The Plasmid Colb-P9 Antisense Inc RNA Controls Expression of the RepZ Replication Protein and Its Positive Regulator repY with Different Mechanisms*

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The autonomous replication region of plasmid Colb-P9 contains repZ encoding the RepZ replication protein, and inc and repY as the negative and positive regulators of repZ translation, respectively. inc encodes the antisense Inc RNA, and repY is a short open reading frame upstream of repZ. Translation of repY enables repZ translation by inducing formation of a pseudoknot containing stem-loop I, which base pairs with the sequence preceding the repZ start codon. Inc RNA inhibits both repY translation and formation of the pseudoknot by binding to the loop I. To investigate control of repY expression by Inc RNA, we isolated a number of mutations that express repY in the presence of Inc RNA. One class of mutations delete a part of another stem-loop (II), which derepresses repY expression by initiating translation at codon 10 (GUG), located within this structure. Point mutations in stem-loop II can also derepress repY translation, and the introduction of compensatory base-changes restores control of repY translation. These results not only indicate that suppressing a cryptic start codon by secondary structure is important for maintaining the translational control of repZ but also demonstrate that the position of start site for repY translation is critical for its control by Inc RNA. Thus, Inc RNA controls repZ translation by binding in the vicinity of the start codon, in contrast to the control of repZ expression at the level of loop-loop interaction.

The initiation phase of translation is rate-limiting in protein synthesis and often the target of control of gene expression (1). It is well established that the selection of translational start sites in prokaryotes is stimulated by base pairing between the 3′-end of the small ribosomal RNA (16 S RNA) and the Shine Dalgarno (SD)1 sequence in the mRNA, located ~7 bases upstream of the initiation codon (2). The ribosome-binding site (RBS) of the mRNA contains the SD sequence and the initiation codon (3). Translational control can be achieved by sequencing or opening the RBS, either by binding of trans-acting factors (RNA or protein) or by modulating the higher-ordered structure of the mRNA (2). We have been studying the various aspects of translational control found in the replication control system in plasmid Colb-P9 (4).

The replication of the Colb-P9 plasmid (93 kilobases, IncIa group) depends on expression of the replication initiator protein RepZ, encoded by its autonomous replication region (5), shown schematically in Fig. 1A. The level of repZ expression is strictly controlled at the translational level by the actions of positive and negative regulatory elements, repY (6) and inc (5, 7), respectively. repY is a short open reading frame encoding 29 amino acids, the 3′-end of which overlaps with the 5′-end of repZ (6) (see Fig. 1A). An amber mutation of repY codon 11 (rep57) did not allow repZ to be translated, indicating that repY translation is required for repZ translation (6). The process of repY translation and its termination, rather than the RepZ polypeptide, is required for the induction of repZ translation (8). inc is situated 5′ to repY and encodes the antisense Inc RNA with a size of ~70 bases (5, 7) (see Fig. 1A). Inc RNA represses repZ expression by binding to a complementary region within the repZ mRNA (7).

Inc RNA represses the translation of both repY and repZ at different rates (9). In the presence of Inc RNA, the level of repZ expression is kept constant regardless of changes in copy number of the repZ reporter plasmid, although repY expression is increased with an increase in plasmid copy number (10). Thus, Inc RNA appears to help maintain the constant level of repZ expression, by repressing repZ expression more efficiently than repY expression. Because the level of repZ expression is linearly correlated with the copy number of Colb-P9, it was proposed that the negative feedback loop conferred by this differential control plays the major role in establishing the constant copy number of Colb-P9 (4).

To understand the mechanism of this unique differential control by Inc RNA, it was important to analyze the structure of the repZ mRNA leader region and its changes during the regulation. Fig. 1B shows the secondary structures of the repZ mRNA leader, deduced from the ribonuclease cleavage experiments in vitro (10). We found that two stem-loop structures, I and III, play important negative roles. Structure I is the target site of the Inc RNA: base pairing between the 5′-rGGC-3′ sequence in the loop I (positions 327–329) and its complementary sequence in the Inc RNA is rate-limiting for the binding of Inc RNA (11). Structure III sequesters the repZ RBS (Fig. 1B): its disruption by mutations derepresses repZ expression, independently of the actions of repY and Inc RNA (8). These stem-loop structures are drawn schematically in Fig. 1C, panel a.
These revertants, except for those isolated from plaque-purified three or four such revertants from each parent and sequenced them to determine the site of mutation as described previously (9).

The revertants were classified into four types, as shown in Table 1, according to whether or not they carried repY-repZ fusion mutations (column 5), or disrupted stem-loop II (column 9). λrep2006 (inc2 G327A) generated two groups of revertants showing different burst sizes on W3110 (typical fused) versus CH11, one with that of ~30, and the other with that of ~80. The former, consisting of type 3 mutations, is isolated more frequently than the latter consisting of type 4 mutations. A(rep1030) (inc1 G327A) generated type 3 mutations more frequently (column 5), because C403 insertion occurred predominantly after four consecutive cytosine residues, including one altered by inc1 at position 400. Because λrep2044 (inc2 G438A) generated both type 1 and type 4 mutations in a similar frequency, we isolated and sequenced ~30 revertants in a single isolation, and part (this table) or all (Fig. 2B) of the isolated sup mutations are reported.

