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Regulatory mechanisms employed by *cis*-encoded antisense RNAs

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Bacterial small regulatory RNAs that act by base-pairing can be divided into two classes: *cis*-encoded and *trans*-encoded antisense RNAs. The former — mainly discovered in plasmids, phages and transposons — are encoded in the same DNA locus and are therefore completely complementary to their targets over a long sequence stretch. Regulatory mechanisms employed by these RNAs encompass inhibition of primer maturation or RNA pseudoknot formation, transcriptional attenuation, inhibition of translation or promotion of RNA degradation or cleavage. Although the final product of antisense RNA/target RNA binding is a full duplex that is degraded by RNase III, inhibition does not require complete duplex formation. By contrast, in many cases, partially paired binding intermediates have been shown to be sufficient for the biological function.

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Introduction

A quarter of a century ago, the first naturally occurring antisense RNA was discovered in plasmid ColE1 [1]. Meanwhile, small regulatory RNAs that act by base-pairing (termed antisense RNAs) have been found in both prokaryotes and eukaryotes. Two classes of prokaryotic antisense RNAs can be distinguished: *cis*-encoded RNAs are located in the same DNA region and are, therefore, fully complementary to their targets over a long sequence stretch, whereas *trans*-encoded RNAs are located in another chromosomal location, and are only partially complementary to their target RNA(s). The majority of *cis*-encoded antisense RNAs have been found in plasmids, phages and transposons [2] but, more recently, a few of them have been also discovered in the bacterial chromosome (Table 1). However, because previous searches mainly focused on *trans*-encoded chromosomal small RNAs from intergenic regions, it can be expected that future analyses in different prokaryotic species will increase our knowledge of *cis*-encoded antisense RNAs from chromosomal locations. One example was a cloning-based screen focused on 30–65 nt long RNAs in *Escherichia coli*, where a few *cis*-encoded RNAs were found [3•], among them RyjC RNA that is complementary to the 5′ end of the SOS induced *yjiW* mRNA, which is currently under investigation (G Storz, personal communication). Another recent genomic SELEX approach resulted in a series of novel Hfq binding RNAs in *E. coli*, ~80% of which were clearly *cis*-encoded, that is counter-transcripts were within known ORFs (R Schroeder, personal communication).

Biological functions of *cis*-encoded small RNAs

The major role of *cis*-encoded antisense RNAs discovered to date is to regulate fundamental processes as replication initiation, conjugation efficiency, suicide, transposition, mRNA degradation and translation initiation. However, in a few cases, metabolic processes are subject to antisense control, too. Table 1 provides an overview on all currently known *cis*-encoded RNAs and their biological functions.

Characteristics of *cis*-encoded antisense RNAs

Antisense RNAs are small (~50 to 300 nt), diffusible, mostly untranslated and highly structured (one to four stem-loops) molecules that bind their target RNAs (sense RNAs), thereby regulating target gene expression. This overview covers only *cis*-encoded antisense RNAs and emphasizes on the mechanisms of action and sense/antisense RNA binding pathways.

Detailed analyses of CopA regulating replication of plasmid R1 revealed that efficient antisense RNAs have 5–8 nt GC-rich loops [4]. Stems that are important for metabolic stability are often interrupted by bulges to prevent degradation by RNase III and to promote melting upon interaction with the sense RNA [5] or progression of loop–loop intermediates to a stable inhibitory complex [6]. Recognition loops of either sense or antisense RNA were frequently found to contain a 5′ YUNR motif proposed to form a U-turn structure, a sharp bend in the RNA phosphate backbone, that provides a scaffold for the rapid interaction with the complementary RNA [7,8]. In some cases, the degradation pathways of antisense RNAs have been studied (hok/Sok, CopA/CopT of R1, RNAI/RNAII of ColE1) and half-lives determined

Table 1

Overview of *cis*-encoded antisense RNAs.

