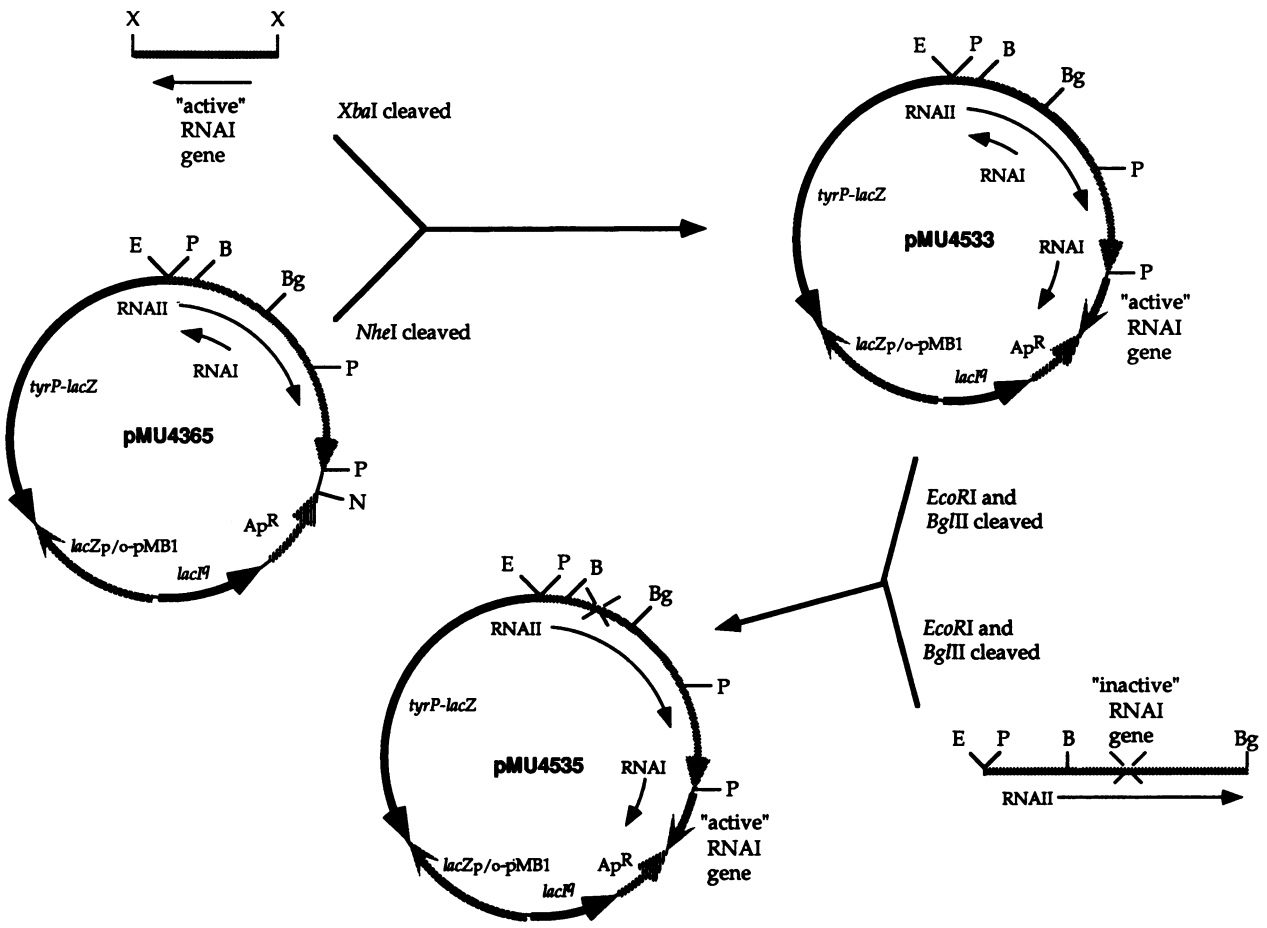

FIG. 2. Construction of the pMU4535 derivatives used to determine the viability and relative copy numbers of various reconstituted IncB replicons. Details of the constructions are given in Materials and Methods. Features important for the construction and use of the plasmids are shown. B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; N, *Nhe*I; X, *Xba*I.

first of these is GGGA and the second is TAAG, located 9 and 5 nt, respectively, from the *repB* start codon (Fig. 3). In order to identify which of these sequences was important for translational initiation, site-directed mutagenesis was used to change GGGA to GCCA and TAAG to CCAG. The effects of these mutations were measured by using low-copy-number plasmids (approximately one to two copies per chromosome) carrying translational *repB-lacZ* fusions in which codon 29 of *repB* was fused in phase with codon 8 of *lacZ*. To evaluate the effect of the mutations on the regulation of *repB* by RNAI, assays were performed in the presence of multicopy plasmids (20 to 30 copies per chromosome) carrying either the gene for RNAI (producing saturating levels of RNAI, resulting in maximal inhibition) or the gene for the RNA complementary to RNAI (i.e., target RNA to titrate out RNAI, resulting in a fully derepressed state). Although both RNAI and RNAII are expressed constitutively, the DNA fragment used to construct the *repB-lacZ* translational fusion was also introduced into a *lacZ* transcriptional fusion to confirm that the mutations had no effect on transcription.

As can be seen in Fig. 3, the first putative SD sequence is located two bases downstream of and the second one overlaps the −10 region of the RNAI promoter. Because it was possible that the *repB* SD sequence mutations might differentially affect

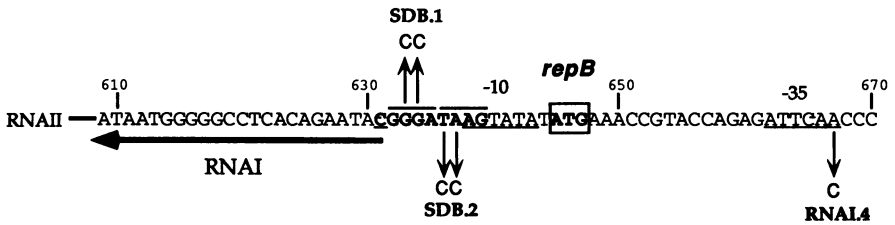

FIG. 3. Partial nucleotide sequence of the replication control region of pMU720 with mutations that alter the putative SD sequences of *repB*. The *repB* start codon is boxed with the putative SD sequences overlined and in boldface type, and the −10 and −35 RNAI sequences are underlined. The RNAI transcript is denoted by an arrow. The site and base changes of the mutations introduced are indicated.