The third structural element is the pseudoknot formed with structure I and the base pairing between the 5′-RGGCG-3′ (loop I at positions 327–329) and 5′-RGGCG-3′ sequences (stem III) at positions 437–440 (bracketed in Fig. 1B). The formation of this pseudoknot is absolutely required for repZ translation (4, 9, 10) and is induced in vitro when structure III is disrupted by mutations (10). We proposed that the translation of repY induces the pseudoknot formation during the termination process, thereby stimulating the access of the ribosome to the repZ RBS (Fig. 1C, panel b), whereas Inc RNA inhibits it immediately by binding to the loop of structure I (Fig. 1C, panel c). Subsequently, duplex formation between the 5′-end of Inc RNA and a region proximal to the repY RBS inhibits repY translation (Fig. 1C, panel d). We provided the biochemical evidence for the loop-loop inhibition model in panel c by showing that the pseudoknot formation and the initial base pairing of Inc RNA to the loop I are competitive (4). Importantly, the model in Fig. 1C can explain the differential control of repY and repZ expression by Inc RNA.

IncFII plasmids also encode both antisense RNA and leader peptide upstream of their replication initiator genes as negative and positive regulatory elements, respectively (Refs. 12 and 13; for review, see Ref. 14). In the case of plasmid R1 of this group, it was demonstrated that the bound antisense CopA RNA inhibited the access of 30 S ribosome to the leader peptide RBS in vitro (15). However, these plasmids do not require mRNA pseudoknot for the expression of their replication initiator genes (13, 16). Thus, the IncIα plasmid ColIb-P9 and its close relatives in IncB group (17) provide unique opportunities to study a single antisense RNA that controls expression of two different genes.

Here, we study the regulation of repY expression by Inc RNA through isolating a number of mutations that derepress repY expression in the presence of Inc RNA. The members of one class of mutations delete a part of the stem-loop structure II (see Fig. 1B) and allow repY translation from its codon 10, GUG, normally embedded in this structure. Point mutations in structure II can also derepress repY translation, and the introduction of compensatory base-changes restores control of repY translation. These results indicate that the position of the start site for repY translation is critical for control by Inc RNA. In contrast, the control of repZ expression by Inc RNA is not constrained spatially, because the site of Inc RNA binding is ~90 bases upstream of the RepZ start codon. Furthermore, by identifying the importance of structure II for suppressing a cryptic start codon, we strengthen the idea that mRNA structure is critical for maintaining the integrity of repZ translational control.

### MATERIALS AND METHODS

**Bacteria, Phages, and Plasmids—** *Escherichia coli* K-12 strains W3110 and W3110inc1 (4) were used as the hosts of λColIb-P9 hybrid plasmids. Strain MC1061 (lacX74) (18) was employed for lacZ fusion studies and as the host for mini-ColIb-P9 plasmids. *λCH10* (5) was a hybrid between *λVIII* (clam) (19) and the 3.0-kilobase EcoRI fragment of ColIb-P9 (5) (Fig. 1A) that shows autonomous replication. *λCH10–1* and *λCH10–2* are derivatives of *λCH10* carrying the Inc-promoter mutations inc1 (A400C) and inc2 (T374C),

### TABLE I

| Strain | Source | Sup (7) | Disrupt Stem-loop II? (8) | Type (10) |
|--------|--------|---------|--------------------------|-----------|
| ACH100W | 11 (324C) | Yes | Yes | 4 |
| repY amber mutation (RepY codon-11 to UAG) | | | | |
| ACH10rep77 | Ref. 6 | rep57 | | |
| λrep2060 | Ref. 9 | inc2 | rep57 | |
| Pseudoknot mutation | | | | |
| λrep2044 | Ref. 9 | inc2 | G438A | |
| λrepZ | Ref. 9 | inc2 | G327A | |
| λrep2134 | Ref. 9 | inc2 | G327A | |
| λrep1030 | This study | inc1 | G327A | |
| λrep2041 | This study | inc2 | G320A | |
| λrep2006 | Ref. 9 | inc2 | G327A | |
| λrep2056 | Ref. 9 | inc2 | G327A | |
| λrep2004 | This study | inc1 | G327A | |
| λrepY-repZ fusion? (8) | | | | |
| λrep2006-1 | Yes | Yes | 4 |
| λrep2056-1 | Yes | Yes | 4 |
| λrep2134-1 | Yes | Yes | 3 |
| λrep1030-2 | Yes | No | 3 |
| λrep2041-2 | Yes | Yes | 4 |
| Typical sup mutants isolated | | | | |
| ACH10del1 | D20 (420–439) | Yes | Yes | 4 |
| ACH10del2 | D5 (421–425) | Yes | Yes | 4 |
| AW21 | Δ11 (421–431) | Yes | Yes | 4 |
| ACH10del57 | D5 (403–407) | Yes | Yes | 4 |
| λrep2060-4 | D5 (405–409) | Yes | Yes | 4 |
| AR2 | ΔC–466 | No | No | 1 |
| AR21 | D4 (405–408) | Yes | Yes | 1 |
| AR42 | ΔA422 | Yes | Yes | 1 |
| AR52 | Δ11 (421–431) | Yes | Yes | 3 |
| AR72 | D4 (405–408) | Yes | Yes | 4 |
| λsup1060-3 | Δ11 (421–431) | Yes | Yes | 4 |
| λsup1068-1 | D11 (421–431) | Yes | Yes | 4 |
| λsup1053-2 | C464A | No | No | 2 |
| λsup2176-2 | C464A | No | No | 2 |
In most cases, we located additional mutations in the control region. Such revertants were purified to homogeneity and analyzed for selections that show excessive replication in the presence of the Inc RNA, we plated these phages on W3110(inc2) (6) and W3110(inc2 G438A IT454; repY-repZ-lacZ Pin*) (9). The purified RNA polymerase and T7 RNA polymerase, respectively.