Antisense RNA/target RNA	Length of As RNA	Location	Biological function	Mechanism of action	Peculiarity
Plasmid encoded RNAs		Plasmid			
RNAI/RNAII	108 nt	ColE1	Replication control	Inhibition of primer maturation	Rom protein promotes kissing
RNAI, II/rep	115 nt	ColE2 and relatives	Replication control	Translation inhibition	
CopA/CopT	90 nt	R1 and other IncFII relatives	Replication control	Translation inhibition	Leader peptide, translation coupling
RNAIII/RNAII	136 nt	pIP501 and *Inc18* relatives	Replication control	Transcription attenuation	Unusually stable antisense RNA
RNAI,II/repC	84 nt, 141 nt	pT181	Replication control	Transcription attenuation	2 antisense RNAs
Inc/repZ	71 nt	Inc1α/IncB relatives	Replication control	Translation inhibition	Leader peptide
RNAII/rep	50 nt	pLS1	Replication control	Translation inhibition	
RNAI/rep	82 nt	pSK41	Replication control	Translation inhibition	
Incα/repC	54-57 nt	pTiR10 relatives	Replication control	Transcription attenuation[a]	
Incα/repC	67 nt	pRmeGR4	Replication control	mRNA stability[a] Transcriptional interference[a]	
Sok/hok	64 nt	R1	Segregational stability	Translation inhibition	Activity in absence of gene locus
RNAII/RNAI	64 nt	pAD1	Segregational stability	Translation inhibition	2 complementary regions involved in complex
FinP/traJ	79 nt	F, R1 and relatives	Conjugation control	Translation inhibition	FinO protein stabilizes duplex
Qa, mD/prgX		pCF10, pAD1	conjugation control	Transcription attenuation[a]	Pheromone represses Qa/mD
RNAα/fatA,B	650 nt	pJM1	Iron transport regulation	mRNA stability[a]	Regulation by chromosomal Fur protein, RNAα stabilized by iron
Transposon encoded RNAs		Transposon			
RNAout/RNAin		IS10/Tn10	Transposition	Translation inhibition	
RNA-C		IS30	Transposition	Inhibition of translation elongation[a]	
Phage encoded RNAs		Phage			
OOP/CII	77 nt	λ	Switch lysis/lysogeny	mRNA stability	
C4, CI/icd-ant	77 nt	P1, P4	Switch lysis/lysogeny	Transcription termination	
Sar/arc-ant	68 nt	P22	Switch lysis/lysogeny	Translation inhibition	
Sas/sieb-esc	105 nt	P22	Superinfection override	Switch of translation start site	
T_{ant}/T1	151 nt	ΦH	Switch lysis/lysogeny	RNA processing	Archaebacterial
Chromosomally encoded RNAs		Species			
p3 RNA/glnA	43 nt	*C. acetobutylicum*	Glutamine synthetase	Translation inhibition[a]	
Sof/gef		*E. coli*	Toxin/antitoxin system	Translation inhibition[a]	
Isf/sulA	350 nt	*E. coli*	SOS response?	?	
RdlD/ldrD	66 nt	*E. coli*/relatives	Toxin/antitoxin system	mRNA stability[a]	
GadY/gadXW	105 nt/90 nt/59 nt	*E. coli*	Acid response regulation	mRNA stabilization	3 GadY species of different length
RatA/TxpA	222 nt	*B. subtilis*	Toxin/antitoxin	mRNA degradation	75 nt overlap
IsrR/isiA	176 nt	*Synechocystis* species	Photosynthesis component	mRNA degradation[a]	Regulation by iron stress
RyjC/yjiW	77 nt	*E. coli*	?	?	SOS induced
RyjB/sgcA	90 nt	*E. coli*	Phosphotransferase II comp	?	
RyeA, B/pphA	100 nt, 275 nt	*E. coli*	Phosphatase I	?	

For plasmid-encoded RNAs, the antisense RNA of best studied example is given.

[a] Mechanism proposed but not experimentally substantiated.?, no mechanism proposed. References and details of mechanisms can be found in the text and in [2]. Sar, Sas, C4, T_{ant}, Sof and Isf are reviewed in [45]. Incα, RNAI (pSK41), RNAII/I (pAD1),Qa and mD are reviewed in [20]. Sok is reviewed in [19].

(summarized in [2]). Whereas antisense RNAs that regulate plasmid replication or post-segregational killing are mostly unstable with half-lives of ~1 min, transposon-encoded or a few phage-encoded antisense RNAs are stable. By contrast, the half-life of the chromosomally encoded RNAs can vary considerably ranging between 2 and >30 min [9]. For proper control, the inhibitor must be in excess over the target. This has been confirmed by calculations of the intracellular concentrations for RNAI/RNAII of ColE1 (1 μM versus 7 nM) [10] and RNAIII/RNAII of pIP501, (1–2 μM versus 50 nM) [11].

