Structural Analysis of Late Intermediate Complex Formed between Plasmid ColIb-P9 Inc RNA and Its Target RNA

HOW DOES A SINGLE ANTISENSE RNA REPRESS TRANSLATION OF TWO GENES AT DIFFERENT RATES?∗

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The antISENSE Inc RNA encoded by the inclα ColIb-P9 plasmid replicon controls the translation of repZ encoding the replication initiator and its leader peptide repY at different rates with different mechanisms. The initial loop-loop base pairing between Inc RNA and the target in the repZ mRNA leader inhibits formation of a pseudoknot required for repZ translation. A subsequent base pairing at the 5′ leader of Inc RNA blocks repY translation. To delineate the molecular basis for the differential control, we analyzed the intermediate complexes formed between RepZ mRNA and Inc RNA², a 5′-truncated Inc RNA derivative. We found that the initial base pairing at the loops transforms into a more stable intermediate complex by its propagation in both directions. The resulting extensive base pairing indicates that the inhibition of the pseudoknot formation is established at this stage. Furthermore, the region of extensive base pairing includes bases different in related plasmids showing different incompatibility. Thus, the observed base pairing is important for determining the incompatibility of the low-copy-number plasmids. We discuss the evolution of replication control systems found in inclα, inclβ, and inclFII group plasmids.

Antisense RNAs bind to the complementary regions (or “sense” strands) of their target RNAs and exert a variety of regulatory functions (1, 2). Some of them are encoded in the autonomous replication regions of plasmids and negatively control their replication and copy number (3). The copy number of the plasmid is proportional to the intracellular concentration of its antisense RNA, which, in turn, down-regulates the frequency of plasmid replication, thereby establishing a negative feedback loop for the maintenance of constant copy number. These antisense RNAs, when expressed in a bacterial cell, repress the replication of identical or closely related plasmids introduced by means of transfer or transformation. This phenomenon is called incompatibility (Inc) and is used to classify plasmids: i.e., if two different plasmids are incompatible in a single bacterial cell, they belong to the same Inc group (4).

There are essentially two types of antisense RNAs that show the highest binding rate constants of ~10⁶M⁻¹s⁻¹ (1). High copy number plasmids including pMB1 and ColE1 encode one type (designated here as class H for high copy) of such antisense RNAs. These antisense RNAs have the size of ~100 bases, form three stem-loops with a single-stranded 5′ leader, and bind to the preprimer RNAs for the leading strand DNA synthesis, thereby inhibiting formation of mature primer RNAs (1). The other type of rapid binders (class L for low copy) are encoded by low-copy-number plasmids including the members of inclFII, inclα (incl₁) and inclβ groups (1, 2). These antisense RNAs are ~70–100-base long, fold essentially into a single large stem-loop of ~50 bases, and control the expression of the plasmid replication initiator (rep) genes at the translational level. Specifically, they block the translation of a rep leader peptide (RLP) coupled to the translation of rep (5–9), by binding to the vicinity of the ribosome-binding site (RBS) for the RLP (10–12). In the case of inclα ColIb-P9 and inclβ pMU720 plasmids, the translation of RLP (repY and repB) induces formation of a pseudoknot between the target stem-loop and a sequence preceding the rep (repZ and repA, respectively) RBS, which is required for rep translation (13–15). The antisense RNA additionally inhibits the pseudoknot formation by binding to the loop of the target stem-loop structure (16). Because of the different mechanisms, the antisense RNA represses rep expression more efficiently than RLP expression, thereby establishing the constant level of Rep protein and hence the plasmid copy number (16). We have been studying this unique differential control exerted by the antisense Inc RNA of the ColIb-P9 plasmid.

