Positive and Negative Regulations of Plasmid ColIb-P9 repZ Gene Expression at the Translational Level*

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Expression of the repZ gene involved in DNA replication of the ColIb-P9 plasmid depends on translation of a transcribed repZ leader sequence (repY) and is negatively regulated by Inc RNA, the product of the inc gene and a countertranscript to RepZ mRNA. To further understand the regulatory loop of repZ expression, we isolated and characterized replication-defective ColIb-P9 mutants that affected the level of repZ expression. Here we report that mutations occurring in two complementary sequences, one (5'GGCG3') in the inc region and one in the repY region, reduce the level of repZ expression without affecting transcription. The mutations in one complementary sequence were suppressed by compensatory base changes in the other sequence, restoring the ability of repZ expression. These results indicated that interaction by base-pairing between the two complementary sequences of RepZ mRNA was essential for repZ translation. The two sequences, separated by 107 bases from each other, have a potential to form a novel pseudoknot in the RepZ mRNA leader. We also found that some mutations in the 5'GGCG3' sequence altered the specificity of Inc RNA, thereby reducing significantly its regulatory activity. Thus, this single specific sequence is involved in both positive and negative regulations for repZ expression. Possible regulatory mechanisms of repZ expression are discussed.

Gene expression, in some cases, is controlled at the level of translational initiation. Various mechanisms have been known to be involved in this regulation (for reviews, see Refs. 1 and 2). In the case of some Escherichia coli ribosomal protein operons (for reviews, see Refs. 3 and 4), bacteriophage T4 gene 32 (5), or bacteriophage Mu mom gene (6), interactions between regulatory proteins and specific sites on mRNA molecules are responsible for the control of gene expression. Especially for the E. coli α (7) or rpsO (8) operon, ribosomal protein S4 or S15 has been shown to bind to an mRNA pseudoknot, respectively, thereby regulating autogenously the synthesis of ribosomal proteins. In the case of the plasmid IncFI repA gene (9–11) or insertion sequence IS10 transposase gene (12), small RNA binds to mRNA essential for gene expression at the complementary sequence to regulate the translational initiation. Yet, in the case of the ermC gene of some Gram-positive bacteria, translation of a transcribed ermC leader has been proposed to induce a conformational change in mRNA secondary structure for expression (13, 14). Perhaps, a common feature of these regulatory systems appears to be a dynamic alteration of mRNA secondary structure as a requirement for translational initiation. However, the precise mechanism(s) involved in this process remains to be studied.

The ColIb-P9 plasmid belonging to the IncIa (= IncI,) group is a large plasmid with a size of 39 kilobases (kb).† The basic ColIb-P9 replicon consists of a 1.8-kb DNA segment which contains the information essential for autonomous replication and copy number control (15). The frequency of ColIb-P9 replication depends on the level of expression of the repZ gene encoding a 39-kDa protein, which probably reacts with the replication origin to promote replication. Previous studies showed that the expression of repZ was regulated by the actions of the inc and repY genes, both of which were located in the leader region of repZ (15–17). The inc gene that governs the phenotype of incompatibility encodes an RNA of approximately 72 bases. This small RNA, termed Inc RNA, is transcribed opposite to RepZ mRNA and forms a hybrid with it at the complementary sequence, thereby inhibiting or regulating repZ expression at the translational level (16). However, the precise mechanism of this regulation is unclear. The repY gene resides between the repZ and inc genes and encodes a small polypeptide of 29 amino acids (Ref. 17; also see Fig. 1). It has been suggested that repY translation per se rather than the RepY polypeptide functions as a positive regulator for repZ expression. The role of repY translation has been deduced to disrupt a stem-loop structure sequestering the ribosome binding site for repZ translation (17).

To explore the regulatory loop of repZ expression, we undertook isolation and characterization of replication-defective ColIb-P9 mutants that affected the level of repZ expression. In this report, we describe that two specific short complementary sequences of RepZ mRNA, one in the inc region and the other in the repY region, are required for repZ expression. We also show that some mutations in the inc region alter the specificity of Inc RNA, thereby reducing its regulatory activity. Evidence is presented indicating that Inc RNA regulates the expression of not only repZ but also repY at different rates. Based on these observations, the regulatory loop of repZ expression is discussed.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Phages, and Plasmids—E. coli K12 strains W3110supE, W3110(hinc2), and C600supE (18) were used as hosts of phages λCH10–2 and its mutant derivatives. DM2555 (mutDS zaf13::Tn10) was used for in vivo mutagenesis of λCH10–2. MV1184 (19) was the host of plasmid M13. BW313 (dut ung) (20) and BMH71–

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‡ The abbreviations used are: kb, kilobase(s); bp, base pair(s); SDS, sodium dodecyl sulfate.
18mutS (21) were employed for site-directed mutagenesis. MC1061 (lacX74) (22) was used for rep-lacZ fusion studies. XCH10-2, a composite replicon of the XVIII phase and the 3.0-kb mini ColIb-P9 fragment of XCH10-2 and its rep or sup mutants, respectively, to the Tn5 1.3-kb nonreplicable Km' fragment of pCH71. New England Nuclear Research Products.

