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# Mutations Affecting Pseudoknot Control of the Replication of B Group Plasmids

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**The translational initiation region of the mRNA for the replication initiation protein (RepA) of pMU720 is predicted to be sequestered in an inhibitory secondary structure designated stem-loop III. Activation of *repA* translation requires both the disruption of stem-loop III by ribosomes involved in the translation and termination of the leader peptide RepB and the formation of a pseudoknot, a tertiary RNA structure. Disruption of stem-loop III by site-directed mutagenesis was found to be insufficient to allow high *repA* expression in the absence of pseudoknot formation, indicating that the pseudoknot acts as an enhancer of *repA* translation. Furthermore, extending the length of the leader peptide RepB and changing the distance between the pseudoknot and *repA* Shine-Dalgarno sequence were found to have major effects on the translation of *repA*.**

Plasmid replication in prokaryotes is, in many cases, dependent on a replication initiation protein (Rep), whose expression determines a plasmid's copy number and stability. In the case of pT181 and IncFII plasmids, the regulators of Rep synthesis are small countertranscript RNAs which inhibit Rep expression by binding with their target RNAs. In pT181, binding of the countertranscript RNA to the mRNA for the Rep protein is proposed to cause premature transcriptional termination by altering the folding of the RNA (9, 13). The mechanism by which the countertranscript RNAs of the IncFII plasmids R1 and NR1 regulate Rep expression is not clear. However, it is thought that they indirectly regulate Rep expression by sterically inhibiting the translation of a leader peptide. Translation of the Rep protein is believed to be dependent on the translation of the leader peptide, and the two genes are said to be translationally coupled (4, 23).

The replication frequency of the B group miniplasmid pMU720 is thought to be dependent on the expression of the *repA* gene, which is negatively regulated primarily at the posttranscriptional level by a small countertranscript RNA, RNAI (14, 15). RNAI is transcribed from the opposite strand of, and is complementary to, the leader region of the mRNA coding for *repA* (RNAII) (see Fig. 1). Computer analysis of the folding of RNAII indicates that the translational initiation region (TIR) of *repA* is sequestered within a secondary structure designated stem-loop III (see Fig. 1 and Fig. 2). It is postulated that stem-loop III inhibits ribosome access to the *repA* TIR. Previous studies have revealed that for *repA* to be expressed, stem-loop III must be disrupted by the translation and termination of a small leader peptide RepB, and a pseudoknot has to form (15). Pseudoknot formation is essential for the translation of *repA* and involves pairing between complementary sequences in RNAII. One of these sequences lies in the loop of a large structure called stem-loop I (proximal pseudoknot sequence), which is complementary to RNAI (see Fig. 2), and the other involves bases adjacent to the Shine-Dalgarno (SD) sequence of *repA* (distal pseudoknot sequence). RNAI is thought to regulate the translation of *repA* primarily by pairing with stem-loop I to form an RNA-RNA duplex. The major consequence of duplex formation for *repA* expression is the sequestering of the proximal bases required for the formation of the pseudoknot, although this duplex formation also interferes with the access of ribosomes to the *repB* TIR (15). In support of its primary role in the inhibition of pseudoknot formation is the recent finding that the initial site of RNAI-RNAII interaction in pMU720 involves three of the four proximal bases essential for the formation of the pseudoknot (20).

A similar model for control of Rep synthesis has been proposed for *repZ* expression in the closely related IncI1 plasmid ColIb-P9 (1, 2, 5, 19). It was in this system that pseudoknot formation was first reported through the isolation of replication-deficient mutants and their second-site revertants (1). Recently, Asano et al. (2) confirmed the existence of a structure sequestering the *repZ* TIR (designated structure III) and found that disruption of this structure allowed low but significant *repZ* expression which was independent of both the translation of the leader peptide *repY* and the formation of the pseudoknot. They also proposed that the pseudoknot acts as a translational enhancer of *repZ* expression, promoting selection of the *repZ* TIR via a pseudoknot-ribosome interaction.

In this report, we describe the effects of mutations that disrupt the secondary structure of stem-loop III on the translation of *repA*. We present evidence supporting the notion that the pseudoknot acts as a translational enhancer of *repA* and show that the length of *repB* and the spacing between the pseudoknot and the *repA* SD sequence are crucial for this enhancement.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and phages.** The strains of *Escherichia coli* K-12 used in this study are given below. JM101 [Δ(*lac-proAB*)*supE thi* F′(*traD36 proA*$^+$*B*$^+$ *lacI*$^q$*ZΔM15*)] (10) was used for cloning and propagating M13 derivatives. 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 undergone mutagenesis as described by Vandeyar et al. (22). 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 DNA sequencing and mutagenesis were M13tg130 and M13tg131 (8). The plasmids used are described in Table 1.

