Antisense RNAs in plasmids: control of replication and maintenance

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Abstract

The search for small RNAs which might act as riboregulators became successful over the past two years both in prokaryotes and in eukaryotes. Moreover, artificially designed antisense RNAs have become powerful tools to downregulate the expression of targeted genes. It seems that antisense RNAs as regulatory molecules are most likely to be found everywhere. However, the first naturally occurring antisense RNAs were identified in plasmids and other prokaryotic accessory DNA elements. The thorough and detailed analyses of these systems have provided deep insights into structure and function of prokaryotic antisense RNAs and the kinetics of antisense/sense RNA interaction. Here, I focus on the role of antisense RNAs in plasmid replication and maintenance.

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1. Introduction

The antisense principle is rather widespread in nature. In eukaryotes, processes like splicing, editing or rRNA modification, developmental regulation or the more recently discovered RNA interference (RNAi), (Fire et al., 1998) make use of complementary small RNAs or stretches thereof. However, the first naturally occurring antisense RNA was discovered in 1981 in a prokaryote—in the replication control of the Escherichia coli plasmid ColE1 (Tomizawa et al., 1981). During the following two decades, it became clear that antisense RNAs are principal regulators in prokaryotic accessory DNA elements (for a review see Wagner et al., 2002), where they regulate replication, segregational stability or conjugation of plasmids, fine-tune the decision between lysis and lysogeny in phages or regulate transposition. Over the past six years, a few small chromosomally encoded RNAs with hitherto unknown function have been characterized as antisense RNAs, too, like OxyS, DsrA, and 6S RNA (reviewed in Brantl, 2002). Recently, three independent computer searches in E. coli resulted in the detection of in total >30 new small RNAs, the function of which is currently investigated in the corresponding laboratories (Argaman et al., 2001; Rivas et al., 2001; Wassarman et al., 2001). This indicates that antisense RNAs are most likely to be found everywhere as small regulatory molecules.

Antisense RNAs are small, diffusible, untranslated, highly structured (one to four stem-loops) molecules that bind to their target RNAs (sense RNAs) thereby controlling expression of
the target genes. Typically, they are encoded in \textit{cis} from the opposite DNA strand, but recently, a number of \textit{trans}-encoded antisense-RNAs have been found. Detailed analyses revealed that efficient antisense RNAs have 5–8 nt GC-rich loops. Stems that are important for metabolic stability are often interrupted by bulges to prevent dsRNAse degradation and to facilitate melting upon antisense/sense RNA interaction (reviewed in Wagner et al., 2000). Recognition loops of the antisense RNA or the sense RNA often contain a 5′-YUNR motif proposed to form a U-turn structure, a sharp bent in the RNA phosphate backbone, thus providing a scaffold for the rapid interaction with the complementary RNA (Franch and Gerdes, 2000). Recognition loop of the antisense RNA orthosense RNA often contain a 5′-YUNR motif proposed to form a U-turn structure, a sharp bent in the RNA phosphate backbone, thus providing a scaffold for the rapid interaction with the complementary RNA (Franch and Gerdes, 2000).

In this minireview, I emphasize on antisense-RNAs regulating replication, segregational stability, and conjugation of plasmids.

Plasmids are selfish genetic elements that normally constitute a burden for the bacterial host cell that, consequently, tries to eliminate the “intruder.” To prevent this elimination, plasmids have evolved copy number control and maintenance systems. They control unavoidable copy number fluctuations and prevent great decreases or increases of copy numbers. Principally, two control modes can be distinguished: iteron-mediated control (reviewed in Chattoraj, 2000) and antisense-RNA-mediated control discussed below.

In plasmid copy number control the antisense RNAs act both as measuring devices and as regulators, and regulation occurs in all cases by inhibition. Antisense-RNAs are constitutively synthesized and metabolically unstable (one exception: pIP501, see below). Therefore, any change in plasmid concentration will be reflected by the corresponding concentration changes of the regulating antisense-RNA. These concentration changes are “sensed” leading to altered replication frequencies. Increased plasmid copy numbers lead to increasing antisense-RNA concentrations, which, in turn, lead to inhibition of a function essential for replication (e.g., replication initiator protein or primer). On the other hand, decreased plasmid copy numbers entail decreasing concentrations of the inhibiting antisense-RNA, thereby, increasing the replication frequency.