It is established that the process of binding of antisense RNA I (A) to its target RNA II (T), encoded by ColE1 (class H), comprises a series of reactions producing more stable intermediates:

$$A + T \rightleftharpoons C^* \rightleftharpoons C^{**} \rightarrow C^S$$

(Eq. 1)

where C* is the stable final product, C** is characterized by its altered Rnase sensitivity and C* is the unstable complex, the formation of which is inferred from the inhibition kinetics. The C* complex appears to consist of multiple contacts at loops and 5′-single stranded leader, with the inhibitory dissociation constant ($K_d$) of 11 nM (17, 18). In the C** complex, base pairing in each loop-loop interaction may stack on the stems of the antisense and target RNAs, which altogether provides the equilib-

∗ This research was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture (Monbu-sho) of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: Inc, incompatibility; RLP, rep leader peptide; RBS, ribosome-binding site.
one of the bases critical for C* formation in IncIα ColIb-P9 is altered in IncFII plasmids (16). Despite sharing the bases critical for C* formation, IncIα and IncB plasmids are compatible for several generations although they show weak incompatibility thereafter (22). Therefore, the reduced stability of the complex formed later than C*, most likely C**, must be responsible for the observed compatibility between IncIα and IncB plasmids.

In this report, we focus on the secondary structure of a late intermediate complex (C**) formed between Inc RNA and its target, encoded by IncIα ColIb-P9 (highlighted in Fig. 1, step g). We find that the initial transient interaction (C*) between RepZ mRNA and Inc RNA, a derivative of Inc RNA lacking its 5′-single-stranded leader, transforms into a more stable complex altering the sensitivity of the target RNA region to a variety of RNases. Our results show that the region of extensive base pairing encompasses the bases different from the IncB plasmids, consistent with the idea that C** is an important determinant of plasmid incompatibility. Furthermore, we find that the extensive intermolecular base pairing is separated at the site of initial base pairing, suggesting a stacking trend between each intermolecular helix and the stem of Inc RNA or its target. This unusual structure at the late intermediate step provides insights into the molecular basis for the unique differential control by Inc RNA.

**EXPERIMENTAL PROCEDURES**

**RNA Preparation**—Preparation of Inc RNA, Rep RNA, and Inc RNA derivatives was chemically synthesized and purified as described (16).

**Binding of Inc RNA to RepZ mRNA in Vitro**—Binding of Inc RNA to RepZ mRNA was conducted in the binding buffer (50 mM HEPES-KOH, pH 7.6) at 37 °C and analyzed by denaturing polyacrylamide gel electrophoresis in the presence of 8.3 M urea (16, 19). Inc RNA-Rep Z RNA hybrid was detected as a unique differential control by Inc RNA. A radioactive RNA was employed for the cleavage experiments as above, and the amount of RNase T1 products cleaved at G-327 and G-328 was quantitated together with the cleavage product at G-360 as an internal standard. For the measurement of Kd, 0, 25, 50, 100, and 400 nM Inc RNA was employed for the cleavage experiments as above, and the amount of RNase T1 products cleaved at G-327 and G-328 was quantitated together with the cleavage product at G-360 as an internal standard. Kd was calculated as [Inc RNA] or [Ps14] -1/2 = 20 nm, where y is the relative intensity of the G-327 and G-328 bands.

**RESULTS**

**Inc RNA** Binds to Stem-Loop I in Two Steps—We previously synthesized three RNA species, Inc RNA, Rep RNA, and Inc RNA derivatives (see under “Experimental Procedures”) and examined their effects on the interaction between Inc RNA and Rep RNA, a 293-base RNA corresponding to the repZ mRNA leader (16). Inc RNA is an Inc RNA derivative lacking the 5′-single-stranded leader and did not form a stable complex with Rep RNA under the denaturing gel conditions. Ps14 is a single-stranded 14-base portion of Rep Z mRNA with a 7-base sequence complementary to loop I, and its binding to the target stem-loop (designated structure 1) mimics the repZ mRNA pseudoknot. Sl54 is a 54-base RNA corresponding to the stem-loop I of Rep Z mRNA. Fig. 2 shows a reproduced result of the kinetic analyses on the inhibition of Inc RNA binding to Rep RNA by these three inhibitory RNAs. As shown by the filled squares in Fig. 2,
A and B, 32P labeled Rep RNA293 or Inc RNA reduced the fraction of unhybridized species, due to its hybridization to excess unlabeled Inc RNA or Rep RNA293, respectively. As shown by open symbols in Fig. 2, A and B, the presence of inhibitory RNAs decreased the initial rate of the hybridization with K_i values of 6–10 nM, as reported previously (16). Thus, the three RNAs formed transient, reversible complexes with the partners, and competed with the rate-limiting step of the hybridization reaction (complex C**, see the second steps in Fig. 2, C and E).

