Antisense RNA-mediated transcriptional attenuation: an in vitro study of plasmid pT181

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Summary
Antisense RNAs regulate plasmid replication by several different mechanisms. One of these mechanisms, transcriptional attenuation, was first described for the staphylococcal plasmid pT181, and later for the streptococcal plasmids pIP501 and pAMβ1. Previously, we performed detailed in vitro and in vivo analyses of the pIP501 system. Here, we present an in vitro analysis of the antisense system of plasmid pT181. The secondary structures of antisense and sense RNA species of different lengths were determined. Binding rate constants for sense/antisense RNA pairs were measured, and functional segments required for complex formation were determined. A single-round transcription assay was used for in vitro analysis of transcriptional attenuation. A comparison between pT181 and pIP501 revealed several differences; whereas a truncated derivative of pIP501 antisense RNA was sufficient for stable complex formation, both stem–loop structures of pT181-RNAI were required. In contrast to the sense RNA of pIP501, which showed an intrinsic propensity to terminate (30–50% in the absence of antisense RNA), the sense RNA of pT181 required antisense RNA for induced termination. Rate constants of formation of pT181 sense–antisense RNA complexes were similar to inhibition rate constants, in striking contrast to pIP501, in which inhibition occurred at least 10-fold faster than stable binding.

Introduction
Antisense RNA-mediated control of gene expression is ubiquitous in nature but has been best characterized in prokaryotic accessory DNA elements: phages, plasmids and transposons (Wagner and Simons, 1994; Zeiler and Simons, 1998). Antisense RNAs are the principal copy number control elements of many plasmids. These inhibitors are constitutively transcribed and metabolically unstable (exception pIP501; Brantl and Wagner, 1996). Antisense RNAs bind and inhibit target RNAs to affect the replication frequency of the plasmid, by negative feedback. The mechanisms by which this occurs differ in detail, e.g. inhibition of primer formation (in ColE1-related plasmids; Tomizawa, 1986), inhibition of Rep protein translation (IncFII-related plasmids; Blomberg et al., 1992; Wu et al., 1992; and pLS1, del Solar et al., 1997), inhibition of formation of an activator RNA pseudoknot required for Rep protein synthesis (IncB/Incα plasmids; Asano et al., 1991; Wilson et al., 1993) or induction of premature termination, attenuation, of a rep mRNA (pT181, pIP501, pAMβ1; Novick et al., 1989; Brantl et al., 1993; Le Chatelier et al., 1996). In all these mechanisms control is kinetic; the rate of antisense RNA binding determines the fraction of the target RNA that becomes functionally inactivated. Consequently, emphasis has been placed on in vitro studies of antisense–target RNA interaction. Binding rate constants for these pairs were shown to be remarkably similar, usually in the range of 10⁶ M⁻¹ s⁻¹ (ColE1, Tomizawa, 1984; R1, Persson et al., 1988; Incα, Asano et al., 1998; IncB, Siemering et al., 1994; ColE2, Sugiyama and Itoh, 1993; pIP501, Brantl and Wagner, 1994). This indicates a similar rate-limiting step in the binding process, in spite of great differences with respect to sequences and structures of antisense and target RNAs, as well as of the details of the binding pathways used.

A particular property of antisense RNA regulation in bacteria reflects the need for rapid binding; antisense RNAs will most often interact with a 'moving target'. This is most pronounced in the plasmid cases in which interaction with a target structure has to occur within a short time window to result in inhibition. In ColE1, RNAI is able to bind to RNAII (target RNA) species of different lengths but fails to inhibit when the nascent target RNA exceeds 360 nucleotides (Tomizawa, 1986). The same applies to transcriptional attenuation systems. Here, the nascent target RNA can fold into two mutually exclusive structures. The formation of one of these permits read-through of the attenuator sequence; the other one forms a Rho-independent terminator. Productive interaction with an antisense RNA forces the nascent target RNA into the
termination-proficient folding pathway (Novick et al., 1989; Brantl and Wagner, 1994), i.e. the regulatory decision must occur after the correct target structure has been formed but before the terminator sequence is encountered.