Plasmid-encoded antisense RNAs involved in the regulation of replication, conjugation or segregational stability, are expressed constitutively. By contrast, chromosomally encoded antisense RNAs have been found to

be expressed only under certain conditions, for example in stationary phase (e.g. GadY [12]) or under iron stress (e.g. IsrR [13•]).

Mechanisms of action

In the majority of cases, antisense RNA action entails post-transcriptional inhibition of target RNA function but, in a few cases, activating mechanisms have been found too. The currently known regulatory mechanisms employed by *cis*-encoded antisense RNAs are discussed below and summarized schematically in Figure 1.

Transcription attenuation

This mechanism was first discovered for the replication control of staphylococcal plasmid pT181 and later for the streptococcal plasmids pIP501 and pAMβ1 (reviewed in [2]). It has not been found in Gram-negative bacteria. The nascent *rep* mRNA can adopt two mutually exclusive conformations depending on the presence or absence of the antisense RNA. In its presence, a terminator stem-loop is induced in the nascent *rep* mRNA and, consequently, transcription is terminated prematurely upstream of the *rep* Shine–Dalgarno (SD) sequence preventing Rep protein synthesis, and hence, replication. In the absence of the antisense RNA, the *rep* RNA can refold by complementary base-pairing between two alternative sequences preventing terminator formation and allowing transcriptional read-through, Rep protein synthesis, and consequently, replication. The antisense RNA binds and exerts its inhibitory effect only during a short time window and without a helper protein. As the pIP501 antisense RNA is unusually long-lived [11], a second control element (repressor CopR) is needed for proper regulation (reviewed in [2]). The pT181 and pIP501 systems have been studied *in vitro* in terms of secondary structures, binding kinetics and sequence requirements for efficient inhibition [8,14–16].

Translation inhibition

The most trivial mechanism used by an antisense RNA is direct blocking of the ribosome binding site (RBS), and has been found in the control of plasmid replication and maintenance. In plasmid pLS1, RNAII, which is complementary to the *repB* RBS, directly inhibits ribosome loading. The same mechanism is used by the FinP antisense RNA that blocks the RBS of *traJ*, an activator of the plasmid F and R1 conjugal transfer operons. The FinO protein promotes the *traJ*–FinP duplex and prolongs FinP half-life by protecting it against RNase E (for a review and structural requirements, see [2,17]). Additionally, Sok and RNAI, encoded by plasmids by R1 and pAD1, respectively, and RNA-OUT, encoded by insertion element IS10, use translational inhibition as a control mechanism. In the former two cases, the synthesis of the killer toxin that kills plasmid-free cells is inhibited, in the latter one, the expression of transposase mRNA is regulated [2,18–20]. In the replication control systems of plasmid R1 and the IncB and IncIα plasmids, the antisense RNAs CopA and Inc inhibit translation of a leader peptide that is required — through translational coupling — for efficient Rep translation [2].

Inhibition of primer maturation

This mechanism has been only found for ColE1 and its related plasmids (summarized in [21]) that require a plasmid encoded replication primer that is synthesized as a 550 nt pre-primer (RNAII). For the formation of a persistent RNAII/DNA hybrid within the origin, RNAII must acquire specific secondary and tertiary structures which form during RNAII synthesis in a well-characterized series of events. Subsequently, the mature primer, which can be extended by DNA polymerase I, is generated through cleavage of the RNA strand of the RNAII/DNA hybrid by RNase H. The binding of the antisense RNA (RNAI), which must occur within a short time window, induces a change in the nascent primer, thereby preventing primer maturation. The kissing complex between RNAI and RNAII is stabilized by the plasmid-encoded Rom protein.