Regulation is achieved by a variety of mechanisms: inhibition of translation of the essential Rep protein (R1 and pMV158 family), transcriptional attenuation (pT181 and \textit{inc}18 family) or inhibition of pseudoknot formation (IncB and Inc19). In some cases, the antisense RNA(s) act alone (pT181 family, IncB/IncI\(\alpha\) family, and ColE2). In other cases, they are accompanied by regulatory proteins, which are either transcriptional repressors or RNA binding proteins. These regulatory proteins can either play an auxiliary role, as in the case of R1 or ColE1, or can be necessary for proper regulation (\textit{inc}18 family and pMV58 family). All cases are discussed briefly below.

2. Plasmid replication control

2.1. Antisense-RNA mediated transcriptional attenuation: the \textit{inc}18 and the pT181 families

Regulation of plasmid replication by antisense-RNA mediated transcriptional attenuation has been described first in 1989 (Novick et al., 1989) for the staphylococcal plasmid pT181 replicating by the rolling-circle mechanism. Later on, this mechanism was also found for two plasmids belonging to the \textit{inc}18 family of streptococcal plasmids that replicate via the theta mechanism: pIP501 (Brantl et al., 1993) and pAM\(\beta\)1 (Le Chatelier et al., 1996). Attenuation mechanism and regulation in pIP501 and pT181 are as follows (reviewed in Brantl, 2002, submitted):

Target for copy number control is the \textit{rep}-mRNA encoding the essential rate limiting replication initiator protein. During transcription, this RNA can adopt two mutually exclusive structures depending on the presence or absence of the antisense RNA. Antisense-RNA binding leads to formation of a \(p\)-independent transcriptional terminator (attenuator) and, consequently, premature termination of \textit{rep}-mRNA transcription. Escape from antisense RNA binding permits re-folding (see Fig. 1) silencing the transcriptional terminator. Transcription proceeds resulting in a full-length \textit{rep}-mRNA and, hence, Rep protein synthesis. Thus, the antisense RNA affects gene expression by aborting a transcript for an essential protein.

Binding of the antisense-RNA must occur within a short time frame (estimated to be 10–20 s) to be effective. Based on this estimate of the time-window, the inhibition rate constant of the pIP501 antisense-RNA (RNAIII) has been calculated to be \(1–2 \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}\) which is 10 times higher than...
Fig. 1. Examples for antisense-RNA mediated control in plasmid replication and maintenance. Antisense RNAs are drawn in red, sense RNAs in blue; (+) and (−) stands for activation and inhibition, small black triangles denote promoters; SD, Shine Dalgarno sequence; fbi, fold back inhibition; term, bidirectional terminator. The four upper panels show examples for different control mechanisms found in plasmid replication. The three lower panels illustrate control mechanism found in plasmid maintenance (segregational stability and conjugation). Details are described in the text. Based on Brantl (2002) and Greenfield et al. (2001).
the pairing rate constant of the sense/antisense-RNA pair indicating that full duplex formation is not required for inhibition. Apparently, steps preceding formation of a complete duplex between sense- and antisense-RNA are sufficient for inhibition (Wagner and Brantl, 1998). In the case of pT181, inhibition rate and the pairing rate constants were found to be in the same range.

For pIP501, quantitative Northern blotting used to determine the intracellular concentrations and half-lives of sense- and antisense-RNAs led to two surprising results: (1) the antisense-RNA (RNAIII) is unusually stable (half-life of ≈30 min) and its concentration is identical in copR+ and copR− plasmids, replicating at 10-fold different copy number. The unusually long half-life of RNAIII should cause problems upon downward fluctuations of copy numbers: the high concentration of the inhibitor would lead to further decreases of the replication frequency and, finally, to plasmid loss.

