An RNA Pseudoknot as the Molecular Switch for Translation of the repZ Gene Encoding the Replication Initiator of IncIα Plasmid ColIb-P9*

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Evidence has been accumulating that RNA pseudoknots, stem-loop structures containing an extended double helix with a complementary sequence outside the loop (for review, see Refs. 1 and 2), play an important role in control of gene expression at the translational level. In the Escherichia coli ribosomal protein α and rpsO operons, RNA pseudoknots act as the binding sites for proteins S4 and S15, respectively, being responsible for autoregulation of the respective operon genes (3, 4). In some mammalian retroviruses and in the mRNA for the rat ornithine decarboxylase antizyme, RNA pseudoknots function as regulatory signals for +1 or −1 frameshifting or translational read-through to allow translation of sequences downstream of stop codons (5–7). In IncIα ColIb-P9 and IncB pMU720 plasmids, possible RNA pseudoknots have been proposed to be the molecular switches for translation initiation of the replication initiator genes, thereby controlling plasmid copy number in the host cells (8–10).

ColIb-P9 is a low-copy number and self-transmissible plasmid with a size of 93 kilobases (kb). The basic replication of ColIb-P9 consists of a 3-kb DNA fragment which contains sufficient information required for autonomous replication and copy number control (Ref. 11, also see Fig. 1). The frequency of ColIb-P9 replication is limited by the degree of expression of the repZ gene encoding a 39-kDa replication initiation protein, RepZ, which is thought to react with the replication origin (11). Previous studies revealed that repZ expression was negatively controlled by the antisense Inc RNA of about 70 bases, the product of the inc gene that governs the phenotype of plasmid incompatibility (11, 12). Inc RNA is transcribed from the non-coding strand of the leader region of repZ and binds to RepZ mRNA at the complementary region to form an RNA-RNA duplex (12). Two promoter down mutations, inc1 and inc2, altered the −35 and −10 regions of the inc gene promoter, Pinc, and increased the level of repZ expression without affecting significantly the amount of RepZ mRNA (Refs. 11 and 12; see Fig. 1B for positions of inc1 and inc2). Thus, Inc RNA negatively controls repZ expression at the translational level.

In an effort to elucidate the molecular events required for repZ expression, we also isolated replication-defective (rep) mutations (8, 13). Two types of rep mutations were isolated that were located in the leader region of repZ. One type including rep57 disrupted an upstream open reading frame called repY which encodes a polypeptide of 29 amino acids. rep57 was an amber mutation of repY codon-11 (13) and reduced the level of repZ expression without affecting the amount of RepZ mRNA (8, 13). Changing the amino acid composition of the reading frame by frameshift mutations did not affect repZ translation, whereas repZ translation was abolished profoundly when the position of the repY stop codon, located 7 bases downstream of the repZ start codon, was shifted either by frameshift mutations or changing the stop codon to a sense codon, indicating that the translational termination event of the repY reading frame, not the RepY polypeptide itself, is required for repZ translation (9). Since the level of repY expression was inversely correlated with the amount of endogenous Inc RNA (13) and the 5′-end of Inc RNA was located 3 bases upstream of the repY
mRNA between the 5′-rGGCG-3′ and 5′-rCGCC-3′ sequences, distantly separated at positions 327–330 and 437–440, respectively (Ref. 8, see Figs. 1B and 2C). rep2006 (G327A) and rep2041 (G330A) changed the first and fourth guanine residues of the former, whereas rep2044 (G438A) altered the second guanine residue of the latter (8). Besides, compensatory base changes to each of the rep mutations in the other sequence restored the ability to produce RepZ (8). This intramolecular base pairing had the potential to form a pseudoknot with a stem-loop structure designated I which was predicted as the target site of Inc RNA. On the other hand, another stem-loop structure designated III sequestered the 5′-rCGCC-3′ sequence together with the repZ RBS. Based on these findings, we proposed a regulatory model (Fig. 1B), in which translation and termination of repY induces the intramolecular base pairing, leading to form a novel RNA pseudoknot for repZ translation. Inc RNA inhibits the formation of the pseudoknot, both directly and indirectly by inhibiting repY translation. Consistent with this model, disruption of structure III by two mutations (AG451 and C459A) resulted in partial derepression of repZ expression (9). In addition, Inc RNA inhibited repZ expression more efficiently than repY expression (8). However, biochemical evidence for the proposed pseudoknot had not been provided.