**RESULTS**

**Isolation of Constitutive ColIb-P9 Replication Mutations from the λColIb-P9 Hybrid Phages**—The method of isolation of ColIb-P9 replication control mutations using the λColIb-P9 hybrid phage system was described previously (5–9). In this study, we isolated ColIb-P9 mutations that show excessive replication in the presence of the inc gene in trans. We employed a wide variety of the λColIb-P9 species as parents (Table I, columns 1–4). The parental phages included ACH10, ACH10W (334C wild-type), and ACH10 rep phages defective in the pseudoknot formation or the repY or repZ start codon, thereby reducing repZ expression. These phages did not form visible plaques on W3110(λind*) cells, because the replication from the λ portion was inhibited by the λ repressor encoded by the λind– prophage, and the replication frequency from the ColIb-P9 portion was not sufficient to support the lytic growth of the hybrid phage. To eliminate possible revertants, the replication of which was repressible by Inc RNA, we plated these phages on W3110(λind*) harboring pCH11 (inc) (or pCH11W for ACH10W). In this condition, they generated visible plaques at frequencies between $10^{-5}$ and $10^{-6}$ (Table I, column 5), a value significantly higher than the frequency ($10^{-10}$) at which the λVIII vector generated visible plaques. Such revertants were purified to homogeneity and analyzed for sequencing the entire replication control region of ColIb-P9 as described (9). In most cases, we located additional mutations in the control region that were responsible for increasing repZ expression, and designated them as sup mutations. In rare occasions, however, we did not find any mutation in the ColIb-P9 control region. This was because mutations occurred in the λ portion, as confirmed by the analysis of the λVIII vector excised from the revertant phage DNA (5). AC phages, listed in Fig. 6A, were isolated as pseudorevertants by plating 2sup2044-13 on W3110(λind*) harboring pCH11.

**Measurement of ColIb-P9 Replication and repZ Expression**—The replication ability of the λColIb-P9 hybrid phages was determined by one-step growth on W3110(λind*) cells as described previously (5). The burst size was calculated by the number of progeny phages divided by the number of adsorbed phages. The level of repZ expression was estimated by measuring the β-galactosidase activity expressed from MC1061 (Lac–) carrying pKa140 or its derivative (9). The specific activity of the enzyme was expressed as Miller's units (22). The method of determination of the copy number (4, 11) of mini-ColIb-P9 plasmids was described previously. The values reported in this study are averages from at least three independent experiments.

**In Vitro Synthesis of the RepZ Protein and the Inc RNA**—Polypeptides directed by pCH11 or its derivative were synthesized by the in vitro coupled transcription-translation methods and analyzed by SDS-PAGE, followed by autoradiography, as described previously (5). Quantitative analysis of synthesis of the Inc RNA from pCH11 or its derivative in vitro was conducted also as described (5).

The binding of the Inc RNA to the repZ mRNA—Inc RNA and the RepZ mRNA leader were synthesized in vitro from pKa100 or its derivative by E. coli RNA polymerase and T7 RNA polymerase, respectively, and purified as described previously (11). The purified RNAs were allowed to bind at 37 °C in a binding buffer (10 mM MgCl2, 100 mM NaCl, 20 mM Tris-HCl, pH 7.6) and analyzed by denaturing PAGE (8.3 M urea) as described (11). Inc RNA-RepZ mRNA hybrid was detected as a complex that persisted the gel electrophoresis and migrated anomalously compared with free RNA species. Secondary structures in the in vitro transcribed RNAs were analyzed with partial ribonuclease cleavage, as described previously (10).

**Plasmid**

| Plasmid | Description |
|---------|-------------|
| pBR322  | Cloning vector (20) |
| pCH11   | 1.1-kb EcoRI-SalI fragment of ACH10 into pBR322 (5) |
| pCH11-1 | pCH11 carrying inc1 (5) |
| pCH11-2 | pCH11 carrying inc2 (5) |
| pCH11-R57 | pCH11 carrying rep57 (6) |
| pCH11-57-1 | pCH11 carrying rep57 sup57-1 (ΔΩA399) (6) |
| pCH11-1C | pCH11 carrying del2 |
| pCH11W  | pCH11 carrying 334C |
| pKA1    | lacZ fusion vector (9) |
| pKA140  | 1.1-kb EcoRI-HincII fragment of ACH10 into pKA1; repZ-lacZ Pin* (9) |
| pKA140-W21 | pKA140 carrying del2; repY-repZ-lacZ Pin* |
| pKA140-12 | pKA140 carrying inc6; repZ-lacZ Pin* (9) |
| pKA140-12sup2044-13 | pKA140 carrying inc2 G438A IT454; repY-repZ-lacZ Pin* (9) |
| pKA140-34 | pKA140-12sup2044-13 carrying G412C |
| pKA140-34* | pKA140-12sup2044-13 carrying G421G |
| pKA140-34A | pKA140-12sup2044-13 carrying G405A |
| pKA140-34A398 | pKA140-12sup2044-13 carrying G405A/C428T |
| pTZ19R  | Cloning vector with T7 promoter (21) |
| pKA100  | 281-bp Nsp(7524)I-Sau3AI fragment of CH10 containing a moderate deletion |
| pKA100del2 | pKA100 carrying del2 |
| pKA10  | pKA100 carrying 334C (11) |
| pKA10-W21 | pKA100 carrying 334C W21 |

