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Mutations Affecting Translational Coupling between the *rep* Genes of an IncB Miniplasmid

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The nature of translational coupling between *repB* and *repA*, the overlapping *rep* genes of the IncB plasmid pMU720, was examined. Mutations in the start codon of the promoter proximal gene, *repB*, reduced the efficiency of translation of both *rep* genes. Moreover, there was no independent initiation of *repA* translation in the absence of *repB* translation. The position of the *repB* stop codon was crucial for the efficient expression of *repA*, with the wild-type positioning being optimal. Translational coupling was found to be totally dependent on the formation of a pseudoknot structure. A model which invokes formation of a pseudoknot to facilitate initiation of *repA* is proposed.

Replication of prokaryotic plasmids is in many cases regulated by small countertranscript RNA molecules which on hybridizing with the target RNA molecules block their activity. The role of the target molecule and the mechanism of inhibition of its function by the countertranscript differs from plasmid to plasmid. Thus, in ColE1 plasmids, the target is a preprimer which, if duplexed with its countertranscript RNA, cannot be processed to the active form (12, 28–30). In pT181, binding of the countertranscript RNA is proposed to so alter the folding of its target, an mRNA for an essential replication protein, as to cause it to terminate prematurely (10, 17). In FII plasmids, complexing with the countertranscript inhibits translation of the target RNA (11), but the mechanism of this inhibition is not understood. Recent study suggested that the target countertranscript duplex is cleaved by RNase III and that this cleavage may somehow affect translation of the *rep* mRNA (4).

The main regulator of IncB plasmids is also a small countertranscript molecule, RNAI (16, 19). This molecule is made from the opposite strand of and is complementary to the leader region of a second RNA molecule, RNAII, whose product, the RepA protein, is thought to be essential for replication (19, 20). Interaction of RNAI with RNAII leads to inhibition of translation of the *repA* gene. The mechanism by which formation of this RNA-RNA hybrid inhibits translation initiating 91 nucleotides (nt) downstream is unclear, since computer-aided analyses (7, 8, 35) of the folding of RNAII predict that the translation initiation region of *repA* is sequestered within a secondary structure whose stability is unaffected by binding of RNAI. Recently, we reported that translation of *repA* is coupled to that of the upstream gene, *repB*, since mutations in *repB* have a profound effect on the efficiency of expression of *repA* (32).

Studies on the regulation of a related plasmid, ColIb-P9, have also demonstrated that translation of the gene for the putative replication protein, which is called *repZ*, is dependent on the translation of an upstream gene, *repY*, whose open reading frame overlaps the start of *repZ* (6). Furthermore, it was recently suggested that 4 bases in the loop of RNAII (GGCG) interact with a complementary 4-base sequence just preceding the putative Shine-Dalgarno sequence of *repZ* mRNA to form a pseudoknot. This structure is thought to stabilize the translation initiation region of *repZ* in its unfolded state, thus facilitating ribosome access to initiate *repZ* translation (2). In this paper, we describe the effects of changing the position of the *repB* stop codon relative to the initiation codon of *repA* on the translational coupling in *repB-repA*. We also describe effects of pseudoknot mutations on *repA* expression. These data not only provide evidence in support of pseudoknot formation but also suggest that the translational coupling observed in *repB-repA* is totally dependent on pseudoknot formation and indicate that *repA* translational initiation may require a pseudoknot-ribosome interaction.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The *Escherichia coli* K-12 strains used in this work are given below. JM101 [*supE thi* Δ(*lac-proAB*) F′(*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *ZΔM15*)] (13) was used for propagating derivatives of bacteriophage M13. SDM [*hsdR17 mcrAB recA1 supE44* Tet^r Δ(*lac-proAB*) F′(*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15*)] was used to grow M13 derivatives which had been subjected to a mutagenic procedure based on that of Vandeyare et al. (31). JP3923 (*thr-1 leuB6 thi-1 lacZΔM15 lacY1 gal-351 supE44 tonA21 hsdR4 gyrA379 rpsL743 recA56 srl-1300*::Tn*10 aroL513*) was used for all β-galactosidase assays.

Bacteriophage vectors used to clone fragments for sequencing and mutagenesis were M13mp18 (34), M13tg130, and M13tg131 (9). Plasmids are described in Table 1.

Media and chemicals. The minimal medium used was half-strength buffer 56 (15) supplemented with 0.2% glucose, thiamine (10 μg/ml), and necessary growth factors. Chemicals and enzymes were purchased from commercial suppliers and used without further purification. Ampicillin was used at a final concentration of 50 μg/ml, trimethoprim was used at 10 μg/ml, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 25 μg/ml, and isopropylthiogalactoside (IPTG) was used at 1 mM. α-^{35}S-dATP (1,200 Ci/mmol) for sequencing was obtained from Amersham Corp.

