Copy number control of IncI α plasmid Collb-P9 by competition between pseudoknot formation and antisense RNA binding at a specific RNA site

Katsura Asano^{1,2,3} and Kiyoshi Mizobuchi⁴

¹Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo, Tokyo 113 and ⁴Department of Applied Physics and Chemistry, University of Electro-Communications, Chofu-shi, Tokyo 182, Japan

²Present address: Laboratory of Eukaryotic Gene Regulation, NICHD, National Institutes of Health, Bethesda, MD 20892, USA

³Corresponding author
e-mail: kasano@aghamac1.nichd.nih.gov

Replication of a low-copy-number IncI α plasmid Collb-P9 depends on expression of the *repZ* gene encoding the replication initiator protein. *repZ* expression is negatively controlled by the small antisense Inc RNA, and requires formation of a pseudoknot in the RepZ mRNA consisting of stem-loop I, the Inc RNA target, and a downstream sequence complementary to the loop I. The loop I sequence comprises 5'-rUUGGCG-3', conserved in many prokaryotic antisense systems, and was proposed to be the important site of copy number control. Here we show that the level of *repZ* expression is rate-limiting for replication and thus copy number, by comparing the levels of *repZ* expression and copy number from different mutant Collb-P9 derivatives defective in Inc RNA and pseudoknot formation. Kinetic analyses using *in vitro* transcribed RNAs indicate that Inc RNA binding and the pseudoknot formation are competitive at the level of initial base pairing to loop I. This initial interaction is stimulated by the presence of the loop U residue in the 5'-rUUGGCG-3' motif. These results indicate that the competition between the two RNA–RNA interactions at the specific site is a novel regulatory mechanism for establishing the constant level of *repZ* expression and thus copy number.

Keywords: antisense RNA/plasmid replication/RNA loop structure/RNA pseudoknot/translational coupling

Introduction

The copy number of bacterial plasmids is strictly controlled in host cells through a variety of negatively acting regulatory mechanisms (for review, see Scott, 1984; Novick, 1987). In plasmids R6K, RK2 and P1, interactions *in trans* between short repetitive DNA sequences, designated iterons, control their copy number (McEachern *et al.*, 1989; Abeles and Austin, 1991; Kittell and Helinski, 1991; for review, see Nordström, 1990). In other types of plasmids, small antisense RNAs regulate replication in different ways (for review, see Eguchi *et al.*, 1991; Wagner and Simons, 1994). In the case of ColE1, the antisense RNA I inhibits formation of primer RNA for leading strand synthesis (Tomizawa, 1985a), and in the case

of pT181-, IncFII-, IncI α /IncB-type and other types of plasmids, antisense RNAs control the synthesis of the plasmid-specified replication initiator proteins (Kim and Meyer, 1986; Novick *et al.*, 1989; Asano *et al.*, 1991a; Blomberg *et al.*, 1992; Wu *et al.*, 1992; Wilson *et al.*, 1993; del Solar *et al.*, 1997). The IncI α group plasmid Collb-P9 is unique in that an intramolecular RNA–RNA interaction within the replication initiator message plays a critical role in replication control (Asano *et al.*, 1991a).

Collb-P9 is a 93 kb self-transmissible plasmid that is maintained at one or two copies in *Escherichia coli* and other enterobacteria (Clewell and Helinski, 1970; Jacob *et al.*, 1977). Sufficient information to control, initiate and terminate its unidirectional replication is specified by a 3 kb DNA segment as shown schematically in Figure 1A. The replication region contains the *repZ* gene encoding a 39 kDa replication initiation protein (Hama *et al.*, 1990a). RepZ acts on the origin of replication (*ori*), located 3' to *repZ* (Tanaka *et al.*, 1992), presumably recruiting the host replication machineries. In addition to *ori*, transcription termination at *CIS*, located between *repZ* and *ori*, is essential for Collb-P9 replication (Mori *et al.*, 1995).

The *inc* gene resides 5' to *repZ* and encodes the antisense Inc RNA of ~70 bases (Hama *et al.*, 1990a). Inc RNA binds to a complementary sequence within RepZ mRNA and represses *repZ* expression at the translational level (Shiba and Mizobuchi, 1990). The target site of Inc RNA is folded into a stem-loop designated structure I with a size of 51 bases (Asano *et al.*, 1998; see Figure 1B). The base-pairing interaction between Inc RNA and RepZ mRNA at the 5'-rGGC-3' sequence in the loop of structure I at positions 327–329 is critical for the intermolecular interaction probably as an initial recognition reaction (Asano *et al.*, 1998).

Translation of *repZ* depends on translation of *repY*, a short open reading frame (ORF) encoding 29 amino acids. *repY* was identified by isolation of a replication-defective mutation, *rep57*, an amber mutation of *repY* codon-11 (Hama *et al.*, 1990b). We showed that the translation of *repY*, a translation termination event near the *repZ* start codon and a moderate *repZ* Shine–Dalgarno sequence were required for *repZ* translation (Asano *et al.*, 1991b). Translation of *repY* is coupled very efficiently to that of *repZ*; in the absence of Inc RNA, the ratio of translation between *repY* and *repZ* was nearly one (Asano and Mizobuchi, 1998). Similar translational coupling between an upstream reading frame (*tap* or *repA6*) and the replication initiator gene (*repA* or *repA1*) was also reported in IncFII plasmids (R1 or R100, respectively), distant relatives of Collb-P9 (Blomberg *et al.*, 1992; Wu *et al.*, 1992; Kato and Mizobuchi, 1994).

Studies of translational *lacZ* fusions have suggested that *inc* and *repY* cooperate to keep *repZ* expression at a constant level (Asano and Mizobuchi, 1998). In the

presence of the natural amount of Inc RNA, which is in vast excess over that of RepZ mRNA (Shiba and Mizobuchi, 1990), both *repY* and *repZ* expression are reduced >100-fold when compared with *repY* and *repZ* expression in cells lacking Inc RNA. Thus the Inc RNA represses translation of *repY* as well as of *repZ*. However, the ratio of *repY:repZ* translation is now 5:1 when the plasmid copy number is comparable with that of the native ColIb-P9. When the copy number of the reporter system is increased, the level of *repY* expression is increased concomitantly, whereas that of *repZ* is not. Thus, by repressing *repZ* expression more efficiently than *repY* expression, Inc RNA allows the level of *repZ* expression to be kept constant. As the level of *repZ* expression should limit the replication initiation frequency, it was predicted that the constant level of *repZ* expression would lead to constant copy number.

Clues to understanding how Inc RNA controls *repZ* expression came from genetic analyses of additional requirements for *repZ* expression. We found that *repZ* translation also depends on an intramolecular base pairing within RepZ mRNA, which occurs between the 5'-rGGCG-3' sequence (positions 327–330) in the loop of structure I and the 5'-rCGCC-3' sequence (positions 437–440) preceding the *repZ* Shine–Dalgarno sequence (Asano *et al.*, 1991a; see Figure 1B). This base pairing forms an RNA pseudoknot with structure I (Asano and Mizobuchi, 1998). In addition, we found that the 5'-rCGCC-3' sequence and the *repZ* ribosome-binding site (RBS) are masked by another stem–loop designated structure III in the nascent RepZ mRNA (see Figure 1B, and Asano *et al.*, 1991b; Asano and Mizobuchi, 1998). Based on these findings, we proposed that, for the pseudoknot to be formed *in vivo*, structure III must be unfolded by translation and termination of *repY* (Asano *et al.*, 1991b). We presumed that the 3' side of stem–loop III is covered by the ribosome in the termination process, thereby allowing the 5'-rCGCC-3' sequence in the 5' side of the stem to base-pair with the loop I sequence. The RNA pseudoknot thus formed facilitates the recognition of the *repZ* RBS by the ribosome (Asano and Mizobuchi, 1998). Our model, depicted in Figure 1C, explains the requirement for both the termination event near the *repZ* start codon and the *repZ* Shine–Dalgarno sequence (Asano *et al.*, 1991b).