**Fig. 1. Isolation of rep and sup mutants in the leader region of the repZ gene.** a, organization of the replication control region of the ColIb-P9 plasmid. Thick line denotes the region essential for ColIb-P9 replication for which the nucleotide sequence has been determined (15). Open boxes indicate the locations of the repZ, repY, and inc genes. ori indicates the possible replication origin proposed from DNA sequencing. Long and short horizontal arrows represent major transcripts representing RepZ mRNA (about 1400 bases in size) and Inc RNA (about 72 bases), respectively. Vertical arrows denote the recognition sites of appropriate restriction enzymes; B, BglII; P, BstUI (= BstEII); E, EcoRI; H, HincII; S, Sall; and SA, Sau3AI. b, distribution of rep and sup mutations. Arrowheads and thick bars indicate the sites of rep and sup mutations. Base changes are shown above the arrowheads. Two complementary sequences are boxed (see text). Under the nucleotide sequence, the complete replication control region of the ColIb-P9 plasmid. Thick line denotes the region essential for ColIb-P9 replication of Plasmid ColIb-P9.

**Media—**LB medium, minimal A medium (27), and +b medium (0.5% yeast extract (Difco), 2% Trypton (Difco), 20 mM MgSO4) were used for the growth of bacteria. TXA (15) was used for the growth of ACH10-2 and its mutant derivatives. 2 X YT (28) was employed for the growth of M13 in preparation of single-stranded DNA. Solid and [35S]methionine (800 Ci/mmol) were obtained from Du Pont-New England Nuclear Research Products.

Isolation of rep and sup Mutants—The method of isolation of rep and sup mutants of the ColIb-P9 replication region was described previously (17). Briefly, XCH10-2 grown in DM2555 cells or mutagenized in vitro with NH4OH was plated with C600supE cells. Since XCH10-2 formed clear plaques on C600supE cells due to the replication initiation of the ColIb-P9 replicon while vector XVIII formed typical turbid plaques, turbid plaques were isolated as candidates of replication-defective mutants of ColIb-P9. The suspected mutant phages were then plated for measurement of reversion frequency on W3110 (Xind-) cells, in which XCH10-2 formed clear plaques while XCH10-2rep and vector XVIII did not form any visible plaque. Mutants whose spontaneous reversion frequencies were more than 10^{-7} plaque forming unit were selected and their DNA sequences were examined for XCH10-2rep. XCH10-2sup mutants were isolated as spontaneous pseudorevertants by plating the XCH10-2rep phages on W3110 (ind-) cells.

**DNA Manipulation and Sequencing—**Small scale preparation of DNA from XCH10-2 and its mutant derivatives was according to Sills et al. (29). Transformation of plasmid DNA was carried out according to Maniatis et al. (31). Nucleotide sequence of the 724 bp EcoRI-BstPI fragment of rep and sup mutants was determined by the dideoxy chain-termination method (32) using [α-32P]dCTP and oligo-2 (5'CTTCAGTCTTCTGACT'3' at positions 276-259 in Fig. 1), oligo-3 (5'GACCTCAGTCTCTCGG3' at positions 600-648), and oligo-4 (5'TGGCCAGGCTCTGCTG3' at positions 746-730) as the primers (16). After subcloning the EcoRI-SalI fragment of each XCH10-2rep or XCH10-2sup mutant into the EcoRI-SalI sites of pTZ19R, single-stranded DNA as the template was prepared by infection of M13K07 (19) and served for DNA sequencing.