However, this plasmid loss is prevented by a second control mechanism provided by the dual function of the small (10.6 kDa) transcriptional repressor CopR that binds at two consecutive sites in the major groove immediately upstream of the −35 box of the (sense) repR promoter pII (reviewed in Brantl, submitted) leading to a 10–20-fold decrease of transcription of the repR-mRNA and, consequently, of the pIP501 copy number (first function of CopR). The second function of CopR is to prevent convergent transcription from the sense and the antisense promoters pII and pIII, respectively, thereby indirectly increasing the initiation frequency at pIII. The scenario for copy number control is as follows: if copy numbers increase, RNAIII is sufficient to downregulate replication; higher RNAIII concentrations lead to increased premature termination. If copy numbers decrease, the intracellular CopR concentration decreases, too, resulting in derepression of repR transcription. At the same time, convergent transcription from pII and pIII results in a decrease in the initiation at pIII and, thus, less inhibition by RNAIII. Hence, replication frequency increases. Furthermore, the now higher amounts of repR-mRNA titrate the remaining long-lived RNAIII. In summary, pIP501, and its related plasmids pAMBl and pSM19035 (all inc18 family) have evolved a very efficient mechanism to cope with copy number fluctuations: the concerted action of two control components, an unusually long-lived antisense-RNA and a Cop protein with a dual function.

In the case of pT181 and its related plasmids, the antisense-RNAs are fairly unstable and, therefore, no second control component is needed to ensure an efficient regulation of plasmid replication.

In contrast to the situation in gramnegative bacteria, deletion of either copy number control element in pIP501 or pT181 does not result in “runaway replication” (Uhlin and Nordström, 1978), but only in a significant copy number increase. We hypothesize that in grampositive bacteria a limiting host factor is responsible for this behaviour.

Interestingly, antisense-RNA mediated transcriptional attenuation has not been found as a replication control mechanism for plasmids of gramnegative hosts. Recently, we could show experimentally that this mechanisms principally functions in E. coli, albeit with a much lower efficiency than in B. subtilis or S. aureus (Brantl and Wagner, 2002). This lower efficiency might be due to some nucleolytic or processing activity present in E. coli which will affect the concentrations of the interacting RNAs and probably the distribution of their inactive and active processing products. Our data, however, ruled out the possibility that properties of the transcriptional machinery make this mechanism nonfunctional in E. coli. We suggest that antisense-RNA mediated transcriptional attenuation which leads to a much broader copy number distribution than a control mechanism based on inhibition of translation (as e.g., in R1, see below) can only be tolerated by plasmids which do not tend to “runaway replication.”

In the case of plasmid pSM19035 (inc18 family) it was found that the plasmid encoded ω protein presents a link between copy number control and better than random segregation (Hoz et al., 2000). The ω protein, the crystal structure of which was determined recently (Murayama et al., 2001) acts as transcriptional repressor at three promoters: its own promoter, the copS promoter and the δ promoter. It represses copS transcription about 8-fold.

2.2. Translational inhibition

2.2.1. Inhibition of leader peptide translation: R1 and related plasmids

The best studied example is the E. coli IncFII plasmid R1. Its basic replicon contains the ORFs, copB, tapA, and repA encoding the transcriptional repressor CopB, a 24 aa leader peptide TAP and the essential initiator protein RepA, respectively,
the latter of which is rate-limiting for replication. Replication control is reviewed in Wagner et al., 2000: The replication origin oriR is located downstream of the repA gene. Within the leader region of the repA-mRNA, a ≈90 nt long antisense-RNA, CopA, is transcribed from the complementary strand. Regulation occurs at two levels. The main control element is CopA, which contains two stem-loops and is unstable (Fig. 1). Its target, CopT, is part of the repA-mRNA leader region. Binding of CopA to CopT sterically blocks initiation of translation of the tap leader peptide and also results in RNase III-dependent cleavage. The latter one has, however, only minor effects on control. Tap translation is required for repA translation (translational coupling) since a stable RNA secondary structure blocks the repA RBS. Consequently, the CopA antisense-RNA inhibits repA translation via inhibition of translation of the tap leader peptide. Recently, the binding pathway between CopA and CopT has been elucidated in detail: binding starts with the interaction of two single loops of CopA and CopT. Later, a single stranded region is required to overcome the torsional stress created upon progressing of this loop–loop interaction. Next, a partial duplex is formed which contains a four-helical junction (Kolb et al., 2000a,b). This intermediate is converted into the stable inhibitory complex which is only a partial duplex and is only slowly converted into a stable duplex, the substrate for RNase III degradation (for a figure, see Wagner et al., 2002). Recently, the importance of a putative U-turn motif in the major loop of CopT for efficient sense/antisense-RNA interaction and, hence, regulation, has gained experimental evidence. When the orientation of the 5′-YUNR sequence was inverted, CopA/CopT binding rates in vitro were decreased more than 10-fold, paralleled by a corresponding regulation defect in vivo. By contrast, loop swapping between CopA and CopT (putative U-turn now present in CopA) maintained wild-type-like behaviour (J.G. Slagter-Jäger and E.G.H. Wagner, personal communication).