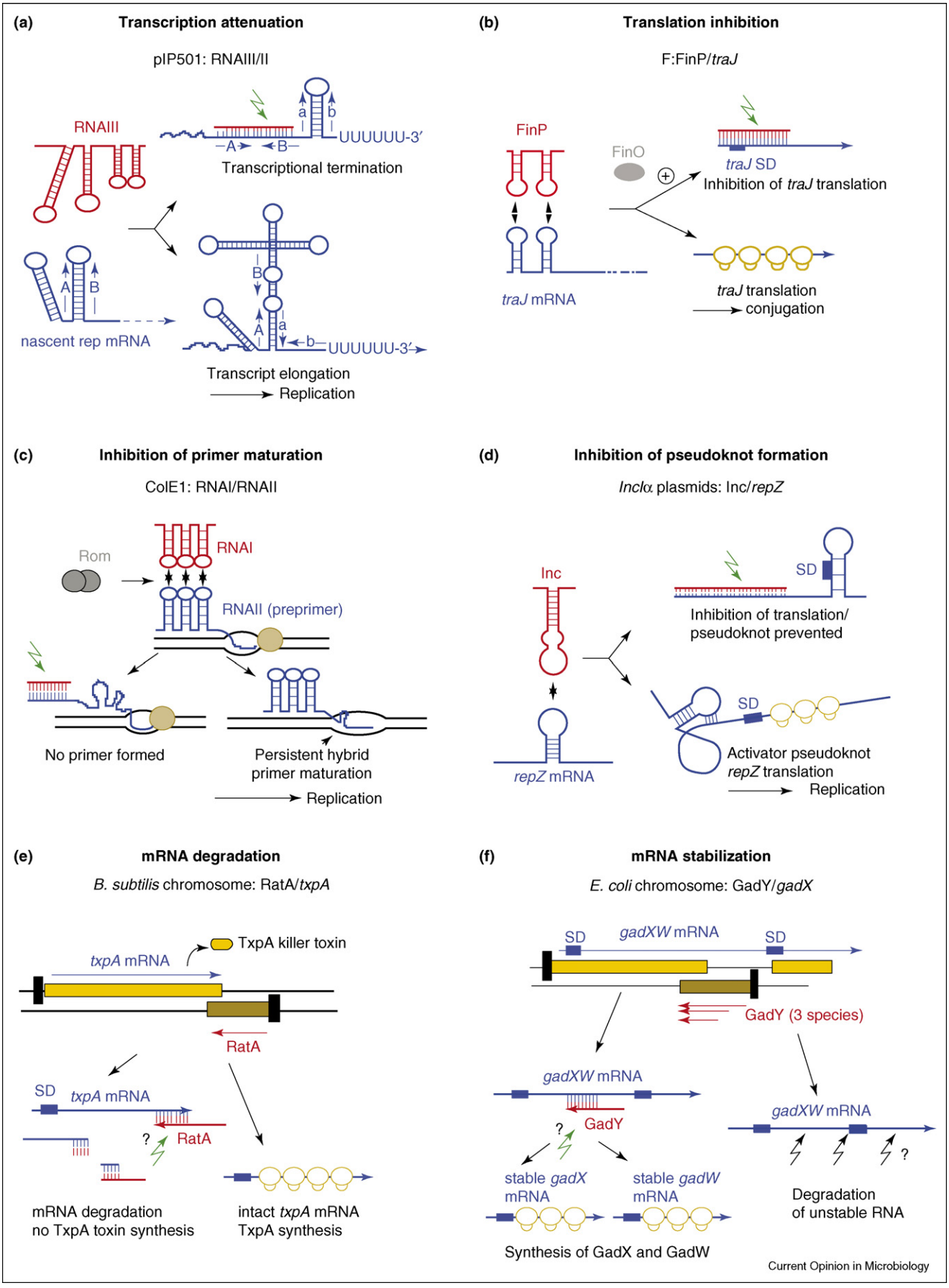
Prevention of formation of an activator RNA pseudoknot

Rep expression in IncB, IncIα, IncK, IncL and IncM plasmids involves a long-distance activator RNA pseudoknot. As in R1, a leader peptide ORF, *repY*, must be translated in order to enable RepZ synthesis to disrupt an inhibitory stem-loop at the *rep* RBS. This permits formation of a short helix between the target loop and disrupted stem, located 100 nt apart. This long-distance pseudoknot activates *repZ* translation. The corresponding antisense RNAs have a dual function: they block leader peptide translation and pseudoknot formation (see also [22,23]).