As the proposed site of initial base pairing with the Inc

RNA and the site of formation of the pseudoknot overlap in the loop of RepZ mRNA structure I, we hypothesized that the binding of Inc RNA at the loop of structure I would inhibit formation of the pseudoknot, thereby inhibiting *repZ* translation (Asano *et al.*, 1991a, 1998). This model accounts for the differential control of *repY* and *repZ* expression as described above (Figure 1C). It also suggests the importance of structure I as the site of competition between the two distinct RNA–RNA interactions. Indeed, the loop sequence of structure I, 5'-rUUGGCG-3', is conserved in a wide variety of prokaryotic antisense systems, and may comprise a specific loop conformation to stimulate its binding to complementary RNAs (Asano *et al.*, 1998).

In this study, we directly examine the role of the unique RNA pseudoknot and the Inc RNA in replication control of the ColIb-P9 plasmid. We find that the intra- and intermolecular RNA–RNA interactions play counteracting roles in determining *repZ* expression and copy number. In addition, we analyze the critical initial steps in these RNA–RNA interactions by simple *in vitro* assays, and provide evidence that the early steps in these two processes are competitive. Based on these and other findings, we propose that competition between the two RNA–RNA interactions at the initial step is important for establishing the constant level of *repZ* expression, and thereby constant plasmid copy number.

Results

Effect of mutations affecting negative control of *repZ* expression on copy number of ColIb-P9

To explore the effects of changes in the RNA–RNA interactions on the copy number of plasmid ColIb-P9, we introduced mutations, known to reduce these interactions, into the mini-ColIb-P9 plasmid, pAK10. Mutations employed in this study are depicted in Figure 1B. The copy number of the resulting mutant plasmids was measured as described in Materials and methods. *repZ* expression was also monitored by constructing the corresponding mutant derivatives of a translational *repZ-lacZ* fusion plasmid pKA140W, and measuring β -galactosidase activity (see Materials and methods).

We first examined the effects of three different types

Fig. 1. The autonomous replication segment of plasmid ColIb-P9 and RNAs responsible for the stringent copy number control. (A) The autonomous replication segment of ColIb-P9 carried by plasmid pAK10. Boxes denote the functional segments *inc*, *repY*, *repZ*, *CIS* and *ori* (see Introduction for details). The unidirectional replication (dotted, hooked arrow) starts from the G-site (Tanaka *et al.*, 1992) and terminates at the *ter* site. Long and short horizontal arrows denote two major transcripts, RepZ mRNA of 1.4 kb to the right and Inc RNA to the left. Open and closed circles on the transcripts represent the 5'-rGCC-3' and 5'-rGGC-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; Bp, *Bst*PI; E(B), *Eco*RI site converted from the original *Bgl*III site; EO, *Eco*O109I; H, *Hinc*II; Ha, *Hae*III; N, *Nsp*(7524)I; S, *Sal*I; Sa, *Sau*3AI. (B) Nucleotide sequence and secondary structures in Inc RNA and RepZ mRNA leader region. Six secondary structures, Ia, I, IIa, II, III and IV, were identified *in vitro* in Rep RNA₂₉₃, corresponding to the RepZ mRNA sequence depicted here (Asano and Mizobuchi, 1998). The 3' ends of *in vitro* transcribed *repZ* mRNA species are indicated by hooked arrows. The Shine–Dalgarno sequence for *repZ* is shown by asterisks. Start codons of *repY* and *repZ* are underlined. The stop codon of *repY* is double-underlined. The two largest complementary sequences, present in the loop I and stem III regions, are boxed and connected by a dotted line. The –10 and –35 regions of Inc RNA promoter in the complementary strand are overlined. The nucleotides in parenthesis at the 5' end of Inc RNA are those present in *in vitro* transcription but not present *in vivo*. The triple bases critical for the pseudoknot formation and Inc RNA pairing are shown by brackets. Nucleotide numbers are coordinated from the *Bgl*III site at the left end of the replication region. Arrows indicate the sites of the mutations used in this study. The mutation in parentheses refers specifically to a base change in RepZ mRNA. For mutations in Inc RNA, we added a prime to the coordinate to indicate bases in the antisense strand. (C) The proposed regulatory circuit of *repZ* expression. Each panel represents the possible state of RepZ mRNA. In (a), the nascent RepZ mRNA blocks *repZ* translation due to the presence of stem–loop III, which sequesters the *repZ* RBS (open boxes for the Shine–Dalgarno sequence and the GUG initiation codon). Translation and termination of *repY* (whose reading frame starts and ends at filled and open circles, respectively) induce the pseudoknot for *repZ* translation by unfolding stem–loop III, thereby allowing the two complementary sequences (black boxes) to base-pair (b). Inc RNA represses *repZ* translation by inhibiting the pseudoknot formation at the level of the initial loop–loop contact (c), and by blocking *repY* translation at the level of duplex formation (d).

Fig. 2. Relationship between the level of *repZ* expression and copy number of Collb-P9. (A) The copy numbers of each plasmid, measured by an electrophoretic method as shown in (B), are plotted as a function of the RepZ activity using the data presented in Table I. The dashed line indicates the linear regression with a slope of 3.9 per unit of the RepZ activity, calculated with the lower 16 points which are shown on an enlarged scale (C). The closed circle represents the wild-type. (B) Plasmid DNA samples prepared in the presence of external reference (11 kb DNA) were linearized with restriction enzyme digestion, electrophoresed on a 0.7% agarose gel in the presence of ethidium bromide and photographed under UV light. Arrows indicate the position of mini-Collb-P9 (4.3 kb) and pBR322 (4.4 kb). Lanes 1 and 9, pBR322; 2, pAK10; 3, pKA101; 4, pA1; 5, pI2; 6, pR44; 7, pA3; 8, pI2-44; 10, pA5; and 11, pA9. See Table I for their genotypes. (D) Relationship between the copy number and the growth rate of the host cells (numbers of cell divisions per hour measured at A_{600}).

of *inc* mutations, *inc2*, *C334A* and *C329T* (see Figure 1B for the mutation site). Mutation *inc2* (Hama *et al.*, 1990a) occurred at the -10 region of the *inc* promoter and reduced the synthesis of Inc RNA to 18% of the level of the wild-type (K.Asano and K.Mizobuchi, unpublished observations). Mutations *C334A* and *C329T*, located within the *inc* gene, reduced the rate constant of binding of the mutant Inc RNA to the cognate RepZ mRNA by 10- and 5-fold, respectively, even though the mutant Inc and RepZ RNAs are complementary to each other (Asano *et al.*, 1998). The copy number of each plasmid was estimated with respect to that of pBR322. The copy number of pBR322 had been determined to be 15 copies per host chromosome in exponentially growing *E.coli* cells (Lin-Chao and Bremer, 1986). Figure 2B shows examples of the DNA patterns for determination of copy numbers, and the results of all the experiments are summarized in Table I. The wild-type mini-Collb-P9 plasmid, pAK10, exhibited 1.7 copies per host chromosome, a value comparable with that of the intact Collb-P9 (lane 2, Figure 2B; Clewell and Helinski, 1970). The three individual *inc* mutations caused a 5- to 9-fold increase in copy number (Figure 2B, lanes 3, 4 and 10; and Table I, lines 2, 3 and 5). Combination of two or three *inc* mutations elevated their copy numbers substantially with a concomitant increase in the level of *repZ* expression (Figure 2B, lane 5; Table I, lines 4 and 6–8). These results together indicate that Inc RNA controls the copy number of the Collb-P9 plasmid as well as the level of *repZ* expression.