The second copy number control element of plasmid R1 is the tetrameric 11 kDa transcriptional repressor CopB the deletion of which leads to 8-fold copy number increase (reviewed in Brantl, submitted). The cloned copB gene does not exert incompatibility against wild-type R1, indicating that it is only an auxiliary control component. Although CopB prevents convergent transcription from repA and copA promoters, this has not the same importance as in the case of pIP501. Therefore, CopB is a rescue device at dangerously low-copy numbers and/or after conjugal transfer of R1. During normal steady-state conditions, CopA is sufficient to cope with copy number deviations.

2.2.2. Inhibition of translation of the rep-mRNA: the pMV158 family

The promiscuous plasmids of the pMV158 family apparently use the same copy number control mechanism (reviewed in del Solar and Espinosa, 2000): the replication of these RC plasmids is controlled by two components, an antisense RNA and a transcriptional repressor.

In the case of the best characterized plasmid of this series, pLS1, the 50 nt long antisense RNA, RNA II, is complementary to a region covering the RBS of the mRNA of the essential 24.5 kDa RepB protein. It was shown that RNA II is the main incompatibility determinant of the plasmid. Direct experimental evidence was provided that RNA II inhibits RepB translation. The second control component is the transcriptional repressor CopG (formerly RepA). By binding to its own promoter, the homodimeric protein CopG (45 aa, 5.1 kDa) represses its own synthesis and that of RepB. CopG is not essential, since its deletion does not affect plasmid replication or maintenance. An increase in CopG dosage does not result in incompatibility towards pLS1 or in any significant reduction in its copy number, thus, CopG is not able to efficiently correct big fluctuations in plasmid copy number, probably because of its autoregulatory role. CopG binds at two successive major grooves on one face of the DNA to a 13 bp element forming an imperfect repeat, within which lies the −35 box of the repAB = copG − repB promoter. Recently, the crystal structure of CopG has been solved both alone and in complex with a 19 bp DNA-target: the CopG dimer has a ribbon-helix–helix-structure and uses its HTH region for oligomerization instead of DNA recognition.

2.3. Inhibition of primer formation: the ColE1 replicon

The multi-copy E. coli plasmid ColE1, does—in contrast to all other plasmids reviewed here—not require a plasmid-encoded replication initiator protein, but only an RNA primer, RNAII, which is the target for copy number control. Regulation occurs as follows (reviewed in Eguchi et al., 1991):
First, a 550 nt long pre-primer is synthesized by host-RNA polymerase. During synthesis, this pre-primer undergoes specific conformational changes which are required for its activity. The active conformation forms a persistent hybrid with the DNA in the origin region. The RNA of the RNA–DNA hybrid is cleaved by host RNase H and converted to a mature primer for replication that delivers the free 3′-OH end required by DNA polymerase I. Replication control is mediated by the 108 nt long RNAI (composed of three stem-loops and an ss 5′ tail), which is transcribed constitutively from the complementary strand in the pre-primer region (Fig. 1). Binding of RNAI to RNAII prevents refolding of the nascent pre-primer and, therefore, formation of a persistent RNA–DNA hybrid within the origin region. Consequently, primer maturation is prevented. As in the case of pIP501/pT181, a time window exists, during which inhibition can occur.