*References are given in parentheses for plasmids constructed previously.
FIG. 1. Control of replication of plasmid ColIb-P9 involving antisense RNA and the repZ mRNA pseudoknot. A, the autonomous replication segment of ColIb-P9. Boxes denote the functional segments inc, repY, repZ, CIS, and ori. RepZ is presumed to act on ori and stimulate the initiation of unidirectional replication (dotted arrow) from the G-site (36). Transcription termination of RepZ mRNA at CIS is additionally required for ColIb-P9 replication (37). ter denotes the replication termination site. Short, thick arrow represents Inc RNA. Open and closed circles on the transcripts represent the 5'→3' and 5'→3' sequences, respectively, critical for the positive (+) and negative (−) control of repZ translation. The recognition sites for restriction enzymes used in this study are shown by arrowheads; Bs, BstPI; E, EcoRI; E/B, EcoRI site converted from the original BglII site; H, HincII; N, NspI(7524)I; S, SalI; Sa, Ssa3AI. B, nucleotide sequence and secondary structures in RepZ mRNA leader region. Roman numerals indicate secondary structures identified in the RepZ mRNA in vitro (10). The SD sequences for repY and repZ are shown by asterisks. Start codons of repY and repZ are underlined. The stop codon of repY is double-underlined. The two largest complementary sequences are boxed. The bases critical for the pseudoknot formation are shown by brackets. C, the proposed regulatory model of repZ expression. Panels a, b, c, and d represent the possible states of RepZ mRNA. Roman numerals indicate stem-loop structures. The region complementary to Inc RNA is bracketed. Open boxes represent the repZ RBS. Filled boxes represent the complementary sequences for the pseudoknot formation. Filled and open circles represent start and stop codons for repY, respectively. See the Introduction for details on this model. Panel a depicts the nascent RepZ mRNA. Panel b describes conformational changes induced by repY translation. Panels c and d describe the steps of Inc RNA binding.
Control of ColIb-P9 Replication Genes by Inc RNA

Column 5) when plated on W3110(\(T-374\) and G-438 are altered to C and A (listed in Table I except ones from designated here as ical of lytic phages (data not shown) and is consistent with the revertants identified showed burst sizes of \(\text{repY}\) indicate base substitutions or single-base deletions or insertions.\(\text{repY}\) functions.

Fig. 2. Locations of sup mutations that derepress repY or repZ expression in the repZ leader region. A, locations of the sup mutations, listed in Table I except ones from \(x2\text{rep2044}\), are indicated on the nucleotide sequence of the repZ leader region, numbered as in Fig. 1. Arrowsheads indicate base substitutions or single-base deletions or insertions. Thick horizontal bars denote the positions of deletions. Amino acid sequences for repY and repZ are shown below the nucleotide sequence.

B, locations of all the sup mutations isolated from \(x2\text{rep2044}\) are described as in A. Note that T-374 and G-438 are altered to C and A (boldfaced) in the nucleotide sequence, due to the parental mutations \(inc2\) and G438A, respectively.

Column 5) when plated on W3110(\(\text{ind}^-\))\(p\)CH11, an \(E.\) \(c\)oli strain expressing the wild-type Inc RNA. The spontaneous revertants identified showed burst sizes of \(\geq30\), which is typical of lytic phages (data not shown) and is consistent with the growth phenotype. They were found to contain mutations (designated here as sup) in the repZ leader region, as described in Fig. 2 and Table I, columns 6-9, and classified into four types according to the nature of the mutations (Table I, column 10). These sup mutations exhibited repZ expression more than 100 times higher than their parents, when examined using a translational \(\text{lacZ}\) fusion (see under “Materials and Methods”). A subset of the results is summarized in Table III. These high expression levels were not affected by the presence of the \(\text{inc}\) gene in trans, as was expected from the mutant selection scheme (data not shown). Thus, all the identified mutations derepress repZ expression regardless of the Inc RNA inhibitory function.

The type 1 and type 2 sup mutations, isolated only from specific rep phages, were found to derepress translation from the repZ reading frame (Table I). Type 1 mutations, reported previously (8), deleted C-446 or changed it to a guanine residue (Fig. 2). These mutations strengthen the SD sequence for repZ and weaken structure III in the context of the parental G438A, thereby derepressing translation from the natural repZ start codon (8). The novel type 2 sup mutations altered C-464 to adenine and created a new AUG codon at RepZ codon 4 (Fig. 2). Thus, the type 2 sup mutations derepressed repZ expression by initiating translation from the AUG codon outside of the replication control region (see under “Discussion”).