Several other fundamental questions have arisen during the course of studies on the control of repZ translation: does pseudoknot formation simply expose the repZ RBS to the ribosome, or also enhance repZ translation through specific interaction with the ribosome? And why does a single base substitution in the proposed 5′-rGGCG-3′/5′-rCGCC-3′ duplex affect profoundly the intramolecular base pairing, when the complementary sequences consist of 7 bp or more (see Fig. 2C)? What is the efficiency with which repY translation stimulates the initiation of repZ translation? To answer some of these questions, we set out to characterize biochemically the pseudoknot. In this report, we demonstrate that RepZ mRNA synthesized in vitro formed structures I and III, but not the pseudoknot. However, disruption of structure III by means of base substitution/deletion resulted in the formation of a unique RNA pseudoknot in vitro. Evidence is also presented indicating that the pseudoknot formed in vitro is generated in vivo, affecting directly the level of repZ expression.

**EXPERIMENTAL PROCEDURES**

**Bacteria, Phages, and Plasmids**—The E. coli K12 strains W3110 and W3110 (inc−) were used as the hosts of λ-ColIb-P9 hybrid phages. Strains MV1184 (14) and NM522 (15) were the hosts of phage M13. Strains BW313 (dut ung) (16) and BMH71-18mutS (17) were used for site-directed mutagenesis of the rep mRNA leader region. Strain MC1061 (lacX74) (18) was employed for lacZ fusion studies. λ-ColIb-P9 hybrid phages and plasmids used in this study were listed in Table I. λW3 (inc1) was isolated as a clear-plaque forming mutant from λCH10W by plating with W3110 (inc−) cells as described (11). The original mini-ColIb-P9 replicon pCH10 (11) was found to have a copy-up mutation by changing cytosome to adenine at position 334 (C334A) within the inc gene region. Therefore, we re-constructed the wild type mini-ColIb-P9 as pK10 (92) and pDX14-12 (inc1 rep57 A29) and pDX14-12 (inc1 rep57 A29) were prepared by site-directed mutagenesis as described (8) using oligo-A25, 5′-GTATCTTACAGATCATTTTCTTGTGCGTATAGG-3′ and oligo-A28, 5′-CGCTTATGGCGGAGGTTGGCTGCGGTATT-3′, respectively. pDX14-A33 was constructed by introducing rep57, an amber mutation of repY codon-11 (13), into pDX14-W3. pK3A40-W3 was a translational repZ-lacZ fusion and was used to measure the level of repZ translation activity in the absence of Inc RNA. pK3A40-A52 carrying a repY-repZ-lacZ fusion in-frame was constructed by insertion of a cytosome between positions 403 and 404 into the repZ-lacZ fusion and was used to measure the level of repZ translation activity in the absence of Inc RNA.

**Measurement of β-Galactosidase Activity**—The β-galactosidase activity expressed from translational lacZ fusions was assayed as described (8). The specific activity of the enzyme was expressed as Miller units (23). The values reported here are the results of at least three inde
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| Phage or plasmid | Description | Source |
|------------------|-------------|--------|
| M13KO7 | Helper phage for the preparation of single-stranded DNA | Ref. 14 |
| λVI | Cloning vector | Ref. 19 |
| λCH10W | 3.0-kb mini-ColIb-P9 into λVI | This paper |
| λW3 | λCH10W carrying the incI mutation | This paper |
| Plasmid | pTZ19R | Cloning vector |
| pMC1403 | lacZ fusion vector | Ref. 20 |
| pXX564 | Mini-F with a Km’ fragment | Ref. 21 |
| pAK10 | 3.0 kb mini-ColIb-P9 with a Km’ fragment | S. Hiraga |
| pDX14-W3 | 1120-bp EcoRI-SalI fragment of AW into pTZ19R | Ref. 22 |
| pDX14-A33 | pDX14-W3 carrying the rep57 mutation | This paper |
| pDX14-A25 | pDX14-A33 carrying the A25 mutation | This paper |
| pDX14-A28 | pDX14-A25 carrying the A28 mutation | This paper |
| pKA10 | 281-bp NspI-Sau3A fragment of pAK10 into pTZ19R | This paper |
| pKA10-A25 | 281-bp NspI-Sau3A fragment of pDX14-A25 into pTZ19R | This paper |
| pKA10-A28 | 281-bp NspI-Sau3A fragment of pDX14-A28 into pTZ19R | This paper |
| pKA15 | pKA10 deleting the HindIII site | This paper |
| pKA16 | pKA15 carrying the A444T mutation | This paper |
| pKA16-A10 | pKA16 carrying the rep2044 mutation | This paper |
| pKA16-A5W | lacZ fusion vector derived from pMC1403 | Ref. 8 |
| pKA140W | repZ-lacZ fusion in multi-copy: 1118-bp EcoRI-SalI fragment of pKA140W into pXX564 | This paper |
| pKA340 | repZ-lacZ fusion in low copy: 7-kb EcoRI-SalI fragment of pKA140W into pXX564 | This paper |
| pKA340-W3 | pKA340 carrying incI | This paper |
| pKA340-A33 | pKA340 carrying incI rep57 | This paper |