Effect of mutations affecting positive control of *repZ* expression on copy number of Collb-P9

We next examined the effects of two mutations, *G327A* (previously called *rep2006*) and *G438A* (previously called *rep2044*), which reduce the pseudoknot interaction between 5'-rGGCG-3' (positions 327–330) and 5'-rCGCC-3' (positions 437–440) (Asano *et al.*, 1991a; see Figure 1B for the mutation sites). These four base pairs were the most critical for *repZ* expression measured in the absence of Inc RNA; compensatory base changes to each of mismatched base pairs restored the level of *repZ* expression (Asano and Mizobuchi, 1998; also see

Figure 5). Here we wished to examine the effects of the two representative mutations specifically on the replication of the mini-Collb-P9 system.

When the replication region of Collb-P9 excised from Collb-P9:p15A composite replicon pTT10-A10 (*G438A inc*⁺) was ligated to a non-replicating kanamycin resistance (*Km*^r) fragment and introduced into *E.coli*, no *Km*^r transformants were observed (Table I, line 9). When we introduced a secondary mutation *C329T*, predicted to restore complementarity between the 5'-rGGCG-3' (positions 327–330) and 5'-rCGCC-3' (positions 437–440), the replication segment supported autonomous replication and exhibited copy number higher than that of the wild-type due to the reduced Inc RNA binding as described above (Figure 2B, lane 11; and Table I, line 14). Thus, the pseudoknot is essential for Collb-P9 replication.

However, when the *G438A* mutation was combined with either *C334A* or *inc2* or both, the mutant Collb-P9 segment supported autonomous replication, and exhibited copy number and RepZ activity higher than those of wild-type (Table I, lines 10–12). These results suggest that the essential function of the pseudoknot is to enhance *repZ* expression. [Our previous finding that the transcription termination of *repZ* at *CIS* was essential for Collb-P9 replication (Mori *et al.*, 1995) would also exclude a second function for the pseudoknot in pre-primer synthesis.] To confirm that the destabilized pseudoknot was responsible for increasing replication, another pseudoknot mutation (*G330A*, see Figure 1B) was introduced into the *G438A C334A* double mutant. As expected, the resulting Collb-P9 replicon (in pTT10-A17) did not support replication (Table I, line 13).

Similar results were also obtained for *G327A* (Table I, lines 18–25) except that, in contrast to pTT10-A10 (*G438A*), the copy number of pA4 (*G327A*) was 1.3 per host chromosome, a value slightly less than, but comparable with that of the wild-type (Table I, line 18). This unexpected result is due to compensation of defective pseudoknot formation by a reduced rate of binding between mutant Inc RNA and RepZ mRNA (Asano *et al.*, 1998). Our results with *G438A* and *G327A* mutations together indicate that a defect in formation of the RepZ mRNA pseudoknot reduces the copy number of Collb-P9, by

Table I. Effect of replication control mutations on copy number, *repZ* expression and transformation frequency in Collb-P9

	Mini-Collb plasmid	Mutations affecting			Copy number	RepZ activity	Transformation frequency (%)
		Inc ^a	Ps/Inc ^a	Ps ^a			
1	pAK10	wild-type			1.7	0.7	(100)
2	pA1	<i>inc2</i>			11	2.4	113
3	pKA101	<i>C334A</i>			15	2.5	116
4	pI2	<i>C334A inc2</i>			128	179	114
5	pA5		<i>C329T</i>		7.9	1.1	151
6	pS44	<i>C334A</i>	<i>C329T</i>		16	1.7	106
7	pA7	<i>inc2</i>	<i>C329T</i>		64	22	123
8	pV24	<i>C334A inc2</i>	<i>C329T</i>		89	82	95
9	pTT10-A10			<i>G438A</i>	ND ^b	NT ^c	<0.1
10	pR44	<i>C334A</i>		<i>G438A</i>	3.3	1.6	8
11	pA3	<i>inc2</i>		<i>G438A</i>	4.9	2.5	18
12	pI2-44	<i>C334A inc2</i>		<i>G438A</i>	27	6.1	10
13	pTT10-A17	<i>C334A</i>	<i>G330A^a</i>	<i>G438A</i>	ND ^b	NT ^c	<0.1
14	pA9		<i>C329T</i>	<i>G438A</i>	8.2	2.2	107
15	pRS44	<i>C334A</i>	<i>C329T</i>	<i>G438A</i>	12	3.0	127
16	pA8	<i>inc2</i>	<i>C329T</i>	<i>G438A</i>	75	43	128
17	pI2-S44	<i>C334A inc2</i>	<i>C329T</i>	<i>G438A</i>	109	171	78
18	pA4		<i>G327A</i>		1.3	0.6	12
19	pI11	<i>C334A</i>	<i>G327A</i>		3.4	0.8	13
20	pA6	<i>inc2</i>	<i>G327A</i>		22	3.1	12
21	pI2-6	<i>C334A inc2</i>	<i>G327A</i>		35	5.7	10
22	pA14		<i>G327A</i>	<i>C440T</i>	9.3	2.4	93
23	pA18	<i>C334A</i>	<i>G327A</i>	<i>C440T</i>	22	7.8	108
24	pA15	<i>inc2</i>	<i>G327A</i>	<i>C440T</i>	64	61	97
25	pI2-S6	<i>C334A inc2</i>	<i>G327A</i>	<i>C440T</i>	151	254	61

^aMutations affecting Inc RNA binding (Inc), the pseudoknot interaction (Ps) or both (Inc/Ps). The effect of *G330A* on Inc RNA binding is minimal.

^bND, Collb-P9 replication was not detected.

^cNT, not tested.

reducing the level of *repZ* expression. Together with the effect of the *inc* mutations, we conclude that the Inc RNA and the pseudoknot play counteracting roles in determination of *repZ* expression and copy number of Collb-P9.

Linear relationship between *repZ* expression and copy number in Collb-P9

When we plotted copy numbers as a function of the level of *repZ* expression using the data in Table I, the copy number was roughly proportional to *repZ* expression up to 30 copies, with a slope of 3.9 per unit of the RepZ activity (Figure 2C). This correlation strongly suggests that the level of *repZ* expression is rate-limiting for determination of the copy number within this range. However, when the copy number increased to >50, this linear relationship was lost, and the growth rate of the host cells was severely retarded (Figure 2D). These last findings indicate that maintaining too many copies of Collb-P9 was deleterious for the host cell growth.

To estimate the replication ability of plasmids at the initial establishment stage, we measured frequencies of transformation using purified pAK10 and its derivatives by a conventional RbCl method (Hanahan, 1985). As shown in Table I, column 5, the frequency of transformation was reduced by *G438A* or *G327A* defective in pseudoknot formation, and was not affected by the altered action of Inc RNA. Thus, replication of Collb-P9 at the initial stage of transformation is not controlled by the copy number control system, and the pseudoknot plays a pivotal role in the former process (see Discussion).

The RepZ mRNA pseudoknot inhibits Inc RNA binding

To analyze biochemically the competition between the Inc RNA binding and the pseudoknot formation, we prepared two different species of the *repZ* mRNA leader *in vitro*, Rep RNA₂₉₃, which did not form the pseudoknot due to the presence of structure III, and Rep RNA₂₁₄, which was expected to form the pseudoknot due to the deletion of nucleotides beyond C-452 including the 3' side of structure III (see Figure 1B). As shown in Figure 3, ³²P-labeled Rep RNA₂₁₄ bound to unlabeled Inc RNA at a rate reduced by 8-fold (Figure 3A), compared with Rep RNA₂₉₃ (Figure 3C). The reduced binding observed with the Rep RNA₂₁₄ was restored by disruption of the pseudoknot in the *G438A* mutant (Figure 3B), although the same mutation did not affect Inc RNA pairing with Rep RNA₂₉₃ (Figure 3D). Thus, the pseudoknot inhibited Inc RNA binding.