RNAI/RNAII binding follows a two-step pathway. It initiates between one or two loop-pairs (out of three). A reversible unstable kissing complex, C\textsubscript{r}, which is likely to involve a single pair of stem-loops, initiates binding. Subsequently, a kissing complex possibly involving all three RNAI loops is formed. Finally, stable complex formation (complex C\textsubscript{r}) occurs at a rate constant of 10⁶ M⁻¹ s⁻¹. RNAI lacking its 5′-tail is arrested at this stage, but inhibits primer formation in vitro and in vivo, which implies that a full RNA duplex is not required for control. The duplex is formed very slowly, concomitant with stepwise loss of loop–loop contacts and unfolding of the stem-regions. It was concluded that all seven loop bases are base-paired to each other, creating a co-axial stack of the two stems bent at the loop–loop helix.

In summary, in the ColE1 family the antisense RNA does not affect the expression of a protein-coding gene, but the activity of a target RNA by induction of a non-functional conformation.

The second plasmid-encoded control component is the small RNA one modulator (Rom) protein (63 aa) encoded downstream from the origin of replication (reviewed in Brantl, submitted): the Rom dimer increases the interaction between RNAI and RNAII, i.e., the conversion of the unstable RNAI–RNAII complex to a stable complex, thereby increasing inhibition of replication. Its effect on replication frequency is with two to 5-fold relatively low, since the inhibition rate is primarily determined by the binding rate constant and not the binding affinity between loop–loop complexes. Extra copies of rom do not increase incompatibility demonstrating that Rom is not a primary inhibitor of ColE1 replication, but only an auxiliary factor, as it exerts its maximum effect at the wild-type concentration. The intracellular concentrations of RNAI, RNAII, and the Rom protein, have been determined with 1 μM, 7 nM, and 1 μM, respectively, and it is suggested that plasmid copy number is little affected by the rate of RNA II synthesis but is strongly dependent on that of RNA I.

2.4. Inhibition of pseudoknot formation: the IncI2l IncB case

The low-copy number IncB and IncI2l plasmids of gramnegative bacteria are similar to R1 but use another inhibitory mechanism. The two best characterized examples are ColIb-P9 (IncI2l) and pMU720 (IncB). Here, a 70 nt antisense-RNA as the only regulator inhibits formation of a long-distance RNA pseudoknot that is required for efficient translation of the essential, rate-limiting replication initiator protein Rep (Asano et al., 1991a,b; Wilson et al., 1993). The mechanism has been reviewed recently (Wagner et al., 2000): as in plasmid R1, a leader peptide ORF—repY (in ColIb-P9)—must be translated to permit synthesis of RepZ (see Fig. 1). Two stem-loop structures in the repZ mRNA that have been mapped in vitro and are located upstream (structure I) of the repY RBS and in the middle (structure III) of the repY gene are necessary for replication control. Structure III occludes both the repZ RBS and a short sequence complementary to a region in the loop of structure I. Appropriate termination of repY translation unfolds structure III which in turn allows the formation of a short helix between the target loop and the disrupted stem, located ≈100 nt apart thus inducing the formation of the activator pseudoknot by intramolecular pairing of loop I with its complementary sequence. Pseudoknot formation facilitates ribosome binding to the repZ RBS. The pseudoknot could be mapped in vitro using mutations that disrupt structure III.

The antisense-RNA (RNAI) is encoded upstream of the repY-ORF and has a dual function: (i) by sequestering the repY RBS it blocks directly repY translation and (ii) it prevents activation of repZ translation, since the site of RNA I/repZ mRNA interaction involves the nucleotides in structure I required for pseudoknot formation. In this way, RNA I can repress repZ translation at the level of a transient interaction with its target before
a complete duplex is formed—similar to the R1 and the pIP501 case (see Wagner and Brantl, 1998).

In the case of the IncIa-plasmids, a hexanucleotide which includes the structure I sequence involved in the initial interaction presumably supports a U-turn (see above).

The early stages in pseudoknot formation and binding of RNA I are similar. However, RNA I represses repY translation much less efficiently than repZ translation. Repression of repZ and repY expression are accomplished at different stages during the pairing between RNA I and rep mRNA (Asano and Mizobuchi, 2000). This differential repression allows RNA I to keep the total level of repZ expression constant thereby ensuring a constant copy number value.