Recombinant DNA techniques. The isolation and manipulation of DNA were performed as described by Sambrook et al. (22). DNA sequence was determined by the chain termi-

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TABLE 1. Plasmids

Plasmid	Relevant characteristics[a]	Reference or source
pBR322	ColE1 derivative, Ap Tc	26
pMU720	Gal IncB, miniplasmid	3
pMU617	pBR322 carrying nt 438 to 718 of pMU720, Ap IncB	19
pMU2422	pMU617 with G-596→T substitution in insert, Ap	K. Siemering
pMU2423	pMU617 with G-594→T substitution in insert, Ap	K. Siemering
pMU662	pBR322 carrying first 637 nt of pMU720, Ap	19
pMU525	*lac'ZYA'* Tp IncW, low-copy-number translational-fusion vector	19
pMU575	*galK'-lac'ZYA* Tp IncW, low-copy-number transcriptional-fusion vector	33
pMU2385	*galK'-lac'Z* Tp IncW, low-copy-number transcriptional-fusion vector derived from pMU575	This study
pMU1550	*repA-lacZ* translational fusion carrying nt 1–779 of pMU720, Tp IncW IncB	This study
pMU1551	Transcriptional-fusion version of pMU1550	This study
pMU1578	*repB-lacZ* translational fusion carrying nt 1–730 of pMU720, Tp IncW IncB	This study

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

nation method of Sanger et al. (23). However, T7 DNA polymerase was used instead of the Klenow fragment, and the products were labeled with α-^{35}S-dATP.

Site-directed mutagenesis. In vitro mutagenesis with synthetic oligonucleotides was performed by using commercially available kits (Amersham Corp. and United States Biochemical Corp.). Oligonucleotides were synthesized by using Gene Assembler Plus from Pharmacia LKB Biotechnology. Mutations were confirmed by DNA sequence analysis.

Construction of promoter cloning vector pMU2385. pMU2385 was made by modifying the promoter cloning vector pMU575 (33) as follows. The 1.0- and 0.9-kb *Dra*I fragments, which contain the coding regions of *lacY* and *lacA*, were deleted. A transcription terminator was inserted into the remaining *Dra*I site to prevent readthrough from *lacZ* into the vector. In addition, the polylinker cloning site was modified, so that pMU2385 contains nine unique sites for insertion of promoter-bearing DNA fragments upstream of the *galK'-lac'Z* fusion. These sites, starting from the one farthest away from *galK'*, are *Pst*I, *Sal*I, *Bam*HI, *Hin*dIII, *Xba*I, *Kpn*I, *Sma*I, *Eco*RI, and *Bgl*II.

Construction of the *lacZ* fusion plasmids. The *lacZ* fusion plasmids consist of DNA fragments from pMU720 inserted into the promoter cloning vectors pMU525 and pMU2385 (Table 1 and Fig. 1). In these plasmids, transcription from the RNAII promoter on the cloned fragment is directed towards the *lacZ* gene. Because there are no convenient sites in pMU720 that would enable us to move the mutations we generated into *repB* and *repA* fusions, we used polymerase chain reaction to create appropriate restriction enzyme sites at the 5′ and 3′ ends of the desired DNA fragments. The forward primer used in these reactions corresponded to nt 1 to 18 of pMU720 and contained an *Eco*RI linker at its 5′ end. The reverse primers corresponded to nt 730 to 712 (for *repB* fusions) or 789 to 773 (for *repA* fusions) of pMU720 and contained a *Bgl*II linker at the 5′ end (see Fig. 2). The fragments generated by polymerase chain reaction were cloned into M13 vectors, and their sequences were checked for the presence of misincorporated nucleotides. Clones carrying error-free inserts were used as sources of DNA fragments for construction of *lacZ* fusions. The translational fusions were constructed by inserting *Eco*RI-*Bgl*II fragments into *Eco*RI- and *Bam*HI-cleaved pMU525; transcriptional fusions were made by inserting *Pst*I-*Bgl*II fragments into *Pst*I- and *Bgl*II-cleaved pMU2385 (Fig. 1).

pBR322 derivatives. The construction of these plasmids has been described previously (19). pMU617 is pBR322 carrying an intact RNAI gene. Consequently, this plasmid expresses RNAI from its own promoter and is used to deliver extra copies of RNAI. pMU662 carries the promoter and promoter-proximal region of the RNAII coding sequence but not the promoter for RNAI. This plasmid expresses the leader region of RNAII, which is the target for RNAI, from its own promoter and is used to titrate out RNAI molecules synthesized by other plasmids. pMU662 does not express RNAI.

β-Galactosidase assays. β-Galactosidase activity of mid-log-phase cultures was measured as described by Miller (14). Each sample was done in duplicate, and each assay was performed at least three times.