**Site-directed Mutagenesis—**Site-directed mutagenesis of the ColIb-
were used instead of MV1184. The transformants of BMH71-l8mutS by DNA sequencing. In some experiments, BMH71-l8mutS cells transformants. Then single-stranded DNA was prepared from each NaC1, 0.5 mM dithiothreitol) at 65 °C for 15 min. After cooling at 25 °C for 2 h by the addition of T4 DNA polymerase (1 60 mM ammonium acetate, 37 °C for 15 min, 25 μM 13-bp EcoRI-Sau3A fragment of ColIb-P9 from XCH10-2 into pBR322.

pDX14-I2 was ligated with a 1.3 kb Km fragment of pCH71.

pKA100-I2 3-kb EcoRI (EcoRI leader region of ColIb-P9 from XCH10-2 was ligated with a 1.3 kb Km fragment of pCH71.

pCH11-12 1120-bp EcoRI-Sau3A fragment of ColIb-P9 from XCH10-2 into pBR322.

pKA101-I2 3-kb EcoRI (EcoRI leader region of ColIb-P9 from XCH10-2 was ligated with a 1.3 kb Km fragment of pCH71.

pCH11-12 1120-bp EcoRI-Sau3A fragment of ColIb-P9 from XCH10-2 into pBR322.

pMC1403 lacZ fusion vector pMC1403 deleting the 516-bp BetP1 fragment. This work.

pKA104 repZ-lacZ fusion; 1118-bp EcoRI-HincII fragment of ColIb-P9 into pKA1.

pKA104-I2 pKA140 carrying inc2 1.38 kb inc* Sau3A fragment of ColIb-P9 into pACYC184.

pCH1847. This work.

The RepZ1 site is converted into an EcoRI site.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>Cloning vector</td>
<td>Ref. 23</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cloning vector</td>
<td>Ref. 24</td>
</tr>
<tr>
<td>pTZ19R</td>
<td>Cloning vector</td>
<td>Ref. 25</td>
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<tr>
<td>pCH71</td>
<td>1.3-kb Km fragment of Tn5 into pACYC184</td>
<td>Ref. 17</td>
</tr>
<tr>
<td>pDX14-I2</td>
<td>1120-bp EcoRI-Sau3A fragment of ColIb-P9 from XCH10-2 into pBR322. This work.</td>
<td></td>
</tr>
<tr>
<td>pKA100-I2</td>
<td>3-kb EcoRI (EcoRI leader region of ColIb-P9 from XCH10-2 was ligated with a 1.3 kb Km fragment of pCH71. This work.</td>
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<tr>
<td>pCH11-12</td>
<td>1120-bp EcoRI-Sau3A fragment of ColIb-P9 from XCH10-2 into pBR322.</td>
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<tr>
<td>pMC1403</td>
<td>lacZ fusion vector</td>
<td>Ref. 26</td>
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<tr>
<td>pKA1</td>
<td>pMC1403 deleting the 516-bp BetP1 fragment. This work.</td>
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<td>pKA104</td>
<td>repZ-lacZ fusion; 1118-bp EcoRI-HincII fragment of ColIb-P9 into pKA1. This work.</td>
<td></td>
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<tr>
<td>pKA104-I2</td>
<td>pKA140 carrying inc2 1.38 kb inc* Sau3A fragment of ColIb-P9 into pACYC184. Ref. 15</td>
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The results of the sites of mutations were determined by DNA sequencing (Fig. 1b). Each mutant acquired a single base substitution besides the inc2 mutation. Two mutations, rep2044 and rep2060, were assigned to the repY region. In addition, rep2060 (C → T at position 408) was found to be the same as rep57, a amber mutation of codon-11 of RepY (17) and rep2044 (G → A at position 438) was a missense mutation of codon-21 (Ala → Thr). However, as described later, the change in the amino acid sequence of the RepY polypeptide by the rep2044 mutation was not important for repZ expression. Four mutations, rep2006, rep2041, rep2056, and rep2134, were located within the inc region. Since rep2006, rep2056, and rep2134 had the same base change, rep2006 served as a representative for further examinations.

The defects in replication were first examined by one-step growth of λCH10—rep mutants upon infection to W3110(λind’) cells, in which the parental λCH10—2 phage exhibited burst size of about 70 even in the presence of λ repressors, the products of the cI gene, while vector XVIII did not grow (burst size of 0.004). All the mutants isolated were found to exhibit the burst size of less than 0.01. To determine more directly the defect in replication, the 3.0-kb mini-ColIb-P9 rep1 mutation was isolated from λCH10—2 or λCH10—2 rep mutants and ligated to the 1.3-kb nonreplicable Km’ fragment of pCH71. The resulting plasmids, pKA101-I2 (inc2 rep57), pKA104-I2, and pKA104-I2 were introduced into MC1061 cells to estimate frequency of Km’ transformation. When compared with the inc2 strain (pKA101-I2), the rep mutations reduced the frequency of transformation to 2.5—7.5% of the level of the parental inc2 strain (column 3 in Table II). These results, coupled together, indicated that the mutations were defective in ColIb-P9 replication.
TABLE II

