Antisense RNA-mediated transcriptional attenuation in plasmid pIP501: the simultaneous interaction between two complementary loop pairs is required for efficient inhibition by the antisense RNA

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Streptococcal plasmid pIP501 uses antisense RNA-mediated transcriptional attenuation to regulate its replication. Previous in vitro assays suggested that binding intermediates between RNAII (sense RNA) and RNAIII (antisense RNA) are sufficient for inhibition, and a U-turn structure on RNAII loop L1 was found to be crucial for the interaction with RNAIII. Here, sequence and structural requirements for an efficient RNAII–RNAIII interaction were investigated. A detailed probing of RNA secondary structure combined with in vitro single-round transcription assays indicated that complex formation between the two molecules progresses into the lower stems of both loop pairs of the sense and antisense RNAs, but that the complex between RNAII and RNAIII is not a full duplex. Stem-loops L3 and L4 were required to be linked to one other for efficient contact with the complementary loops L2 and L1 of the sense RNA, indicating a simultaneous interaction between these two loop pairs. Thereby, the sequence and length of the spacer connecting L3 and L4 were shown not to be important for inhibition.

INTRODUCTION

Antisense RNA-mediated gene regulation has been found and studied in prokaryotic accessory DNA elements such as plasmids, phages and transposons, and a broad variety of regulatory mechanisms has been observed (reviewed by Brantl, 2004). During the past 5 years, a growing number of recently identified chromosomally encoded small RNAs have been included in such studies (e.g. Zhang et al., 2002; Rasmussen et al., 2005; Udekwu et al., 2005). Independently of whether binding initiates by loop–loop contacts (plasmid copy number control systems) or linear region–loop contacts (e.g. hok/sok of plasmid R1; Thisted et al., 1994), a rapid interaction between antisense and target RNA has been shown to be crucial for regulation (reviewed by Wagner et al., 2002). Structural requirements for efficient antisense RNAs have been defined (Hjalt & Wagner, 1992, 1995) and pairing rate constants for sense/antisense RNA pairs calculated to be mainly in the range of $10^6$ M$^{-1}$ s$^{-1}$.

Only for a few plasmid-encoded antisense RNA systems has a detailed biochemical analysis been performed to investigate the structural and sequence requirements for inhibition (e.g. Asano & Mizobuchi, 2000; Greenfield et al., 2001). For CopA (antisense RNA) of plasmid R1, the multistep pathway of interaction with its sense RNA (CopT) has been elucidated in detail, and a four-helix junction has been identified as the inhibitory intermediate (Kolb et al., 2000a, b). A subsequent study on Inc RNA/repZ mRNA of ColIb-P9 suggests that similar pathways are used to form inhibitory antisense–target RNA complexes (Kolb et al., 2001). Apparently, in many cases, kissing is sufficient for inhibition, and inhibitory intermediates as in R1 are only slowly converted into full duplexes (Wagner & Brantl, 1998; Malmgren et al., 1997).

Streptococcal plasmid pIP501 exerts its replication control by the concerted action of a small antisense RNA (RNAIII, 136 nt) and a transcriptional repressor, CopR (Brantl & Behnekhe, 1992). The deletion of either control component causes a 10- to 20-fold increase in plasmid copy number; a simultaneous deletion has, however, no additive effect. RNAIII functions by transcriptional attenuation of the repR mRNA (RNAII) that encodes the rate-limiting replication initiator protein (Brantl et al., 1993). CopR acts as a transcriptional repressor at the essential repR promoter (Brantl, 1994). Additionally, it has a second function: since RNAIII, with an half-life of ~30 min, is unusually stable (Brantl & Wagner, 1996), it would not be able to correct downward fluctuations in copy number. Therefore, CopR is
required to prevent convergent transcription from the sense promoter pII and the antisense promoter pIII, thereby indirectly increasing the amount of RNAIII (Brantl, 1994; Brantl & Wagner, 1997). When copy number decreases, decreased CopR synthesis will derepress pII. This results in increased transcription of RNAII and convergent transcription, which decreases transcription of RNAIII. Both effects increase RepR synthesis and, consequently, the replication frequency. Fig. 4(A) presents a model of replication control of pIP501.