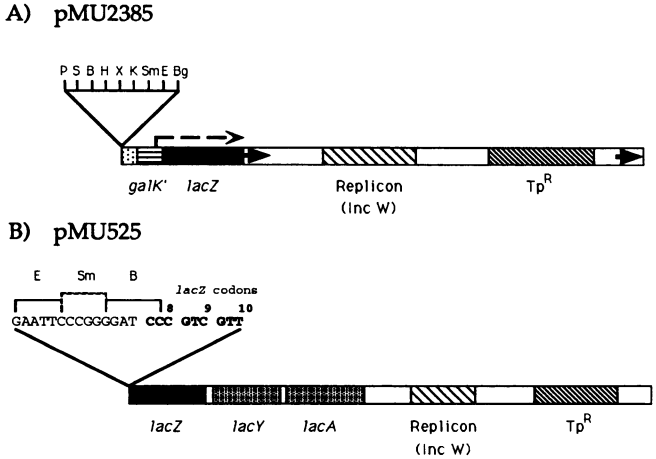

FIG. 1. Transcriptional *lacZ* fusion vector pMU2385 and translational *lacZ* fusion vector pMU525. (A) pMU2385 is a *galK'-lac'Z* fusion vector in which the amino terminus of *galK'* is fused in phase with *lacZ* such that translation but not transcription of *lacZ* initiates from *galK* (broken arrow). Termination codons present in all three reading frames between the polycloning site and the *galK* gene are indicated by a dotted region. Transcriptional terminators (filled arrows) terminate transcription from *lacZ* and transcription entering the polycloning site. (B) pMU525 is a *lacZ* fusion vector in which codon 8 of *lacZ* is linked to the polycloning site. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Tp^r, trimethoprim resistance.

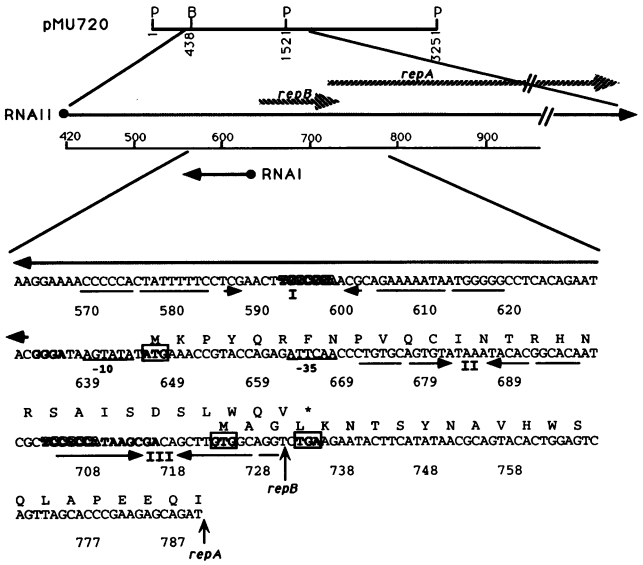

FIG. 2. Replication control region of pMU720 (19). RNAI and RNAII transcripts and the coding regions of the two *rep* genes (hatched arrows) are indicated. The nucleotide sequence between base positions 561 and 789 is shown together with the amino acid sequence of *repB* and the amino-terminal end of *repA*. The putative Shine-Dalgarno sequences of the two genes are shown in boldface, and the initiation and termination codons are boxed. The promoter region of RNAI and the putative stem-loop structures I, II, and III are indicated. The region of complementarity between stem-loops I and III is indicted by bases shown in outline. The vertical arrows indicate the 3′ ends of the pMU720 fragments inserted into the fusion vectors. P, *Pst*I; B, *Bam*HI. The *Pst*I site used in construction of the transcriptional fusions is the one shown at position 1 in the diagram of pMU720.

Prediction of RNA secondary structures. The computer programs of Zuker and his colleagues (7, 8, 35) were used to predict the secondary structures of RNA molecules.

RESULTS

Mutations in the start codon of *repB*. We have shown previously that in pMU720, translation of *repA* is dependent on translation of the upstream gene, *repB* (32). It was therefore of interest to examine the effects of a decreasing translational activity of *repB* on expression of *repA*. The approach used was to change the initiation codon of *repB* by site-directed mutagenesis and then analyze the effect of each mutation on expression of both *repB* and *repA*. This analysis was carried out using translational fusions in which codon 29 of *repB* or codon 23 of *repA* (Fig. 2) was fused in phase with codon 8 of *lacZ*. The expression of *lacZ* in both sets of fusions was from a wild-type *rep* (RNAII) promoter, and the *rep* mRNA leader sequence was intact. The DNA fragments used to construct *repA-lacZ* translational fusions were also inserted into the promoter cloning vector pMU2385 to produce transcriptional fusions. pMU2385 is a *galK'-lac'Z* fusion vector in which the amino terminus of *galK*, including the translation initiation region but not the promoter, is fused in phase with codon 8 of *lacZ*. This vector has termination codons present in all three reading frames between the polycloning site and the *galK* gene to minimize the effect of translational activity within the inserted DNA on the expression of *lacZ*. Thus, whereas expression of β-galactosidase from the translational fusions is dependent on both the activity of the RNAII promoter and the efficiency of translation of the gene (*repA* or *repB*) fused in phase with *lacZ*, expression of β-galactosidase from the transcriptional fusions is dependent solely on the number of RNAII molecules reading into *lacZ*. By comparing the data from the two types of *repA-lacZ* fusions, it was possible to ascertain whether the effect of a mutation was at the translational or the transcriptional level.