Properties of rep and sup mutations

Four mutants, rep2006, rep2041, rep2044, and rep2060, were isolated from λCh10-2 carrying the inc2 mutation. Mutations T326C, G328A, C329T, G331A, and A332T were prepared from pDX14-12 (inc2) by site-directed mutagenesis. sup2044-6c, sup2044-13, and sup2044-18 were isolated as class I suppressor mutants, and sup2006-10, sup2041-7, and sup2044-2 were isolated as class II suppressor mutants (see text). The transformation frequency was measured by using plasmids pKA101-12 and its rep or sup derivatives and expressed as the number of Km<sup>+</sup> transformants per μg of DNA. RepZ activity was measured by the β-galactosidase activity of translational fusion plasmids using pKA140-12 (inc2) and its mutant derivatives. Values of the transformation frequency and the RepZ activity are average of at least three experiments.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Base change (position)</th>
<th>Transformation frequency</th>
<th>RepZ activity&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>inc2 (parent)</td>
<td>T → C (374)</td>
<td>7.1 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>T326C</td>
<td>T → C (326)</td>
<td>4.3 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>181</td>
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<tr>
<td>rep2006</td>
<td>G → A (327)</td>
<td>3.8 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5.7</td>
</tr>
<tr>
<td>G328A</td>
<td>G → A (328)</td>
<td>6.5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>9.3</td>
</tr>
<tr>
<td>C329T</td>
<td>C → T (329)</td>
<td>5.9 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>82</td>
</tr>
<tr>
<td>rep2041</td>
<td>G → A (350)</td>
<td>1.8 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>23</td>
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<tr>
<td>G331A</td>
<td>G → A (331)</td>
<td>4.4 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>41</td>
</tr>
<tr>
<td>A332T</td>
<td>A → T (332)</td>
<td>5.3 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>36</td>
</tr>
<tr>
<td>rep2060</td>
<td>C → T (408)</td>
<td>2.8 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.5</td>
</tr>
<tr>
<td>rep2044</td>
<td>G → A (438)</td>
<td>5.3 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.1</td>
</tr>
<tr>
<td>sup2006-10</td>
<td>G → A (327)</td>
<td>4.4 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>201</td>
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<tr>
<td>sup2041-7</td>
<td>G → A (330)</td>
<td>5.8 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>85</td>
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<td>sup2044-2</td>
<td>C → T (329)</td>
<td>6.7 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>132</td>
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<td>sup2044-13</td>
<td>G → A (438)</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>704</td>
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<tr>
<td>sup2044-18</td>
<td>G → A (438)</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1092</td>
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<tr>
<td>ΔCG(432-433)</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1393</td>
<td></td>
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</table>

*Miller units (27).

<sup>a</sup>Single base insertion occurs after the position indicated.

<sup>b</sup>ND, not detected.

Fig. 2. Identification of RepY-RepZ fused protein synthesized by the sup2044-13 mutant. DNAs of pCH11-12, pCH11-12sup2044, pCH11-12sup2044-13, and pBR322 (vector, a negative control) were used as templates for in vitro protein synthesis. [<sup>35</sup>S] Methionine-labeled proteins were separated by electrophoresis on an SDS, 12.5% polyacrylamide gel and visualized by autoradiography. Y′ and Z′ denote the truncated RepY-RepZ fusion and RepZ proteins, respectively, whose sizes, expected from the amino acid sequences, are 29,438 and 26,348, respectively. In rep2044, the synthesis of RepZ′ protein is reduced, when compared with inc2.

to 1–4% (1.5–6.1 units) of the activity of the inc2 strain (column 4 in Table II). Surprisingly, when similar experiments were performed for rep2006 and rep2041, RepZ expression was reduced as well. This unexpected observation indicates that the inc gene region as well as the rep gene functions as a positive regulatory element for rep expression. Furthermore, these results also demonstrate that the replication defect of the rep mutants results from the decrease in the level of rep expression.

Isolation of Suppressor Mutants—The rep mutations were suppressed by additional mutations. Such suppressor (sup) mutants were spontaneously isolated as pseudorevertants at a frequency of 10<sup>−5</sup>–10<sup>−6</sup>/plaque forming unit by plating λCh10-2 mutants on W3110(lind<sup>−</sup>) cells. Here we denote, for example, sup2044-1, sup2044-2, etc., as suppressor mutations isolated independently from rep2044. Analysis of DNA sequences showed that the sup mutations were classified into two groups, classes I and II, by mutation types. Mutants of class I were either of the single base insertions or two base deletions. Such mutants were isolated from all four rep mutants and mapped exclusively in the rep Y region (Fig. 1b). Since the rep Y coding frame overlaps the rep Z one in the −2 frame with respect to rep Y (see Fig. 1b), these suppressor mutations will bring about the formation of a rep Y-rep Z fused gene without changing the amino acid sequence of the RepZ protein, as had been shown in the sup57 mutations that were derived from rep57 (17). Indeed, when sup2044-13, for example, was examined for in vitro protein synthesis, the single base insertion between positions 454 and 455 resulted in the synthesis of a fused gene product with a size expected from the deduced amino acid sequence (Fig. 2). These results indicate that the rep mutations are suppressed by integration of rep Z into a part of rep Y in structure regardless of the location of the rep mutations.