TABLE 2. Effects of mutations in putative *repB* SD sequences on the expression of β-galactosidase from *repB-lacZ* fusions

Mutation present in *repB-lacZ* fusion	β-Galactosidase activity (U) from *repB-lacZ* fusion with coresident plasmid present in *trans*[a]			
	Translational fusions with:			Transcriptional fusions with pBR322
	pBR322	pMU617 (RNAI)	pMU662 (target)	
None	953	122	4,186	769
RNAI.4	14,677	72	13,136	1,408
SDB.1[b]	2,736	27	2,873	1,268
SDB.2	12,073	69	10,354	1,398

[a] β-Galactosidase activities were measured by the method of Miller (16), and the results shown are the averages of at least three independent determinations. pMU617 (RNAI) carries nt 438 to 718 of pMU720 and therefore expresses RNAI but not RNAII (18). pMU662 (target) carries nt 1 to 637 of pMU720 (18) and thus expresses the leader sequence of RNAII (SLI) which is complementary to RNAI but does not express RNAI. These plasmids do not carry *lacZ*.
[b] SDB.1 and SDB.2 were present in a *repB-lacZ* fusion carrying the RNAI.4 mutation.

the efficiency of the RNAI promoter, we inactivated the promoter by changing T-667, the first base of the −35 sequence, to a nonconsensus residue G (RNAI.4 mutation in Fig. 3). The RNAI.4 mutation resulted in a complete derepression of *repB-lacZ*, as shown by the observation that saturating amounts of target RNA had no effect on expression, indicating that little RNAI was synthesized from this fusion plasmid (Table 2). Accordingly, the *repB* SD sequence mutations were introduced into the RNAI.4 *repB* translational *lacZ* fusion. Of the changes to the two putative SD sequences, only the changes to SDB.1 (GGGA) had a significant effect on *repB* expression, and we conclude that this is the functional SD sequence for *repB*. As can be seen in Fig. 3, SDB.1 is the sequence which lies immediately adjacent to the nucleotide encoding the 5′ end of RNAI.

Is there a critical distance between the *repB* SD sequence and the 5′ end of RNAI for inhibition? If RNAI controls *repB* translation by blocking ribosome access to the SD sequence when it binds RNAII, then it should be possible, by inserting DNA between the SD sequence and the nucleotide corresponding to the 5′ end of RNAI (the downstream junction of RNAI-RNAII interaction), to move the two far enough apart so that ribosome binding is no longer affected. To do this, we created a unique *Sma*I restriction site by changing two bases immediately upstream of the *repB* SD sequence TACGGG to CCCGGG (Fig. 4) and then inserting different linker DNA sequences at this site.

As one consequence of these insertions could be to change the transcription initiation site for RNAI, we again inactivated the RNAI promoter, this time with the mutation RNAI.3 and

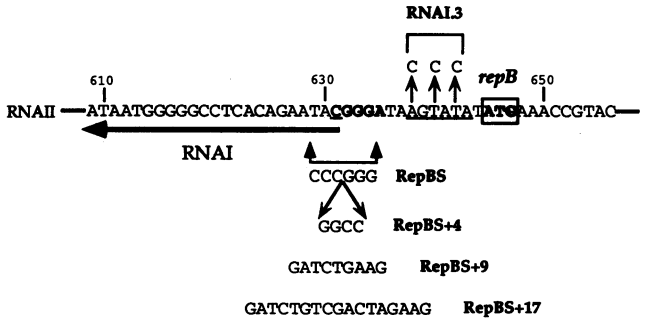

FIG. 4. Partial nucleotide sequence of the replication control region of pMU720 with mutations that alter the distance between the start point of RNAI transcription and the *repB* SD sequence. The *repB* start codon is boxed with the SD sequence in boldface type, and the initiation of RNAI transcription is underlined and in boldface type. The site and base changes of the mutations introduced are indicated.

provided an RNAI gene in *trans* on a multicopy plasmid. This RNAI gene also carried the *Sma*I site sequence (RepBS mutation) to ensure complementarity between RNAI and RNAII.

The inactivating mutation RNAI.3 changes the −10 sequence of the RNAI promoter from TATAcT to TgTgcg (Fig. 4) and causes complete derepression of a *repB-lacZ* translational fusion, as shown by the finding that the addition of saturating amounts of target RNA in *trans* had no effect on expression (10,882 U). Although the effects of the two RNAI promoter mutations on the expression of *repB-lacZ* were similar, data obtained with the *repA-lacZ* translational fusion indicated that RNAI.3 has an even more severe effect on RNAI transcription than RNAI.4 (data not shown). The creation of the *Sma*I site (RepBS) had practically no effect on *repB* expression (Table 3). An insertion of 4 bases, however, reduced RNAI inhibition from 137-fold in RepBS to 35-fold, and an insertion of 9 or 17 bases reduced it further to 4- and 2-fold, respectively. In other words, as the SD sequence is moved further away from the RNAI-RNAII complex, the ability of RNAI to inhibit *repB* translation is diminished. Table 3 also shows the impact of these insertions on translational control of a *repA-lacZ* fusion.

The change to *repA-lacZ* expression shows a fold decrease in RNAI inhibition similar to that with *repB-lacZ*. In this case, however, effective control of *repA* expression is still maintained by the inhibition of pseudoknot formation by RNAI. Because pseudoknot formation is prevented when RNAI is introduced in *trans* on a multicopy plasmid, we conclude that the decreased ability of RNAI to inhibit the translation of *repA* when the distance between the *repB* TIR and RNAI-RNAII complex was increased reflects some pseudoknot-independent translational coupling between *repB* and *repA*. The decreasing inhibition of *repA* expression is therefore a result of the increasing derepression of *repB* expression. The reduced expression of *repA-lacZ* in the absence of RNAI in the case of the +17 insertion may be a consequence of the separation of the proximal and distal regions of the pseudoknot. Such effects have been previously observed (29).