During the course of these analyses, we realized that the binding between Inc RNA and Rep RNA293 was significantly retarded within 2 or 3 min in the presence of Inc RNA54 and SI14, carrying a large stem-loop structure (Fig. 2, A and B). Because Inc RNA54 and SI14 are present in large excess over 32P-Rep RNA293 and 32P-Inc RNA, respectively, we hypothesized that this secondary inhibition is due to their rapid, irreversible formation of a different complex with the complementary region in the 32P-Rep RNA293 or 32P-Inc RNA. We presumed that such a complex did not withstand the denaturing gel electrophoresis for the detection of Inc RNA-Rep RNA hybrid (see the third steps in Fig. 2, C and E). In contrast, the secondary inhibition was not observed with the single-stranded Ps14 RNA in this time frame (Fig. 2A). However, the preincubation (>15 min) of excess Ps14 (400 nM) with 32P-Rep RNA293 completely blocked Inc RNA binding (data not shown). Thus, the initial base pairing with Ps14 may transform into an irreversible complex more slowly than the complex with Inc RNA54 (Fig. 2D).

Secondary Structure Analyses of the Late Complex between RepZ mRNA and Ps14—To examine the possibility that the initial base pairing (C*) between Inc RNA and its target transforms into an irreversible intermediate interaction corresponding to C**, we analyzed the secondary structure of 5′-32P labeled Rep RNA293 using RNases V1 (specific to double-stranded RNA), T1, U2, PhyM, and CL3 (specific to an unpaired G, A, U/A, or C residue, respectively), after the preincubation (30 min) of 32P-Rep RNA293 with Inc RNA54 or Ps14. Fig. 3 shows the gel electrophoretic patterns of the cleaved products of Rep RNA293. Fig. 4 describes the deduced secondary structure of the target stem-loop I in RepZ mRNA. Cleavage experiments with 32P-Rep RNA293 alone (Fig. 3, lanes 1, 7, 13, 16, 20) confirm the previously proposed structure of stem-loop I (14, 19), as shown in Fig. 4A. G-327 and G-328 at the top of the loop were strongly cleaved by RNase T1. These two bases are most critical for both the pseudoknot formation (13, 14) and Inc RNA binding (19).

In the presence of Ps14, the structure of the top of stem-loop I was altered substantially. The RNase T1 cleavage at G-327 and G-328 was lost with excess Ps14 (Fig. 3, lane 4; Fig. 4C). Alternatively, the enzyme cleaved G-321 strongly. In addition, U-318, A-319, A-322 were cleaved by RNase PhyM, and A-323 and C-320 were cleaved by RNase CL3. However, the cleavage patterns of the stem I region were not changed. These results indicate that Ps14 and Rep RNA293 form a complex by base pairing between their complementary sequences. This complex is very similar in structure to RNA290, a derivative of Rep RNA293 truncated 3′-terminally at the same residue as Ps14 (14). RNA290 was unable to bind Inc RNA (19), consistent with our finding that the preincubation of Rep RNA293 with Ps14 blocked its binding to full-length Inc RNA.

The results shown in Fig. 3, lanes 2–4, indicate that the loss of RNase T1 cleavage at G-327 and G-328 (located at the top of structure I) is a good indicator of formation of the late complex with Ps14. In order to measure the equilibrium dissociation constant (K_d) for this Ps14-Rep RNA293 complex, we incubated a fixed amount (40 nM) of 32P-Rep RNA293 with 25–400 nM Ps14...
and conducted partial cleavage with RNase T1. The amount of cleavage products at G-327 and G-328 was quantitated relative to that of cleavage product at G-360 as an internal standard (see Fig. 3). As shown in Fig. 5, the cleavage signal at G-327 and G-328 was reduced to 50% when [Ps14] is 80 nM, giving the $K_d$ value of 60 nM. Thus, the formation of the extended pseudoknot is probably a slow process. These results support the idea that the pseudoknot induced in vivo by repY translation is unfolded by the inhibitory secondary structure (III) before it reaches the more extended pseudoknot, as illustrated in Fig. 1, steps b–d.