One of the characteristics of the class I sup mutations was that the level of rep Z expression increased significantly, when compared with the inc2 strain. Such an example can be seen in sup2044-6c, sup2044-13, or sup2044-18 (column 4 in Table II). Since the rep Z gene of class I suppressors behaves structurally as a part of rep Y, this phenomenon can be explained as indicating that the level of rep Y expression is severalfold higher than that of rep Z expression. In addition, estimating the transformation frequency in the class I suppressors was not successful because introduction of their plasmid DNAs into host cells did not produce Km<sup>+</sup> transformants. Conversely, when λCh10-2 sup mutants were infected to W3110(lind<sup>−</sup>) cells, all the class I sup mutants showed a full growth with the burst size of about 100 (data not shown). These observations indicate that the excessive replication of Colb-P9 probably due to a high activity of the functional RepZ protein is deleterious to the host cells. A similar observation was also reported for some sup57 mutations (17).

Class II Suppressor Mutants—Mutants of class II were of the base substitution type. In contrast to class I suppressors, this type of mutation seemed to be specific to particular rep mutations. This indication was based on two observations: (i) the base substitutions were detected only at positions 329, 437, and 440 for rep2044, rep2041, and rep2006, respectively (Fig. 1b). In the case of rep2044, we isolated 12 independent sup mutants (8 class I and 4 class II) from 10 different clones. In contrast to class I sup mutants which were placed in 7 different positions, the 4 class II sup mutants were placed only in one specific position, C-329. Note that all these mutants acquired the same type of base substitution (C → T). Similar results were also obtained in rep2006 and rep2041, and (ii) class II sup mutants were not isolated from rep2060. Since this mutation was of the amber type, conversion of the nonsense codon to sense codon was required for suppression. This was achieved by the single base insertions between the rep Y translation initiation codon and the mutation site of rep2060 as shown by sup2060-1, sup2060-2, or sup2060-3 (see Fig. 1b).

Of particular interest were patterns of base changes be-

![Fig. 2. Identification of RepY-RepZ fused protein synthesized by the sup2044-13 mutant.](image-url)
tween the three rep mutations and their class II suppressors. The DNA sequence of the repZ leader region shows presence of two complementary sequences, 5'TGGCGGA3' and 5'TCCGCCA3' at positions 326-332 and 435-441, respectively (Fig. 1b). These two sequences are separated by 103 bases. In this context, the rep2006 mutation (G → A at position 327) was suppressed by the compensatory base change of C → T at position 440 (sup2006-3 and sup2006-10). Likewise, both rep2041 (G → A at position 330) and rep2044 (G → A at position 438) were suppressed by the compensatory base changes of C to T at positions 437 (sup2041-7) and 329 (sup2044-1 to sup2044-4), respectively. Furthermore, the results of the experiments on transformation and RepZ activity in these class II suppressor mutants were quantitatively comparable with those of the inc2 strain (Table II). Accordingly, these lines of information strongly indicate that the two complementary sequences, if not all of the bases in the sequences, interact with each other through base pairing, functioning as a positively regulatory element for the expression of repZ, and that the maintaining of the base pairing between the two complementary sequences is more important than the specificity of the base sequence. The isolation of class II suppressor mutants also indicate that the amino acid substitution of the RepY polypeptide by the rep2044 mutation is not important for repZ expression.

**Minimal Size of the Complementary Sequences**—To clarify the minimal size of the complementary sequences required for repZ expression, 5 bases in one of the complementary sequences, 5'TGGCGGA3', were changed one by one with site-directed mutagenesis and examined for repZ expression. The conversion of G → A at position 329 (mutation G329A) reduced repZ activity to 6% (0.3/157) of the level of the inc2 strain, a value comparable with those observed in the rep mutants (column 4 in Table II). This base change also significantly decreased the transformation frequency. On the other hand, the conversion of T-326 to C (T326C) did not affect either repZ expression or transformation. Changes at positions 329 (C → T) (C329T), 331 (G → A) (G331A), and 332 (A → T) (A332T) caused the reduction to some extent, 23-52% (36-82 units). However, these three changes were found not to influence significantly the replication ability of ColIb-P9, when judged by the transformation frequency. In contrast to C329T, the change of G → A at position 438 (rep2044), which is complementary to C-329, reduced substantially repZ expression. However, this apparent contradiction may be explained by that the sequence 5'UGGUGGA3' in C329T can still pair efficiently with 5'UCGCACCA3' at the mRNA level (see below). Accordingly, it is concluded from these results that at least four (GGCG) out of the 7 bases in the sequence play a crucial role in repZ expression.