As can be seen in Table 2, the 953 U of β-galactosidase activity expressed from a *repB-lacZ* translational fusion in the presence of pBR322 increased to 4,186 U when the plasmid present in *trans* was the one encoding that part of the RNAII molecule which is the target for RNAI interaction. Expression of β-galactosidase decreased to 122 U when the plasmid present in *trans* encoded the RNAI molecule itself. In the presence of pBR322, expression of β-galactosidase from the *repA-lacZ* translational fusion was approximately eightfold lower than that from the *repB* fusion. However, expression from the *repA* fusion was more sensitive to RNAI than expression from the *repB* fusion, showing a greater increase when RNAI was titrated out by the RNAII molecules transcribed from the target plasmid and a total loss of activity when the gene dosage for RNAI was increased. Changing the *repB* start codon from ATG to GTG or CTG progressively diminished expression from both the *repB* and the *repA* translational fusions without abolishing the RNAI effect. Since these mutations did not decrease β-galactosidase activity from the *repA-lacZ* transcriptional fusions, they must act at the level of translation. These data indicate that translation of *repA* is tightly coupled to that of

TABLE 2. Effect of mutations in start codon of *repB* gene on expression of β-galactosidase from *repB-lacZ* and *repA-lacZ* translational and *repA-lacZ* transcriptional fusions

Mutation present in *rep-lacZ* fusion	β-Galactosidase activity (U) with pBR322 or its derivatives present in *trans*[a]						
	Translational fusion						*repA-lacZ* transcriptional fusion with pBR322
	repB-lacZ			*repA-lacZ*			
	pBR322	Target	RNAI	pBR322	Target	RNAI	
None	953	4,186	122	124	1,283	0.1	113
ATG→GTG	123	248	6	38	106	<0.1	195
ATG→CTG	57	155	0.6	12	56	<0.1	97
ATG→CCG	<0.1	<0.1	<0.1	0.4	1.8	<0.1	29

[a] β-Galactosidase activities were measured by the method of Miller (14). The results shown here are the average of at least three independent determinations. Vector (pBR322) or its derivatives were present in *trans*. Target (pMU662) carries nt 1 to 637 of pMU720 (19) and thus expresses the leader sequence of RNAII, which is the target for RNAI. This plasmid does not express RNAI, because it lacks the necessary promoter sequence. RNAI (pMU617) carries an intact RNAI gene and expresses RNAI (19). These plasmids do not carry *lacZ*.

repB and raise the possibility that there is no *repB*-independent initiation of *repA*. To test this possibility, CCG, which is not known to function as a start codon, was used to replace the ATG codon. This substitution reduced expression of *repA-lacZ* 310-fold, to 0.4 U, and even titration of RNAI by the product of the target plasmid raised this activity only slightly, to 1.8 U. Although this mutation reduced *repA-lacZ* expression from the transcriptional fusion from 113 to 29 U, the effect was minor when compared with the overall decrease in the expression of this gene. It is thus apparent that there is no *repB*-independent translation of the *repA* message.

One possible explanation for the data in Table 2 is that the RepB protein itself is a positive regulator of *repA*. To test this possibility, we introduced a double mutation into a *repA-lacZ* translational fusion: an insertion of C at sequence position 669, which causes a frameshift in repB, and a compensating deletion of nucleotide 701, which shifts *repB* back into its normal reading frame some 20 bases upstream of the *repA* start codon. Although the size of the resulting RepB protein is unchanged, it differs from the wild-type protein in 10 of the 29 amino acid residues (residues 10 to 19 inclusive). Despite these differences, the *repA-lacZ* translational fusion carrying the altered *repB* produced 83 U of β-galactosidase activity. Moreover, expression of *lacZ* from this fusion was fully regulated, since addition of extra copies of RNAI reduced β-galactosidase activity to <0.1 U, whereas removal of RNAI by titration increased this activity to 1,113 U. These data indicate that the importance of *repB* lies in the process of its translation rather than in the product of this translation.