Formation of the pseudoknot in Rep RNA₂₁₄ was confirmed by RNase cleavage experiments; G-327 and G-328 at the top of structure I (see Figure 1B) in Rep RNA₂₁₄ were protected from cleavage by RNase T1 (specific to guanine residues in single-stranded regions), although they were excellent targets for this enzyme in the other RNAs employed in Figure 3 (data not shown). As the two guanine residues are the most critical for formation of the pseudoknot (Asano and Mizobuchi, 1998; see also Figure 5), these results strongly suggest that the pseudoknot inhibited Inc RNA binding at the loop of structure I.

Fig. 3. The pseudoknot inhibits Inc RNA binding *in vitro*. Time course of binding of labeled Inc RNA (0.25 nM) to unlabeled Rep RNA₂₉₃ or its mutant or deletion derivative (2.5 nM) was monitored as described in Materials and methods. The positions of Rep RNA–Inc RNA duplexes and free Inc RNA are indicated by arrowheads.

Analysis of initial steps in Inc RNA pairing and the pseudoknot formation

Next, we set out to analyze the initial steps in these two distinct RNA–RNA interactions. We reasoned that these interactions might share common initial step(s), and therefore be competitive, even before Inc RNA forms a complete duplex with RepZ mRNA. The predicted competition could account for the copy number control by Inc RNA (see Introduction). To investigate the initial steps, we prepared two different RNA species, Inc RNA₅₄ and Ps₁₄, and allowed them to compete with stable duplexes formed between Inc RNA and the *repZ* mRNA leader. Inc RNA₅₄ is an Inc RNA derivative lacking the 5' single-stranded region (Figure 4A). This RNA does not form a persistent duplex with [³²P]Rep RNA₂₉₃ under physiological conditions unless annealed at 95°C (Figure 4B). Ps₁₄ is a part of RepZ mRNA corresponding to positions 430–443 and contains a sequence complementary to the loop of structure I (Figure 4A). The complex formed with structure I and Ps₁₄ would be analogous to the pseudoknot.

Although Inc RNA₅₄ and Ps₁₄ did not form a stable complex with Rep RNA₂₉₃, the presence of 0.01 μM Inc RNA₅₄ or Ps₁₄ inhibited binding of [³²P]Rep RNA₂₉₃ to unlabeled Inc RNA ~3-fold in the initial rate of binding (Figure 4C and D). These results indicate that an unstable interaction between Rep RNA₂₉₃ and Inc RNA₅₄ or Ps₁₄ inhibited stable binding between Rep RNA₂₉₃ and Inc RNA. As we did not pre-incubate Rep RNA₂₉₃ with Inc RNA₅₄ or Ps₁₄, the inhibitory interactions took place transiently and rapidly (<1 min) before the stable duplex was detected.

To measure the affinity of the transient interactions as inhibitory dissociation constant (K_i), binding experiments similar to those in Figure 4C and D were performed with various concentrations (i_0) of Inc RNA₅₄ or Ps₁₄. From the initial slopes of the curves, the ratio of the initial rates of binding in the presence (v_i) and absence (v_0) of these RNAs was calculated. As shown in Figure 4E, $v_0/v_i - 1$ was proportional to i_0 , in agreement with the equation of Tomizawa (1985b) (equation 1 in Materials and methods). The lines intercept with the i_0 axis at $-K_i$, giving similar K_i values, 0.008 μM for Ps₁₄ and 0.006 μM for Inc RNA₅₄. These results strongly suggest that Inc RNA₅₄ or Ps₁₄ forms a transient inhibitory complex with Rep RNA₂₉₃ with similar efficiency. Thus, the initial steps in the Inc RNA pairing and the pseudoknot formation are competitive, even before the stable duplex is formed between RepZ mRNA and Inc RNA.

Inc RNA pairing and the pseudoknot compete at the loop of structure I

As Inc RNA₅₄ and Ps₁₄ are complementary to the loop of structure I, the inhibitory interactions most likely took place at the loop of structure I. To investigate directly the involvement of the loop I sequence, we examined the effect of loop I mutations on the K_i values of these inhibitory interactions. First, we synthesized a derivative of Inc RNA₅₄ altering the G-329' residue to A (in G329'A Inc₅₄), and employed it for challenging the Inc RNA–Rep RNA₂₉₃ hybridization reaction (see Table II for changes in loop sequences). We found that the K_i value was increased 100-fold due to a mismatch at the loop (Figure 4E and Table II). When the same Inc RNA₅₄ derivative was used for challenging hybridization between C329U Rep RNA₂₉₃ and G329'A Inc RNA, K_i was recovered partially to 0.02 μM (Table II). As the stability of the base pairing at position 329 (G:C>A:U>A:C) correlated with the affinity of the complex represented by K_i (Table II), we concluded that the inhibitory interaction observed with Inc RNA₅₄ occurred through base pairing at the loop of structure I.

Likewise, when an analogous mutation, G438A, was introduced into Ps₁₄, the K_i relative to the wild-type pair was increased >300-fold (Figure 4E and Table III) whereas K_i relative to the C329U:G329'A pair was recovered to 0.02 μM (Table III). In addition, when the wild-type Ps₁₄ was challenged with the hybridizing pair bearing C329U:G329'A or G327A:C327'U, we obtained the value of 0.3 or 1.7 μM, respectively (Table III). The fact that the K_i values decreased in the order of stability of base pairing at the loop I region (G:C>A:U>G:U>C:A) indicates that the inhibitory action of Ps₁₄ also depends on base pairing at loop I. In addition, similar effects of analogous mutations, G329'A in Inc RNA₅₄ and G438A in Ps₁₄, strengthen the idea that the initial interactions in the Inc RNA binding and the pseudoknot formation are competitive, sharing the same target.

Of note is the finding that we obtained an inverse proportionality between the K_i values for the different Ps₁₄–loop I complexes and the level of *repZ* expression *in vivo* from the corresponding mutant derivatives (Table III). On log/log scale, the slope of the repression was -0.84 , close to -1 (shown by dotted and solid lines, respectively), with the correlation coefficient of -0.97 (Figure 4F). Formation of the pseudoknot opens the *repZ* RBS, and the dissociation constant value can be regarded as a function of the ratio of the pseudoknot formed in the cell (see equation 2 in Materials and methods). Therefore,