**Regulation of repZ Expression at the Translational Level**—To elucidate at which level the complementary sequences are involved in repZ expression, transcription or translation, RNA dot-blot analysis was performed. Fig. 3 shows the autoradiograms of the RNA-DNA hybridization of wild type (inc+ rep+, pKA140) and its mutant derivatives. Relative amounts of RepZ mRNA synthesized were estimated by intensity of the autoradiograms. When compared with the wild type, the amounts of RepZ mRNA were increased 2.5- to 4.6-fold in inc2 and its sup derivatives but were not changed significantly in the rep mutants. On the other hand, when the levels of repZ translation, measured by the β-galactosidase activity of the translational repZ-lacZ fusions, were compared, inc2 and its sup derivatives increased 71- to 168-fold to that of the wild type. These results demonstrate that translation is effected to a much higher degree than transcription. In addition, since the primary structure of the repZ gene promoter, located at positions 121-148 (16), was not altered by either rep or sup mutations, the increase in amounts of RepZ mRNA in the inc2 and sup strains was probably due not to the activation of repZ transcription but rather to the protection or stabilization by active translation. If this interpretation is correct, it can be said that repZ expression is controlled only at the translational level and hence that the interaction of the two complementary sequences takes place within the RepZ mRNA molecule.

**A Possible Pseudoknot Structure Is a Signal for repZ Translation Initiation**—The leader sequence of RepZ mRNA has been predicted to form three possible stable stem-loop structures termed I, II, and III (Ref. 17; also see Fig. 1). Structure I (−22.6 kcal/mol) corresponds to the inc gene region. Structures II (−11.3 kcal/mol) and III (−10.0 kcal/mol) reside in the repY region. We have also reported that the sequestering of the ribosome binding site for repZ translation in structure III is probably disrupted by repY translation (17). In this context, it is interesting to point out that one of the two complementary sequences, 5'GGCG3' at positions 327-330, is located in the loop region of structure I, and the other sequence (5'GCCC3' at positions 437-440), preceding by 1 bp the repZ ribosome binding site (Shine-Dalgarno sequence), is located in the stem of structure III. Based on these structural features and the results presented in this and other studies (17), it is conceivable that the 5'GGCG3' sequence of the loop of stem-loop structure I and the 5'GCCC3' sequence, sepa-
rated 107 bases in length from each other, have a potential to form an extended double helix termed a pseudoknot (34) during the repZ translation initiation reaction (Fig. 4). Probably, such a novel structure transiently stabilizes the structure III region disrupted by repY translation and consequently, facilitates ribosome access to initiate the repZ translation reaction. Thus we propose that the possible pseudoknot structure functions as a signal for repY expression. However, it should be noted that this putative structure is not required for repY expression because of the isolation of class I suppressor mutants from rep2006, rep2041, and rep2044 in addition to rep2060.

**Regulation of repZ Expression by Inc RNA**—It has been reported previously that Inc RNA regulates the expression of not only repZ but also repY at the translational level (16, 17). In this report, we have described that one of the two complementary sequences essential for repZ expression is located in the inc gene region. In addition, repZ expression depends on repY translation as has been observed in the rep2060 mutation. At this point, a pertinent question was how Inc RNA regulated repZ expression, especially the formation of the putative pseudoknot structure. To approach this problem, we examined first if the mutations occurring in the inc gene region altered the property of Inc RNA. After conversion of the inc2 mutation site of the mutants, described in Table II, to the wild type inc promoter sequence (Pinc*), the 418-bp Sau3A fragment containing the whole inc gene region was cloned into vector pACYC184. The resulting plasmids, pCH1847 (wild type, inc*) and its mutant derivatives, were introduced into MC1061 cells harboring pKA140-12, which was compatible with pACYC184, and tested for their effects on repZ expression by measurement of the β-galactosidase activity. Fig. 5 shows the results of the experiments. The presence of the inc* gene in trans repressed repZ expression to 11.1% of the level of repZ expression in the absence of the inc* gene (= vector), a value comparable with the reported one previously (15). When compared with the wild type, at least four mutations, T326C, rep2006, G328A, and C329T, those of which are located at positions 326–329, reduced significantly the inhibitory activity of Inc RNA. Other mutations including rep2044 repressed repZ expression at the same extent as that of pCH1847 did. These results together with the observation that Inc RNA binds to RepZ mRNA at the complementary sequence (16) indicate that some mutations in the inc region indeed alter the specificity of Inc RNA and that the base pairing of Inc RNA with RepZ mRNA at positions 326–329 plays a critical role in the regulation of repZ expression. Furthermore, it is interesting that these mutation sites overlap with the 5′GGCG3′ sequence responsible for the formation of the putative pseudoknot structure.