In order to measure the effects of this deregulation of *repB* expression on IncB plasmid replication, the insertions were introduced into a derivative of the chimeric plasmid pMU4535 whose second replicon is fully repressed in the absence of the inducer IPTG (Fig. 2). This derivative carries the RepBS mutation (*Sma*I site) both in the IncB replicon in which the RNAI promoter had been inactivated by the RNAI.3 mutation and in a separate sequence, depicted as active RNAI gene in Fig. 2, which provides the RNAI that regulates replication of pMU4535. As before, insertions were introduced into the *Sma*I site adjacent to the inactive RNAI gene. Although the active

TABLE 3. Effects of insertions between the start site of RNAI transcription and the *repB* SD sequence on the expression of β-galactosidase from *repB-lacZ* and *repA-lacZ* fusions carrying the RNAI.3 mutation and on IncB plasmid replication

Mutation present in *rep-lacZ* fusion	β-Galactosidase activity (U) from *rep-lacZ* with coresident plasmid present in *trans*[a]					Relative plasmid copy no.[b]
	Translational fusion				Transcriptional fusion	
	repB-lacZ		*repA-lacZ*		*repB-lacZ*	
	pBR322	pMU4528 (RNAI)	pBR322	pMU4528 (RNAI)	pBR322	
RNAI.3	11,790	81 (146)	4,229	0.2 (21,145)	1,170	
RepBS[c]	14,562	106 (137)	3,751	0.3 (12,503)	800	1.0
RepBS+4	7,414	215 (35)	2,670	0.5 (5,370)	591	0.9
RepBS+9	20,837	4,731 (4)	3,296	1.7 (1,939)	1,625	0.9
RepBS+17	17,902	7,687 (2)	1,102	4.7 (234)	1,498	2.2

[a] β-Galactosidase activities were measured by the method of Miller (16), and the results shown are the averages of at least three independent determinations. Values in parentheses are fold inhibitions obtained by dividing β-galactosidase activity obtained from the translational fusion in the absence of RNAI (pBR322 column) with the β-galactosidase activity obtained in the presence of RNAI. Vector (pBR322) or its derivatives were present in *trans*. RNAI (pMU4528) carries nt 553 to 789 of pMU720 and therefore expresses RNAI but not RNAII. These plasmids do not carry *lacZ*.

[b] For copy number determinations, the mutations present in the *rep-lacZ* fusions were introduced into the IncB replicon of pMU4535 (Fig. 2), and replication control was provided by an active RNAI gene containing the RepBS mutation (*Sma*I site). Copy number determinations were carried out with strain JP8042 grown in minimal medium containing glucose as the source of carbon and ampicillin. β-Galactosidase activities obtained with mutant derivatives of pMU4535 were normalized to the activity obtained with pMU4536. β-Galactosidase activities were measured by the method of Miller (16), and the results shown are the averages of at least three independent determinations.

[c] The RepBS mutations were present in *repB-lacZ* and *repA-lacZ* fusions carrying the RNAI.3 mutation.

RNAI gene in *cis* contained a *Sma*I site to provide homology between the 5′ and 3′ ends of RNAI and RNAII, it contained no inserts. The copy number of each of these plasmid derivatives was determined by growing cells in the absence of IPTG (to prevent activation of the pMB1 replicon) and measuring β-galactosidase (expressed from a constitutive promoter on pMU4535). Levels of β-galactosidase in cells carrying this plasmid with no insertions into the *Sma*I site were taken as equivalent to a copy number of one. The copy numbers of the various insertional derivatives tested (Table 3) were derived by comparing β-galactosidase values. The interesting finding is that although regulation of expression of *repB* is almost completely abolished in the case of the +17 insertion, the effect of this insertion on relative copy number is minor, causing only a twofold increase. There is no runaway replication, confirming that if there is sufficient expression of *repB*, the regulation of *repA* expression via the pseudoknot ensures adequate control.

Is the distance between SLI and the TIR of *repB* also critical for *repB* expression? Having concluded that translation of *repB* was controlled by the RNAI-RNAII complex blocking ribosome attachment, we wondered whether the existence of a paired structure such as SLI adjacent to a SD sequence could, in the absence of a complementary molecule, inhibit translation. Obviously, this is not the case with the wild-type sequence, but we wished to determine if moving SLI closer to the SD sequence would impede translation initiation. We used site-directed mutagenesis to delete bases between SLI and the *repB* SD sequence, as depicted in Fig. 5. Once again, to avoid uncontrolled effects on RNAI expression, we inactivated the RNAI promoter with the previously mentioned RNAI.3 mutation. The gene for wild-type RNAI was again introduced in *trans* on a multicopy plasmid, and transcriptional fusions were constructed to test for any effects on transcription.

The results which are shown in Table 4 indicate that in the absence of RNAI (pBR322 column), *repB* translation is increasingly inhibited as SLI is moved closer to the SD sequence. The deletion of 12 bases which leaves the SD sequence only 1 base away from SLI lowered *repB* expression approximately 50-fold. The minimal level of *repB* expression in the presence of wild-type RNAI remained fairly constant in all these constructs. With the exception of RSD-9, transcriptional effects were negligible. These data suggest that the presence of an intrastrand secondary structure in close proximity to the *repB* TIR can have a similar effect as the formation of the interstrand complex between RNAI and RNAII. An alternative explanation is that the bases between SLI and the *repB* SD sequence are actively involved in *repB* expression and that their deletion causes a drastic reduction in the translation of *repB*. At present, we cannot distinguish between these two possibilities.

In order to determine whether the reduced level of *repB* expression in these deletion plasmids would be sufficient to allow enough *repA* expression to maintain the plasmid, the mutations were inserted into the IncB replicon of the previously described chimeric plasmid pMU4535 and replication control was provided by active wild-type RNAI (Fig. 2). From Table 4, it can be seen that despite severe reductions in *repB* expression, none of the deletions inactivated the IncB replicon, and unexpectedly the RSD-7, RSD-9, and RSD-12 mutations actually resulted in increased relative plasmid copy numbers. These increases in relative copy numbers are not fully understood but may result from the reduced ability of wild-type RNAI to interact with a SLI in which the region complementary to the RNAI 5′ tail is deleted. Thus, even when the expression of *repB* is inhibited 50-fold, as is the case in RSD-12, the residual expression is sufficient to enable enough RepA synthesis for replication.