**Media, enzymes, and chemicals.** The minimal medium used

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TABLE 1. Plasmids

| Plasmid | Relevant characteristics[a] | Reference |
|---|---|---|
| pBR322 | pMB1 derivative; Ap Tc | 21 |
| pMU720 | Gal IncB; miniplasmid | 3 |
| pMU617 | pBR322 carrying nt 438 to 718 of pMU720; Ap IncB | 14 |
| pMU662 | pBR322 carrying nt 1 to 637 of pMU720 | 14 |
| pMU525 | *lac'ZYA'* Tp IncW; low-copy-number translational fusion vector | 14 |
| pMU575 | *galK'-lac'ZYA* Tp IncW; low-copy-number transcriptional fusion vector | 24 |
| pMU2385 | *galK'-lac'Z* Tp IncW; low-copy-number transcriptional fusion vector derived from pMU575 | 15 |
| pMU1550 | *repA-lacZ* translational fusion carrying nt 1 to 779 of pMU720; Tp IncW IncB | 15 |
| pMU1551 | *repA-lacZ* transcriptional fusion carrying nt 1 to 779 of pMU720; Tp IncW IncB | 15 |

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

was 0.5× buffer 56 (12) supplemented with 0.2% glucose, thiamine (10 μg/ml), and necessary growth factors. Enzymes and chemicals of a suitable grade were purchased commercially and not purified further. [$^{35}$S]dATPαS (1,000 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. (17). The method used for DNA sequencing was as described by Sanger et al. (18), except that T7 DNA polymerase was used instead of the Klenow fragment and terminated chains were uniformly labelled with [$^{35}$S]dATPαS.

**Site-directed mutagenesis.** In vitro mutagenesis was performed with the commercially available United States Biochemical Corp. kit. Oligonucleotides were synthesized with 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.** Because there are no convenient sites in pMU720 that would allow the movement of DNA fragments into the *lacZ* fusion plasmids pMU525 and pMU2385 (Table 1) to create *repA* fusions, polymerase chain reaction was used to generate a fragment with appropriate restriction enzyme sites as described previously (15). The fragment consisted of nucleotides (nt) 1 to 789 of pMU720 (Fig. 1) with an *Eco*RI linker at the 5′ end and a *Bgl*II linker at the 3′ end. This fragment was cloned into M13 vectors, and its sequence was checked for the presence of misincorporated nucleotides. Clones carrying error-free inserts were used as the source of DNA for site-directed mutagenesis. The translational fusions were constructed by inserting *Eco*RI-*Bgl*II fragments into *Eco*RI-and-*Bam*HI-cleaved pMU525. In this plasmid, codon 23 of *repA* is fused in phase with codon 8 of *lacZ*; thus, β-galactosidase activity is dependent on transcription from the RNAII promoter and *repA* translational initiation. The transcriptional fusions were made by inserting *Pst*I-*Bgl*II fragments into *Pst*I-and-*Bgl*II-cleaved pMU2385 (15). pMU2385 is a *galK'-lac'Z* fusion vector in which the amino terminus of *galK*, including the translational 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 prevent translational activity within the inserted DNA reading through into *lacZ*. Therefore, in this plasmid β-galactosidase expression is dependent solely on transcription from the RNAII promoter and *galK* translational initiation.

**pBR322 derivatives.** The construction of these plasmids has been described previously (14). 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 stem-loop I, which is the target for RNAI, but does not express RNAI. This plasmid is used to titrate out RNAI molecules synthesized by other plasmids. Neither pMU617 nor pMU662 carries *lacZ*.

**Measurement of β-galactosidase activity.** β-Galactosidase activity of mid-log-phase cultures was assayed as described by Miller (11). 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 (6, 7, 25) were used to predict RNA secondary structures.

## RESULTS

**Disruption of stem-loop III.** The *repA* TIR is predicted to be sequestered within stem-loop III, and it is postulated that this secondary structure blocks access by ribosomes, making the translation of *repA* dependent on the translation of *repB* and the formation of the pseudoknot. It was therefore of interest to determine how destabilizing stem-loop III affected *repA* expression. Stem-loop III was disrupted by site-directed mutagenesis as shown in Fig. 2, and the effect on *repA* expression was assayed, using low-copy-number plasmids (approximately one or two copies per chromosome) with translational fusions in which codon 23 of *repA* was fused in phase with codon 8 of *lacZ*. The effects of the various mutations on the regulation of *repA* by RNAI were determined by performing assays with either the gene for RNAI (producing saturating RNAI levels) or the gene for the RNA complementary to RNAI (i.e., "target" RNA to titrate out RNAI) in *trans* on a multicopy-number plasmid (~20 to 30 copies per chromosome) (14, 15). To ascertain whether the effect of the mutation was at the translational or transcriptional level, the DNA fragment used to construct the *repA-lacZ* translational fusion was also inserted into the promoter cloning vector pMU2385 (15), in which expression of *lacZ* is solely dependent on the number of RNAII molecules reading into *lacZ*. By comparing the data obtained from the two fusions, the effects of the mutation at both the transcriptional and translational levels could be obtained.