In vitro assays show that RNAIII-mediated inhibition occurs faster than complete duplex formation, suggesting that binding intermediates between RNAII and RNAIII are sufficient for inhibition (Brantl & Wagner, 1994). Furthermore, the deletion of stem–loops L1 and L2 at the 5′ end of RNAIII has no effect on the inhibitory function of RNAIII in vivo (Brantl et al., 1993), whereas mutations in loop L3 of RNAIII lead to new incompatibility groups (Brantl & Wagner, 1996), indicating that L3 is the recognition loop. However, since a U-turn structure on loop L1 of RNAII complementary to L4 of RNAIII proves to be important for an efficient interaction with RNAIII, we have suggested that L3 and L4 are of equal importance for the initial contact (Heidrich & Brantl, 2003).

Here, we investigate the sequence and structural requirements for an efficient RNAII–RNAIII interaction of plasmid pIP501 by a combination of secondary-structure probing and attenuation assays with wild-type and mutated RNAIII species, as well as single stem–loops. Our results demonstrate that helix formation progresses into the lower parts of stems L3 and L4, whereas the 6 nt spacer separating them remains unpaired. The sequence and length of this spacer are not important for efficient inhibition, and the exclusive function of the spacer is to present both stem–loops simultaneously for interaction with the complementary loop pair L1/L2 of RNAII.

METHODS

Enzymes and chemicals. Chemicals used were of the highest purity available. T7 RNA polymerase and T4 polynucleotide kinase were purchased from NEB, Firepol Taq polymerase from Solis BioDyne, and Thermoscript reverse transcriptase from Invitrogen. Bacillus subtilis RNA polymerase was prepared by J. M. Sogo, Universidad Autónoma de Madrid. 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate (CMCT) and lead acetate from Merck, and DMSO from Fluka, were used for the chemical probing.

In vitro transcription. In vitro transcription experiments were performed as described previously (Brantl & Wagner, 1996; Heidrich & Brantl, 2003). Templates for in vitro transcription of mutated RNAIII species were generated by PCR on plasmid pPR1 as template (Brantl & Behnke, 1992), with oligonucleotide SB1 (Brantl & Wagner, 1994) and one of the following mutagenic oligodeoxynucleotides:

- SB547: 5′ TTA ATT GAT TGG TGA TCA ATT AAC CGA TAC AGT TAA AGT TTC TCA GGC TGT AAT AAC TGG TCG TGG CTC TT 3′
- SB548: 5′ TTA ATT GAT TGG TGG TAA TCA ATT AAG CGG AGT TAA AGT TTC TCA GGC TGT AAT AAC TGG TCG TGG CTC TT 3′
- SB 570: 5′ TTA ATT GAT TGG TGG TAA TCA ATT AAC AGT TAA AGT TTC TCA GGC TGT AAT AAC TGG TCG TGG CTC TT 3′
- SB571: 5′ TTA ATT GAT TGG TGG TAA TCA ATT AAG CGT CCA CAC GGC AGT TAA AGT TTC TCA GGC TT TTT AAC TGG TCG TGG CTC TT 3′
- SB619: 5′ AAT ATT GAT TGG TGG TAA TCA ATT AAG CGG AGT TAA AGT TTC TCA GGC TGT AAT AAC TGG TCG TGG CTC TT 3′
- SB620: 5′ AAT TTT GAT TGG TGG TAA TCA AAA TTG GCT CGG TCA TAA AGT TTC TCA GGC TT TTT AAG AGC ACG TGG TGG CTC TT 3′
- SB645: 5′ AAT TTT GAT TGG TGG TAA TCA AAA TTG GCT CGG AGT TAA AGT TTC TCA GGC TT TTT AAG AGC ACG TGG TGG CTC TT 3′
- SB619: 5′ AAT ATT GAT TGG TGG TAA TCA ATT AAG CGG AGT TAA AGT TTC TCA GGC TGT AAT AAC TGG TCG TGG CTC TT 3′
- SB645: 5′ AAT TTT GAT TGG TGG TAA TCA AAA TTG GCT CGG AGT TAA AGT TTC TCA GGC TT TTT AAG AGC ACG TGG TGG CTC TT 3′
- SB 576: 5′ AAT TTT GAT TGG TGG TAA TCA AAA TTG GCT CGG TCA TAA AGT TTC TCA GGC TT TTT AAG AGC ACG TGG TGG CTC TT 3′