Promotion or inhibition of mRNA degradation

For many years, only a few antisense RNAs were known to influence mRNA stability, among them the λ-OOP RNA that facilitates RNase III dependent decay of the *cII* mRNA [24,25] and RNAα expressed from plasmid pJM1 that affects the stability of both *fatA* and *fatB*-mRNA in *Vibrio anguillarum* [26]. Interestingly, to date nearly all known *cis*-encoded antisense RNAs from the bacterial chromosome seem to affect either translation or mRNA stability, although the detailed mechanism has not yet been elucidated. The RdlD antitoxin RNA of *E. coli* was reported to exert its function post-transcriptionally, and to regulate the expression of the *ldrD* toxin gene [27]. It is still unclear, whether RdlD affects translation or stability of *ldrD* mRNA by base-pairing. Similarly, for the IsrR/*isiA* system involved in photosynthesis in *Synechocystis* sp. PCC 6803 [13•], IsrR-mediated *isiA*-mRNA degradation has been suggested, but not yet experimentally confirmed. By contrast, the experimental data for the RatA/TxpA antitoxin/toxin system from *Bacillus subtilis* support antisense RNA mediated mRNA degradation, although an

Figure 1


(a)
Transcription attenuation
pIP501: RNAIII/II
RNAIII
a
b
A
B
UUUUUU-3′
Transcriptional termination
nascent rep mRNA
Transcript elongation
Replication
(b)
Translation inhibition
F:FinP/traJ
FinP
FinO
traJ SD
Inhibition of traJ translation
traJ mRNA
traJ translation
conjugation
(c)
Inhibition of primer maturation
ColE1: RNAI/RNAII
Rom
RNAI
RNAII (preprimer)
No primer formed
Persistent hybrid
primer maturation
Replication
(d)
Inhibition of pseudoknot formation
Incα plasmids: Inc/repZ
Inc
SD
Inhibition of translation/
pseudoknot prevented
Activator pseudoknot
repZ translation
Replication
repZ mRNA
(e)
mRNA degradation
B. subtilis chromosome: RatA/txpA
TxpA killer toxin
txpA mRNA
RatA
SD
mRNA degradation
no TxpA toxin synthesis
intact txpA mRNA
TxpA synthesis
(f)
mRNA stabilization
E. coli chromosome: GadY/gadX
gadXW mRNA
GadY (3 species)
GadY
stable gadX
mRNA
stable gadW
mRNA
Degradation
of unstable RNA
Synthesis of GadX and GadW
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involvement of RNase III is still elusive: deletion of the *ratA* promoter and 5′ region led to a dramatic increase in *txpA*-mRNA levels, and a truncated 177 nt *txpA* RNA was detected in the presence of RatA that might result from RNaseIII-cleavage of the RatA/*txpA* duplex [28•].

So far, one exception is the *E. coli* GadY/*gadX* system, for which an mRNA stabilizing effect by the antisense RNA has been proposed [12]: GadY and *gadX* mRNA encoding a transcription factor involved in acid response overlap at their 3′ regions. A *gadY* overexpressing strain displayed a 20-fold increase in levels of *gadX*-mRNA, whereas a strain with a mutation in the *gadY* promoter showed 4.5-fold reduction in *gadX*-mRNA levels, and the 3′ UTR of *gadX* was required for this effect. The most recent data suggest that base-pairing between GadY and the *gadX* mRNA stimulates cleavage of a longer *gadXW* mRNA resulting in two products that are more stable than the full-length transcript (G Storz, personal communication).

Binding kinetics, binding pathway and requirement of RNase III

For many systems, antisense/sense RNA binding pathways have been studied in detail and binding kinetics measured. Analysis of pairing rate constants usually yielded values of $\sim 10^6\ M^{-1}\ s^{-1}$. The initial contact between antisense and sense RNA that often form complementary structures, can either occur between two complementary loops (many replication control systems, reviewed in [2]) or between a loop and a single-stranded region (e.g. RNA-IN/RNA-OUT, [29] and hok/Sok, [30]). In the first case, simple helix progression in both directions is topologically impossible because of accumulating torsional stress. Therefore, loop–loop initiating systems require a subsequent interaction at a distal site to circumvent this limitation. Independent of a one-step or multi-step pathway (Figure 2), the final result of the interaction is a complete duplex that is degraded by the double strand specific RNase III (shown for CopA/CopT, [31]; RNA-OUT/RNA-IN of IS10, [32] and hok/Sok, [33]). However, despite the fact that *cis*-encoded antisense RNAs are fully complementary to their targets, formation of complete duplexes is too slow to account for the observed biological effects. Instead, many antisense RNAs mediate inhibition by forming complexes that involve limited numbers of base-pairs with their targets. As has been shown for R1, ColE1, pIP501 and the IncB/Inc1α type plasmids, full duplex formation is not required for control (reviewed in [34]). Consequently in these cases, RNase III cleavage is not necessary for control, as has been experimentally confirmed for R1 [34] and IS10 [32]. For replication control of R1, a partially paired binding-intermediate is sufficient for inhibition *in vivo* [35]. This was supported by *in vitro* analyses of two transcription attenuation systems mediated by antisense RNA, pT181 [15] and pIP501. In pIP501, complexes between the complementary loop pairs of sense and antisense RNA form and progress into the stems, but the spacer between the two stem-loops remains single-stranded and is unimportant for inhibition [16].