Fig. 4. Initial phases of the Inc RNA pairing and the pseudoknot formation are competitive. (A) Inhibitory RNAs employed in this study. The secondary structure of Inc RNA₅₄ is based on the previous report (Asano *et al.*, 1998). A dotted line indicates weak base pairing. Ps₁₄ (positions 430–443) contains the sequence complementary to loop I; bases not complementary are marked with asterisks. The 5'-rGCC-3' sequences complementary to 5'-rGGA-3' at positions 327–329 are boxed. Analogous mutations G329'A for Inc RNA₅₄ and G438A for Ps₁₄ are indicated by arrows. The 5'-GGA-3' sequence derived from the T7 RNA polymerase transcription template is shown in black. (B) Inc RNA₅₄ does not form a persistent hybrid with Rep RNA₂₉₃ under physiological conditions. [³²P]Rep RNA₂₉₃ (40 nM) was mixed with the indicated concentrations of Inc RNA₅₄ in standard binding buffer. After incubation at 37°C for 30 min, the sample was analyzed by electrophoresis on an 8.3 M urea–16% polyacrylamide gel at 200 V for 12 h. Lane H, [³²P]Rep RNA₂₉₃ (40 nM) annealed to Inc RNA₅₄ at 95°C for 3 min. (C) Inhibition of hybridization between [³²P]Rep RNA₂₉₃ and Inc RNA by Inc RNA₅₄ or Ps₁₄. [³²P]Rep RNA₂₉₃ (0.25 nM) and Inc RNA (2.5 nM) were incubated in the absence (upper panel) or presence of 10 nM Inc RNA₅₄ (middle panel) or Ps₁₄ (lower panel) at 37°C in 50 μ l of standard binding buffer. Every 10 μ l of the mixture was withdrawn at the times indicated and analyzed as described in Materials and methods. (D) Time course of the inhibition experiments described in (C). The ratio of free and bound Rep RNA₂₉₃ was determined and plotted using a Fuji BAS2000 BioImage Analyzer. (E) Determination of the K_i values. The values v_o/v_i are calculated from the inhibition experiments with different concentrations (i_o) of Inc RNA₅₄ (a) or Ps₁₄ (b) and are plotted against i_o . Derivatives of Inc RNA₅₄ (G329'A in c) or Ps₁₄ (G438A in d) were also examined. (F) The K_i values of Ps₁₄–structure I complexes with different mismatches at loop I were plotted against the levels of *repZ* expression in the absence of Inc RNA, based on the values presented in Table III.

this correlation strongly suggests that the stability of the pseudoknot assayed here *in vitro* corresponds to that of the pseudoknot formed *in vivo* required for *repZ* translation.

Specific structure in loop I is important for the initial interactions

The fact that we obtained similar K_i values with two differently structured RNAs, Inc RNA₅₄ or Ps₁₄ (Figure 4),

suggests strongly that unstructured complementary sequence is the only requirement. Thus, we turned our attention to loop I for investigating the structural requirement for the transient interactions. By previous analyses, we reached two important conclusions regarding the requirement for base pairing for the RepZ mRNA pseudoknot formation (Asano and Mizobuchi, 1998). First, as summarized in Figure 5, the pseudoknot required for *repZ* expression depended on base pairing

Table II. Effect of loop mutations on the transient inhibitory interaction of Inc RNA₅₄ with loop I

Rep RNA ₂₉₃ :Inc RNA ^a	Loop I sequence ^b	Inc RNA ₅₄ ^c	Inc loop sequence ^d	K_i (μ M)
Wild-type	5'-GGC-3'	wild-type	3'-CCG-5'	0.006 ^e
Wild-type	5'-GGC-3'	G329'A	3'-CCA-5'	0.6 ^e
C329U:G329'A	5'-GGU-3'	G329'A	3'-CCA-5'	0.02

A pair of Inc RNA (2.5 nM) and [³²P]Rep RNA₂₉₃ (2.5 nM) or their indicated derivatives were incubated in the presence of different concentrations of Inc RNA₅₄ or its G329'A derivative, and the K_i values for each set of reactions were calculated as described in the text.

^aMutations in the pair of Rep RNA₂₉₃ and Inc RNA employed in the reaction, respectively.

^bLoop I sequence in Rep RNA₂₉₃ at positions 327–329.

^cMutation in Inc RNA₅₄.

^dInc loop sequence in Inc RNA₅₄ at positions 327'–329'.

^eTaken from Figure 4E.

Table III. Effect of pseudoknot mutations on the transient inhibitory interaction of Ps₁₄ with loop I

Rep RNA ₂₉₃ :Inc RNA ^a	Loop I sequence ^b	Ps ₁₄ ^c	Stem III sequence ^d	K_i (μ M)	<i>repZ</i> expression ^e
Wild-type	5'-GGC-3'	wild-type	3'-CCG-5'	0.008 ^f	1912
Wild-type	5'-GGC-3'	G438A	3'-CCA-5'	3.0 ^f	23
C329U:G329'A	5'-GGU-3'	G438A	3'-CCA-5'	0.02	1041
C329U:G329'A	5'-GGU-3'	wild-type	3'-CCG-5'	0.3	170
G327A:C327'U	5'-AGC-3'	wild-type	3'-CCG-5'	1.7	10

A pair of Inc RNA (2.5 nM) and [³²P]Rep RNA₂₉₃ (2.5 nM) or their indicated derivatives were incubated in the presence of different concentrations of Ps₁₄ or its G438A derivative. The K_i values for each set of reactions were calculated as described in the text and compared with the level of *repZ* expression from the corresponding mutants *in vivo*.

^aMutations in the pair of Rep RNA₂₉₃ and Inc RNA employed in the reaction, respectively.

^bLoop I sequence in Rep RNA₂₉₃ at positions 327–329.

^cMutation in Ps₁₄.

^dStem III sequence in Ps₁₄ corresponding to positions 440–438.

^eMiller's units of β -galactosidase from the *repZ-lacZ* fusion carrying *incI* (which removes Inc RNA) and corresponding mutation(s), encoded by a derivative of pKA340-W3: data from Asano and Mizobuchi (1998).

^fTaken from Figure 4E.

Fig. 5. Bases involved in the pseudoknot formation *in vivo*. The line drawing in the upper panel describes the pseudoknot structure required for plasmid ColIb-P9 *repZ* translation (not drawn to scale). Only the two complementary sequences are described as nucleotides, numbered as in Figure 1. The hooked arrow indicates the start position of *repZ*. The effect of substitution of each base pair (indicated by dotted arrows with substituted bases emptied) on *repZ* expression was examined by translational *repZ-lacZ* fusion studies using derivatives of pKA340-W3 (*incI*) lacking Inc RNA. The reduction in *repZ* expression is expressed as the ratio of β -galactosidase activity relative to 1912 Miller units from the parental pKA340-W3 plasmid, based on the values reported previously (Asano and Mizobuchi, 1998). Base pairings encompassed in brackets were judged to be involved in the pseudoknot formation *in vivo*.

at 5'-rUGGCGG-3' (positions 326–331). The effect of mutations differed for each base pair, and *repZ* expression increased in the order of stability of base pairing (G:C>A:U>G:U>C:A or C:U). However, this was not the case when we changed U-326 at the 5' end to a G residue (see Figure 5). This last observation led us to the second conclusion: U-326 is important in a base-specific manner. As U-326 and the most critical 5'-rGGC-sequence (positions 327–329) comprise a small internal loop (see Figure 1B for the secondary structure), we presumed that a specific conformation in loop I stimulates pairing with complementary RNA not only for the pseudoknot formation but also for the binding of Inc RNA (Asano and Mizobuchi, 1998; Asano *et al.*, 1998; also see Discussion).

To examine if these requirements are observed at the level of the initial interactions, we synthesized SI₅₄, an RNA consisting of the entire structure I, and allowed it to compete with binding between Inc RNA and Rep RNA₂₉₃. When K_i was estimated, we obtained a value of 0.010 μ M (Figure 6A), close to the K_i measured for the interaction between Rep RNA₂₉₃ and Inc RNA₅₄ (Figure 4E). When we synthesized SI₂₁, which contains only the 21 base loop of structure I and did not exhibit any secondary structure (data not shown), it failed to inhibit Inc RNA–Rep RNA₂₉₃ interaction as efficiently as SI₅₄ (Figure 6B; compare the results with a and b in Figure 4E). This result suggests that a specific, unstable

Fig. 6. Dissociation constants (K_i) for transient complexes between Inc RNA and stem-loop structure I RNA derivatives. The K_i values of the transient inhibitory complexes formed with Inc RNA and the indicated structure I derivatives are measured as described in Materials and methods, and shown with their secondary structures. Introduced base changes are highlighted. Nucleotides not original to the ColIb-P9 sequence are in lower case. The secondary structures of the inhibitory RNAs are confirmed as described in Materials and methods (data not shown). The 5'-rGGCGG-3' sequence is boxed.

loop conformation in structure I favors the inhibitory complex formation, and that this loop conformation is only conferred within the entire structure of stem-loop I. To confirm this idea, we synthesized SI₁₉ which exhibited a moderate secondary structure (Figure 6C). The K_i for SI₁₉ was 0.05 μM , comparable with that for SI₂₁. Thus, having a secondary structure is not sufficient for stimulating the inhibitory interaction.