The template for RNAIIIp2 was generated as described previously (Brantl & Wagner, 1994).

The template fragment for RNAII complementary to SB645 was generated by a two-step PCR on pPR1 as template, with outer primers SB6 and SB7 (Brantl & Wagner, 1994) and the following mutagenic oligonucleotides as inner primers:

- SB646: 5′ TTT AAC TGC GAG CCA ATT TTT GCT AGA CCC 3′
- SB647: 5′ GGG TCT CCA ATT TTT GCT AAG TTT GCT ACC AAT CAA AAT TAG ACG TGA AGA CCA 3′
- SB649: 5′ CGG TCT CCA ATT TTT GCT AAG TTT GCT ACC AAT CAA AAT TAG ACG TGA AGA CCA 3′
- SB650: 5′ GGG TCT CCA ATT TTT GCT AAG TTT GCT ACC AAT CAA AAT TAG ACG TGA AGA CCA 3′
- SB651: 5′ GGG TCT CCA ATT TTT GCT AAG TTT GCT ACC AAT CAA AAT TAG ACG TGA AGA CCA 3′

Secondary structure analysis. Secondary-structure probing with chemical probes (Pb²⁺, CMCT and DMSO) using 2 pmol of unlabelled RNAIII in a total volume of 20 μl was carried out according to Brunel & Romby, 2000, as follows. Reaction buffers of the following final concentrations were used: for CMCT, 25 mM borate-NaOH, pH 8.0, 5 mM magnesium acetate, 75 mM potassium acetate, 5 mM β-mercaptoethanol; for DMSO, 25 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 75 mM KCl, 5 mM β-mercaptoethanol; for Pb²⁺, 25 mM Tris-acetate, pH 7.5, 5 mM magnesium acetate, 25 mM sodium acetate. Denaturing buffers were: for CMCT, 25 mM borate-NaOH, 1 mM EDTA; for DMSO, 25 mM Tris/HCl, pH 7.5, 1 mM EDTA. RNA-removal buffers for the CMCT and DMSO reactions contained 10 mM Tris/HCl, pH 7.5, 1.5 mM EDTA and 0.1 % SDS. A subsequent reverse-transcription (RT) reaction was used to visualize the products. Primer hybridization was done in a total volume of 12 μl containing 9 μl of the modified RNA, 1 μl with 100,000 c.p.m. of 5′-[³²P]-labelled oligonucleotide SB2 (Hartmann Analytic) (Brantl & Wagner, 1994) and 2 μl 10 mM dNTPs for 5 min at 65 °C, followed by RT with ThermoScript reverse transcriptase (2 U, Invitrogen) in 20 μl for 45 min at 55 °C. Partial digestions of in vitro-synthesized, unlabelled RNAIII and RNAII species with ribonucleases T1, T2 and V were performed in the same way as described previously for 5′ end-labelled species (Heidrich & Brantl, 2003), except that digestions were followed by an RT reaction with 5′ end-labelled primer SB2 (see above). All reaction products were subjected to electrophoresis in 8% denaturing polyacrylamide gels.
Single-round transcription assays and calculation of inhibition rate constant $k_{\text{inhib}}$. Single-round transcription assays were performed as described previously (Brantl & Wagner, 1994), using PCR-generated 500 bp DNA fragments of pPR1 (comprising promoters pII and pIII, the attenuator and 100 bp downstream) as templates and *B. subtilis* RNA polymerase prepared by J. M. Sogo. The protocol of the attenuation experiments was as described previously (Brantl & Wagner, 1994), with one alteration: the concentration of the unlabelled RNAIII species included was determined by UV spectrophotometry. The inhibition rate constants were calculated as described previously (Brantl & Wagner, 1994).