Is the 5′ RNAI tail involved in inhibition of the translation of *repB*? The interaction between RNAI and RNAII is believed to occur in three steps (Fig. 6) (25). The initial step, which consists of base pairing between complementary sequences in the hairpin loops of RNAI and SLI (initial kissing complex), is followed by intrastrand melting and interstrand pairing of the upper stem, a step facilitated by the interior loop structures (extended kissing). The last step involves stabilization of the extended kissing complexes by pairing between the complementary single-stranded tail regions of RNAI and RNAII (stable complex). It has recently been shown that the extended kissing complex is sufficient for the inhibition of *repA* translation in vivo and that the 5′ RNAI tail is not required for *repA* control (25). This result is consistent with the model for the

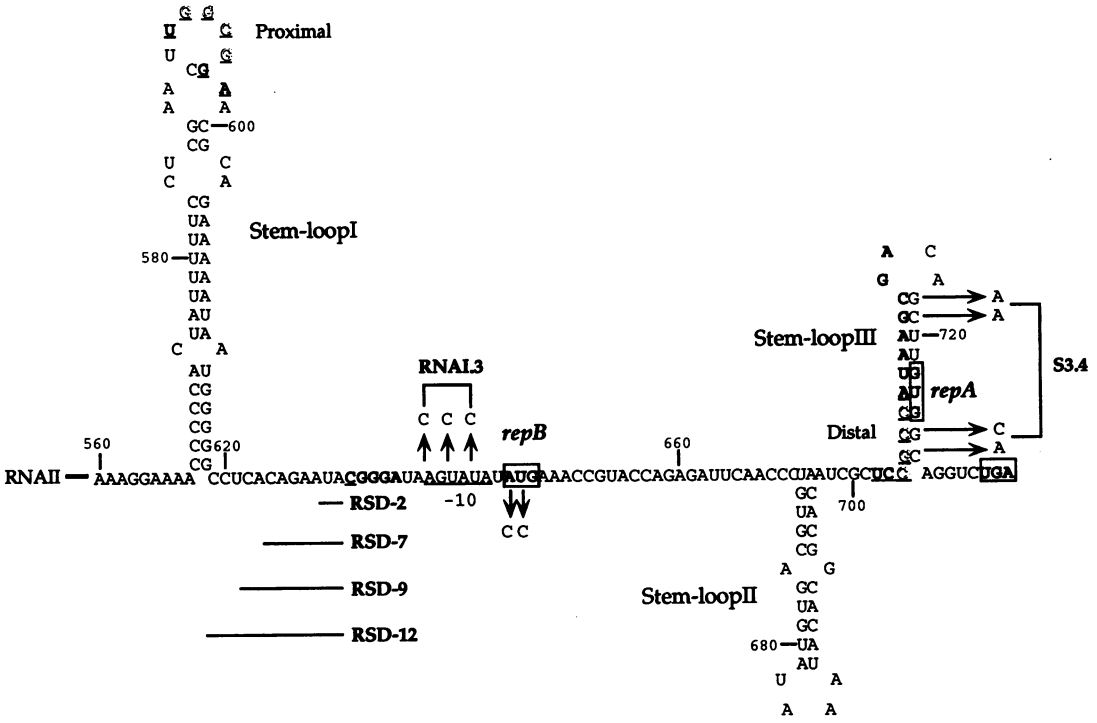

FIG. 5. Predicted secondary structure of the replication control region of RNAII with mutations that alter the distance between SLI and the TIR of *repB*. The initiation and termination codons are boxed, and the putative SD sequences are shown in boldface type. The complementary pseudoknot sequences are shown underlined and in boldface type, with the bases indispensable for pseudoknot formation in outline type. The site and base changes of the mutations introduced are indicated, with the regions deleted denoted by a line.

regulation of *repA* in IncB plasmids, since formation of an extended kissing complex is sufficient to sequester the proximal bases required for pseudoknot formation, and without the pseudoknot, *repA* cannot be expressed above a very low basal level (20, 29). By contrast, the 5′ tail region of RNAI is postulated to be involved in the control of *repB*, although the effect of tailless RNAI molecules on the translation of *repB* has not been tested. To determine whether the 5′ RNAI tail is indeed involved in the regulation of the expression of *repB*, various 5′ deletions were introduced into the RNAI gene by site-directed mutagenesis (Fig. 7). These deletions were created between the initiation site and the stem of RNAI, so that the initiation point of RNAI was unchanged and the mutated RNAI molecules were fully complementary over their entire length with wild-type RNAII molecules. The design of these experiments also took into account the possibility that changes to the structure of RNAI might affect the stability of the countertranscript or its ability to interact with RNAII. Thus, to minimize any effects due to alterations in the stability of the mutant RNAI molecules, the genes encoding them were inserted into a multicopy plasmid (pBR322) and introduced in *trans* to *lacZ* fusions carried on low-copy-number plasmids, ensuring that the levels of RNAI should remain saturating even if RNAI half-lives had been severely reduced. To monitor the abilities of these RNAI molecules to hybridize with RNAII, we took advantage of the fact that RNAI also directly regulates the expression of *repA* via a mechanism which is independent of the regulation of *repB*. Because RNAI regulates *repA* expression primarily by interacting with SLI to prevent formation of the pseudoknot, the ability of RNAI to control *repA* expression was used as an indicator of its ability to bind to SLI. The *repB* and *repA* translational *lacZ* fusions used in these experiments carried the RNAI.3 mutation to reduce the transcription of the RNAI gene present on the *rep-lacZ* fusions to a level low enough not to obscure the effect of the mutant RNAI molecules added in *trans*.