Excessive ColIb-P9 Replication Mutations That Derepress Translation from the repY Reading Frame—Type 3 and type 4 sup mutations fused repY in-frame with repZ (Table I and Fig. 2) and hence are expected to derepress the repY-repZ fusion expression by impairing the inhibitory action of Inc RNA on repY expression. Type 3 sup mutations were single-base insertions distributed throughout repY and were only identified in rep phages carrying the G327A pseudoknot mutation in the loop I (Table I). Such repY-repZ fusion mutations were isolated also from other rep phages on W3110(\(\text{ind}^-\))\(p\)CH11 (inc) (6, 9). We previously showed that the wild-type Inc RNA binds RepZ mRNA carrying G327A \(>50\)-fold more slowly than it binds the wild-type RepZ mRNA, due to the C:A mismatch at position 327 (11). Therefore, the isolation of type 3 mutations from the G327A mutants reinforces the idea that Inc RNA represses repY expression in a manner requiring the loop-loop interaction with structure I.

Type 4 sup mutations included more dramatic alterations characterized by deletions of up to 23 bases and therefore occurred at the lowest frequency of \(10^{-9}\) (Table I).

Interestingly, these mutations commonly disrupted a part of stem-loop structure II (Fig. 2). In contrast to other sup mutations, the type 4 mutations were isolated from a variety of \(\lambda\)-ColIb-P9 hybrid phages. Thus, disruption of structure II alone appears to be sufficient for the lytic growth of \(\lambda\)-ColIb-P9. Because involvement of structure II is novel, we further characterized these mutations.

First, we examined whether type 4 sup mutations impaired the action of Inc RNA against repY translation. When the repZ leader DNAs of three representative phages, \(\lambda\)CH10del1, \(\lambda\)CH10del2, and \(\lambda\)CH10del57, were subcloned into \(p\)BR322 and examined in vitro protein synthesis, we observed polypeptides (Fig. 3, lanes 5, 6, and 9, respectively, arrowheads), slightly smaller than the RepY-RepZ fusion protein (29 kDa) (lane 3) but significantly larger than the RepZ protein (26 kDa) (open triangles) from the wild-type or \(inc\) (lane 1, 2, or 8). Because translation from the natural repZ start codon was not observed, type 4 sup mutations bypassed the control of repY expression by Inc RNA. However, it is not clear by this analysis whether the repY-repZ fusion proteins observed in the type 4 sup mutants were synthesized exclusively from the natural repY start codon (see below).
Burst sizes of representative λ-ColIb-P9 sup mutants and their parents were measured on W3110(λind−) as described previously (5) and shown below. To measure the level of repZ expression, pKA140 derivatives carrying the sup mutations were constructed and introduced into MC1061 (lac−) for determination of β-galactosidase activity. The value in Miller’s unit (22) is presented here as RepZ activity.

| Strain         | Mutation       | Burst size | RepZ activity |
|---------------|---------------|------------|---------------|
| Type 1 mutations | λ2rep2044 (Parent) | <0.5       | 6.1*         |
|               | ΔR21          | 77         | 3863         |
|               | ΔR11          | 76         | 3960         |
| Type 2 mutations | λ2rep2176 (Parent) | <0.5       | 8.7          |
|               | Δ2sup2176-2   | 132        | 1386         |
| Type 3 mutations | λ2rep2006 (Parent) | <0.5       | 5.7*         |
|               | Δ2sup2134-1   | 88         | 1405         |
| Type 4 mutations | ΔCH10 (Parent) | <0.5       | 2.5*         |
|               | ΔCH10de12     | 74         | 2110         |
|               | ΔCH10W (Parent) | <0.5       | 0.7*         |
|               | ΔW21          | 102        | 1062         |

* Data from Ref. 4.

RepZ mRNA leader depended only on the base in the loop of structure I (at position 334), but not on bases in structure II, deleted in del2 or W21. Finally, primer extension analyses of the Inc RNA-RepZ mRNA hybrid indicated that the movement of reverse transcriptase on RepZ mRNA was blocked at U-363 by Inc RNA (7) (see Fig. 5A for the position of hybrid formation) regardless of the presence of del1 or del2. These results together indicate that both the binding rate and the final product of Inc RNA-RepZ mRNA duplex formation were not affected by the type 4 sup mutations.

We also conducted ribonuclease cleavage experiments using the in vitro transcribed RepZ mRNA leader from del2 (employed in Fig. 4B) and confirmed that stem-loop II in this mutant was indeed interrupted without inducing additional stem-loops surrounding the deleted region (data not shown).

The Type 4 sup Mutations Derepress the Translation of repY from Its Codon 10 GUG—At this point, a likely possibility was that the type 4 sup mutations derepressed a cryptic start codon in the repY reading frame that is normally sequestered in structure II. repY codon 10 GUG could be such a start codon, because it is located 8 bases downstream of a possible SD sequence 5′-rAGAGAU-3′ at positions 391–396 (see Fig. 5A). This hypothesis accounts for the following two contradictions, brought about if repY-RepZ expression from the type 4 sup mutants is initiated only from the natural repY start codon: (i) a type 4 sup mutant was isolated from the repY start codon mutant λ1rep1060 (Table I); and (ii) a four-base deletion in λR72 disrupted a part of structure II, but did not fuse repY in-frame with repZ, and hence can be classified as a subtype of type 4, type 4* (Table I and Fig. 2B). The AR72 mutation may derepress RepZ translation from the GUG codon at positions 410–412 (in-frame with repY) by bringing it closer to the presumed SD sequence (positions 391–396) for the repY codon 10.