RESULTS AND DISCUSSION

Pairing between RNAII and RNAIII does not yield a full duplex

Previously, the secondary structures of RNAIII and RNAII were probed with RNases T1 (single-stranded Gs), T2 (single-stranded region with a slight preference for As) and V (double-stranded and stacked regions) (Brantl & Wagner, 1994). To obtain more detailed information about the 3’
single-stranded region 5’ of stem–loop L3 were corroborated by a combination of enzymic and chemical probing.

Since Pb²⁺ is a sensitive probe for single-stranded sequences, it was used to probe the structure of a complex between the 5’ 184 nt of RNAII (containing the target for RNAIII) and 5’-labelled full-length RNAIII₆₁₆. For comparison, unpaired RNAIII was probed with T₁, T₂, V and Pb²⁺. As shown in Fig. 2, cleavage positions indicated that the RNAII–RNAIII complex is not fully base-paired in the single-stranded region between L₃ and L₄ of RNAIII. Four significant Pb²⁺ cuts within the large single-stranded region 5’ of L₃ that were not reduced upon pairing with RNAII suggested that at least part of this region also remained single stranded. By contrast, the loops were found to be almost completely paired, with the exception of the 3’ outermost U of L₃. Interestingly, 2 nt of the 5’ half of stem L₄ became single stranded (asterisks in Fig. 2). Lead cleavage cannot be used to assess whether the stems of L₃/L₂ and L₄/L₁ are engaged in intramolecular or intermolecular helices.

To answer this question and to evaluate the role of the spacer region between L₃ and L₄, single-round transcription experiments were performed to determine the inhibition rate constants of mutated antisense RNAs with either wild-type or complementary sense RNAs. In all these experiments, RNAIII₇₂, consisting only of stem–loops L₃ and L₄ with their 6 bp spacer region, was used as a ‘wild-type’ species, since previous experiments had shown that inhibition does not require stem–loops L₁ and L₂ and the large single-stranded region (Brantl & Wagner, 1994).

The stems are involved in the formation of intermolecular helices

In RNAIII of pIP501, the stems of L₃ and L₄ consist of only 10 and 9 bp, respectively, and are not interrupted by bulges that, in longer helices, protect the antisense RNAs against degradation by RNase III and are required for efficient strand opening (Hjalt & Wagner, 1995). To find out whether pairing is restricted to the 6 and 9 nt loops L₃ and L₄ with their 6 bp spacer region, was used as a ‘wild-type’ species, since previous experiments had shown that inhibition does not require stem–loops L₁ and L₂ and the large single-stranded region (Brantl & Wagner, 1994).

stem–loops L₃ and L₄ and the spacer in between them, additional structure-probing experiments were performed with CMCT (Us), Pb²⁺ (single-stranded regions) and DMSO (Cs and As), as described in Methods. The results are shown in Fig. 1. Four cuts for Pb²⁺ and one cut each for DMSO and CMCT within the spacer region, as well as three cuts for V in the lower stem of L₃, confirmed that the spacer between L₃ and L₄ is indeed 6 nt long. Furthermore, the sizes of L₃ with 6 nt and of L₄ with 9 nt as well as that of the large

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through the top portion of the stems to a stable duplex (Gubbins et al., 2003). By contrast, in the inhibitory intermediate of CopA and CopT of plasmid R1, only the upper parts of the stems of the decisive 3′ CopA stem–loop pair containing a bulge region are involved in intermolecular helices, whereas the lower parts remain paired intramolecularly (Kolb et al., 2000a).