**FIG. 4. A possible pseudoknot structure of the RepZ mRNA leader region.** A stable form of stem-loop structure I predicted is shown. Four bases (5′GGCG3′) of the loop region interact with the complementary sequence, 5′GC GCC3′ at positions 437–440, to form an extended helix, as indicated by box (see text). Transcription initiation site of RepZ mRNA is either T-157 or A-158 in coordinate for RNA and initiation codons for the repY and repZ genes are indicated by dots and underlines, respectively. The repY gene termination codon is represented by a double underline.

**FIG. 5. Alteration of specificity of Inc RNA in mutants T326C, rep2006, G328A, and C329T.** To test the alteration of the specificity of Inc RNA by mutations occurring in the inc gene region as well as in the repY region, the inc2 site was converted to the wild type inc promoter sequence by site-directed mutagenesis using pDX14-I2 (inc2) or its rep derivatives as the templates. As a result, the amounts of Inc RNA synthesized recovered to the level of the wild type strain regardless of the mutation types. After cloning the 418-bp Sau3A fragment of each strain into vector pACYC184, the resulting plasmids, pCH1847 (wild type; inc*) and its mutant derivatives, were introduced into MC1061 cells carrying pKA140-12, a translational repZ-lacZ fusion derivative of pMC1403. The β-galactosidase activity of pKA140-12 in the presence of pACYC184 (vector), pCH1847 (wild type), or mutant plasmids was assayed for estimating the alteration of the Inc RNA specificity.

**Table III**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fusion</th>
<th>β-Galactosidase activity in the presence of</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pACYC184 (vector)</td>
</tr>
<tr>
<td>inc2</td>
<td>repZ-lacZ</td>
<td>117</td>
</tr>
<tr>
<td>sup2044-13</td>
<td>repY-repZ-lacZ</td>
<td>751</td>
</tr>
</tbody>
</table>

Accordingly, the overlapped region has profound effects on both positive and negative regulations for repZ expression.

To determine whether Inc RNA regulated repZ expression through controlling repY translation, we compared the effect of Inc RNA on the expression between repZ and repY using pKA140-12 and pKA140-12sup2044-13. These two plasmids had the same translational repZ-lacZ fusion except that the latter contained an additional translational repY-repZ-lacZ fusion due to the single base insertion (see Fig. 2). Such an insertion probably does not affect the activity of the inc gene promoter locating at positions 372–400 (16). As shown in Table III, the presence of the wild type inc gene in trans inhibited differentially the expression of repZ and repY to levels of 11.1% (13/117) and 28.6% (215/751), respectively, in the absence of inc, an indication that repZ expression is more sensitive to Inc RNA than repY expression is. We have shown in this report that the interaction of the two complementary sequences in the RepZ mRNA leader region is essential for repZ expression and that a part of sequence 5′GGCG3′ in the inc region is critically involved in the base pairing with Inc RNA. These lines of information, coupled together, strongly indicate that Inc RNA prevents the formation of the putative pseudoknot structure by binding to RepZ mRNA at positions 326–
DISCUSSION

In this report, we have presented genetic evidence that interaction between the two complementary sequences of RepZ mRNA, 5′GGCC3′ and 5′CGCC3′ at positions 327-330 and 437-440, respectively, is essential for the expression of repZ. These sequences are separated by 107 bases from each other and have a potential to form a novel pseudoknot with stem-loop structure I. We have also shown that some mutations occurring in the 5′GGCC3′ sequence decreased considerably the activity of Inc RNA. Since the synthesis of the RepZ protein is rate-limiting for the replication frequency of the ColIb-P9 plasmid, the 5′GGCC3′ sequence in the inc gene region plays a central role in both positive and negative regulations of ColIb-P9 replication. To our knowledge, this might be a first example indicating that a single specific sequence is positively and negatively involved in regulation of gene expression for DNA replication in vivo.