The antisense/sense RNA binding pathway of the IncIa and IncB group plasmids is a two-step pathway and very similar to that of Copa/CopT of plasmid R1 (e.g., Asano and Mizobuchi, 2000). Recent data suggest that antisense/sense RNA complexes of ColIb-P9, R1 and of many other distantly related plasmids may share the same overall topology including the position of the junction (Kolb et al., 2001). Apparently, efficient inhibitory antisense RNAs initiate interactions by loop–loop contacts, but the subsequent steps of helix progression to stable inhibitory complexes depend on topological constraints in order to keep topological stress at a minimum.

3. Control of segregational stability

Plasmid maintenance is ensured by post-segregational killing (PSK) systems, the best characterized of which is the (host killing) hok/sok system of R1 that has been studied in detail by G erdes’ group (reviewed in Gerdes et al., 1997). The hok/sok locus of R1 encodes a small protein, Hok that causes damage to the bacterial membrane leading to cell death. Translation of hok requires translation of the overlapping ORF mok (modulator of killing). The antitoxin is the sok antisense RNA (Fig. 1). The system functions as follows: the hok-mRNA accumulates as a stable and inert RNA, that is neither translated nor target for sok. At a low rate, 3’ end processing generates a truncated translationally active RNA that is also able to bind sok. In plasmid carrying cells, an active, translation competent hok-mRNA will be attacked by sok, followed by inhibition of translation and decay of the message. In contrast, in plasmid-free segregants sok will be lost rapidly due to its metabolic instability, whereas the stable activatable hok-mRNA persists. When 3’ truncation occurs, hok is translated and the host cell killed. Inhibition occurs by a combination of effects exerted on the level of translation and enhanced mRNA degradation.

Recently, the first antisense-RNA regulated PSK system was identified in a Gram-positive organism. The par stability locus of the Enterococcus faecalis plasmid pAD1 encodes two small, convergently transcribed RNAs, designated RNAI and RNAlII, which are the toxin and the antitoxin of the par PSK, respectively. Both RNAs overlap in a bi-directional terminator and, additionally, contain a set of direct repeat sequences (DRa and DRb) located far apart (Fig. 1). Interaction between RNAI and RNAlII within these two complementary regions and in the complementary terminators blocks ribosome binding and thus fst (toxin) translation (reviewed in Brantl, 2002). The pAD1 par system contains both properties of cis and trans encoded antisense systems. As in the hok/sok system, RNAlII is unstable and RNAI is stable and initially translationally inert. RNAI/RNAlII binding occurs in two steps (Greenfield et al., 2001; Fig. 1): first, a kissing complex between the terminator stem-loops is formed, followed by the pairing of the complementary DR sequences and, finally, complete hybridization of the 5’ nucleotides to stabilize the RNAI–RNAlII complex.

4. Control of plasmid conjugation

The best studied examples for conjugal control are the E. coli plasmids R1 and F (reviewed in Brantl, 2002). Control is exerted by the 79nt long antisense RNA FinP (Fig. 1). This RNA is complementary to the RBS of the traJ-mRNA which encodes an activator for the conjugal transfer operon that comprises ≈30 genes. Thus, binding of FinP blocks directly traJ-mRNA translation. The 20kD FinO protein promotes duplex formation between traJ-mRNA and FinP about 50-fold and prolongs the FinP half-life by protecting it against RNase E. Recently, the structural requirements for the FinP antisense RNA were analysed, and, in this context, the role of a putative U-turn structure was investigated (see L. Frost, Abstracts of Plasmid Biology 2002).

The future will probably show that the role of small RNAs in gene regulation has been under-
estimated for a long time. The lessons which can be learned from the well-studied naturally occurring antisense RNAs reviewed here can be applied to design artificial antisense RNAs for the inhibition of gene expression.

Acknowledgments

Due to the limited space for this minireview, only a few original articles could be selected as references. Therefore, I would like to apologize to all colleagues whose original data I did not quote. These data can be found in all detail in Brantl, 2002 and in Brantl, submitted.

References