**The sequence and length of the spacer between L4 and L3 do not affect the inhibitory function of RNAIII**

In sense–antisense systems containing two complementary loop pairs, the length of the spacer between the two stem–loops is found to be different (for examples, see Brantl, 2004). To analyse the influence of the length and sequence of the spacer between L3 and L4, mutated RNAIII species with varying spacer lengths and with a heterologous spacer were investigated in the attenuation assay. As shown in Fig. 3(B), RNAIII547, which contains a heterologous spacer, was slightly more inhibitory than the wild-type, indicating that the sequence of the spacer is not important. This corresponds well with the results of the Pb++-based secondary-structure probing of the RNAII–RNAIII complex, in which this spacer was still lead sensitive, i.e. single stranded, although the loops were almost completely paired (Fig. 2). Both RNAIII548 with a 3 nt spacer and RNAIII571 with a 12 nt spacer were as efficient in inhibition through the top portion of the stems to a stable duplex (Gubbins et al., 2003). By contrast, in the inhibitory intermediate of CopA and CopT of plasmid R1, only the upper parts of the stems of the decisive 3′ CopA stem–loop pair containing a bulge region are involved in intermolecular helices, whereas the lower parts remain paired intramolecularly (Kolb et al., 2000a).

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**The sequence and length of the spacer between L4 and L3 do not affect the inhibitory function of RNAIII**

In sense–antisense systems containing two complementary loop pairs, the length of the spacer between the two
as the wild-type with a 6 nt spacer, suggesting that the spacer length can be varied. Interestingly, RNAIII$_{570}$, which does not contain a spacer between L3 and L4, exhibited an increase of 1.4-fold in $k_{\text{inhib}}$ compared with the wild-type. These results prove unequivocally that neither the sequence nor the length of the spacer between L3 and L4 influences the inhibitory function of RNAIII.

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Characteristics</th>
<th>Inhibition rate constant $k_{\text{inhib}}$ (M$^{-1}$ s$^{-1}$)</th>
<th>Relative $k_{\text{inhib}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA619</td>
<td>Heterologous 3 bp stem base in L3/L4</td>
<td>$0.75 \times 10^6$</td>
<td>0.5</td>
</tr>
<tr>
<td>RNA620</td>
<td>Heterologous 4 bp stem base in L3/L4</td>
<td>$0.4 \times 10^6$</td>
<td>0.25</td>
</tr>
<tr>
<td>RNA645</td>
<td>Heterologous 4 bp stem base in L3 wild-type template</td>
<td>$0.37 \times 10^6$</td>
<td>0.23</td>
</tr>
<tr>
<td>RNA645</td>
<td>Heterologous 4 bp stem base in L3 complementary template</td>
<td>$0.6 \times 10^6$</td>
<td>0.38</td>
</tr>
<tr>
<td>RNA547</td>
<td>Heterologous 6 nt spacer</td>
<td>$1.9 \times 10^6$</td>
<td>1.2</td>
</tr>
<tr>
<td>RNA548</td>
<td>3 nt spacer</td>
<td>$2.0 \times 10^6$</td>
<td>1.25</td>
</tr>
<tr>
<td>RNA570</td>
<td>No spacer between L3 and L4</td>
<td>$2.2 \times 10^6$</td>
<td>1.4</td>
</tr>
<tr>
<td>RNA571</td>
<td>12 nt spacer</td>
<td>$1.8 \times 10^6$</td>
<td>1.1</td>
</tr>
<tr>
<td>RNAIII$_{72}$</td>
<td>Wild-type L3 and L4, 6 bp spacer</td>
<td>$1.6 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td>Stem–loop L3</td>
<td>Synthetic RNA oligonucleotide</td>
<td>$1.4 \times 10^5$</td>
<td>0.1</td>
</tr>
<tr>
<td>Stem–loop L4</td>
<td>Synthetic RNA oligonucleotide</td>
<td>$3.0 \times 10^5$</td>
<td>0.2</td>
</tr>
<tr>
<td>Stem–loops L3 + L4</td>
<td>Mixed 1 : 1</td>
<td>$1.25 \times 10^5$</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 1. Inhibition rate constants of wild-type and mutated RNAIII species

Values represent the means of at least three independent determinations. The inhibition rate constant for RNA$_{72}$ is the mean of 33 independent determinations.