Due to the presence of a pseudoknot structure in the leader region of ColIb-P9, we prepared in vitro RNA samples labeled with $^{32}$P at the 5’-end as described under “Experimental Procedures.” This RNA corresponds to the wild-type ColIb-P9 sequence from positions 244 to 524 that covered the entire region containing structures I to III. After the RNA sample was partially digested by RNases T1 (specific for guanine residues in single-stranded regions), RNase Bc (specific for pyrimidines in single-stranded regions), or RNase V1 (specific for double-stranded regions), the resulting products were analyzed by electrophoresis on a denaturing polyacrylamide gel. The patterns of the autoradiography are shown in Fig. 2, A and B, and the cleavage sites identified are summarized in Fig. 2C, in which the deduced secondary structures are also presented. We observed three stem-loop structures, I, II, and III as predicted previously (13). In addition, we found three new stem-loop structures designated Ia, Iib, and IV, which could not be predicted computationally. Note that RNase Bc appeared to cleave any ribonucleotides in the 5’ side of loop sequences in the assay condition employed in this study.

When we looked at structure I and III regions which contained the two complementary sequences, 5’-rGGCGG-3’ (position 327–330) and 5’-rCGCC-3’ (position 437–440), required for the intramolecular base pairing, two characteristic cleavage patterns were observed. First, both G-327 and G-328 residues in structure I were strongly cleaved by RNase T1 while both G-330 and G-331 residues were hardly cleaved (Fig. 2, lane 1). Additionally, C-324, U-325, and U-326 were weakly cleaved by RNase Bc. We inferred from these patterns that the 5’-rUGGC-3’ of the leader sequence is folded in structure I as well. Thus, the data showed that the translational initiation signals of repZ are embedded within structure III (Fig. 2C). Likewise, the data showed that the 5’-rGCGG-3’ sequence is folded in structure III as well. Thus, these data, taken together, revealed that the pre-mRNA leader region contains 7 and 11 nucleotides at the 5’- and 3’-end respectively that are included in the wild-type structure, and that the two structures are folded as a single pseudoknot.

RESULTS

Secondary Structures of RepZ mRNA Leader Region—To characterize biochemically secondary structures in the RepZ mRNA leader region, we prepared in vitro RNA samples labeled with $^{32}$P at the 5’-end as described under “Experimental Procedures.” This RNA corresponds to the wild-type ColIb-P9 sequence from positions 244 to 524 that covered the entire region containing structures I to III. After the RNA sample was partially digested by RNases T1 (specific for guanine residues in single-stranded regions), RNase Bc (specific for pyrimidines in single-stranded regions), or RNase V1 (specific for double-stranded regions), the resulting products were analyzed by electrophoresis on a denaturing polyacrylamide gel. The patterns of the autoradiography are shown in Fig. 2, A and B, and the cleavage sites identified are summarized in Fig. 2C, in which the deduced secondary structures are also presented. We observed three stem-loop structures, I, II, and III as predicted previously (13). In addition, we found three new stem-loop structures designated Ia, Iib, and IV, which could not be predicted computationally. Note that RNase Bc appeared to cleave any ribonucleotides in the 5’ side of loop sequences in the assay condition employed in this study.