Secondary Structure Analyses of the Late Complex between RepZ mRNA and Inc RNA54—In the presence of Inc RNA54, the structure of stem-loop I was altered more dramatically (Fig. 4B). The RNase T1 cleavage at G-327 and G-328 was significantly reduced by increasing the amount of Inc RNA54 (Fig. 3, lanes 5 and 6). However, the RNase T1 cleavage at these residues remained even with excess Inc RNA54, whereas it was lost completely with excess Ps14 (Fig. 3, lanes 4 and 6). Consistent with this observation, only RNase PhyM weakly cleaved at U-326 adjacent to guanine residues, among other single-strand specific RNases (Fig. 3, lane 21). The RNase V1 cleavage at C-312−U-314 completely disappeared and instead shifted to loop bases at G-321 and A-322 (Fig. 3, lane 11). These results suggest that Inc RNA54 and Rep RNA293 form a novel X-shaped complex with two intra- and intermolecular helices, as shown in Fig. 4B. It is conceivable that the initial base-pairing propagates in both directions until it meets with the stems of Inc RNA and the target and, thereafter, the long intermolecular helix was separated at the center of the loops. This separation would allow the intramolecular stems to stand side by side, being stimulated by a stacking trend between each of inter- and intramolecular helices. Furthermore, the loss of RNase V1 sensitivity at C-312−U-314 strongly suggests that the region of extended base pairing covers the 21-base Inc loop region plus a few bases each from the upper stem and includes the bases different from IncB pMU720 plasmid (Fig. 4B). Because the bases in the 5′ single-stranded leader of the antisense RNAs are identical between IncI and IncB plasmids, we proposed that the stability of the complex as depicted in Fig. 4B is responsible for determining different incompatibility between IncI and IncB plasmids (see under “Discussion”).

Next we attempted to estimate the $K_d$ value for this late Inc RNA54 complex by plotting the relative intensity of the RNase T1 cleavage products at G-327 and G-328 against [Inc RNA54]. Fig. 5 shows that $K_d$ for the Inc RNA54 complex is much smaller than that for the Ps14 complex and is close to the detection limit for this experiment. Nevertheless, we could tentatively estimate the $K_d$ value of ~3 nM. Although determining the accurate $K_d$ value awaits further experiments, the results in Fig. 5 suggest that the late Inc RNA54 complex is more stable than the transient initial interaction (C*) and corresponds to the C** complex. Thus, the whole structural data support our model that the initial base-pairing between the stem-loops of Inc RNA54 and the target in Rep RNA293 (C*) rapidly transforms into a more stable, irreversible complex (C**), leading to block full-length Inc RNA binding, as observed in Fig. 2A.

Why Is the Late Inc RNA54 Complex Unstable?—The intermolecular base pairing observed in the late Inc RNA54 complex was quite extensive as shown in Fig. 4B. Nevertheless, it was not stable in denaturing gels, probably because the interacting loops are closed by stable stems: annealing of two strands of RNA generates torsion as they form a helical structure. Fixing the ends of these strands by a stable stem would remove the way to release such torsion, thereby providing a stress into the structure of the resulting complex. To test this idea briefly, we prepared Inc21, a 21-base RNA corresponding to the loop of Inc

![Diagram](image-url)
pseudoknot formation should be established at this stage, because the positions of free or complexed 32P-Rep RNA293.

32P-Rep RNA293 (0.25 nM) was incubated with Inc RNA (absent in Inc RNA 54) to base pair efficiently with a region step, as depicted in Fig. 1, reaction. Second, for the control of observed irreversible base pairing would trap the initial tran-

sumed side-by-side configuration of the complex would bring the ends of the stems closer, thereby allowing the Inc 5 to RepZ mRNA also supports our previous proposal that there is little requirement in the structure of Inc RNA for its efficient binding to RepZ mRNA (16, 19).