From comparisons of several antisense RNA systems, it was concluded that full antisense–target RNA duplex formation most often is not required for control (reviewed in Wagner and Brantl, 1998). Regulation of pIP501 replication is such a case. We showed previously that inhibition occurred at least 10 times more rapidly than stable RNAIII–RNAII complex formation (Brantl and Wagner, 1994). Hence, steps preceding stable complex formation are sufficient to trigger inhibition.

Plasmid pT181 replicates via a rolling circle mechanism and is the best-characterized representative of a family of five staphylococcal plasmids (Projan and Novick, 1988). The pioneering work of the Novick group demonstrated that pT181 regulates its copy number by a novel mechanism, transcriptional attenuation (Novick et al., 1989). The synthesis of the replication rate-limiting RepC protein is controlled post-transcriptionally by two antisense RNAs, RNAI ($\approx 85$ nt) and RNAII ($\approx 150$ nt) (Carleton et al., 1984; Kumar and Novick, 1985). Genetic analyses had indicated that the antisense RNAs, by interacting with the nascent repC-mRNA, induce transcriptional termination at an inverted repeat structure located upstream of the repC reading frame (Novick et al., 1989); for a schematic representation of the attenuation mechanism see Fig. 1.

Results

Secondary structures of the antisense RNAs, RNAI and RNAII and three species of the sense RNA, repC-mRNA

Experimentally determined secondary structures of antisense RNAs from Gram-positive bacteria have so far been reported only for pIP501 (RNAII; Brantl and Wagner, 1994) and showed some deviations from computer-predicted structures, as also found in many other cases (unpublished). Because for the antisense and sense RNAs of pT181 only computer-generated RNA structures were available (Novick et al., 1989), we performed limited digestions with structure-specific ribonucleases in vitro. The antisense RNAs, RNAI (84 nt) and RNAII (146 nt), and repC-mRNA species of different lengths (112 nt, 132 nt and 184 nt) were 5'-end labelled, gel purified and treated with RNases T1 (cleaves 3' of unpaired G residues), T2...
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(unpaired nucleotides with a slight preference for A residues), V1 (double-stranded or stacked regions), A (unpaired U and C residues), or U2 (unpaired A residues). Figure 2A shows such analyses of RNAI and RNAII, and Fig. 3A of the repC mRNAs. Schematic representations of structures consistent with the cleavage data are presented in Figs 2B and 3B–D. The experimentally determined structure for RNAI deviates from the predicted structure mainly in that the putative recognition loop L2 carries only 6 nt, i.e. is much smaller than predicted (16 nt). This is strongly supported by G residues at positions 67, 68 and 69 being refractory to cleavage by T1 and by the inaccessibility of A residues 55–57 to RNases A, T2 or U2. Because the same conclusion was supported when 3′-end-labelled RNAI was used, we propose that the upper stem region (Fig. 2B) carries two non-canonical A-G basepairs.

RNAII (146 nt), whose 5′-proximal sequence is identical

Fig. 2. Secondary structures of antisense RNAs.
A. Secondary structure probing of RNAI and RNAII with RNases. Purified, 5′-end-labelled RNAI or RNAII was subjected to limited cleavage with the RNases indicated. The digested RNAs were separated on 8% denaturing gels. Autoradiograms are shown. RNase concentrations used were: 7.2 \times 10^{-3} \text{ U ml}^{-1} (V1); 0.34 \text{ \mu g ml}^{-1} (A); 10^{-4} \text{ U ml}^{-1} (T1); 2.1 \times 10^{-2} \text{ U ml}^{-1} (T2); 2.0 \text{ U ml}^{-1} (U2). L, alkaline ladder; M, pBR322 MspI size marker; C, incubation control.
B. Proposed secondary structure of RNAI. A structure consistent with the cleavage data in Fig. 2A and additional experiments (data not shown) is depicted. Major and minor cuts are indicated by symbols (see box).
C. Proposed secondary structure of RNAII. Proposed structure of RNAII, as in B.
Fig. 3. Secondary structures of target RNAs.
A. Secondary structure probing of the repC-RNAs of different lengths with RNases. Purified, 5'-end-labelled repC-RNAs were subjected to limited cleavage with RNases as indicated and separated on 8% denaturing gels. Autoradiograms are shown. RNase concentrations used were as in Fig. 1. L, alkaline ladder; M, pBR322 MspI size marker; C, incubation control. Important nucleotide positions are indicated on the right margins of the corresponding autoradiograms and, in the case of repC-RNA184, the positions of the pBR322 MspI-marker bands are denoted.
B, C and D. Proposed secondary structures of repC-RNA112, RNA132, and RNA184, respectively, based on the cleavage data in Fig. 2A and additional experiments (data not shown). Symbols are as in Fig. 1. The horizontal line (in B) indicates T2 cleavages throughout this region. The locations of the two target loop sequences and the terminator sequence are indicated in D.