The results are shown in Table 5, in which it can be seen that decreasing the length of the 5′ tail of RNAI results in a progressive loss of inhibition of *repB-lacZ* translation by the truncated molecules. Wild-type RNAI causes a 146-fold inhibition, RNAI-2 causes a 103-fold inhibition, and RNAI-7 and RNAI-9 cause only about 15-fold inhibition. The changes in inhibition of *repA-lacZ* expression almost parallel the effects on *repB*, but because of the extraordinarily tight control of *repA* expression, its reduction to 11% of the wild-type level still leaves a considerable residual inhibition of about 2,000-fold. We conclude that the effect on *repA* expression reflects the derepression of *repB* expression, which results in increases in pseudoknot-independent translational coupling between *repB* and *repA*, as previously mentioned. In order to test our conclusion that the truncated RNAI molecules were unaltered in their abilities to bind SLI, we used a third *lacZ* fusion called *repA*^{III}-*lacZ*. In this construct, SLIII of RNAII has been disrupted by a number of base changes (S3.4 mutation [Fig. 5]). The effect of these mutations is to make pseudoknot formation and its enhancement of translation independent of *repB* translation (29). This S3.4 mutation was introduced into a plasmid in which endogenous RNAI expression is reduced (RNAI.3) together with a mutation which eliminates *repB* translation by changing its initiation codon from ATG to CCG (Fig. 5). The net effect of these various changes is to uncouple the translation of *repA* completely from that of *repB*, permitting direct measurement of the abilities of RNAI species to interact with SLI. As can be seen in Table 5, the −2 and −7

TABLE 4. Effects of deletions between SLI and the *repB* SD sequence on the expression of β-galactosidase from *repB-lacZ* fusions carrying the RNAI.3 mutation and on IncB plasmid replication

Mutation present in *repB-lacZ* fusions	β-Galactosidase activity (U) from *repB-lacZ* with coresident plasmid present in *trans*[a]			Relative plasmid copy no.[b]
	Translational fusions with:		Transcriptional fusions with pBR322	
	pBR322	pMU617 (RNAI)		
None	953	122	769	
RNAI.3	11,790	81	1,170	1.0
RSD-2[c]	6,306	81	1,165	0.4
RSD-7[c]	2,640	65	1,200	3.4
RSD-9[c]	456	38	647	3.6
RSD-12[c]	230	74	1,258	3.1

[a] β-Galactosidase activities were measured by the method of Miller (16), and the results shown are the averages of at least three independent determinations. Vector (pBR322) or its derivatives were present in *trans*. RNAI (pMU617) carries nt 438 to 718 of pMU720 and therefore expresses RNAI but not RNAII (18). This plasmid does not carry *lacZ*.

[b] For copy number determinations, the mutations present in the *rep-lacZ* fusions were introduced into the IncB replicon of pMU4535 (Fig. 2), and replication control was provided by an active wild-type RNAI gene. Copy number determinations were carried out with strain JP8042 grown in minimal medium containing glucose as the source of carbon and ampicillin. β-Galactosidase activities obtained with mutant derivatives of pMU4535 were normalized to the activity obtained with pMU4535. β-Galactosidase activities were measured by the method of Miller (16), and the results shown are the averages of at least three independent determinations.

[c] The four RSD mutations were present in a *repB-lacZ* fusion carrying the RNAI.3 mutation.

deletions retain 84 and 80%, respectively, of the wild-type ability to inhibit $repA^{III}$-*lacZ* expression, and the −9 deletion retains 58% of this activity. These results suggest that although deleting the 5′ tail of RNAI can have some effect on RNAI-RNAII interactions, this effect is significantly less than the effect on inhibition of *repB* translation. The relatively high residual level (24 U) of $repA^{III}$-*lacZ* activity in the presence of wild-type RNAI is a result of the disruption of SLIII which allows some constitutive, pseudoknot-independent *repA* translation to occur (20, 29).

To determine how effectively the shortened RNAI molecules controlled replication, these mutations were introduced into the active RNAI gene of the chimeric plasmid pMU4535 (Fig. 2). The −7 and −9 deletions resulted in an increase in relative copy number to 3 and 7, respectively (Table 5). Unexpectedly, the −2 deletion resulted in an unstable plasmid with an apparent relative copy number 10-fold lower than that of pMU4535. The basis for this result is unknown but may indicate that RNAI-2 has some other change, such as an increase in its half-life.

What effect will extending the 5′ RNAI tail have on the control of the translation of *repB*? The ability of RNAI to regulate *repB* is extremely poor compared with its control of *repA* expression (146-fold repression compared with 20,650-fold). Since the countertranscript RNA does not actually overlap the TIR of *repB*, the relatively inefficient regulation may reflect the inability of the RNAI-RNAII complex to completely exclude ribosomes from this region. To examine this possibility, the 5′ tail region of RNAI was extended to overlap the *repB* TIR (Fig. 7). Insertions were introduced by site-directed mutagenesis immediately beyond the start point of RNAI transcription and were designed so that the resulting countertranscript was fully complementary over its entire length to the RNAII produced from the RNAI.3 mutation (Fig. 7). The genes encoding these extended RNAI molecules were then inserted into a multicopy plasmid and introduced in *trans* to *repB* and *repA* translational *lacZ* fusions carrying the RNAI.3 mutation. Increasing the 5′ tail of RNAI so that the RNAI molecules overlap the *repB* TIR did not result in any increased ability of RNAI to regulate *repB* (Table 6). Even when the 5′ tail of RNAI was made to overlap not only the *repB* SD sequence but also the *repB* initiation codon