We had previously shown that single mutations were insufficient to seriously perturb stem-loop III (15), and therefore multiple mutations were used. The first set of mutations, located in the 5′ stem of stem-loop III, was predicted to not

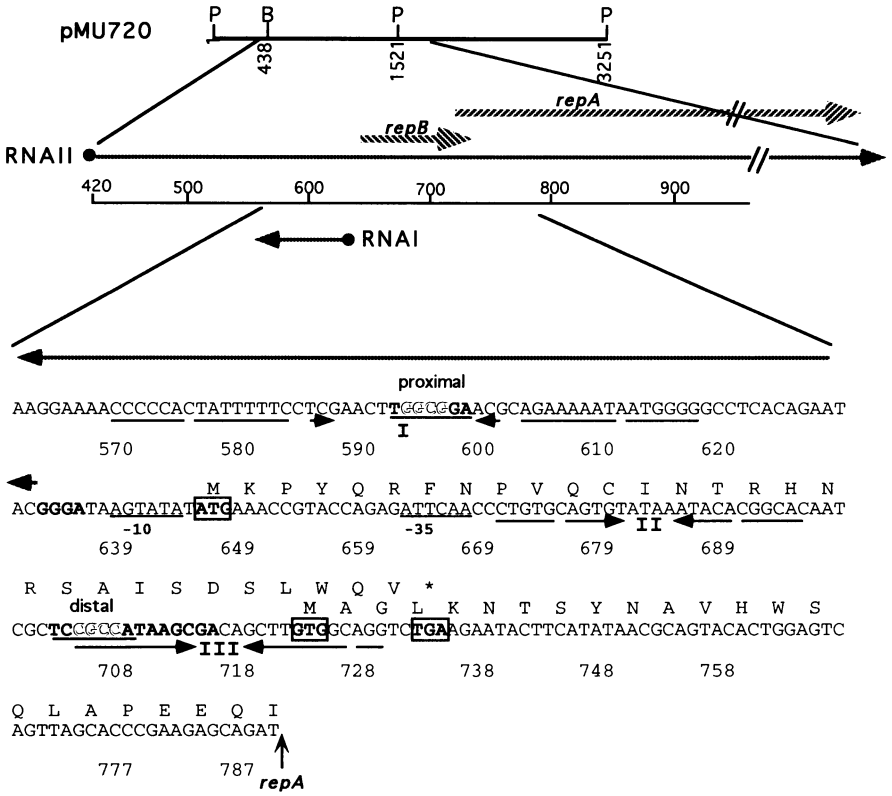


FIG. 1. Replication control region of pMU720 (14). RNAI and RNAII transcripts and the coding regions of the two *rep* genes (hatched arrows) are indicated. The nucleotide sequence between base positions 561 and 789 is shown together with the amino acid sequence of *repB* and the amino-terminal end of *repA*. The initiation and termination codons are boxed, and the putative SD sequences of the two genes are shown in boldface type. The promoter region of RNAI and the putative stem-loop structures I, II, and III 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. The *Pst*I site used in construction of the transcriptional fusions is the one shown at position 1 in the diagram of pMU720. P, *Pst*I; B, *Bam*HI.

only disrupt this secondary structure but also to abolish the pseudoknot (Fig. 2). As shown in Table 2, the S3.1 mutation resulted in an approximately fourfold decrease in β-galactosidase expression from the *repA-lacZ* translational fusion in the presence of the vector alone (pBR322). This expression was, however, no longer subject to RNAI control, because the addition of extra RNAI molecules or the titration of RNAI by the addition of target RNA in *trans* did not significantly affect *repA* expression. Data obtained with the transcriptional fusion indicated that the reduced *repA* levels resulted from changes in translation rather than transcription. This pseudoknot-independent expression of *repA* in S3.1 was also independent of the translation of *repB*, because the introduction of an ochre mutation of *repB* codon 18 (RepB-26), which prematurely terminates *repB* translation and which had previously been shown to result in almost complete loss of *repA* activity (Table 2) (15) did not significantly alter *repA* expression from either the *repA-lacZ* translational or transcriptional fusion (Table 2, S3.1-RepB-26).

To disrupt stem-loop III without affecting the distal bases involved in pseudoknot formation, mutations were inserted into the 3′ stem of stem-loop III (Fig. 2). Introduction of the S3.2 and S3.3 mutations singly was found not to significantly alter the level of *repA* expression that was insensitive to RNAI (data not shown). Combining these two sets of mutations to form S3.4 resulted in a 210-fold increase in the level of *repA* expression in the presence of excess RNAI, which was similar to the RNAI-insensitive *repA* expression observed in S3.1 (Table 2). In addition, S3.4 retained a high level of expression that was regulated by the presence or absence of RNAI. This suggests that stem-loop III had been significantly perturbed, as was predicted by computer analysis (data not shown). Introduction of the Pk.1 mutation which prevents pairing between the essential pseudoknot bases 596 and 704 (15) resulted in the loss of all RNAI-sensitive translation. Although this mutant showed fairly high basal levels of *repA* expression (49 to 63 U), this was 23-fold lower than the fully derepressed level (with target) obtained from S3.4, indicating that the formation of the pseudoknot is essential for high *repA* expression even when stem-loop III is significantly disturbed.