For the replication control system of plasmid R1, the binding pathway between antisense RNA CopA and sense RNA CopT has been elucidated in detail (Figure 2a). Binding initiates with an unstable loop–loop interaction (kissing complex), that is converted into an extended kissing complex. Later, a single-stranded region is required to overcome the torsional stress created upon the unidirectional progression of this loop–loop interaction. Next, a partial duplex is formed that contains a four-helical junction [36,37]. This intermediate is transformed into a stable inhibitory complex that is a partial duplex and is slowly converted into a stable duplex, the substrate for RNase III. Apparently, this step-wise binding pathway is conserved, and the four-helix junction, although comprising different sequences, is not restricted to R1 and its relatives, but is a binding intermediate in the IncIα and other related plasmids as well [38].

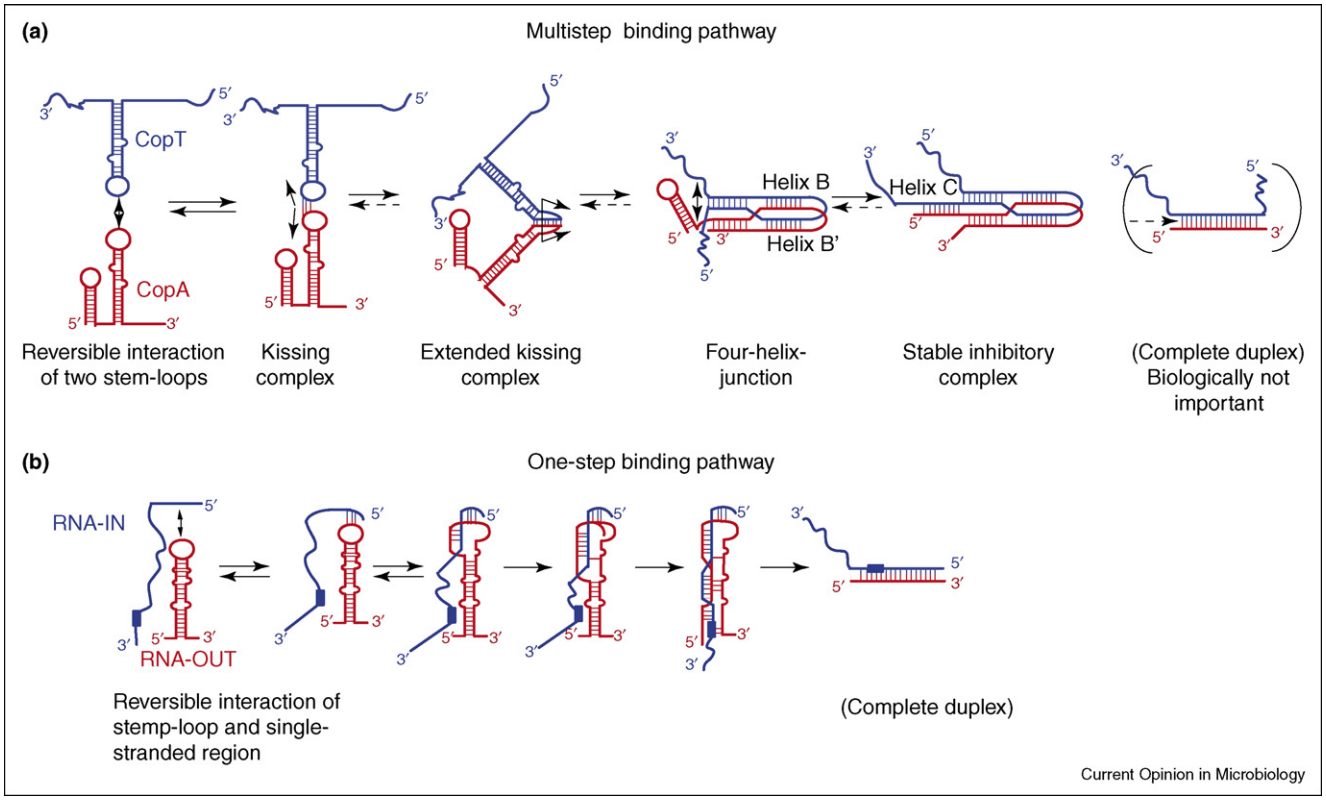
In contrast to R1 and IS10, in λ-OOP RNA, RNase III cleavage was found to be necessary for control [24,25], most probably, because the mechanism exerted by OOP RNA is mRNA degradation, whereas in the other systems steps preceding degradation are inhibited.