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adenine residues without changing the repZ RBS (the substituted bases are underlined in the sequence). The resulting multiple mutation was designated as A25. Secondary structures of A25 RNA293 synthesized in vitro were analyzed in conjunction with mutation rep2041 (G330A) and rep2044 (G438A) defective in the intramolecular base pairing (8). When compared with the wild-type RNA293, RNase T1 sensitivities at G-327 and G-328 in A25 RNA293 were substantially reduced, although the cleavage pattern of the sequence corresponding to the 3' side of the structure I stem was not changed (A25 in Fig. 3A). Furthermore, the RNase V1 sensitivity at C-440 disappeared, indicating that structure III of A25 RNA293 was indeed disrupted by the base changes. On the other hand, when mutation rep2041 or rep2044 was introduced into A25 RNA293, the degree of the RNase sensitivities of G-327 and G-328 was restored to that observed in the wild-type RNA293 (A26 and A32 in Fig. 3A). Since C-329 and G-330 had been shown to interact with G-327 and G-328 by base pairing, respectively (8), these results can be explained by suggesting that, in A25 RNA293, at least 5'-rGGGC-3' in the loop of structure I base pairs with its complementary sequence 5'-rCGCC-3' located in the unfolded structure III region to form an RNA pseudoknot as shown in Fig. 3C (panel A25).

However, we also observed in A25 RNA293 that G-438 and G-433 were weakly cleaved by RNase T1 and V1, respectively, whereas G-447 became insensitive to RNases T1 and Bc (A25 in Fig. 3B, lanes 1–3). In addition, the degree of cleavage of G-438 and G-433 as observed in A25 was enhanced in both A26 and A32 RNA293 except that in the latter the RNase T1 sensitivity of G-438 was lost due to the base change by mutation rep2044 (Fig. 3B). These results can be explained by proposing that A25 RNA293 contains a mixture of at least two types of base pairing. One type occurs between the 5'-rGGGC-3' and 5'-rCGCC-3' to form the pseudoknot, as mentioned above, and the other occurs between 5'-rUGCGCU-3' (positions 431–435) and 5'-rAGCGA-3' (position 444–448) to produce a new stem-loop structure designated IIIa, as shown in Fig. 3C. We do not know if these types of base pairings coexist in a single RepZ mRNA molecule, or occur separately in two groups of messages. By contrast, both A26 and A32 RNA293 formed only structure IIIa.

To separate the pseudoknot from structure IIIa, we constructed a mutant A28 from A25. This mutant deleted three bases, 5'-rUGCG-3' (positions 433–435) at the 5' side of the structure IIIa stem of A25 without affecting the repZ RBS. When cleavage patterns of A28 RNA293 were compared with those of A25 RNA293, the RNase T1 sensitivities at G-327 and G-328 were reduced as observed in A25 RNA293 (A28 in Fig. 3A). By contrast, G-445 and G-447, and U-442 were strongly cleaved by RNases T1 and Bc, respectively (A28 in Fig. 3B, lanes 1 and 2), an indication that structure IIIa was disrupted by the deletion. When mutation rep2041 was introduced into A28, G-327 and G-328 residues were strongly cleaved by RNase T1 while G-445 and G-447 were not (A29 in Fig. 3, A and B). Similarly, when mutation rep2044 was introduced into A28, both G-327 and G-328 were cleaved by RNase T1. In addition, G-445 and G-447 were also cleaved by RNase T1 as observed in A28 RNA293 (A34 in Fig. 3, A and B). We inferred from these results that A28 RNA293 predominantly formed the pseudoknot that was observed in A25 RNA293 (Fig. 2C). Conversely, A29 RNA293 does not form the pseudoknot, and instead, generates a small stem-loop structure designated IIIb (A29 in Fig. 3C).

However, A34 RNA293 produced neither the pseudoknot nor structure IIIb. It should be noted that both structures IIIa and IIIb cover partially the Shine-Dalgarno sequence for repZ translation.

A Pseudoknot Formed in RNA206—The sequence of the wild-type RepZ mRNA leader region showed that 10 out of 11 bases of the 5'-rUGCGGAAAGA-3' sequence (positions 326–336) in structure I were complementary to the downstream sequence just preceding the repZ RBS. Since A28 RNA293 deleted the 3 complementarity bases from the sequence, we examined whether these bases also contributed to the pseudoknot formation. For this, we prepared RNA206, a deletion of RNA293 beyond A-443 (see Fig. 2C for the position of 3'-end of RNA206). This RNA
lacked the sequence responsible for structure III, IIIa, or IIIb formation. When RNA206 was digested by RNases, the cleavage pattern of the structure I region was found to be quite different from that of the wild-type RNA293. (i) Both G-327 and G-328 were not cleaved by RNase T1; (ii) instead, G-321 was strongly cleaved by RNase T1; and (iii) RNase Bc sensitivity at U-317 was lost (Fig. 4A, lanes 1–3). However, the cleavage patterns of the sequence corresponding to the stem region in structure I were identical to those of RNA293 (Fig. 4A). Surprisingly, when rep2044 was introduced into RNA206, the cleavage patterns of structure I region became identical to those of RNA293 (Fig. 4A, lanes 4–6, and also see, Fig. 2B, lanes 1–3). These results indicate strongly that the 10 complementary bases in the top region of structure I had the potential to pair with those in the downstream sequence, generating a unique RNA pseudoknot as shown in Fig. 4B. Of further significance was the finding that this seemingly strong base pairing was abolished by introduction of a single mismatch mutation, rep2044. This phenomenon will be studied later in relation to the role of each base pair in repZ expression.