Abolition of the *repB* stop codon. As can be seen in Fig. 2, part of the putative Shine-Dalgarno sequence and the initiation codon for *repA* are predicted to be sequestered in the stem of structure III. The *repB* stop codon is located 10 nt downstream of the *repA* initiation codon. Translation of *repB* should open up structure III to expose the translation initiation region and also the sequence CGCC preceding the Shine-Dalgarno sequence and postulated to be essential for pseudoknot formation (2). In order to determine whether the transient opening up of structure III by a translating ribosome was by itself sufficient to allow translation of *repA*, we used site-directed mutagenesis to change the *repB* stop codon. We changed TGA at position 732 to AGA, CGA, or GGA. These changes substituted codons for arginine or glycine for the stop codon and caused translation of *repB* to continue until it reached the next in-phase stop codon at position 771.

The consequence of these changes was almost complete loss of β-galactosidase activity in the *repA-lacZ* fusions, reducing levels ~300-fold, to ~0.4 U, in the presence of pBR322 and ~640-fold, to ~2 U, in the presence of the target plasmid. This loss of activity must have been due to failure to initiate translation of *repA*, since expression from transcriptional fusions was enhanced in the mutants. Moreover, given that all three mutations had the same effect, it was the loss of the stop codon and not the change in the primary sequence which affected *repA* translation.

This result means that transient melting of the secondary structure III is not sufficient to allow translation initiation of *repA* and suggested that the position of the *repB* stop codon may also be important for *repA* translation.

The effective window for coupling. We examined this question further by introducing into the strain in which the original *repB* stop codon had been changed new stop codons further downstream, at positions 735, 738, 744, and 753, and

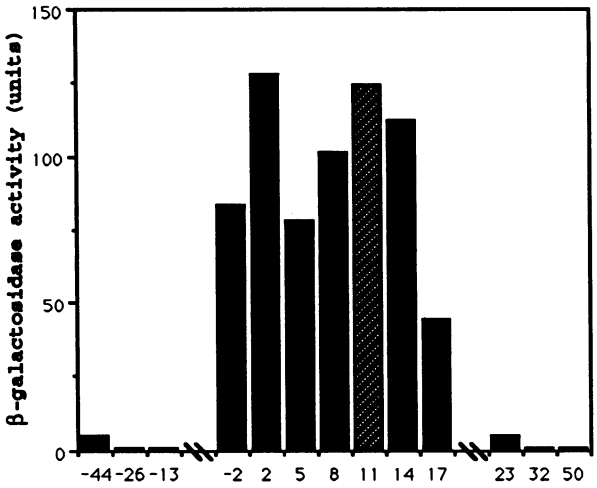

FIG. 3. Effect of the position of the *repB* stop codon on the expression of β-galactosidase from *repA-lacZ* translational fusions. The position of the stop codon is expressed relative to that of the first G of the GTG start codon of *repA*, which is considered +1. Thus, −1 denotes the adjacent position on the 5′ side, and +2 denotes the one on the 3′ side (i.e., the T of the GTG). The position of the stop codon in the wild type is indicated by the hatched column. Changes to stop codon positions between −2 and +17 are in increments of one codon.

by introducing into the wild type, upstream of the wild-type stop codon, other stop codons at positions 678, 696, 709, 720, 723, 726, and 729. The levels of expression of β-galactosidase in these strains are shown in Fig. 3. Results obtained with the target or RNAI coding sequence in *trans* confirm that β-galactosidase synthesis was still subject to control by RNAI, and the results obtained with the transcriptional fusions confirm that decreased levels of expression are the result of translational not transcriptional inhibition (data not shown). As can been seen in Fig. 3, whereas moving the stop codon from its normal position at +11 (nt 732) within the *repA* coding region to position +14 had little effect, moving it 3 bases further (to +17) reduced *repA* expression 2.8-fold, to 44 U. Moving the stop codon 12 bases to position +23 reduced expression to 5 U, and moving it 21 (to +32) or 38 (to +50) bases abolished *repA* expression.

By contrast, the *repB* stop codon could be moved at least 12 bases upstream of its normal position without reducing the *repA-lacZ* expression below 78 U (Fig. 3). However, the introduction of a stop codon at position −13 (nt 709, i.e., 23 bases upstream of the normal position) or −26 abolished *repA* expression, and a stop codon at position +44 (nt 678) reduced expression ~25-fold, to 5 U. It is noteworthy that, despite its distance from the *repA* start codon, the normal position of the *repB* stop codon appears to be optimal for *repA* expression. The stop codons at positions +2 and −2, which establish the very close coupling reported in many systems (1, 5, 18, 24, 27), resulted in unchanged or reduced *repA* expression (Fig. 3).

The mutations at positions −44, −26, and −2 were also introduced into *repB-lacZ* translational fusions to check for any leakiness in termination. These strains showed β-galactosidase activity of less than 1% of that of wild-type (data not shown), confirming that *repA* expression in these strains was not the result of inefficient termination at the introduced stop codons.