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to that of RNAI, folds into the same secondary structure motifs in this segment (Fig. 2C). Its 3’-terminal extension forms two additional stem–loop structures. The third, A-U-rich stem–loop appears to be metastable, indicated by the simultaneous presence of single-strand-specific cleavages and V1 cleavages. The 3’-most stem–loop has the characteristics of a transcriptional terminator.

Three species of repC-mRNA were probed. The shorter species were chosen to represent two of several size classes of nascent transcripts of a length at which the regulatory decision between termination and refolding/read-through occurs. RNA184 extends from the 5’ end to the sequence that, in the attenuation-proficient state, can form the terminator. In order to propose secondary structures, we used both the STAR genetic algorithm (van Batenburg et al., 1995) and Mfold (Mathews et al., 1999; Zuker et al., 1999), and subsequently refined the structures based on probing results. The 112-nt-long repC mRNA (Fig. 3B) consists of two prominent stem–loops flanked by single-stranded extensions. The 5’-proximal loop is much larger than the corresponding antisense loop (14 unpaired nt versus 6 nt), and is supported by T1 and T2 cleavages. The weak V1 cleavages may indicate that a fraction of the RNA can form two loop–internal base pairs (U-A, C-G), in good agreement with the structure suggested by Novick et al. (1989).

Upon extension of repC-RNA to 132 nt, a very stable long stem–loop structure is formed, topped by a four-base loop (Fig. 3A,C). The probing results indicate that the second stem–loop may differ slightly between repC-RNA112 and repC-RNA132. Because these structural elements are energetically almost identical, this difference may be insignificant.

Interestingly, the enzymatic reactivities of repC-RNA184 (Fig. 3A and D) are mainly consistent with the read-through-proficient folding of the sense RNA and inconsistent with the attenuated form. First, the stem region of the target loop of RNA112/RNA132 is accessible to single-strand-specific cleavage in RNA184. Second, the region between nt 54 and 59 was mainly cut by V1 and, thus, is stacked or paired. Third, the 3’-half of the terminator sequence, which has to form for attenuation to occur (indicated in Fig. 3D; see also Novick et al., 1989), appears single stranded, supported by the absence of V1 cuts and the presence of T1 cuts in the 3’ tail region. Thus, RNA184 in vitro shows characteristics of a refolded RNA, which agrees well with the general scheme suggested by Novick et al. (1989); the terminator cannot form, because its 5’ half is sequestered by the so-called pre-emptor (see bottom stem segment; Fig. 3D). The sequences complementary to the two antisense loops remain largely unpaired, although they are now located between stem segments. The upper helical segment containing the target loop sequence (Fig. 3D) is fairly unstable and may be unpaired in a fraction of the molecules.

**Binding kinetics of sense–antisense RNA pairs**

To analyse the kinetics of stable complex formation in vitro, we studied binding between either of the two antisense RNA species, RNAI84 and RNAII146, and either a short or a long target RNA (repC-RNA112, repC-RNA184). Some time course experiments are shown in Fig. 4, and calculated second-order binding rate constants are presented in Fig. 5. Two conclusions can be drawn. Both antisense RNAs are equally effective in target RNA binding. The k_{app} value of $\approx 1 \times 10^6$ M$^{-1}$ s$^{-1}$ obtained with repC-RNA112 is equally high as values reported for, for example, CopA/CopT of plasmid R1 or RNAI/II of plasmid ColE1. However, both antisense RNAs show a fourfold decrease in their binding rate to repC-RNA184. This could be explained by a less favourable target loop presentation in the conformation shown in Fig. 3D.