Interestingly, SI₁₉ appears to convey part of the important structural information, as changing the loop residue corresponding to U-326 to any residue increased the K_i value significantly (Figure 6D–F). Given that any of the altered bases mismatch with A-326' in the Inc RNA, the results indicate that pyrimidine residues are favored at position 326, thereby supporting the model that the initial interaction is stimulated by a specific loop conformation conferred by U-326.

Finally, we wished to delimit the bases required for the initial interaction by introducing base changes to SI₁₉ without affecting its secondary structure. Exchanging two consecutive A:U base pairs in SI₁₉ without altering 5'-rGGCGG-3' (positions 327–331 in the original structure) increased K_i by only 2-fold (Figure 6G). However, exchanging the G:C pair involving the fifth residue of the 5mer increased K_i by 65-fold (Figure 6H), an

increase comparable with that caused by G329'A, G438A or G327A (Tables II and III). Since the U to C change at a base 5' of the 5mer (in U326C SI₁₉) increased K_i by 3-fold (Figure 6F), we conclude that the initial base pairing interaction involves the 5'-rGGCGG-3' sequence (positions 327–331), which is nearly identical to the bases required for the pseudoknot formation *in vivo* (Figure 5) (We did not include U-326 for base pairing in the K_i complex, but a mismatch at this position had the smallest effect on *repZ* expression among bases at positions 326–331.)

The results presented in Figure 6 are consistent with the proposed model for loop I, and emphasize its importance as the site of competition. By delimiting the bases involved in the transient interaction to those nearly identical to the site of the pseudoknot formation *in vivo* (Figure 5), we strengthened the idea that the initial interaction between Inc RNA and loop I is competitive to the step of pseudoknot formation as required for *repZ* translation. Thus, competition between the RNA–RNA interactions is important for determining the level of *repZ* expression and thereby the copy number of ColIb-P9.

Discussion

Control of plasmid copy number by competition between RNA–RNA interactions

The ColIb-P9 antisense Inc RNA represses the translation of *repZ* by inhibiting formation of the RepZ mRNA pseudoknot required for *repZ* translation. By doing so, Inc RNA allows the level of *repZ* expression to be kept constant irrespective of changes in plasmid copy number throughout the cell cycle. In this study, we found that formation of the pseudoknot is essential for ColIb-P9 replication by showing that the G438A mutation, which reduces the pseudoknot interaction, is defective in ColIb-P9 replication, and that the secondary mutation, C329T, which restores the pseudoknot, recovered the replication ability (Table I, lines 9 and 14). As the G438A mutation was also suppressed by the reduced action of Inc RNA (Table I, lines 9–12), the pseudoknot and the Inc RNA play counteracting roles in determining plasmid copy number. We also showed that the level of *repZ* expression is roughly proportional to the copy number of the corresponding mini-ColIb-P9 derivatives (Table I and Figure 2). This finding suggests that the level of *repZ* expression is rate-limiting for plasmid copy number, and explains the negative feedback loop by Inc RNA for the stable maintenance of plasmid copy number.

Nordström previously predicted that the constant copy number of the IncFII R1 plasmid can be maintained by keeping a constant level of the initiator, RepA, and thereby a constant number of replication initiation events in the cell (Nordström, 1990). We believe that our results provide experimental support for this '+n mode' of replication. R1 is a distant relative of the IncI α ColIb-P9 with the autonomous replication segment similar to ColIb-P9 in gene organization. However, R1 additionally encodes a transcriptional repressor for *repA* mRNA synthesis (Nordström, 1990), and its replication does not require formation of a pseudoknot analogous to that in ColIb-P9 (Blomberg *et al.*, 1992).

In Table I, we also showed that the transformation

frequency of mini-ColIb-P9 correlated with the ability to form the pseudoknot, and not with the action of Inc RNA. We speculate that, in the initial stage of transformation, the pseudoknot is formed predominantly to stimulate *repZ* expression until the plasmid DNA can establish its own replication. Thus, a defect in the pseudoknot would impair the ability to establish replication, thereby leading to a reduced chance of the mutant plasmids' survival. These observations suggest that the process of transformation of the mini-ColIb-P9 plasmid consists of two distinct steps, establishment of the replication system as the initial step, and subsequent maintenance of the replicon at the constant copy number.

Having observed that the Inc RNA and the pseudoknot play counteracting roles in copy number control, we considered the importance of competition between the RNA–RNA interactions. We found that the *repZ* mRNA leader by itself formed a pseudoknot and was capable of inhibiting Inc RNA binding *in vitro* (Figure 3). Furthermore, we obtained evidence that the RNA–RNA interactions compete at the level of initial base pairing. To analyze the initial steps in the Inc RNA binding or the pseudoknot formation, we prepared an Inc RNA derivative (Inc RNA₅₄) or a part of the *repZ* mRNA leader (Ps₁₄), respectively (Figure 4A). These RNAs did not form a stable complex with RepZ mRNA, but instead inhibited the binding between RepZ mRNA and Inc RNA probably by their transient interactions with loop I (Figure 4B–D). By measuring the inhibition dissociation constant (K_i) values, we showed that the inhibitory interactions occur with similar efficiencies and thus are competitive (Figure 4E). As expected, both these transient interactions depended on base pairing at loop I (Tables II and III).

We believe that the initial interaction analyzed here with Ps₁₄ mimics the pseudoknot and is equivalent to the pseudoknot required for *repZ* translation *in vivo*. This was supported by the direct correlation of the destabilization of the pseudoknot (measured by K_i) with reduced *repZ* expression (Table III and Figure 4F). In addition, the bases involved in both of the interactions were nearly identical (Figures 5 and 6). As the initial loop–loop complex between stem–loop I and Inc RNA has an affinity similar to the pseudoknot, Inc RNA can repress formation of the pseudoknot at this initial step. We propose that the observed competition accounts for the inhibition of *repZ* translation by Inc RNA, important for maintaining the constant level of *repZ* expression.