Confirmation of the role of the pseudoknot in *repA* transla-

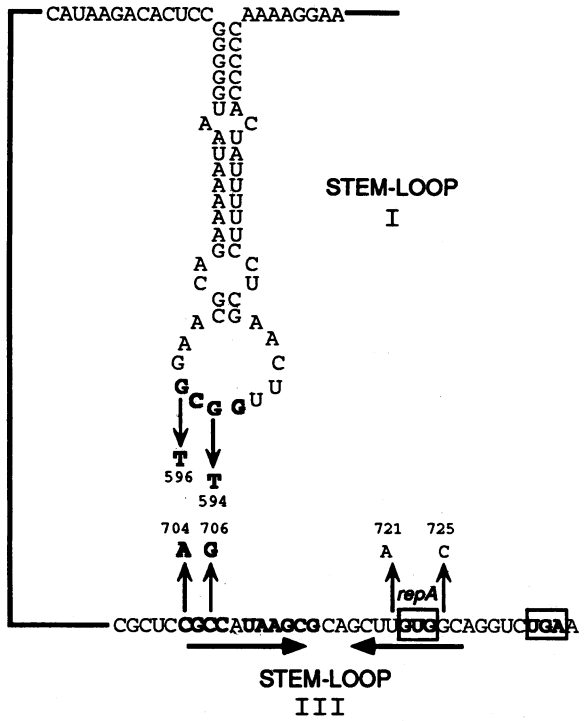

FIG. 4. Diagrammatic representation of the base substitutions used to check the importance of the pseudoknot for *repA* expression. The initiation codon of *repA* and the termination codon of *repB* are boxed, the putative Shine-Dalgarno sequence is in boldface, and bases indispensable for pseudoknot formation (2) are in outline type.

tion. In ColIb-P9, a plasmid closely related to the one described here (pMU720), mutations which decrease complementarity between bases in the loop of stem-loop I and bases adjacent to the Shine-Dalgarno sequence of the downstream gene (*repZ*) of the coupled gene pair reduce the expression of *repZ*. This has been explained in terms of requirement for a tertiary structure, a pseudoknot, which is postulated to stabilize the translation initiation region of *repZ* in its transiently unfolded configuration, thus permitting access by ribosomes (2). Since all the bases thought to be involved in pseudoknot formation in ColIb-P9 are conserved in pMU720, we expected this structure to exist in our system as well. To show that this is indeed the case, we examined the effect on *repA-lacZ* expression of changing two of the bases which had been shown to be important for the formation of the pseudoknot in ColIb-P9.

Substituting A for C at position 704 (Fig. 4) reduced β-galactosidase expression from the *repA-lacZ* translational fusion 62-fold, to 2 U (Table 3). Introduction of a compensating mutation at position 596, which restored the ability of bases 704 and 596 to pair with each other, increased *repA* expression to 268 U.

In the −2 mutant of Fig. 3, the change of T to A at position 721 created the sequence TAGTG. In the case of coupled genes whose stop and start codons overlap ("perfect" coupling), it is thought likely that upon termination of the upstream gene, the ribosome remains bound to the message and reinitiates the downstream gene. Since any secondary or tertiary structures involving the translation initiation region of the downstream gene would have been disrupted by the terminating ribosome, one would not expect these structures to have any role in reinitiation. We investigated whether this

TABLE 3. Effects of mutations predicted to affect formation of pseudoknot on expression of β-galactosidase from *repA-lacZ* translational fusions

Mutation(s) present in *rep-lacZ* fusion	β-Galactosidase activity (U) with pBR322 or its derivatives present in *trans*[a] from *repA-lacZ*			
	Translational fusions			Transcriptional fusions with pBR322
	pBR322	Target	RNAI	
None	124	1,283	0.1	113
C-704→A	2	18	<0.1	155
C-704→A, G-596→T	268	ND	0.3	412
C-704→A, T-721→A	7	20	0.4	241
C-704→A, G-596→T, T-721→A	98	ND	0.9	361
T-721→A	83	823	0.3	88
C-706→G	17	41	3	34
C-706→G, G-594→T	102	ND	6	115
C-706→G, G-725→C	14	36	0.8	68
C-706→G, T-721→A	34	41	34	120
C-706→G, G-594→T, T-721→A	78	ND	49	155

[a] β-Galactosidase activities were measured by the method of Miller (14). The results shown here are the average of at least three independent determinations. ND, not done. Vector (pBR322) or its derivatives were present in *trans*. Target (pMU662) carries nt 1 to 637 of pMU720 (19) and thus expresses the leader sequence of RNAII, which is the target for RNAI. This plasmid does not express RNAI because it lacks the necessary promoter sequence. RNAI (pMU617) carries an intact RNAI gene and expresses RNAI (19). These plasmids do not carry *lacZ*. RNAI added in *trans* to fusions with substitutions at position 594 and 596 carried the appropriate mutation to retain full complementarity with the RNAII target.

same argument applied to pseudoknot function by first creating the overlap (T-721→A) and then introducing the pseudoknot mutation C-704→A. As can be seen in Table 3, the introduction of the latter mutation reduced *repA* expression from 83 to 7 U of β-galactosidase activity. Furthermore, the introduction of the compensating mutation in stem-loop I (G-596→T) restored *repA* expression, confirming that pseudoknot formation was required for *repA* expression even when the stop and start codons overlapped. Although these pseudoknot mutations affected expression from the *repA-lacZ* transcriptional fusions (Table 3), their major impact was on translation.