To test the possibility that type 4 sup mutations derepress translation from the repY codon 10, we altered this codon to CUG (G405C) in pKA140del2 (Fig. 5A). As a control, we employed the sup2044-13 mutant (inc2 G438A OT454) that produces only RepY-RepZ fusion (and not RepZ) protein as described previously (9). We found that changing repY-codon 10 (G405C) in the del2 mutant reduced the level of repY-RepZ expression to 1.6% (Fig. 5B, column 2), whereas it did not alter repY-RepZ expression in sup2044-13 (Fig. 5C, column 2). Conversely, altering the natural repY start codon to ACG (T379C) in del2 reduced repY-RepZ expression to 66% (Fig. 5B, column 3), whereas the same mutation reduced expression in sup2044-13 to 2.1% (Fig. 5C, column 3). In addition, altering

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Fig. 3. In vitro protein synthesis from three type 4 sup mutants, del1, del2, and del57. The 35S-labeled proteins synthesized from pCH11 and its mutant derivatives were separated by 7.5% SDS-PAGE (see under “Materials and Methods”). Arrowheads and open triangles indicate the repY-RepZ fusion and repZ proteins, respectively. Ovalbumin (45 kDa) and carbonic anhydrase (31 kDa) were used as the size markers. Mutations in the plasmids are listed across the top. Lane 8, pBR322 was used instead of pCH11 plasmids.

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K. Shiba and K. Mizobuchi, unpublished observations.
each of three bases of the proposed repY SD sequence (5′-rGGGU-3′) at positions 366–369 (Fig. 5A) reduced repY expression to 51–74% in sup2044-13 (Fig. 5C, columns 4–6). These results not only confirm the location of the repY RBS but also indicate that the translation of the RepY-RepZ fusion protein in del2 is initiated mainly from the repY codon 10.

It was conceivable that the del2 T379C double mutant (Fig. 5B, column 3) expressed only the shorter form of the fusion protein, as expected for sup1060-3 and R72 mutants (see above and Table I). To confirm this idea, we introduced G405C, which alters codon 10 to this double mutant. The resulting triple mutant (G405C T379C del2) showed only 5.5 β-galactosidase units of repY expression, a value 320-fold lower than that of del2 T379C, and even 8-fold lower than the level from del2 G405C. Thus, the RepY-RepZ fusion protein from the del2 T379C double mutant is initiated only from the repY codon 10.

Next, we wished to examine the effect of Inc RNA on repY expression from different mutants. To maximize its effect, we compared repY-RepZ expression from isogenic pKA140 derivatives carrying either Pinc°C or Pinc°; inc2 reduces the amount of Inc RNA in vivo to ~18%. By this approach, repY-RepZ expression in sup2044-13 was reduced to 6% (Fig. 5C, column 7), a reduction much larger than the value (29%) obtained by supplying the inc gene from another plasmid (9). The same is true for control of repZ expression; repZ expression from inc2 was reduced to 1.4% compared with the wild-type (Table IV, wild-type), a reduction larger than the one previously reported, 11% (9). These results confirm that repZ expression is reduced more efficiently than repY expression by the same increase in the amount of Inc RNA (9, 10). Because repY-RepZ expression in del2 was increased only 1.3-fold by the introduction of inc2 (Fig. 5B, column 4), we conclude that the type 4 sup mutations retain high levels of repY-RepZ fusion expression by allowing translation initiation from the repY codon 10. Importantly, this aberrant translation initiation is not repressible by Inc RNA, demonstrating that the Inc RNA binding site must be close to the repY RBS for the normal control of repY expression by Inc RNA.

The Base Pairings in Structure II Suppress Translation from repY Codon 10—It was conceivable, however, that the putative start codon mutations introduced to del2 (see Fig. 5B) might have caused some unexpected alteration in the secondary structure of the RNA, due to the lack of a strong RNA secondary structure surrounding the deleted region. To overcome this problem, we attempted to obtain point mutations in stem-loop II. We plated A2sup2044-13 (repY-RepZ fusion) or W3110(lacI) sup2044-13 and isolated clear-plaque forming mutants. As shown in Fig. 6A, we were able to isolate mutations that were presumed to interrupt structure II. Among eight independent mutations, two altered G-412 to T (G412T), three changed G-412 to C (G412C), two changed G-425 to A, and still another had G-405 altered to A (G405A). Note that the last mutation altered the repY codon 10 to a typical AUG start codon. We constructed pKA140-I2sup2044-13 derivatives, transformed MC1061 (Lac-ind) in order to assay their β-galactosidase activity and found the β-galactosidase levels 2-fold higher than that from sup2044-13 (data not shown; see also Fig. 6, B and D, left columns).