To test whether the translation of *repB* was required for the formation of the pseudoknot when stem-loop III was disturbed, *repB* translation was prematurely terminated by the introduction of the RepB-26 mutation, which prevents ribosomes reading into the stem-loop III region. This change caused no significant alteration in the expression of *repA* in the presence of the vector alone and caused only an approximately twofold reduction in expression when RNAI was titrated out by the target plasmid, showing that when stem-loop III is disrupted, translation of *repB* is not required for pseudoknot formation. Addition of the Pk.1 mutation (S3.4-Pk.1-RepB-26) further confirmed this, because its introduction abolished all RNAI-sensitive expression of *repA*. The higher-than-expected basal level of expression produced when both pseudoknot and

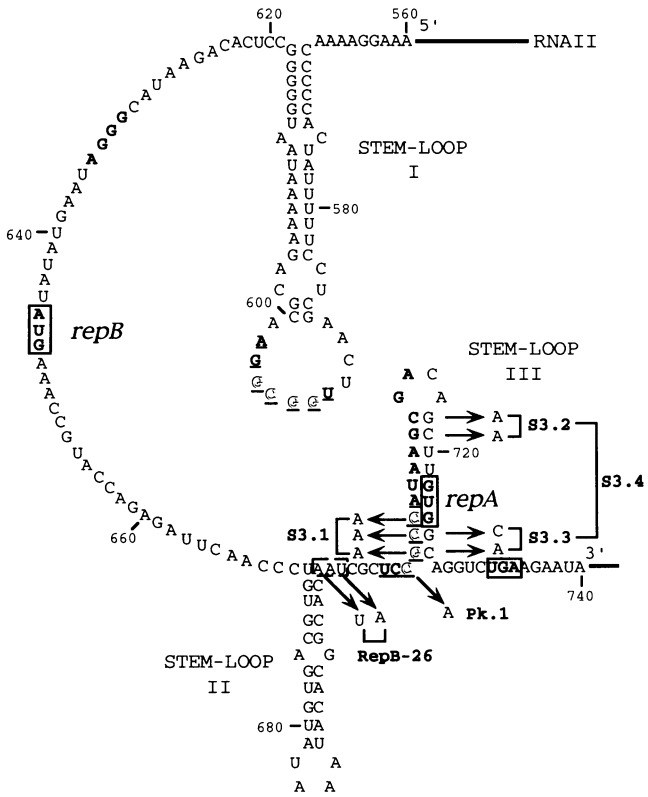


FIG. 2. Predicted secondary structure of the replication control region of RNAII with stem-loop III mutations. The initiation and termination codons are boxed, and the putative SD sequences are in boldface type. The complementary pseudoknot sequences are shown underlined and in boldface type, with the bases indispensable for pseudoknot formation in outline type. The sites and base changes of the mutations introduced are indicated.

chain-terminating mutations were introduced into S3.4 (S3.4-Pk.1-RepB-26) is probably a reflection of the exceptionally large increase in transcriptional expression (998 U) observed for this mutant.

**Role of stem-loop II and the length of *repB* on *repA* expression.** As shown in Fig. 2, the leader region of the *repA* mRNA is predicted to form three structures designated stem-loops I, II, and III. To examine whether stem-loop II had any role in regulation of *repA*, all the bases predicted to be involved in its formation were deleted by site-directed mutagenesis to create S2.Δ (Fig. 3). As shown in Table 3, deletion of stem-loop II does not alter the ability of RNAI to regulate *repA* expression. However, expression is increased ~3.5-fold in the presence of the vector alone and ~1.6-fold with the target plasmid in *trans*. Having established that stem-loop II was not essential for either the translation of *repA* or its regulation, we wished to determine whether the length of *repB* and hence the distance between the proximal and distal pseudoknot sequences may influence *repA* expression or its control. The approach used was to create a unique *Sca*I restriction site between stem-loop II and the distal pseudoknot sequence (RepB*Sca*I [Fig. 3]). Linker DNA of increasing length was then inserted, ensuring that the *repB* reading frame was maintained and that no alteration in the folding of stem-loops II and III was predicted as a result of the insertions (data not shown). Successive increases in the length of *repB* by 12, 33, and 60 bases resulted in successive decreases in the expression of *repA* (Table 3).