Positive Control of repZ Expression—It has been proposed previously that repY translation is required for the disruption of stem-loop structure III sequestering the ribosome binding site for repZ translation (17). The involvement of two specific complementary sequences in repZ expression strengthens arguments in favor of the above mechanism with the possible formation of a pseudoknot from stem-loop structure I and the 5′CGCC3′ sequence during the repZ translation reaction. In addition, our preliminary experiments indicate that the disruption of stem-loop structure III by base changes results in the synthesis of the RepZ protein even in the absence of repY translation.3 In this context, our present hypothesis is that repY translation functions to disrupt stem-loop structure III, facilitating the formation of the pseudoknot, which stabilizes transiently the unfolded structure III. Only under these conditions do ribosomes recognize the repZ-translational initiation signal to proceed the translation reaction, as illustrated in Fig. 6.

The proposed model explains well the properties of all rep and sup mutants. In the case of rep2060, the translation of repY is terminated at the amber codon corresponding to codon-11 of RepY. Such an incomplete translation can not disrupt stem-loop structure III. Accordingly, the pseudoknot is not formed. In the case of rep2006, rep2041, and rep2044, the repY translation proceeds normally. However, the pseudoknot is not efficiently formed unless the compensatory base changes occur in the complementary sequences as has been observed in the class II suppressor mutations. Additionally, all the rep mutations that occurred in the repZ leader region are suppressed by single base insertions or two base deletions to result in translational repY-repZ fused genes. Since repZ in these fusions is integrated into a part of repY in structure, and since repY translation does not require the interaction between the two complementary sequences, the pseudoknot is not necessary for their expression. Thus, our model satisfies all the results described in this report.

Brierley et al. (35) have recently reported that a coronavirus RNA pseudoknot functions as a signal for the translational frameshift in the synthesis of the 95-kDa protein. This information is interesting in view of the role of repY translation

3 K. Asano and K. Mizobuchi, unpublished result.
in repZ expression. Here two possible mechanisms could be considered. One mechanism is that repY translation per se is required only for the disruption of structure III. When ribosomes traverse the structure III region in the course of repY translation, the secondary structure is transiently disrupted. Consequently, the interaction between the two complementary sequences is induced intrinsically for the initiation of repZ translation. The other mechanism is that termination of repY translation is further required for facilitating repZ translation. Indeed, the distance between the repY termination codon and repZ initiation codon is about 10 bases in length, suggesting that repZ translation occurs easily by the switch of ribosomes from the former to the latter. To clarify these possibilities in relation to the pseudoknot formation, studies are now in progress.

Negative Control of repZ Expression by Inc RNA—Inc RNA regulates the expression of both repZ and repY genes at the translational level. The nucleotide sequence of the inc gene region shows that there are 2 and 88 bases in length from the 5′-end of Inc RNA to the possible repY and repZ ribosome binding sites (Shine-Dalgarno sequences), GGGT and TATAAGCGG, respectively (see Fig. 1). These features and the observations that repZ expression depends on repY translation might predict that Inc RNA regulates repZ expression indirectly through controlling repY expression. However, this was not the case, and repZ expression was repressed more preferentially by Inc RNA than repY expression (Table III). To explain these phenomena, here we propose a possible mechanism wherein Inc RNA reacts with RepZ mRNA in a two step manner (Fig. 6). The first reaction is the binding of Inc RNA to RepZ mRNA at the specific sequence(s) including 5′UGGC3′ at positions 326 and 329 in the loop of stem-loop structure I. As a result, Inc RNA prevents the intramolecular interaction between the two complementary sequences of RepZ mRNA, hence directly regulating repZ expression. The second reaction is the formation of an RNA duplex between Inc RNA and RepZ mRNA at the entire complementary region, as has been previously reported (16). This reaction might follow the first reaction although the precise mechanism is unknown. Probably, such a duplex prevents interaction of ribosomes with the repY translation signal, thus regulating repY expression.

Small RNAs whose sequences are complementary to parts of RNAs responsible for replication have been shown to regulate replication in several plasmids. In ColE1 (36, 37), the small RNA termed RNAIL interacts with pre-primer RNAII to inhibit the primer formation for replication. Kinetic analysis indicates that the interaction between RNAIL and RNAII consists of at least two steps, initial recognition reaction, in which the loop regions of RNAIL and RNAII interact with each other, and hybrid formation reaction, in which RNAII forms a duplex with RNAIL at their complementary region (38). In IncP II (8–10), the small RNA regulates the expression of repA at the translational level although the precise mechanism is not known. Yet, in pT181 (39), binding of RNAIL to RNAILIII causes an attenuation of transcription of the downstream region containing the repC gene. Perhaps, a common characteristic in these systems is that binding of small RNAs induces the conformational change of mRNA secondary structures, thereby regulating plasmid replication either at the transcriptional or translational level. Indeed, our results are consistent with this idea because Inc RNA inhibits primarily the formation of the novel pseudoknot structure required for repZ expression.

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