Similar experiments were carried out with a second pseudoknot mutation, C-706→G. This substitution reduced expression 7.3-fold in the translational and 3.3-fold in the transcriptional *repA-lacZ* fusions. Because this mutation is predicted to affect the structure of stem III as well as the interaction with loop I to form the pseudoknot, we changed base 725 from a G to a C, which should reestablish the structure of stem III without reversing the effect on pseudoknot formation. Although the addition of this second substitution had virtually no effect on the levels of β-galactosidase from the translational fusion, it doubled those from the transcriptional fusion, indicating that the main effect of this pseudoknot mutation is also on *repA* translation. On the other hand, introduction of a compensating loop I mutation (G-594→T), which allows a G-U pairing to reestablish the pseudoknot, restored β-galactosidase synthesis to nearly wild-type levels. When the C-706→G mutation was introduced into fusions carrying the overlapping stop and start codons (T-721→A), *repA-lacZ* expression in the translational fusion was reduced only ~2.4-fold, from 83 to 34 U, and this expression was insensitive to RNAI control. Since β-galactosidase levels in the corresponding transcriptional

fusion increased ~1.4-fold, this second pseudoknot mutation also decreases translation of *repA* in the −2 mutant. Introduction of the compensating mutation G-594→T restored *repA* expression almost to the level in the T-721→A parent, although this expression showed very little control by RNAI. The mutations at positions 704 and 706 had only a small effect on β-galactosidase expression from *repB-lacZ* translational fusions (data not shown).

DISCUSSION

The results presented here show that translation of *repA* is absolutely dependent on translation of *repB* and that this coupling requires the ribosomes translating the upstream gene to terminate in close proximity to the *repA* initiation codon. Thus, no coupling was observed when the *repB* stop codon was placed as few as 10 bases upstream or 32 bases downstream of the *repA* initiation codon (positions −13 and +32 in Fig. 3).

The low but anomalous level of *repA* expression (4% of wild-type level) seen with the stop codon at position 678 (−44 in Fig. 3) is almost certainly due to activation by the terminating ribosome of a normally inactive *repA* start codon (a GTG in the same reading frame as the start codon at nt 722) at position 677. This is evident from the finding that the replacement of the normal *repA* start codon at position 722 by CAG did not affect *repA* expression from a plasmid carrying this *repB* termination mutation (data not shown).

An unusual feature of *repA* translation initiation is its requirement for a tertiary interaction (pseudoknot) involving the bases adjacent to the Shine-Dalgarno sequence. This requirement is evident even when the stop and start codons of *repB* and *repA* overlap, as shown with the mutation at position 704. An apparent exception to this is the case of the mutant with both a base change at position 706, which destroys the pseudoknot, and a new *repB* stop codon, which overlaps the *repA* initiation codon. In this case, there is a low level of *repA* expression which is insensitive to RNAI. The finding that the expression of *repB* in the translational fusion carrying the substitution at position 706 was fully regulated by RNAI (data not shown) indicates that in the former strain, translation of *repA* was uncoupled from that of *repB*. Introduction of a compensating mutation at 594 which allows a pseudoknot to form restores *repA* expression to the levels seen in the absence of the pseudoknot mutations and shows partial regulation by RNAI. An explanation for this uncoupling is provided by the prediction that stem-loop III does not form when the substitution at position 706 is combined with the one at 721. By contrast, the 704-721 mutant, which shows no independent expression of *repA*, is predicted to have a largely unchanged stem-loop III. Asano et al. (2) also reported that they have preliminary data suggesting that mutations predicted to disrupt stem-loop III allow *repZ* expression in the absence of *repY* translation.