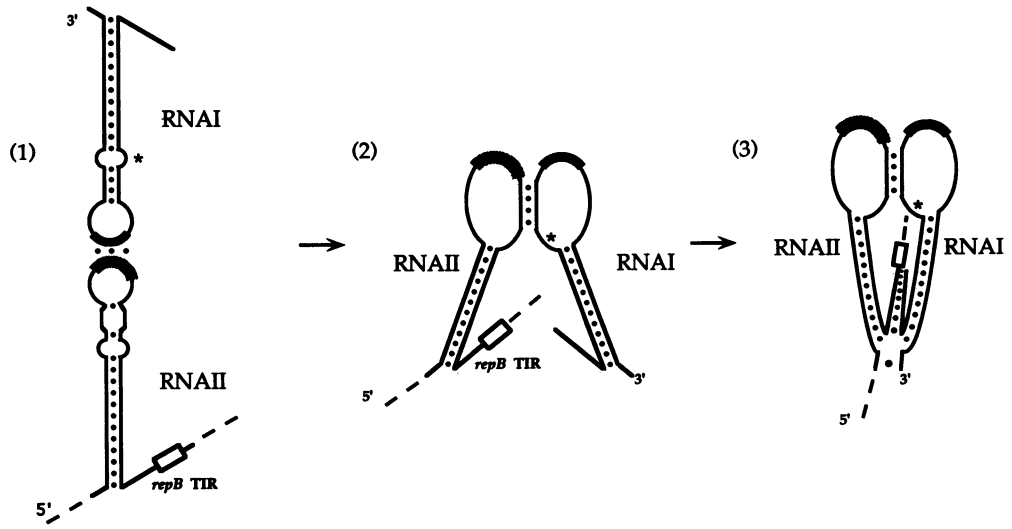

FIG. 6. Diagrammatic representation of the mechanism of binding between RNAI and RNAII which is modified from that of Siemering et al. (25). RNAI and RNAII first interact via the formation of an initial kissing complex between the 5′-GCC-3′ sequence in the hairpin loop of RNAI and its complementary sequence in RNAII (solid boxes) (step 1). Step 2 is facilitated by the interior loop structures in the upper stems of RNAI and RNAII (indicated by an asterisk) and involves intrastrand melting and interstrand pairing of the upper stem regions to form the extended kissing complex. Step 3 quickly follows the second step and involves hybridization of the complementary single-stranded tails of RNAI and RNAII to form the stable complex. The identity of each RNA is indicated. Dots represent base-paired regions. The *repB* TIR is denoted by an open box, and the proximal bases essential for pseudoknot formation are indicated by grey boxes.

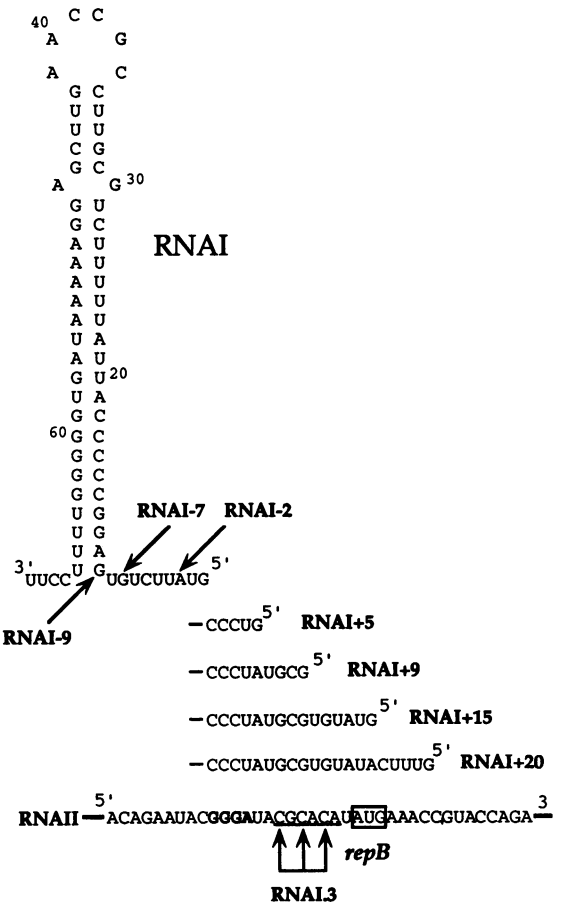

FIG. 7. RNAI mutations constructed to analyze the importance of the 5′ tail of RNAI on the regulation of *repB*. The numbers indicate the nucleotide positions in RNAI relative to the wild-type 5′ end of the molecule. The region of RNAII in an RNAI.3 mutant complementary to the 5′ tail of the various RNAI molecules is shown, with the *repB* initiation codon boxed and the SD sequence in boldface type. Arrows indicate the start sites of the various truncated RNAI molecules; the RNAI-2 species initiates with a G, and therefore retains the wild-type initiation nucleotide while still allowing full complementarity with RNAII by forming a GU base pair. The site and base changes of the mutations introduced are indicated.

(RNAI+15 and RNAI+20), there was no increase in the RNAI-mediated inhibition of *repB*. In fact, RNAI molecules carrying these two insertions showed a slight decrease in their abilities to control the expression of both *repB* and *repA*, indicating that perhaps the longer tail reduced somewhat the ability of the RNAI molecules to bind to SLI. These results clearly show that even though the stable complex formed between the wild-type RNAI and RNAII molecules does not overlap the *repB* TIR, it is optimal for the inhibition of translational initiation from *repB*.

Introduction of these insertion mutations into the active RNAI gene of the chimeric plasmid pMU4535 resulted in an increase in the relative copy number of this plasmid when its replication was driven by the IncB replicon. These changes paralleled the changes in *repA-lacZ* expression reported in Table 6.

DISCUSSION

The results presented here indicate that RNAI regulates the translation of *repB* predominantly by inhibiting the loading of ribosomes to the *repB* TIR. Although the exclusion of ribosome binding at the *repB* TIR by the RNAI-RNAII complex has not been shown, this model for the regulation of *repB* is supported by a number of findings. In particular, physically separating the site of RNAI-RNAII interaction from the *repB* TIR, either by inserting bases between the two or by deleting the 5′ tail of RNAI, was found to significantly decrease the ability of RNAI to inhibit the translation of *repB*. Furthermore, placing the *repB* TIR in close proximity to the large secondary structure SLI (RSD-12) was found to inhibit the translation of *repB* by an amount similar to that observed when RNAI was introduced in *trans*. Assuming that the 12 bases deleted in this construct do not themselves drastically enhance the translation of *repB*, this finding is consistent with the idea that simple inhibition by a secondary structure can mimic the action of RNAI control. Further physical analyses are required to demonstrate conclusively that the RNAI-RNAII complex can prevent ribosome binding.