DISCUSSION

Molecular Basis for Differential Control of repY and repZ by Inc RNA—The plasmid ColIb-P9 Inc RNA controls translation of repY and repZ with different mechanisms (12). By using Inc RNA54 lacking the 5' leader of Inc RNA and P814, a part of RepZ mRNA complementary to loop I, we previously showed that the initial loop-loop base pairing between Inc RNA and the target in the repZ mRNA leader can compete with formation of the pseudoknot required for repZ translation (16). In this report, we show that Inc RNA54 not only reduces the initial rate of the Inc RNA-RepZ mRNA hybridization by its transient base pairing to loop I, but also stops the reaction by its subsequent irreversible binding to the loop (Fig. 2). Intermolecular base pairing found in the latter irreversible complex was quite extensive, spanning probably > 20 base pairs and forms two helices separated at the site of the initial base pairing (Figs. 3 and 4). The equilibrium dissociation constant for this complex was tentatively estimated to be ~3 nM (Fig. 5), a value smaller than that for the transient initial interaction, 6 or 10 nM (16).

Nevertheless, the late complex was unstable in denaturing gels, probably because of a structural stress stored during the complex formation (Fig. 6). Based on these results, we propose that the stepwise model for antisense RNA binding proposed for the ColE1 system (class H) (Ref. 18; see the Introduction) also applies to a class L antisense RNA, but with completely different molecular mechanisms. We believe that the late Inc RNA54 complex characterized here as C** corresponds to an intermediate complex formed between IncB pMU720 RNA T (a 5'-truncated derivative) and its target that was analyzed by native gel electrophoresis (Ref. 23; see below).

In view of differential control of repY and repZ, several important functions could be assigned to the late intermediate step, as depicted in Fig. 1, step g. First, it is conceivable that the observed irreversible base pairing would trap the initial transient interaction in a bound state, thereby driving the binding reaction. Second, for the control of repZ, the inhibition of the pseudoknot formation should be established at this stage, because the bases critical for the pseudoknot formation (G-327–G-330) are still masked (Fig. 4B). Furthermore, the presumed side-by-side configuration of the complex would bring the ends of the stems closer, thereby allowing the Inc 5 leader (absent in Inc RNA54) to base pair efficiently with a region distal to the repY RBS (Fig. 1, steps g and h).

The antisense RNA I of IncB pMU720 replicon is identical to the Inc RNA of IncIa ColB-P9 except at the bases indicated in Fig. 4B. Therefore, the partitioning of these plasmids into different incompatibility groups (22) should be explained at the level of the C** complex formation as analyzed in this study. It is also noteworthy that the mutant IncIa Inc RNA with a loop mutation (G329A in ColB-P9) abolishing the C* formation (16) showed no detectable incompatibility (or interaction) against both wild-type IncIa and IncB plasmids (22). Thus, the mutation in the C* region has more dramatic effect on plasmid incompatibility than that located outside affecting only C** formation, consistent with our previous results (19).

Complex C** Formed by Other Class L Antisense RNAs—Although the presence of C** has not yet been demonstrated for IncFII plasmids, an intermediate complex, called the “extended kissing complex,” was extensively analyzed with the CopA antisense RNA encoded by the R1 plasmid of IncFII group (24–27). This complex, formed between a derivative of CopA lacking its 5' leader and the CopA target, was often characterized by the inhibition secondary structure for the rep RBS is depicted for IncB ColB-P9, and IncFII R1, based on Refs. 6, 14, and 28. Asterisks indicate the Shine-Dalgarno sequences. The rep start codons and RLP stop codons are boxed and underlined, respectively.
allow progression to C**, based on our results (Fig. 2). Second, reduced but remaining RNase T2 sensitivity at a loop G residue found in the 5’-truncated CopA in complex with the target (24) was very well with the weak RNase T1 sensitivity at G-327 and G-328 found in the structure I of RepZ mRNA in complex with Inc RNAe (Fig. 3). Third, a measurement by native gel electrophoresis may underestimate the Kd value, because the complex can dissociate in the gel. In fact, Hjalt and Wagner (26) realized this problem and measured Kd differently by incubating the target RNA with various concentrations of the 5’-truncated CopA and measuring the amount of free form of the former that can bind intact CopA RNA. Kd thus obtained was 1.6 nM consistent with our preliminary estimation for C** (~3 nM; Fig. 5). These values are significantly higher than the Kd obtained for C** in ColE1, 0.1 nM (18). This fact agrees with the idea that C** in ColII-P9 (or R1) is destabilized by a structural stress imposed by the propagation of a single initial loop-loop interaction (Fig. 6). C** in ColE1 should be free of such a stress, because it is composed of multiple loop-loop interactions involving a smaller number of base pairs (18).