TABLE 2. Effects of mutations predicted to disrupt stem-loop III on expression of β-galactosidase from *repA-lacZ* fusions

| Mutation(s) present in *repA-lacZ* fusion | β-Galactosidase activity (U) from *repA-lacZ* with coresident plasmid present in *trans*[a] | | | |
|---|---|---|---|---|
| | Translational fusion | | | Transcriptional fusion with pBR322 |
| | pBR322 | RNAI | Target | |
| None | 124 | 0.1 | 1,283 | 113 |
| S3.1 | 28 | 34 | 29 | 168 |
| RepB-26[b] | 0.5 | <0.1 | 3 | 350 |
| S3.1-RepB-26 | 44 | 50 | 37 | 130 |
| S3.4 | 191 | 21 | 1,448 | 323 |
| Pk.1[b] | 2 | <0.1 | 18 | 155 |
| S3.4-Pk.1 | 49 | 63 | 58 | 366 |
| S3.4-RepB-26 | 209 | 57 | 735 | 259 |
| S3.4-Pk.1-RepB-26 | 106 | 130 | 72 | 998 |

[a] β-Galactosidase activities were measured by the method of Miller (11), and the results shown are the average 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 (14). Target (pMU662) carries nt 1 to 637 of pMU720 (14) and thus expresses the leader sequence of RNAII (stem-loop I), which is complementary to RNAI, but does not express RNAI. These plasmids do not carry *lacZ*.

[b] The mutations Pk.1 and RepB-26 have been described previously (15).

Although this translation could still be completely blocked by excess RNAI, indicating that stem-loop III was not perturbed, the fully derepressed levels in the presence of target were also seen to decrease steadily from 996 to 193 U. Transcriptional controls show that the results we obtained must be due to translational effects. Thus, by changing the length of *repB*, the efficiency of *repA* translation had been affected without loss of its strict control by RNAI. Presumably these changes impinge on the efficiency of pseudoknot formation or function.

**Can pseudoknot dependence be overcome by improving the SD sequence of *repA*?** A possible explanation for the absolute requirement of pseudoknot formation for *repA* translation is that since the predicted SD sequence of *repA* (UAAGCGA) has relatively weak homology with the consensus sequence (UAAGGAGG) (16), it may be poorly recognized by the ribosome. If so, mutations which strengthen the SD sequence should remove dependence on the pseudoknot. To test this hypothesis, the C at position 713 was replaced by a G (mutation SD.1 [Fig. 3]) to increase the SD sequence complementarity with the 16S RNA. This mutation also affects the stem of stem-loop III, so a second mutation was introduced at position 718 from a G to a C to reestablish base pairing at this point. As can be seen in Table 4, the latter mutation by itself (SD.2) had no significant effect on *repA* expression. Mutation SD.1 increased translation overall, in particular increasing the basal level of RNAI-insensitive translation from 0.1 to 16 U. The double mutation (SD.3) resulted in an overall decrease in expression compared with SD.1 but the level is still higher than that of the wild type, especially in RNAI-insensitive expression, despite a twofold decrease in transcription. Introduction of the *repB* terminating mutation into SD.3 (SD.3-RepB-26) almost abolishes RNAI-insensitive translation and reduces expression in the presence of the vector alone and derepressed expression to very low levels, confirming that stem-loop III has not been significantly disrupted in SD.3. When the pseudoknot mutation Pk.1 was introduced into SD.3, there was a sevenfold decrease in the derepressed levels observed in the presence of excess target. This indicates that expression in SD.3 is still predominately pseudoknot dependent. However, if one compares the results of SD.3-Pk.1 with Pk.1 (Table 2), it can be seen that

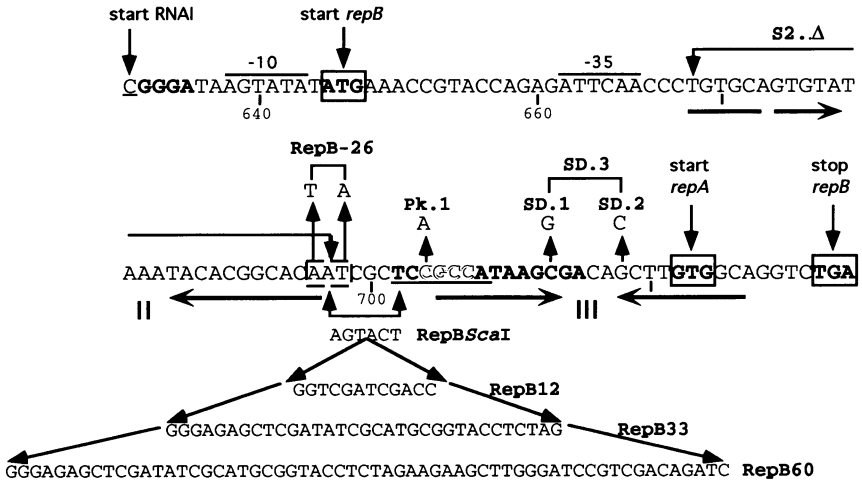


FIG. 3. Partial nucleotide sequence of the replication control region of pMU720 with mutations that alter *repB* length and the *repA* SD sequence. The −10 and −35 RNAI sequences and the putative stem-loop structures II and III are indicated. Start and stop codons of *repA* and *repB* are boxed, and the putative SD sequences are in boldface type. The distal pseudoknot sequence is underlined and in boldface type, with the bases indispensable for pseudoknot formation in outline type. The sites and base changes of the mutations introduced are indicated.