Inc RNA binding and the pseudoknot formation share common initial steps

In addition to complementary sequences, we found that the U-326 residue at the loop of structure I is important specifically for the critical initial interactions to occur (Figure 6). This residue is a part of the loop closed by an internal base pairing between C-324–U-325 and G-330–G-331 (see Figure 1B for the secondary structure). Furthermore, we previously suggested that this internal base pairing together with U-326 is important for the binding of Inc RNA (Asano *et al.*, 1998). Combining these findings with the involvement of 5'-rGGCGG-3' (positions 327–331) for the K_i complex formation, we propose that the initial phase of Inc RNA pairing and pseudoknot formation occurs by two steps, as illustrated in Figure 7. In this

Fig. 7. Stepwise model for formation of the RepZ mRNA pseudoknot and the binding of Inc RNA. Based on the results reported here and elsewhere (Asano and Mizobuchi, 1998; Asano *et al.*, 1998), we propose that the early steps in formation of the RepZ mRNA pseudoknot (A) and the binding of Inc RNA (B) proceed in a similar sequence. The line in each panel represents RepZ mRNA (below) or Inc RNA (above). Panel a describes the two important secondary structures (I and III) in the nascent RepZ mRNA, with the position of *repY* and *repZ* ORFs shown by hooked arrows. The 5'-rGGC-3' (structure I) and 5'-rGCC-3' (Inc RNA or structure III) sequences are indicated by closed and empty boxes, respectively. Bars indicate base pairing interactions. The 5'-rUUGGCG-3' motif, shown by brackets, is conserved in many prokaryotic antisense systems and may confer a specific loop structure for stimulation of binding of single-stranded complementary RNAs (Asano *et al.*, 1998). The step of binding proposed to be as measured by K_i is bracketed. See text for more details.

model, the RNA–RNA interactions are initiated by base pairing at the 5'-GGC-3' sequence (positions 327–329) in loop I (step b), being stimulated by a specific structure surrounding it involving U-326. The initial base pairing then extends two or three more base pairs and transiently stabilizes the complex; the initial interactions assayed here by K_i and the pseudoknot required for *repZ* translation involve base pairing up to this second step (step c). The destabilized loop I structure, as suggested by analyses in Figure 6, would favor this transition by allowing the internal base pairing involving G-330–G-331 (in step b) to be unfolded for pairing with the partner (in step c).

Since the 5'-rUUGGCG-3' loop sequence is conserved

in many prokaryotic antisense systems (Eguchi *et al.*, 1991; Asano *et al.*, 1998), and as a U residue is conserved at a base 5' to the anticodon of tRNAs as a structural element that provides a π -turn to the phosphate backbone (Kim and Sussman, 1976), U-326 may also contribute to a π -turn (represented by a turn at the top of loop I in Figure 7) and lead to better recognition of complementary sequences (Asano *et al.*, 1998). If so, replacement of this residue by a bulky purine residue would disturb the ordered conformation of loop I, and increase the K_i value as observed with U326G SI₁₉ in Figure 6. It would be interesting if the π -turn mediated by a loop U residue, the most classic example of specific loop structures, is identified outside the anticodon loop of tRNAs for the recognition of complementary RNAs. It is important, therefore, to determine the three-dimensional structure of the loop carrying the 5'-rUUGGCG-3' motif.

Subsequent to the common steps, Inc RNA binding and pseudoknot formation follow different pathways (Figure 7). As Inc RNA₅₄ lacking the 5' single-stranded region did not form a stable hybrid with RepZ mRNA *in vitro*, it is conceivable that intact Inc RNA starts duplex formation by another base pairing at the 5' single-stranded region (steps d and e in Figure 7B), as demonstrated for other antisense systems such as plasmid ColEI RNA I and R1 CopA (Eguchi *et al.*, 1991; Wagner and Simons, 1994). Translation of *repY* could be inhibited at these stages (steps d and e) by steric hindrance of the *repY* RBS (Hama *et al.*, 1990b; Asano *et al.*, 1991a). Following the initial contact, a more stable pseudoknot involving more than five base pairs could be formed *in vitro* (step d in Figure 7A), dependent on truncation of the *repZ* mRNA leader beyond the loop of structure III as observed with RNA₂₀₆ (Asano and Mizobuchi, 1998). However, there is no evidence to indicate that the pseudoknot forms to this extent *in vivo*, instead the pseudoknot formed *in vivo* appears to correspond to that described in step c (Figure 7) as discussed above. We believe that the RepZ mRNA pseudoknot is formed only transiently by the refolding of stem-loop structure III after each cycle of *repZ* translation (from step c to o in Figure 7A). This refolding of structure III would be important for Inc RNA to bind to RepZ mRNA during active translation of RepZ, as a pre-formed pseudoknot within RepZ mRNA inhibits binding of Inc RNA (Figure 3).

Differential control of multiple gene expression by a single antisense RNA

Inc RNA represses the translation of both *repZ* and *repY* (uORF coupled to *repZ*) at different rates with different mechanisms. Although this multi-step mechanism is an integral part of copy number control system in the ColIb-P9 plasmid, we believe that our results may help us understand how a single antisense RNA molecule can repress the expression of multiple genes. We imagine that some antisense regulators such as OxyS in *E. coli* (Altuvia *et al.*, 1997) could control different genes at different rates by steric hindrance of the RBS and by inhibiting formation of an RNA structure necessary for gene expression. These alternative mechanisms would enable differential responses for each of the genes controlled. We are also surprised to find that the principle of Inc RNA–RepZ mRNA pairing or the RepZ mRNA pseudoknot formation

is simple, essentially occurring between a specific RNA stem-loop and a single-stranded complementary RNA. This mode of interaction would enable an easier evolution of antisense regulator systems by creating an RNA stem-loop analogous to the structure I of ColIb-P9, in either the target or the regulator itself. The growing list of antisense regulators of non-complementary type (Citron and Schuster, 1990; Altuvia *et al.*, 1997) will test our imagination in the future.

Materials and methods

Bacteria, phages and plasmids

Escherichia coli K12 MC1061 (*lacX74*) (Casadaban and Cohen, 1980) was used as host for mini-ColIb-P9 and *lacZ* fusion plasmids. NM522 (Gough and Murray, 1983) was used as host for phagemids derived from pTZ19R (Mead *et al.*, 1986). BW313 (*dut ung*) (Kunkel, 1985) and BMH71-18*mutS* (Kramer *et al.*, 1984) were used for site-directed mutagenesis. M13KO7 was used as helper phage for preparation of single-stranded DNAs (Vieira and Messing, 1987).

pAK10 (Kato and Mizobuchi, 1994) was a mini-ColIb-P9 plasmid consisting of the 3.0 kb ColIb-P9 *EcoRI* fragment with autonomous replication and a 1.3 kb *Km^r* fragment from pCH71 (Hama *et al.*, 1990b). Mutations employed in this study (Figure 1B) were introduced first into the pDX14W phagemid carrying the 1.1 kb *EcoRI*–*SalI* ColIb-P9 segment (Asano and Mizobuchi, 1998) either by site-directed mutagenesis (Kunkel, 1985) or by replacing the 365 bp *EcoO109I* fragment (positions 350–715) with the corresponding segment of pDX14W or its mutant. All mini-ColIb-P9 plasmids used in this study except pTT10-A10 and pTT10-A17 were constructed by replacing the 724 bp *EcoRI*–*BstPI* fragment of pAK10 with the corresponding segment of pDX14W. Both pTT10-A10 and pTT10-A17 were composite replicons, in which the 3.0 kb *EcoRI* fragment of ColIb-P9 carrying corresponding mutations (see Table I for genotypes) was inserted into the *EcoRI* site of the vector pACYC184 (Chang and Cohen, 1978).

pKA140W (Asano and Mizobuchi, 1998) was a translational *repZ*–*lacZ* fusion plasmid derived from a multi-copy vector pMC1403 (Casadaban *et al.*, 1980). Mutant derivatives of this plasmid were constructed by replacing the 724 bp *EcoRI*–*BstPI* fragment with the corresponding segment of pDX14W. pKA340-W3 (*incI*) was a single-copy translational *repZ*–*lacZ* fusion plasmid to analyze the maximal level of *repZ* expression in the absence of Inc RNA (Asano and Mizobuchi, 1998).