Effect of the Pseudoknot on repZ Expression—To assess the pseudoknot formed in vitro, we examined effects of mutations A25 and A28 on the level of repZ expression in the absence of inc and repY genes, the negative and positive regulatory elements, respectively, as the action of either or both of them might cause conformational changes in the RepZ mRNA leader region (see Fig. 3C for effects of A25 and A28 mutations on RepZ mRNA secondary structure). The RepZ activity was monitored by measuring the β-galactosidase activity of the repZ-lacZ translational fusions, in which codon 221 of the repZ gene was fused in-frame to codon 7 of the promoter-less lacZ gene as the reporter. The vector for this fusion gene was a mini-F plasmid, whose copy number was almost the same as that of ColIb-P9 (data not shown). A summary of the experiments is given in Table II. pKA340-W3 carrying inc1, a promoter-down mutation in the inc gene, showed 1,912 units of RepZ activity. When rep57 (C408T), an amber mutation of repY codon 11 (13), was introduced into pKA340-W3, the resultant plasmid pKA340-A33 exhibited only 2.6 units of the RepZ activity, a value 735-fold (1912/2.6) lower than that of pKA340-W3. In the case of pKA340-A25 carrying the A25 mutation, the level of RepZ activity was 223 units, 86-fold higher than that of pKA340-A33. This level was further elevated to 3,714 units in pKA340-A28 bearing the A26 mutation. The value of the RepZ activity in pKA340-A28 was twice as high as that of pKA340-W3. There results indicate that disruption of structures III and IIIa leads to a higher level of repZ expression.

On the other hand, introduction of mutation rep2041 or rep2044 into pKA340-A25 caused a considerable reduction of repZ expression, yielding a value of 14 or 16 units, respectively. Similarly, the presence of rep2041 in pKA340-A28 decreased the RepZ activity to 265 units. By contrast, when rep2044 was introduced into pKA340-A28, the resulting plasmid pKA340-A34 exhibited almost the same RepZ activity as that of pKA340-W3. Furthermore, we found that this activity was not affected by the action of either Inc RNA or repY translation (pKA340-A34 or -A50 in Table II), an indication that the repZ gene was constitutively expressed.

Of importance was the observation that although mutations rep2041 and rep2044 reduced substantially the level of repZ expression in plasmids pKA340-A26 and -A32, their activities were significantly higher than that of pKA340-A33. In addition, the level of repZ expression in pKA340-A29 was about 18 times higher than those of pKA340-A26 and -A32. These facts and the constitutive expression of repZ in pKA340-A34 suggest strongly that the RepZ activities of these plasmids are inversely correlated with the calculated thermodynamic stabilities of structures III (−11.3 kcal/mol), IIIa (−5.3 kcal/mol), and
IIIb (−1.0 kcal/mol). If this were the case, it could be concluded that these secondary structures observed in vitro are indeed formed in vivo, acting as the negative elements for repZ expression due to sequestering of its Shine-Dalgarno sequence. By the same token, we would conclude that the pseudoknot observed in vitro in A28 RNA293 is also formed in vivo, being responsible for the highest level of repZ expression in pKA340-A28. Furthermore, the data indicate that the role of repY in repZ expression is to disrupt structure III during the process of its translation and termination, inducing the intramolecular base pairing between the two complementary sequences to form the pseudoknot. Thus, the pseudoknot formed by the process of repY translation acts as the molecular switch for translation initiation of repZ expression, as proposed previously as shown in Fig. 1B (8, 9).

RNA293 derivatives near the structure I and III regions are depicted using the same symbols as in Fig. 2.