A simultaneous interaction between both loop pairs of RNAII and RNAIII is required for inhibition

Based on the results with the spacer mutants, we asked whether a mixture of the unlinked stem–loops L3 and L4 is efficient in inhibition. For this purpose, synthetic RNA

![Fig. 4. Model for the interaction between RNAIII and RNAII in the context of replication control of plasmid pIP501. (A) Working model of copy number control of plasmid pIP501 in B. subtilis. Black boxes, promoters; rectangles, ORFs; thick arrows, RNAs; grey/stippled, proteins; oriR, replication origin; stem–loop/ATT, transcriptional attenuator (rho-independent terminator); +, activation; −, repression/inhibition. (B) Putative binding pathway of RNAII and RNAIII of pIP501. A putative RNAII–RNAIII binding pathway was derived from the experimental data presented in Figs 1–3. First, loop pairs L1 (U-turn motif highlighted in black)/L4 and L2/L3 interact simultaneously. Subsequently, basepairing is extended into the lower stem regions, and the large spacer region of RNAIII comes into contact with the complementary region in RNAII. Finally, only the spacer separating L3 and L4 in RNAIII and L1 and L2 in RNAII, as well as the 5' 4 nt of the large single-stranded region 5' of L3, remain single-stranded.](http://mic.sgmjournals.org)
oligonucleotides containing either L3 or L4 were used. First, each stem–loop was investigated separately in the attenuation assay (Fig. 3C). As expected, L3 and L4 alone were 10-fold and fivefold less efficient, respectively, than RNAIII72. This is in good correlation with the previous result, whereby a less than 0.1-fold inhibitory activity was found for RNAIII72 containing only the large single-stranded region and L3 (Brantl & Wagner, 1994). Surprisingly, a 1 : 1 mixture of L3 and L4 was as inefficient in inhibition as L3 alone. Therefore, we can conclude that L3 and L4 have to be attached to one other to ensure efficient inhibition. Apparently, this function is provided by the 6 nt spacer in wild-type RNAIII, which acts as a scaffold for both stem–loops. Consequently, a simultaneous interaction between the two loop pairs of RNAIII and RNAII is required for transcriptional attenuation to occur. This finding is in accordance with our previous result that L4, which is complementary to U-turn loop L1 of RNAII, is important for an efficient contact with the sense RNA (Heidrich & Brantl, 2003). Fig. 4(B) relates the results of this study to the working model of copy-number control. A requirement for both stem–loops of the antisense RNA (RNAI) for efficient complex formation with the sense RNA, repC mRNA, was also found in an in vitro study of the transcription attenuation system of staphylococcal plasmid pT181. Here, two antisense RNAs, RNAI54 (stem loops L1 and L2 connected by an 8 nt spacer) and RNAI146 (stem–loops L1 to L 4) are expressed in vivo that bound equally well to repC mRNA. However, upon deletion of either L1 or L2 or the 3’ part of L2, pairing was reduced 10- to 100-fold (Brantl & Wagner, 2000). By contrast, in plasmid R1, the initial contact with the target RNA is made by the 3’ stem–loop of the antisense RNA CopA, and the 5’ stem–loop is only involved in later pairing intermediates (see Kolb et al., 2000a).

The recently found chromosomally encoded bona fide antisense regulators belong mainly to the trans-encoded RNAs that are only partially complementary to their targets. However, searches for cis-encoded antisense RNAs from different bacterial genomes are under way, and it remains to be seen how new results for such RNAs will expand our knowledge of RNA–RNA interactions involved in prokaryotic gene regulation.

ACKNOWLEDGEMENTS

We acknowledge E. Birch-Hirschfeld (Institute for Virology, Jena) for synthesizing various oligodeoxynucleotides, and Margarita Salas and J. M. Sogo, Universidad Autónoma de Madrid, for providing us with purified B. subtilis RNA polymerase. This work was supported by grant BR 1592/4-4 from the Deutsche Forschungsgemeinschaft (to S.B.).

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Simultaneous interaction of complementary RNA loop pairs


Edited by: L. S. Frost