To determine whether these mutations disrupted base pairing in structure II, we introduced compensatory base-changes to pKA140-C4 (G412C) and pKA140-C9 (G405A). We found that the resulting plasmids, pKA140-C4* and pKA140-C9*, respectively, showed repZ expression slightly lower than but comparable to their parents (Fig. 6, C and E, left columns). When the amount of Inc RNA transcribed from the lacZ fusion plasmids was restored by converting their inc2 mutation to the wild-type, we still observed high levels of repY expression in C4 and C9 (Fig. 6, B and D, right columns); however, C4° and C9° showed repY expression reduced to 6% as compared with the inc2 derivatives of the same strains (Fig. 6, C and E, right columns). In addition, the repY codon 10 mutation G405C, which disrupts the same base pair as that in C9, did not affect repY-RepZ expression in sup2044-13 as shown above in Fig. 5C, but this level of expression was repressible to 5% by increasing in the amount of Inc RNA (Fig. 6F). We conclude that base pairings in structure II allow repY translation to be repressed by Inc RNA through elimination of unregulated translation initiation from the repY codon 10 GUG.

Furthermore, the defective control of repY by Inc RNA, as observed in del2 (Fig. 5B), was restored when we restored the stem-loop II structure disrupted by del2 with a completely different sequence (Fig. 6F). Therefore, only base pairing, not a specific RNA sequence, contributes to suppression of this cryptic start codon.

3 K. Asano and K. Mizobuchi, unpublished observations.
Finally, we examined the effects of deleting the whole structure II, including repY codon 10. Table IV summarizes the level of repZ expression from pKA140 (Pinc1) and pKA140-I2 (Pinc2) derivatives that lack the entire structure II. When 30 nucleotides from position 404 to 433 and encompassing structure II were deleted (in D30; see Fig. 6A for its position), repZ expression from the Pinc+ derivative relative to the Pinc2 derivative was 1.8%, comparable to the ratio observed with the wild-type (Table IV). When 32 nucleotides were deleted to fuse repY in-frame with repZ (in D32), repY-repZ expression from the Pinc+ derivative relative to the Pinc2 derivative was 7.6%, again comparable to
inc RNA Controls Translation of repY and repZ with Different Mechanisms—In this study, we isolated and characterized a number of mutations (sup) that allow excessive repZ expression in the presence of Inc RNA (Tables I and III and Fig. 2). Two classes of sup mutations derepressed repY-repZ fusion expression and provided insight into how Inc RNA represses repY expression. Type 3 sup mutations, isolated only from the mutants altering G-327 in the loop I, were single-base insertions at any place within repY (Table I and Fig. 2A), and we confirmed that Inc RNA repression of repY expression is dependent on the loop-loop interaction between RepZ mRNA structure I and Inc RNA as proposed previously (4, 9, 11).

Type 4 sup mutations disrupted a novel stem-loop in RepZ mRNA (structure II) (Figs. 1B and 2), and caused constitutive repY-repZ fusion expression by allowing translation initiation at repY codon 10 GUG (Figs. 3–5). We provided evidence that base pairing in structure II blocks translation from this cryptic start codon (Fig. 6), implying that repY codon 10 is normally sequestered in structure II. These results indicate that structure II plays an important role in control of repY translation by Inc RNA by blocking unregulated translation from a cryptic start codon. This is probably the only role played by structure II, because deletion of the whole structure II together with the cryptic codon did not significantly affect the level and control of repZ expression (Table IV) and ColIb-P9 replication (data not shown). In agreement with this, the replication control region of the IncZ-group plasmid pMU2200, closely related to ColIb-P9, contains the analogues of repY, inc, structures I and III, and the pseudoknot but lacks structure II (23).

The fact that repY translation from the natural start codon was repressible by Inc RNA whereas that from the codon 10 GUG was not (Fig. 5) indicates that the position of the translational start site of repY is critical for its control by Inc RNA. Using the replicon of a plasmid closely related to ColIb-P9 (IncB-group pMU720), Wilson et al. (17) showed that the binding of RNA I (the Inc analogue) failed to repress repB (the repY analogue) translation, when a foreign nine-base sequence was inserted between the repB SD sequence and the 3′-end of the RNA I binding site. These findings, together with the results presented here, establish that Inc RNA must bind in the vicinity (within 2 bases) of the repY RBS in order to repress repY translation.

Inc RNA consists of a large stem-loop made up of 51 bases and a single-stranded 5′ leader (11). Analyses using in vitro transcribed RNAs and site-directed mutagenesis suggested that Inc RNA binds to the repZ mRNA through two intermediate steps, a transient interaction of its loop with the loop of RepZ mRNA structure I, followed by base pairing between 5′-end of Inc RNA and a region proximal to the repY RBS (4, 11). Our results are consistent with the model that repY translation is inhibited due to the latter step. Similar models were proposed for antisense RNA-mediated inhibition of leader peptide synthesis in the IncFIU R1 (12, 15, 24) and IncB pMU720 (17, 25) plasmids. It is noteworthy that Inc RNA controls repY expression with a mechanism quite different from that revealed for repZ expression, i.e. inhibition of the pseudoknot formation by the transient loop-loop interaction (see the Introduction). Based on our previous and present analyses, it could be proposed that two different parts of Inc RNA, the loop and 5′ leader regions, directly inhibit RNA-RNA interactions critical for repZ and repY expression, respectively.

that seen with sup2044-13 (Fig. 5C). Moreover, we found that a mini-ColIb-P9 plasmid carrying the D30 deletion was stably maintained at a copy number 1.7-fold higher than that of the wild-type (data not shown). Thus, all of structure II could be deleted without affecting control of repY and repZ expression by Inc RNA and replication of the ColIb-P9 plasmid.