expression of SD.3-Pk.1 increased in the presence of the vector alone from 2 to 57 U and showed an 80-fold elevated RNAI-insensitive translation and a 13-fold increase in fully expressed levels. Thus, improving the SD sequence for *repA* has helped to restore some pseudoknot-independent translation. Further changes may be required to establish full independence. When both Pk.1 and RepB-26 mutations are introduced into SD.3 (SD.3-Pk.1-RepB-26), i.e., when pseudoknot formation is prevented and the stem of stem-loop III remains closed, no translation is observed under any conditions.

**Insertions between the pseudoknot and *repA* SD sequence.** The enhancement of *repA* translation which is observed when the pseudoknot forms is postulated to involve a direct interaction between the pseudoknot and the ribosome, which in some way allows recognition of the *repA* TIR (15). Since the distal pseudoknot sequence is immediately adjacent to the SD sequence of *repA* (Fig. 4), we examined whether this spacing was important for translational enhancement by inserting bases between these two sequences (Fig. 4). To retain the correct *repB* reading frame, single and double base insertions were compensated for by deleting a C at base 701 and inserting a C at base 669, respectively. As can be seen in Table 5, increasing the spacing between the SD sequence and the distal pseudoknot sequence by one (PSD.1T and PSD.1A), two (PSD.2), three (PSD.3), and six (PSD.6) bases caused successive decreases in the level of *repA* expression both when the vector was there alone and when excess target removed RNAI by titration. Thus, the spacing between the pseudoknot and the *repA* SD sequence is important for the efficient translation of *repA*. RNAI-insensitive translation remained extremely low, indicating that stem-loop III is not seriously perturbed by the insertions. The higher levels of translation observed when A rather than T was inserted may be explained by the fact that the A can pair with base 591 of stem-loop I, possibly extending the pseudoknot structure.

TABLE 3. Effects of mutations which alter the length of *repB* on expression of β-galactosidase from *repA-lacZ* fusions

| Mutation present in *repA-lacZ* fusion | β-Galactosidase activity (U) from *repA-lacZ* with coresident plasmid present in *trans*[a] | | | |
|---|---|---|---|---|
| | Translational fusion | | | Transcriptional fusion with pBR322 |
| | pBR322 | RNAI | Target | |
| None | 124 | 0.1 | 1,283 | 113 |
| S2.Δ | 446 | 0.1 | 1,993 | 148 |
| RepB*Sca*I | 83 | 0.1 | 996 | 264 |
| RepB12 | 67 | 0.1 | 709 | 615 |
| RepB33 | 25 | 0.1 | 250 | 414 |
| RepB60 | 13 | 0.2 | 193 | 612 |

[a] β-Galactosidase activities were measured by the method of Miller (11), and the results shown are the average 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 (14). Target (pMU662) carries nt 1 to 637 of pMU720 (14) and thus expresses the leader sequence of RNAII (stem-loop I), which is complementary to RNAI, but does not express RNAI. These plasmids do not carry *lacZ*.

## DISCUSSION

The results presented here show that although translation of *repA* is inhibited by the presence of stem-loop III, disruption of this structure alone is insufficient to allow high *repA* expression in the absence of the pseudoknot. This is clearly demonstrated by the 25-fold difference in *repA* translation between the S3.4 and S3.4-Pk.1 mutants, when RNAI was removed by titration. These data support our previous work (15) and that of Asano et al. (2) on *repZ* expression in ColIb-P9, which suggest that the function of the pseudoknot is not simply to keep the *repA* TIR free of inhibitory secondary structures but to actively enhance the translation of *repA*.

The low level of *repA* translation observed in the absence of the pseudoknot in plasmids in which stem-loop III has been disrupted was independent of both RNAI control and the translation of *repB* and probably reflects the inherent inefficiency of the *repA* TIR. It is noteworthy that this independent expression was consistently higher in the presence of excess RNAI, suggesting that ribosomes translating *repB* actually inhibit independent *repA* translation. This confirms previous results (15) which indicated that translational coupling between *repB* and *repA* does not occur in the absence of pseudoknot formation.