If stem-loop III does indeed prevent expression of *repA*, then how does the translation of *repB* and the formation of a pseudoknot overcome this barrier? Asano et al. (2) proposed that ribosomes translating *repB* facilitate formation of the pseudoknot by disrupting stem-loop III and that the pseudoknot in turn inhibits the refolding of structure III. Our data clearly show that it is the process of termination, rather than translation per se, that is crucial for activation of *repA*. This implies that the terminating ribosome has to be positioned on the mRNA to prevent re-formation of structure III but allow formation of the pseudoknot. Examined in this light, the behavior of mutants with new stop codons at positions −44, −26, and −13 is understandable. In the first of these, the terminating ribosome does not reach stem-loop III, whereas in the last two, the ribosome is predicted to cover the sequence essential for formation of the pseudoknot and thus interfere with this tertiary folding. The mutant with the stop codon at −2, i.e., 12 bases downstream of the sequence predicted to be essential for formation of the pseudoknot, does express *repA*. Because of the closeness of the terminating ribosome to this pseudoknot sequence and because this is the perfect-coupling mutant, we expected *repA* expression in this mutant to be pseudoknot independent. As can be seen from Table 3, this is not the case. The simplest explanation for these data is that translational coupling between *repB* and *repA* is totally dependent on formation of the pseudoknot, which can occur when the ribosome terminates at position −2 but not when it terminates at −13 or −26. If we assume that the ribosome covers 12 bases 5′ of its tRNA A site, then the behavior of mutants in which *repB* translation terminates at new sites 3′ of the *repA* start codon is also understandable. Ribosomes terminating at positions +2 to +14 would be predicted to keep bases 704 to 707 unpaired, thus facilitating pseudoknot formation. Stop codons at these positions permitted *repA* expression. Termination at position +17 is predicted to disrupt the lower stem of structure III, whereas termination at +23 is predicted not to interfere with the refolding of structure III. Stop codons at these positions resulted in *repA* expression at 35 and 4% of the wild-type level, respectively. The main difficulty with this interpretation is the observation that a ribosome bound to mRNA protects approximately 20 nucleotides 5′ and 15 nucleotides 3′ to its tRNA A site against nuclease digestion (21, 25). From this observation, one might expect that base pairing, such as that suggested in pseudoknot formation, would also be inhibited over the same range. An alternative explanation is that the pseudoknot forms before and is therefore independent of *repB* translation. More work is necessary to distinguish between these two possibilities.

Both these explanations leave unanswered the question of why the position of the *repB* termination in the wild type offers such excellent coupling despite its distance from the *repA* initiation codon. Evidence that the unusual position of the *repB* stop codon is not the result of the need to maintain the coding sequence of *repB* is provided by our frameshift mutant and by examination of related plasmids. Thus, plasmids belonging to groups IncI1, IncIγ, IncB, IncZ, and IncK have highly homologous systems to control initiation of plasmid replication (20). The pseudoknot sequences are conserved in each, as is the spacing between the downstream pseudoknot sequence, the *repA* initiation codon, and the termination codon for *repB* (Fig. 5). In the case of IncZ, although the length of the coding sequence for *repB* is the same as for the others, the amino acid sequence differs in 7 of the 29 residues. This observation, particularly in light of the considerable divergence of the *repA* sequence of IncZ from that of the other I-complex plasmids (20; Fig. 5), supports the hypothesis that the importance of *repB* is the length of its coding sequence rather than the sequence itself.

The requirement of pseudoknot formation for the translational coupling between *repB* and *repA* appears to be absolute. This is not the result of the seemingly inefficient positioning of the *repA* start codon in relation to the *repB* stop codon, as this positioning appears to be optimal for coupling, and in the absence of major stem-loop III disruption, the perfect coupling is as pseudoknot dependent as

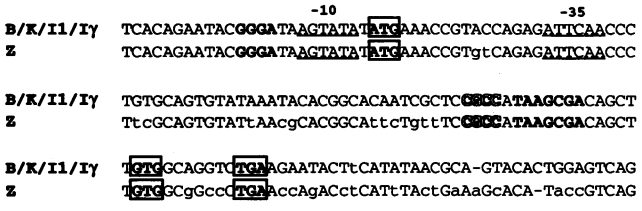

FIG. 5. Comparison of the coding regions (and adjoining sequences) of the *repB* genes from several I-complex plasmids (20). Sequences of group B, K, I1, and Iγ plasmids, which in this region differ at only one position located 9 bases downstream of the *repB* stop codon, are shown on the same line. Nucleotides conserved in all of the I-complex plasmids whose sequences are available are shown in capital letters. The promoter region of RNAI, putative Shine-Dalgarno sequence (boldface), and initiation and termination codons (boxed) of the *rep* genes as well as the bases thought to be indispensible for pseudoknot formation (outlined) are indicated.

wild-type coupling. This is surprising, as it indicates that translational reinitiation does not occur in the absence of pseudoknot formation, unlike the *trpE-trpD* and *trpB-trpA* systems, in which only the overlap between start and stop codons and a functional Shine-Dalgarno sequence is thought necessary for translational coupling (1, 5, 18). The lack of reinitiation at *repA* is not due to the inability of the ribosomes to recognize the *repA* translation initiation region, as disruption of stem-loop III results in significant although lower pseudoknot-independent *repA* translation. The finding that no translational coupling is observed in *repB-repA* in the absence of pseudoknot formation, even when perfect coupling is created, indicates that the function of the pseudoknot is not simply to keep the *repA* translation initiation region free of inhibitory secondary structures, as has been proposed by Asano et al. (2), and that pseudoknot formation actively initiates repA translation, probably via a pseudoknot-ribosome interaction.

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