**FIG. 6.** The repY codon 10 GUG is sequestered in the stem-loop structure II. A, isolation of point mutations in structure II. Deduced RepZ mRNA sequence from position 400 to 435 is shown with the stem-loop II structure. The repY codon 10 is boxed. Point mutations found in λC phages (indicated in parentheses), isolated from λ2sup2044-13 (inc2 G438A T4T454), are shown by arrows and altered bases. Bars below the sequence denote positions of deletions used in Table IV. B–E, the structures of stem-loop II from C4, C9, and their derivatives deduced with the program of Zuker and Stiegler (38) are shown with altered bases in boldface. Histograms show the levels of repY-repZ fusion expression from these mutants (left columns) and their derivatives carrying Pinc* (right columns), measured with the lacZ fusion method (see under “Materials and Methods”). Bars above each β-galactosidase value represent S.D. from three independent measurements. Percentages of expression of the Pinc* derivatives are also indicated. F and G, G405C and a six-base insertion were introduced to pKA140 I2sup2044-13 and pKA140del2, respectively, repY expression from their Pinc2 and Pinc* derivatives is compared as described above for B–E. The 6-base insertion was introduced to pKA140del2 by replacing the 9-base sequence following the repY codon 10 with the 15-base sequence 5′-rUAUGCGAUGUAUGC-3′, without altering the deletion junction found in del2. Bases different from those in wild-type structure II are shown in boldface. The value in the left column in F is taken from column 2 in Fig. 5C.

**DISCUSSION**

Inc RNA Controls Translation of repY and repZ with Different Mechanisms—In this study, we isolated and characterized a number of mutations (sup) that allow excessive repZ expression in the presence of Inc RNA (Tables I and III and Fig. 2). Two classes of sup mutations derepressed repY-repZ fusion expression and provided insight into how Inc RNA represses repY expression. Type 3 sup mutations, isolated only from the mutants altering G-327 in the loop I, were single-base insertions at any place within repY (Table I and Fig. 2A), and we confirmed that Inc RNA repression of repY expression is dependent on the loop-loop interaction between RepZ mRNA structure I and Inc RNA as proposed previously (4, 9, 11).
**Effect of the Translating Ribosome on mRNA Translation**—We have identified two GUG start codons in the coding region of repY; one (repY codon 10) is silenced by structure II, and the other is located in structure III, which serves as the repZ start codon (Fig. 5A). Translation initiation from the latter requires both the termination event of repY translation and the complementarity between 5′-side stem of structure III and the loop of structure I for the pseudoknot formation (8–10). Yet, in the case of the MS2 RNA phage, a simple passage of ribosome through the coat gene region is sufficient to open the RBS for replication and lysis proteins (26).

What determines the accessibility of a certain RBS located in the coding region during the process of translation? It is likely that the balance between the affinity of the ribosome to each RBS and the stability of the secondary structure that blocks it make up this difference. In the case of MS2, the replicase and lysis protein RBSs are blocked with weak (bulged-out) secondary structures (26). Thus, the passage of the ribosome through the coat gene region may be sufficient to trigger ribosome binding to these RBSs and block the formation of RNA secondary structures. In the case of repY codon 10, structure II may refold immediately, and preclude ribosome binding to a relatively weak RBS. In the case of repZ start codon, the repZ RBS is too weak (8) to stimulate ribosome binding during passage of a translating ribosome. Instead, a pseudoknot is induced during the translational termination of repY and serves to keep the repZ RBS accessible for a long enough time to allow ribosome-RBS interaction. Kinetic analyses of mRNA structure formation, such as developed by Ma et al. (27), in combination with coupled protein synthesis, will be important to test these models.

**Importance of mRNA Secondary Structures in Translational Control of repZ**—The identification of a second inhibitory structure (II) also suggests the importance of mRNA structure in the translational control of repZ. This idea is consistent with the previous characterization of structure III with the sup mutations classified here as type 1 (8) and the isolation of the type 2 sup mutations located outside of the replication control region (Tables I and III and Fig. 2A). Interestingly, the isolation of both type I and type 2 mutations appears to require the parental mutations that partially disrupt structure III and creating a stronger SD sequence near the start codon used (8). Thus, we propose that a weak RBS is critical for inhibition of translation by mRNA secondary structures and essential in maintaining the integrity of ColIb-P9 replication control.

A wide variety of translational control mechanisms using mRNA structures evolved in prokaryotes (2), probably because the formation of secondary structures sequestering RBS can directly compete with ribosome binding (28, 29). In contrast, eukaryotic ribosomes scan for the first AUG with the help of numerous initiation factors, as they melt secondary structures found in the 5′ leader of mRNA (30, 31). Accordingly, translational control in eukaryotes occurs by a variety of mechanisms that modulate the activity of initiation factors (32–35).

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