**Fig. 3. Secondary structures of mutant RNA293 derivatives with structure III disrupted.** A and B, 5’-end-labeled RNA293 or its mutant derivatives were synthesized and cleaved with RNase T1, Bc, or V1, and subjected to electrophoresis as in Fig. 2. Mutant RNAs were generated from pKA10-A25 (inc1 rep57 A25) (labeled A25), pKA10-A26 (inc1 rep57 A25 rep2041) (labeled A26), pKA10-A32 (incl rep57 A25 rep2044) (labeled A32), pKA10-A28 (incl rep57 A25 A28) (labeled A28), pKA10-A29 (incl rep57 A25 A28 rep2041) (labeled A29), and pKA10-A34 (incl rep57 A25 A28 rep2044) (labeled A34). incl and rep57 mutations do not change the cleavage patterns of RNA293, data not shown. A part of the RNase T1 cleavage pattern corresponding to the structure I region is shown in panel A, whereas parts of RNase T1, Bc, and V1 cleavage patterns corresponding to the structure III region are shown in panel B (lanes 1–3, respectively). Representation schemes of the RNase cleavage products are the same as in Fig. 2. A, arrows highlighting the nucleotides mentioned in the text are emptied if the signal was weaker significantly than that of the parental species. Positions of structures III, IIIa, and IIIb are shown by vertical bars. C, parts of secondary structures of mutant RNA293 derivatives near the structure I and III regions are depicted using the same symbols as in Fig. 2C. Nucleotides altered in each RNA species are highlighted in black.
that stimulates the interactions involving the rest of the complementary sequences (see Ref. 30 for details).

Requirement for repY Translation in the Induction of repZ Expression—We have previously shown that the translation and termination of the repY reading frame is essential for repZ translation, and that Inc RNA regulates the translation of both repY and repZ at different rates (8). In this paper, the role of repY translation in repZ expression was shown to involve disruption of structure III, inducing pseudoknot formation (Table II). To investigate the effect of repY translation on repZ expression, we compared β-galactosidase activities between pKA340-W3 and pKA340-A52, carrying repZ-lacZ and repY-repZ-lacZ fusions in mini-F derived plasmids, respectively, where in both cases the lacZ gene was connected to the same
position in the repZ gene. The repY-repZ-lacZ fusion in pKA340-A52 was constructed by inserting a cytosine residue between positions 403 and 404 and copy number of this plasmid was the same as that of pKA340-W3. Fig. 5 shows the results of the experiments. pKA340-A52 exhibited 2151 units, a value almost identical to that of pKA340-W3. In addition, we had previously shown that no protein synthesis was initiated from the normal repZ initiation codon in the repY-repZ-lacZ fusions because of the lack of the termination event near the repZ initiation codon (8, 9, 13). Assuming that the $\beta$-galactosidase activity of the repY-repZ-lacZ fusion protein was not affected by the extra 25 amino acids derived from the repY frame, these results indicated that the ratio of repY translation to that of repZ translation was nearly one, implying that ribosome access to the repZ RBS and the subsequent steps in initiation of repZ translation are very efficiently coupled to repY translation and termination.

Finally, we examined whether Inc RNA controls directly translation initiation of the repZ gene. When the inc1 mutation in both pKA340-W3 and pKA340-A52 was converted to the wild-type for restoring the synthesis of Inc RNA, both repY and repZ expressions decreased considerably, and the ratio of repY translation to repZ translation increased to 5 (pKA340-A63 and pKA340 in Fig. 5), consistent with previous results (8). Furthermore, when the copy number of the lacZ fusion genes was increased by re-cloning into the multicopy vector plasmid, pMB1, the repY expression increased in proportion to the gene dosage, whereas the repZ expression did not increase significantly (Fig. 5). These observations indicate that Inc RNA controls repZ translation not only indirectly by inhibition of repY translation, but also directly by uncoupling it from repY translation. In this way, a constant level of repZ expression is maintained irrespective of changes in plasmid copy number.

**DISCUSSION**

In this report, we have presented evidence that the novel RNA pseudoknot formed in the RepZ mRNA leader sequence is involved in translation initiation of the repZ gene, encoding the replication initiator protein of the ColIb-P9 plasmid. Structural analyses of the RepZ mRNA leader sequence synthesized in vitro revealed that it contained several stem-loop structures including structures I and III, but not the pseudoknot (Fig. 2). However, disruption of structure III, without changing the repZ RBS, by means of base substitution and deletion was found to induce new intramolecular base pairing between two distantly separated short complementary sequences located at positions 327–330 and 437–440. These interactions result in formation of a pseudoknot involving structure I (Fig. 3). We have also presented evidence suggesting that the pseudoknot observed in vitro is formed in vivo, affecting significantly the level of repZ expression (Table II). These results strengthen the previously proposed model that the pseudoknot induced by translation and termination of the repY reading frame functions as the molecular switch for translational initiation of the repZ gene.