TABLE 4. Effects of mutations in the SD sequence of *repA* on expression of β-galactosidase from *repA-lacZ* fusions

| Mutation(s) present in *repA-lacZ* fusion | β-Galactosidase activity (U) from *repA-lacZ* with coresident plasmid present in *trans*[a] | | | |
|---|---|---|---|---|
| | Translational fusion | | | Transcriptional fusion with pBR322 |
| | pBR322 | RNAI | Target | |
| None | 124 | 0.1 | 1,283 | 113 |
| SD.1 | 387 | 16 | 1,980 | 145 |
| SD.2 | 116 | 0.3 | 1,139 | 81 |
| SD.3 | 167 | 5 | 1,644 | 53 |
| SD.3-RepB-26 | 12 | 0.6 | 23 | 252 |
| SD.3-Pk.1 | 57 | 8 | 243 | 97 |
| SD.3-Pk.1-RepB-26 | 1 | 0.2 | 4 | 376 |

[a] β-Galactosidase activities were measured by the method of Miller (11), and the results shown are the average 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 (14). Target (pMU662) carries nt 1 to 637 of pMU720 (14) and thus expresses the leader sequence of RNAII (stem-loop I), which is complementary to RNAI, but does not express RNAI. These plasmids do not carry *lacZ*.

Disruption of stem-loop III without affecting bases involved in the pseudoknot allowed the pseudoknot to form without the requirement for the translation of *repB*. This result indicates that the ribosomes translating *repB* are required only for the unfolding of stem-loop III and are not actively involved in pseudoknot formation. However, *repA* expression does appear to be more efficient when *repB* is translated, as in the presence of the target plasmid, translation from S3.4 was ~2-fold higher than was observed for S3.4-RepB-26. This result is in agreement with Asano et al. (2), who found that *repZ* expression in the absence of structure III was significant although decreased when translation of *repY* was prematurely terminated. The exact reason for the increased efficiency is unknown, but the termination of ribosomes translating *repB* may facilitate presentation of the distal pseudoknot bases for pseudoknot formation or the ribosomes translating *repB* may also translate *repA*. Since the pseudoknot can form and activate *repA* translation when *repB* is prematurely terminated and stem-loop III is disrupted, the pseudoknot does not enhance the expression of *repA* via frameshifting of *repB* translation.

The distance between the pseudoknot and the *repA* TIR appears to be crucial for the translation of *repA*, because even small changes in the spacing result in severely reduced *repA* levels. Although such insertions also alter the distance between the pseudoknot and the *repB* stop codon, this is unlikely to account for the reduced *repA* expression, because previous studies have found that extending *repB* translation by either three or six bases results in 90 and 35% of the wild-type *repA* levels, respectively (15), whereas insertions of three and six bases between the pseudoknot and *repA* SD sequence, reduced expression to only 6.5 and 2.0% of wild-type *repA* levels, respectively. The reason for the insertional effect on the translation of *repA* is unknown, but the altered spacing may inhibit either the formation of the pseudoknot or translational enhancement by the pseudoknot. Since there are no physical means, at present, to identify the pseudoknot in pMU720, neither possibility can be dismissed, and further experiments are currently in progress. However, inhibition of pseudoknot formation appears unlikely, because the insertions do not affect either the sequence of the complementary pseudoknot bases or the distance between them, and the abilities of ribosomes translating *repB* to disrupt stem-loop III are not significantly affected in these mutants. Since the pseudoknot is thought to enhance the translation of *repA* via a pseudoknot-ribosome interaction, inhibition of this enhancement by the insertions may indicate that the pseudoknot is recognized in conjunction with the *repA* SD sequence and any separation prevents either ribosome binding or translational initiation.

It is of interest that despite the fact that pMU720 lacks any control at the level of transcriptional initiation from either the RNAI or RNAII promoter (14), several of the mutations used in this study significantly affected expression from the transcriptional *lacZ* fusions. The reason for this is unknown, but, experiments are currently in progress to determine whether these results are the consequence of additional posttranscriptional control mechanisms affecting RNA stability or termination.

The length but not the amino acid sequence of the leader peptide RepB is conserved in plasmids belonging to groups IncI1, IncIγ, IncB, IncZ, and IncK (15). One potential reason for this conservation appears to be the importance of *repB* length on *repA* translation. Shortening *repB* by deleting stem-loop II increased *repA* translation by ~3.5-fold, whereas insertions of increasing length progressively diminished *repA* expression. Although, like the insertions between the pseudoknot and *repA* TIR, *repB* length may affect either pseudoknot formation or function, it is more likely that it is the formation of the pseudoknot which is affected as the distance between the two complementary pseudoknot sequences is altered. Changing the length of *repB* may affect the formation of the pseudoknot either by altering the competition between the binding of the complementary pseudoknot sequences with other sequences within *repB* or by affecting presentation of the sequences for pseudoknot formation. Although stem-loop II is not directly involved in the regulation of *repA*, its presence may increase the probability of pseudoknot formation compared with an equivalent unstructured stretch of RNA, by bringing the complementary pseudoknot bases closer together and sequestering bases which could compete with the pseudoknot sequences for binding.