In the wild-type plasmid, the inhibition of *repB* expression by RNAI appears to depend to a large extent on the 5′ single-stranded tail of these molecules, as deletion of the tail (RNAI-9) results in a 10-fold reduction in the inhibition of *repB*. However, the tailless RNAI species can still inhibit the translation of *repB* by over 14-fold when expressed in *trans* from a multicopy plasmid. This suggests that the extended kissing complex involving RNAI bound to SLI in the loop and upper stem regions not only is sufficient to control the expression of *repA* (25) but also is capable of some inhibition of the translation of *repB*. In the past, it has been assumed that it is the formation of the duplexed RNA structure adjacent to the *repB* SD sequence that is solely responsible for the inhibition of *repB* translation. However, given the structure of the extended kissing complex (Fig. 5) (25) and its close proximity to the *repB* TIR in pMU720, it is not surprising that this complex can impair the access of ribosomes to the *repB* SD sequence. The ability of the extended kissing complex to inhibit *repB* translation also explains why RNAI-9 is more effective in regulating *repB* (14-fold inhibition [Table 5]) than RNAI in regulating *repB* expression from a RepBS+9 *lacZ* fusion (4-fold inhibition [Table 3]), as the deletion of the 5′ tail RNAI (RNAI-9) removes only one component of inhibition: that mediated by formation of an RNA duplex next to the *repB* SD. By contrast, the insertion of nine bases between the RNAI-RNAII complex and the *repB* SD sequence not only relieves the inhibition mediated by the RNA duplex formed immediately adjacent to the *repB* SD sequence but also lessens the inhibition caused by the extended complex itself.

Although most of the inhibition of *repB* by RNAI can be accounted for by the steric hindrance model, the residual inhibition observed even when the RNAI-RNAII complex was separated from the *repB* TIR by 17 bases (Table 3) suggests the involvement of an additional element in regulation of the expression of this gene. This notion is supported by the observation that when SLI was only 1 nt from the *repB* SD sequence, which should maximize steric hindrance by a double-stranded RNA structure, RNAI was still able to inhibit the translation of *repB* by threefold. The most likely mechanism to explain this inhibition, which appears to be dependent only on the interaction of RNAI with RNAII, is RNA processing by a RNase such as RNase III. RNase III cleaves only double-stranded RNA and is known to be involved in the inhibition of gene expression by countertranscript RNAs in many different

TABLE 5. Effects of deletions of the 5′ RNAI tail on the ability of RNAI to inhibit the expression of β-galactosidase from *repB-lacZ* and *repA-lacZ* translational fusions carrying the RNAI.3 mutation and on IncB plasmid replication

RNAI species	β-Galactosidase activity (U) from *rep-lacZ* translational fusions with RNAI species present in *trans*[a]			Relative plasmid copy no.[b]
	repB-lacZ	repA-lacZ	repA^{III}-*lacZ*	
None (pBR322)	11,790	4,130	2,232	
Wild type	81 (146)	0.2 (20,650)	24.0 (93)	1.0
RNAI-2	114 (103)	0.3 (13,767)	28.5 (78)	0.1
RNAI-7	778 (15)	1.2 (3,442)	30.2 (74)	3.2
RNAI-9	841 (14)	1.9 (2,174)	41.0 (54)	7.1

[a] β-Galactosidase activities were measured by the method of Miller (16), and the results shown are the averages of at least three independent determinations. Values in parentheses are fold inhibitions obtained by dividing β-galactosidase activity obtained from the translational fusion in the absence of RNAI with the β-galactosidase activity obtained in the presence of the RNAI species.

[b] For copy number determinations, the mutations present in the RNAI species were introduced into the active RNAI gene of pMU4535 (Fig. 2), which provided the replication control for an IncB replicon containing the RNAI.3 mutation. Copy number determinations were carried out with strain JP8042 grown in minimal medium containing glucose as the source of carbon and ampicillin. β-Galactosidase activities obtained with mutant derivatives of pMU4535 were normalized to the activity obtained with pMU4535. β-Galactosidase activities were measured by the method of Miller (16), and the results shown are the averages of at least three independent determinations.

systems (5, 6, 13). In the IncFII plasmid R1, RNase III has been shown to mediate the cleavage of the countertranscript-Rep mRNA (CopA-CopT) complex at sites in the 5′ tail of the CopA and its complementary region in CopT (5). Although this cleavage has been found to be relatively unimportant in the control of *rep* expression, it did decrease the half-life of the Rep mRNA by a factor of two to three (28). Further experiments are required to determine whether RNase III is involved in the processing of the RNAI-RNAII complex in pMU720. However, even if RNase III is involved, its role in the regulation of *repB* would appear to be relatively minor.

Although the translation of the leader peptide RepB is essential for the expression of *repA* (20), stringent control of *repB* by RNAI is not required for effective control of IncB plasmid replication. Thus, when the expression of *repB* was strongly inhibited when SLI was only one nucleotide from the *repB* SD sequence (RSD-12) or nearly fully derepressed when the *repB* TIR was moved 17 bases downstream of the site of RNAI binding (RepBS+17), the reconstituted IncB replicon was able to replicate and maintain a stable copy number even if slightly elevated. These data support the notions that a stringent control of *repA* translation is the only requirement for viable control of IncB plasmid replication and that this regulation is achieved predominantly by inhibition of pseudoknot formation and not by inhibition of *repB* translation.