Is the disruption of structure III sufficient for pseudoknot

**TABLE II**

| Plasmid       | Mutation | repY | Inc RNA | Secondary structure* | Rep activity* |
|---------------|----------|------|---------|----------------------|--------------|
| pKA340-W3     | inc1     | +    | –       | III                  | 1912         |
| pKA340-A33    | inc1 rep57 | –    | –       | III                  | 2.6          |
| pKA340-A25    | inc1 rep57 A25 | –    | –       | IIIa, Ps             | 223          |
| pKA340-A26    | inc1 rep57 A25 rep2041 | –    | –       | IIIa                  | 14           |
| pKA340-A27    | inc1 rep57 A25 rep2006 | –    | –       | IIIa                  | 12           |
| pKA340-A32    | inc1 rep57 A25 rep2044 | –    | –       | IIIa                  | 16           |
| pKA340-A28    | inc1 rep57 A25 A28 | –    | –       | Ps                    | 3714         |
| pKA340-A29    | inc1 rep57 A25 A28 rep2041 | –    | –       | IIb                   | 265          |
| pKA340-A30    | inc1 rep57 A25 A28 rep2006 | –    | –       | IIb                   | 203          |
| pKA340-A34    | inc1 rep57 A25 A28 rep2044 | –    | –       | None                  | 1391         |
| pKA340-A34+   | rep57 A25 A28 rep2044 | –    | –       | None                  | 1911         |
| pKA340-A50    | inc1 A25 A28 rep2044 | +    | –       | None                  | 2252         |

* Secondary structure(s) observed near the repZ RBS by RNase mapping.

* $\beta$-Galactosidase activity in Miller’s unit (23).
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Effect of pseudoknot mutations on repZ expression

MC1061(lacX74) cells carrying the indicated plasmids were assayed for β-galactosidase activity as described in Table II. Inc-promoter down-mutation incI was employed to measure RepZ activity in the absence of Inc RNA.

| Plasmid | Mutation | Complementary sequences | RepZ activity |
|---------|----------|-------------------------|--------------|
| pKA340-W3 | inc1 | 5′- (325) UUGCCGAAAGCA-3′ | 1912 |
| pKA340-A36 | inc1 T325C | 5′- (325) cUGGGCGAAAGCA-3′ | 2048 |
| pKA340-A37 | inc1 T326C | 5′- (325) UGGCGAAAGCA-3′ | 226 |
| pKA340-A61 | inc1 A441G | 5′- (325) UUGCCGAAAGCA-3′ | 1439 |
| pKA340-A56 | inc1 T326C A441G | 5′- (325) UGGCGAAAGCA-3′ | 1864 |
| pKA340-A38 | inc1 T326G | 5′- (325) UGGCGAAAGCA-3′ | 270 |
| pKA340-A59 | inc1 A441C | 5′- (325) UUGCCGAAAGCA-3′ | 601 |
| pKA340-A51 | inc1 T326G A441C | 5′- (325) UGGCGAAAGCA-3′ | 682 |
| pKA340-A39 | inc1 G327A (rep2006) | 5′- (325) UUGGCGAAAGCA-3′ | 10 |
| pKA340-A62 | inc1 C440T (sup2006-10) | 5′- (325) UUGCCGAAAGCA-3′ | 368 |
| pKA340-A45 | inc1 G327A C440T | 5′- (325) UUGGCGAAAGCA-3′ | 727 |
| pKA340-A40 | inc1 G328A | 5′- (325) UUGGCGAAAGCA-3′ | 8 |
| pKA340-A57 | inc1 C439T | 5′- (325) UUGGCGAAAGCA-3′ | 442 |
| pKA340-A46 | inc1 G328A C439T | 5′- (325) UUGGCGAAAGCA-3′ | 1743 |
| pKA340-A41 | inc1 C329T (sup2044-2) | 5′- (325) UUGGCGAAAGCA-3′ | 170 |
| pKA340-A49 | inc1 G438A (rep2044) | 5′- (325) UUGGCGAAAGCA-3′ | 23 |
| pKA340-A44 | inc1 C329T G438A | 5′- (325) UUGGCGAAAGCA-3′ | 1041 |
| pKA340-A42 | inc1 G330A (rep2041) | 5′- (325) UUGGCGAAAGCA-3′ | 56 |
| pKA340-A58 | inc1 C437T (sup2041-7) | 5′- (325) UUGGCGAAAGCA-3′ | 883 |
| pKA340-A47 | inc1 G330A C437T | 5′- (325) UUGGCGAAAGCA-3′ | 1780 |
| pKA340-A43 | inc1 G331A | 5′- (325) UUGGCGAAAGCA-3′ | 129 |
| pKA340-A60 | inc1 C436T | 5′- (325) UUGGCGAAAGCA-3′ | 227 |
| pKA340-A55 | inc1 G331A C436T | 5′- (325) UUGGCGAAAGCA-3′ | 1509 |
| pKA340-A64 | inc1 A332T | 5′- (325) UUGGCGaaAGCA-3′ | 1326 |
| pKA340-A65 | inc1 T435A | 5′- (325) UUGGCGaaAGCA-3′ | 2227 |
| pKA340-A66 | inc1 A332T T435A | 5′- (325) UUGGCGaaAGCA-3′ | 2007 |
| pKA340-I1 | inc1 C334A | 5′- (325) UUGGCGAAAGCA-3′ | 1226 |