Finally, the secondary structure of the extended kissing complex was analyzed with Ph(II)-induced cleavage experiments (27). Malgren et al. (27) appear to assume that the extended intermolecular base pairing occurs only at the 3’ side of the target loop, even though Ph(II) sensitivity was also reduced at the 5’ side. Using four different RNases that have potential to cleave all four kinds of nucleotides plus a double-strand specific RNase V1, we unambiguously showed that both sides of the partially base paired target loop were protected by Inc RNAe (Fig. 4B). In sharp contrast, the 5’ side of the target loop in complex with Ps1 showed dramatically increased sensitivity to RNases T1 and PhyM (Fig. 4C). Another new aspect concerns the weak single-stand specific RNase sensitivity at U-326—G-328 (Fig. 3). These results raised the interesting possibility that the intermolecular helix is separated at the top of structure I, resulting in a stacking trend between each separated helix and the stem of Inc RNA or structure I (Fig. 4B). We already discussed the biological significance for this unique configuration as above.

Evolution of Control of Plasmid Replication by Class L Antisense RNAs—The whole pathway of Inc RNA binding as shown in Fig. 1 appears to fit best for its differential control of repY and repZ expression. Antisense RNAs encoded by IncII plasmids follow a similar binding pathway (27). Nevertheless, the translation of rep genes encoded by these plasmids does not require formation of a pseudoknot (9, 28), unlike the case for IncI (13) and IncB (15) plasmids. Instead, IncII plasmids additionally encode a transcriptional repressor for the rep mRNA, located upstream of the repressible promoter (3) (see Fig. 7). How did these differences arise during the course of evolution?

We believe that the difference between these related but distinct types of plasmids arose at least partly from difference in RNA secondary structure that blocks the RBS for respective rep genes, as depicted in Fig. 7B. In the case of IncI/IncB-type, the secondary structure that blocks the rep RBS is very tight. Therefore the translation of rep is possible only when the pseudoknot induced during the RLP translation termination transiently keeps the rep RBS accessible to the ribosome (6, 14, 16). On the other hand, the inhibitory secondary structure found in IncII plasmid replicons is less tight around the rep RBS. Thus, the refolding of the inhibitory secondary structure, unfolded during the RLP translation termination, would be slow enough to allow the ribosome to bind the rep RBS. However, this kind of translational coupling could enable leaky, unregulated translation initiation of rep, as demonstrated for a mutant ColII-P9 replicon lacking the pseudoknot and carrying less tight structure III (rep inhibitory stem-loop) (6). It is therefore conceivable that the ancestor of IncII-type plasmids acquired an additional control system at the transcriptional level, in order to complement this regulatory defect.

It would be difficult to solve the evolutionary relationship between these regulatory systems. Nevertheless, we favor the model that the pseudoknot-mediated control in IncII/IncB plasmids is more ancient and was lost in the ancestor of IncII plasmids during recombination between plasmids. In support of this scenario, the “mosaic” structure of large, self-transmissible plasmids is not uncommon (20), and there are even some cases of naturally occurring hybrid replicons carrying the control region of IncIa/IncB-type and the rep gene of IncII-type, or vice versa (9). Evolution of plasmids by recombination may have been stimulated by horizontal transfer of plasmids between a variety of bacteria encoding different restriction-modification systems (29). On the other hand, certain types of plasmids that are transferred only between limited species of enterobacteria and not exposed to harsh natural environment might retain the intact plasmid genome and hence the “molecular fossil” of replication control system. The ongoing genome-wide sequence analyses for different groups of self-transmissible plasmids may answer whether ColII-P9, originally isolated from Shigella sonnei, is one such plasmid.

Acknowledgment—We are indebted to Shigeyuki Yokoyama for inspiring discussion and the experiments conducted by K. A. at the University of Tokyo. We thank Alan Hinnebusch for comments on the manuscript, Tatsuya Niimi for sharing materials used for RNAse cleavage experiments, and members of the Yokoyama and Mizobuchi laboratories for helpful discussion.

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