An unusual feature of the expression of *repA* is that although

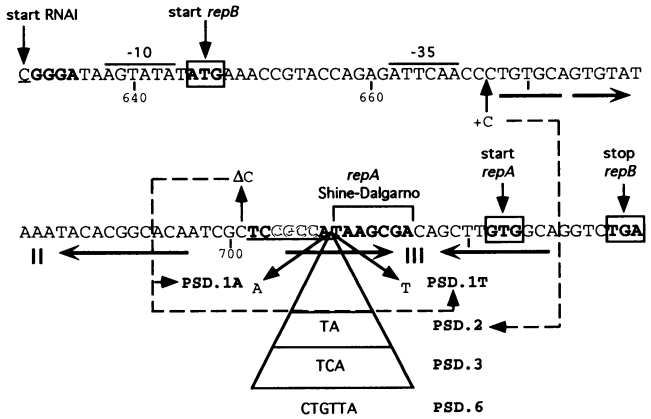


FIG. 4. Partial nucleotide sequence of the replication control region of pMU720 with mutations affecting the spacing between the pseudoknot and *repA* SD sequence. The −10 and −35 RNAI sequences and the putative stem-loop structures II and III are indicated. Start and stop codons of *repA* and *repB* are boxed, and the putative SD sequences are in boldface type. The distal pseudoknot sequence is underlined and in boldface type with the bases indispensable for pseudoknot formation in outline type. The sites and base changes of the mutations introduced are indicated.

TABLE 5. Effects of insertions between the distal pseudoknot sequence and the *repA* SD sequence on expression of β-galactosidase from *repA-lacZ* fusions

| Mutation present in *repA-lacZ* fusion | β-Galactosidase activity (U) from *repA-lacZ* with coresident plasmid present in *trans*[a] | | | |
|---|---|---|---|---|
| | Translational fusion | | | Transcriptional fusion with pBR322 |
| | pBR322 | RNAI | Target | |
| None | 124 | 0.1 | 1,283 | 113 |
| PSD.1T | 44 | 0.2 | 251 | 174 |
| PSD.1A | 82 | 0.5 | 541 | 213 |
| PSD.2 | 19 | 0.3 | 114 | 92 |
| PSD.3 | 8 | 0.3 | 35 | 79 |
| PSD.6 | 2 | 0.3 | 4 | 135 |

[a] β-Galactosidase activities were measured by the method of Miller (11), and the results shown are the average 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 (14). Target (pMU662) carries nt 1 to 637 of pMU720 (14) and thus expresses the leader sequence of RNAII (stem-loop I), which is complementary to RNAI, but does not express RNAI. These plasmids do not carry *lacZ*.

the *repB* and *repA* genes overlap by 10 nt, there is no translational coupling in the absence of the pseudoknot. This is evident even when the termination of *repB* is made to overlap the initiation of *repA* as in the *trpE-trpD* and *trpB-trpA* systems (15). The dependence on the pseudoknot appears to be the result of the poor SD sequence of *repA*, because improving the sequence results in significant *repA* translation in the absence of the pseudoknot. This expression is totally dependent on the translation of *repB* because of the inhibitory nature of stem-loop III. Thus, in SD.3, direct translational coupling between *repB* and *repA* has been established. This conclusion is supported by the degree to which the regulation of *repA* by RNAI in SD.3-Pk.1 resembles that of *repB*. The translation of *repB* is normally repressed approximately eightfold in the presence of excess RNAI and induced approximately fourfold when RNAI is titrated out. Similarly expression of the SD.3-Pk.1 mutant is repressed sevenfold and induced approximately fourfold when RNAI or target was added in *trans*, respectively. SD.3-Pk.1 therefore mimics the situation that is thought to occur in the IncFII plasmids R1 and NR1, because RNAI now indirectly regulates the translation of *repA* via the leader peptide. Establishing direct translational coupling between *repB* and *repA* in the absence of the pseudoknot (SD.3-Pk.1) clearly demonstrates the advantage of directly regulating *repA* expression via inhibition of pseudoknot formation compared with indirect regulation. In a wild-type plasmid, *repA* can normally be expressed to very high levels when RNAI is titrated out (up to 1,283 U) and is regulated over a 10,000-fold range, whereas translation in SD.3-Pk.1 is unable to be expressed above 250 U and is controlled only over a 30-fold range. This advantage is also evident when the expression of *repA* in pMU720 is compared with *repA1* of NR1, whose translation ranges only over 175-fold (23). The ability of the pseudoknot to enhance Rep translation may explain the lack of any transcriptional control in pMU720 and ColIb-P9, since Rep expression can be induced to very high levels when RNAI levels are low, such as when a plasmid first enters a cell. In contrast, the high Rep levels required for establishment in the host cell of the IncFII plasmids occurs through both an increase in translation of the Rep protein and extra transcription resulting from derepression of a second Rep promoter.

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