For preparation of wild-type and mutant derivatives of Inc RNA and Rep RNA₂₉₃ *in vitro*, we employed derivatives of pKA10 with the 271 bp *Nsp*(7524)I–*Sau3A1* fragment (positions 248–519) from the wild-type ColIb-P9 or its corresponding mutants subcloned under the T7 promoter (Asano *et al.*, 1997). For preparation of wild-type and G438A Rep RNA₂₁₄, pKA15 and its G438A derivative were employed (Asano and Mizobuchi, 1998). The *HindIII*–*AluI* site immediately following the T7 promoter was deleted in pKA15 for digestion of this plasmid with *AluI* and subsequent *in vitro* production of Rep RNA₂₁₄.

Copy numbers, transformation frequency and RepZ activity of ColIb-P9

The copy number of pAK10 and its mutants was measured on agarose gels as described (Asano *et al.*, 1998). The transformation frequency of pAK10 expressed as the number of *Km^r* transformants was 7.1×10^6 per μ g of purified covalently closed circular DNA. This value was referred as 100%. The RepZ activity in each strain was measured as the β -galactosidase activity after fusing the 3'-truncated *repZ* gene in-frame to the 5'-truncated *lacZ* gene as described previously (Asano and Mizobuchi, 1998). The activity is expressed as Miller units (Miller, 1972). Values reported in Table I are averages from at least three independent experiments.

Preparation of RNAs

Inc RNA or its mutant derivative was synthesized from corresponding pKA10 or its derivative using *E. coli* RNA polymerase as described (Asano *et al.*, 1998). Rep RNA₂₉₃ or its derivative was synthesized from *SmaI*-digested pKA10 or its corresponding mutant derivatives using T7 RNA polymerase as described (Asano and Mizobuchi, 1998). Rep RNA₂₁₄ or its G438A derivative was synthesized similarly to Rep

RNA₂₉₃ except that *AluI*-digested pKA15 or its *G438'A* derivative was used as template, respectively. Inc RNA₅₄ or its *G329'A* derivative was synthesized using T7 RNA polymerase and the double-stranded DNA template prepared by PCR. This reaction contained a pair of primers, 5'-Inc₅₄ 5'-GGGTAATACGACTCACTATAGGACCCCACTATCTTT-CGTCGTTTC-3' (with the T7 promoter sequence underlined) and 3'-Inc₅₄ 5'-CCCCACTATCTTTCTTACGAAC-3', and pKA10 or its *C329T* derivative as template. SI₅₄ was synthesized using T7 RNA polymerase and the double-stranded DNA template prepared by PCR from pKA10 using primers 5'-SI₅₄ 5'-GGGTAATACGACTCACTATAGGACCCCACTATCTTTCTTACG-3' (with the T7 promoter underlined) and 3'-SI₅₄ 5'-ATTCTGTGAGGCCCACTA-3', followed by digestion with *HaeIII* restriction endonuclease. Oligoribonucleotides Ps₁₄ and *G438A* Ps₁₄ (used in Figure 4 and Table III), and SI₂₁, SI₁₉ and its mutant derivatives (used in Figure 6) were prepared by the CyclonPlus 8700 DNA/RNA synthesizer (Miligen/Bioresearch). All the RNAs employed were purified by gel electrophoresis. The secondary structure of 5'-labeled RNAs was analyzed with RNases T1, Bc and V1 as described previously (Asano and Mizobuchi, 1998). Oligoribonucleotides were radiolabeled at the 5' end by T4 polynucleotide kinase (Takara Shuzo, Japan) in the presence of [γ -³²P]ATP.

Binding of Inc RNA to RepZ mRNA in vitro

Binding of Inc RNA to RepZ mRNA was analyzed as described (Asano *et al.*, 1998). Briefly, 0.25 nM Inc RNA (labeled) and 2.5 nM Rep RNA₂₉₃ or its mutant or deletion derivative (unlabeled) were incubated in standard binding buffer (Tomizawa, 1985a) (50 μ l) containing 10 mM MgCl₂ and 100 mM NaCl in 20 mM Tris-HCl (pH 7.6) at 37°C, and samples were withdrawn at various time points and analyzed by denaturing 8% polyacrylamide gel electrophoresis in the presence of 8.3 M urea. Inc RNA-Rep RNA hybrid was detected as a complex which was stable during the denaturing gel electrophoresis and migrated anomalously compared with free RNA species.

Assaying intermediates in Inc RNA pairing and the pseudoknot formation

To measure the affinity of initial interactions between stem-loop I and Inc RNA₅₄ or Ps₁₄, Inc RNA and [³²P]Rep RNA₂₉₃ were bound as described above except that this reaction was conducted in the presence of these RNAs. After various times of incubation, aliquots were withdrawn and suspended in a gel application buffer for stopping the reaction, and analyzed electrophoretically on a 16% polyacrylamide gel containing 8.3 M urea at 200 V for 12 h. The ³²P radioactivity of the gel was analyzed by Fuji BAS2000 BioImage Analyzer. We started the hybridization reaction without the pre-incubation of Rep RNA species with the inhibitory RNAs, in order to detect intermediates in the shortest time frame, i.e. within 1 min in our conditions before the stable duplex is detected. When the inhibition experiments were conducted with the mutant pair of Inc RNA and Rep RNA₂₉₃, we increased the concentration of Inc RNA so that the rate of hybridization is comparable with that of the wild-type pair.

The inhibitory dissociation constant, K_i , was determined according to the equation of Tomizawa (1985b),

$$v_0/v_i = 1 + i_0/K_i \quad 1$$

where v_0 and v_i were the initial rates of hybridization in the absence and presence of inhibitory RNA, respectively, and i_0 was the initial concentration of inhibitory RNA. We showed that all the conditions required for this equation are satisfied in our experiments (Asano *et al.*, 1998).

The K_i values of initial interactions between [³²P]Inc RNA and structure I-derived RNAs (listed in Figure 6) were calculated similarly except that 2.5 nM Inc RNA and 0.25 nM Rep RNA₂₉₃ were used. Reactions were analyzed by electrophoresis on an 8% polyacrylamide gel at 300 V for 3 h.

As K_i represents the dissociation constant of the inhibitory complex, the following equation can be assumed;

$$K_i = [S][I]/[Ps]$$

where S is the nascent RepZ mRNA containing stem-loop I, I is the inhibitory RNA with a sequence complementary to the loop I, and Ps is the pseudoknot-like complex formed with S and I. *In vivo*, the total amount of S and Ps should be constant as the amount of RepZ mRNA. Thus, when the fraction of the pseudoknot formed *in vivo* is f ,

$$K_i = [I] (1/f - 1)$$

If we assume that [I] is very high and constant (I_0) with regard to a change in f (a reasonable assumption, since I is tethered to S in a single RNA molecule *in vivo*) and that f is small enough, f is inversely proportional to K_i , and the log of the above equation is:

$$\ln K_i = -\ln f + \ln I_0 \quad 2$$

As the ratio of the open RBS correlates with the rate of translation initiation in prokaryotes (de Smit and van Duin, 1990), f is proportional to the level of *repZ* expression in the absence of Inc RNA. Thus, the log of K_i linearly correlates with the log of level of *repZ* expression when f is small. Since the constitutive level of *repZ* expression dependent on the pseudoknot is 3714 β -galactosidase units, twice as high as the highest value in Figure 4F (Asano and Mizobuchi, 1998), we know that f is at least <0.5.

Acknowledgements

We thank Shigeyuki Yokoyama for supporting experiments conducted by K.A. at the University of Tokyo. We are greatly indebted to Gigi Storz, Alan Hinnebusch, Bob Weisberg and Jim Anderson for critical reading of the manuscript, and to Tom Schneider for discussion on the results in Figure 4F and calculating the regression by the program xyplop (written by T.S.). K.A. was affiliated to JSPS Fellowships for Japanese Junior Scientists. This research was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture (Monbusho) of Japan.

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Received May 4, 1998; revised June 29, 1998; accepted July 2, 1998