The genetic organization of the IncFII plasmids R1 and NR1 is similar to that of pMU720, despite the fact that these two groups of plasmids appear to be only distantly related. Like pMU720, the IncFII plasmids encode a small leader peptide which overlaps the translational initiation site of the Rep protein. However, in the IncFII plasmids, the expression of the Rep protein is thought to depend on the direct translational coupling to the leader peptide, and the countertranscript RNA is proposed to regulate the expression of Rep indirectly, only by inhibiting the translation of the leader peptide (4, 31). Despite this difference, the countertranscript RNA of the IncFII plasmids is also postulated to inhibit the translation of the leader peptide by steric hindrance, as the start site of transcription of this RNA is only 2 nt from the SD sequence of the leader peptide. Considering the similarities, it would seem reasonable to speculate that the regulation of the leader peptide in the IncFII plasmids by the countertranscript RNA occurs by a mechanism similar to that elucidated for pMU720. However, recently Wagner et al. (28) found that a tailless countertranscript RNA (CopS) could efficiently regulate the expression of the Rep protein in plasmid R1. Although the ability of the CopS RNA to regulate the leader peptide was not determined, as the expression of the Rep protein is thought to be reliant only on direct translational coupling to the upstream gene, CopS should also efficiently control the expression of the leader peptide. In the case of pMU720, a tailless RNAI species could still adequately regulate the translation of *repB*, because inhibition by the extended kissing complex remained largely unaffected. However, this inhibition was dependent on close proximity of the extended kissing complex to the *repB* SD sequence (nine bases). The countertranscript RNA of R1 (CopA) has a much longer 5′ tail than RNAI, so that the complex formed between CopS and the Rep mRNA would be 32 nt away from the SD sequence of the leader peptide. Our data indicate that this distance is too great for effective control via steric hindrance, implying either that the expression of the Rep protein in the IncFII plasmids can be somehow directly regulated by the countertranscript (as occurs in the IncB and IncI$_1$ plasmids) or that control of the expression of the leader peptide is not determined solely by the countertranscript RNA.

In IncB-like plasmids, the requirement for leader peptides appears to be the result of the inability of the countertranscript RNA to adequately control gene expression directly, by steric hindrance. Thus, IncB replicons, in which *repB* has been fused in phase with *repA*, so that the expression of *repA* is regulated solely via steric hindrance, have a copy number 166-fold higher

TABLE 6. Effects of extending the 5′ RNAI tail on the ability of RNAI to inhibit the expression of β-galactosidase from *repB-lacZ* and *repA-lacZ* translational fusions carrying the RNAI.3 mutation and on IncB plasmid replication

RNAI species	β-Galactosidase activity (U) from *rep-lacZ* translational fusions with RNAI species present in *trans*[a]		Relative plasmid copy no.[b]
	repB-lacZ	*repA-lacZ*	
None (pBR322)	11,790	4,130	
Wild type	81 (146)	0.2 (20,650)	1.0
RNAI+5	91 (130)	0.2 (20,650)	1.5
RNAI+9	92 (128)	0.3 (13,767)	1.9
RNAI+15	117 (101)	0.5 (8,260)	3.3
RNAI+20	124 (95)	0.9 (4,589)	3.1

[a] See footnote *a* to Table 5.

[b] See footnote *b* to Table 5.

than the wild-type plasmid and are highly unstable (data not shown). The inability of RNAI to tightly control *repB* is not the consequence of the positioning of the RNAI-RNAII complex, which forms close to, but does not overlap, the SD sequence of *repB*, since extending the complex to include the entire TIR of *repB* did not improve its regulation. Rather, it is the tight coupling of transcription and translation in prokaryotes which is most likely responsible for the low efficacy of RNAI in the regulation of *repB*. Thus, if regulation of *repB* is envisaged as a race between formation of the RNAI-RNAII complex and the binding of a ribosome to the TIR of *repB*, then the tight coupling between transcription and translation could tip the balance in favor of the ribosome, so that complete inhibition does not occur even in the presence of saturating levels of RNAI. In the case of Rep, this level of regulation would result in the inability of the plasmid to correct the upward fluctuations in its copy number, as replication could never be completely shut down. As a consequence, the number of plasmid molecules per cell would increase progressively with each generation.

The only way to improve regulation of *repB* would be to shift the balance in favor of the RNAI-RNAII complex, by increasing the rate of complex formation or decreasing the efficiency of the translation initiation signals of *repB*. This notion is supported by the observation that the reduction in the rate of translation of *repB*, achieved by alterations to its start codon, was accompanied by an increase in the level of RNAI-mediated inhibition (20). Another way to shift the balance from translation initiation to inhibition would be by introducing (or improving, if one already exists) an RNA polymerase pause site between SLI and the TIR of *repB*, thus extending the window for effective RNAI-RNAII binding. This mechanism had been implicated in the regulation of the synthesis of the Rep protein of NR1 (8) but has not been demonstrated for pMU720. However, none of these measures to improve regulation of *repB* could be used in a low-copy-number plasmid such as pMU720, because all are predicted to reduce the plasmid's ability to correct downward fluctuations in its copy number, and unlike the IncFII plasmids, IncB plasmids do not contain a backup system to correct for downward fluctuations in copy number. In IncFII plasmids there is an additional promoter which is expressed only when the copy number of the plasmid reaches a critically low level (7, 14, 30), and derepression of this promoter has been shown to compensate for significantly decreased Rep expression in NR1 (32). Thus, these measures in IncB-like plasmids would lead to increases in segregational instability.

Direct translational coupling of Rep and the leader peptide may provide a more efficient regulation of replication, since the effect of any noninhibitable expression of the leader peptide can be minimized by a low efficiency of coupling between the two genes. This is the case in the IncFII plasmids, in which expression of the leader peptide is 10- to 20-fold higher than the expression of Rep (4, 31).

The relatively poor SD sequence of *repA* in the IncB-like plasmids ensures that there is little translational coupling between *repB* and *repA* in the absence of the pseudoknot (29). Consequently, the high level of noninhibitable expression of *repB* does not directly translate into a high level of unregulated expression of *repA*. Moreover, the requirement that translation of *repB* be completed before pseudoknot formation can occur increases the time, and therefore the opportunity, for effective interaction between RNAI and RNAII. Thus, when the concentration of RNAI increases in response to an upward fluctuation in the plasmid copy number, there is sufficient time for RNAI to bind to RNAII, so that the pseudoknot cannot form and RepA cannot be synthesized. Removal of the need for prior translation of *repB* would narrow the window for effective RNAI binding, thus shifting the equilibrium towards pseudoknot formation, with the predicted outcome of a high level of noninhibitable expression of *repA*. Therefore, the leader peptide of pMU720 is essential not only because it allows the pseudoknot to form but also because it buys time for the RNAI-RNAII interaction, permitting total shutdown of *repA* when RNAI concentrations raises above the levels set by the copy number control machinery of the plasmid.

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