TABLE III

Formation? The leader regions of the replication initiator (rep) genes of two plasmids, IncB pMU720 and IncK pMU2200, are similar in sequence to the corresponding region of ColIb-P9 (26). These three plasmids commonly have two stem-loop structures, one as the target site of the antisense RNA, and the other that sequesters the rep RBS. Pseudoknots with the target

<sup>a</sup> Complementary sequences involved in formation of the pseudoknot are shown with changed residues in lowercase. G:C pair was connected by “-”.

<sup>b</sup> β-Galactosidase activity in Miller’s unit (23).
stem-loop for antisense RNA binding can also be predicted, and evidence for one in pMU720 is reported (10). However, no other stem-loop analogous to structure II of ColIb-P9 is predicted in pMU720, and in fact, a 30-base long DNA segment encompassing structure II was able to be deleted without affecting the pseudoknot formation in ColIb-P9. In addition, we previously constructed an IncFII R100-ColIb-P9 chimeric mini-plasmid where the ColIb-P9 repZ leader sequence upstream of 5'-rGGGC-3' was replaced with the corresponding region of a ColIb-P9 repY frame (22). The leader regions of rep genes of IncFII-type plasmids encode an antisense RNA and upstream open reading frame such as \textit{inc} and \textit{repA6} in R100, respectively, but the \textit{rep} genes of these plasmids do not require formation of a pseudoknot for coupling translation of upstream open reading frame to \textit{rep} (26). Although the resultant chimeric plasmid did not produce \textit{repZ} due to lack of the pseudoknot structure, the replacement of the Inc RNA-target loop of the R100 portion with that of ColIb-P9, including the 5'-rGGGC-3' sequence, was sufficient for \textit{repZ} in the ColIb-P9 portion to be translated (22). These lines of evidence together suggest that only structure I and the exposed 5'-rGGGC-3' sequence are sufficient for the pseudoknot formation, and hence \textit{repZ} expression.

If so, how does the loop of structure I base pair specifically with a sequence preceding the \textit{repZ} RBS to form a pseudoknot? And why does a single mismatch mutation such as \textit{rep2044} disrupt it profoundly, although possible base pairing occurs between 11 bases of complementary RNA sequences (Fig. 4)? We believe that a transient base paring between a subset of these sequences, 5'-rGGGC-3' and 5'-rGGGC-3', is rate-limiting for the formation of the pseudoknot. Thus, interfering with this transient step by a single mismatch can be sufficient for preventing all subsequent steps in pseudoknot formation. In addition, a certain conformation of the loop region of structure I appears to play a role in stimulating the intramolecular base pairing by giving it higher affinity and specificity, as suggested here by the preference for a pyrimidine residue at a base 5' to the 5'-rGGGC-3' sequence for \textit{repZ} expression (Table III). The same conformation in the loop of structure I may stimulate \textit{RepZ} mRNA-Inc RNA interaction as well. Detailed analyses of the structural basis of the intermolecular interaction in the accompanying paper (30), as well as the analyses of the pseudoknot formation \textit{in vitro} with different RNA species that inhibit binding of Inc RNA to the \textit{RepZ} mRNA leader support this hypothesis.

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