Differences between *cis*- and *trans*-encoded antisense RNAs

The advantage of *cis*-encoded RNAs is their complementarity with their targets over a large nucleotide stretch. Although in two cases, ColE1 and R1/F, plasmid-encoded RNA binding proteins (Rom and FinO, respectively) were shown to have an effect, *cis*-encoded RNAs usually do not require an additional protein to facilitate complex formation with their targets. Rom promotes RNAI/RNAII pairing only fivefold, because the inhibition rate is primarily determined by the binding rate constant and not the binding affinity between the loop–loop complexes. The FinO protein of F plasmid acts by promoting strand exchange between *traJ* and FinP [39], but its key function is to protect FinP against RNase E degradation [40], so that the repression effect of FinO ranges between 5–20-fold. By contrast, for R1 (CopA) and ColE1 (RNAI), replication control *in vivo* was found to be functional in an *E. coli* strain lacking the abundant chromosomally encoded RNA chaperone Hfq, although these two antisense RNAs bound

(Figure 1 Legend) Overview on regulatory mechanisms employed by cis-encoded antisense RNAs. Antisense RNAs are shown in red, sense RNAs in blue. Black rectangles denote promoters, yellow and brown boxes denote sense and antisense RNA genes. Green arrows indicate action of RNase III, black arrows, the putative action of other RNases. Details are described in the text. One example for each mechanism is given. '?' denotes not yet experimentally confirmed. The upper part of the figure is based on a figure from [2].

Figure 2



Binding pathways of antisense/sense RNAs. Antisense RNAs are shown in red, sense RNAs in blue. **(a)** The multistep binding pathway of the sense/antisense RNA system CopT/CopA regulating replication of plasmid R1 (based on [45]). **(b)** The one-step binding pathway of the sense/antisense RNA system RNA-IN/RNA-OUT regulating transposition of IS10 (based on [29]). The initial contact occurs between the first 3G's or C's at the 5′ end of RNA-IN and the complementary bases of RNA-OUT followed by an extension of stable pairing through the loop domain and the stem domain of RNA-OUT.

Hfq (G Wagner, personal communication). In RNAII/III of pIP501, Hfq is not expected to play a role *in vivo*, since the original host of this plasmid, *Streptococcus agalactiae*, does not encode *hfq*. For F plasmid, it was recently demonstrated that Hfq even destabilizes the sense RNA *traJ* by binding 5′ of the stem loops interacting with FinP, but does not bind FinP specifically [41].

By contrast, a large number of *trans*-encoded antisense RNAs need Hfq either to be protected against degradation or for complex formation [42], most probably in order to stabilize the partial duplexes formed with their target RNA(s) (e.g. MicA, [43]). In other cases, the role of Hfq binding to the regulatory RNA is still elusive (e.g. SR1 from *B. subtilis*, [44]) and it is not clear, whether in Gram-positive bacteria another protein fulfils the role of Hfq.

Concluding remarks

Although a multitude of *cis*-encoded base-pairing RNAs is known to date, only a few of them are encoded in the bacterial chromosome. The best-studied examples are still the plasmid-encoded antisense RNAs that use a variety of regulatory mechanisms. It is tempting to speculate that *cis*-encoded RNAs from bacterial chromosomes that will be discovered and investigated in the future might use not only some of the already known mechanisms of action but also new, unexpected ones. Furthermore, it cannot be excluded that some of these RNAs can act both in *cis* and in *trans* on different targets, thereby blurring the border between the two types of regulatory RNAs.

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