Next, we introduced truncations within the antisense RNAs in order to assay the structural elements required for stable complex formation. Some time courses are shown in Fig. 4 and the RNA species analysed are indicated schematically in Fig. 5. Neither stem–loop L1 (RNAI84) nor stem–loop L2 (RNAI109) was sufficient for efficient complex formation. Pairing rates were decreased by 10- to 20-fold compared with those of wild-type RNAI. A similar impairment was observed for RNAI63, composed of stem–loop L1 and half of stem–loop L2. Removal of the 5’-most stem–loop of RNAII (RNAI109) led to a 30-fold reduction in binding rate to the longer target species.

These results, summarized in Fig. 5, indicate that both of the first two stem–loops of RNAI/II are required for rapid binding. The extension carried by RNAII is dispensable for binding in vitro.

**Single-round transcription assay for testing antisense RNA-mediated transcription termination**

In order to measure antisense RNA-mediated termination of nascent repC-mRNAs, we used the assay system developed for pIP501 RNAII/II (Brantl and Wagner, 1994). This system has the following properties: first, sense RNA transcription starts simultaneously, and restarts are prevented, so that the fate of the nascent RNA can be followed in time. Second, antisense RNA is not transcribed in this assay, so that the effect of defined concentrations of added antisense RNA on termination frequency can be assessed. Third, for the calculation of a rate constant of inhibition, the time window during which

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the antisense RNA can exert its effect has to be estimated.

A 420-bp polymerase chain reaction (PCR)-generated DNA fragment (from pIPT2, Experimental procedures) was used as a template. It carried the control region of pT181 but with the pIP501 repR promoter pII in place of the repC promoter. This was necessary because the repC promoter was not efficiently recognized by Bacillus subtilis RNA polymerase. In a preincubation step, repC-RNA transcription was primed by inclusion of the dinucleotide ApA, $\left[\alpha^{32}P\right]$-ATP and RNA polymerase (see Experimental procedures). This confines initiation complex formation to the rep promoter, because ApA is unable to function as a start dinucleotide at the antisense RNA promoter (Novick et al., 1989). The omission of two NTPs stalls the initiation complexes after incorporation of only a few nucleotides. Simultaneous transcript elongation was started by addition of all four unlabelled NTPs. Rifampicin was used to limit the analysis to synchronous single rounds and aliquots were withdrawn at different times for gel electrophoretic analysis. Figure 6 shows time courses of single-round transcription experiments performed, for comparison, on the pT181 template and the previously described 500 bp pIP501 template (Brantl and Wagner, 1994). As a function of time, bands corresponding to RNA species of increasing length appear. Some of these are paused intermediates, i.e. their intensities increase and subsequently decrease (P in Fig. 6). In contrast to the case of pIP501, in which two prominent bands of $\sim 365$ nt (full-length run-off transcript; F) and $\sim 260$ nt (terminated
RNA; T) accumulate, the pT181 reactions yield no detectable T band (expected < 190 nt), but a run-off product of the expected length of < 365 nt (F; Fig. 6), i.e. in the absence of added antisense RNA, the target RNA of pT181 has no intrinsic propensity to terminate, whereas a sizeable fraction of target RNA of pIP501 terminates under those conditions (30–50%; Fig. 5A in Brantl and Wagner, 1994).

From the rate of elongation of nascent target RNAs, a time window for the productive binding of the antisense RNA could be estimated. This window defines the time it takes to elongate from the length of a sense RNA long enough to contain the target for the antisense RNA to one still short enough not to have reached the attenuator. Hence, this time frame corresponds to the maximum time during which the termination of the target can be induced (see also Brantl and Wagner, 1994). The time window was estimated to be ~15 s for both pIP501 and pT181 (Fig. 6), and was used in subsequent calculations.

**Antisense RNA-mediated transcription termination in vitro**

An inhibition rate constant can be determined by using the single-round transcription assay with an addition of defined concentrations of antisense RNA. Because the fate of the nascent transcripts is determined within the first minute of incubation (Fig. 6), and no significant subsequent changes occur, attenuation is conveniently assayed at a 10 min time point. As shown in Fig. 7 (upper panel), increasing concentrations of antisense RNA (shown: RNAII) induced progressively increased termination of the target RNA (T versus F bands, Fig. 7). As control, one incubation was performed in the absence of RNAII (Buffer, Fig. 7). A quantification is shown in the lower panel of Fig. 7. Additional experiments at even higher concentrations of antisense RNA were performed to establish the full range of inhibition rates.
antisense RNA concentrations (7.5 \times 10^{-7} \text{ M}) did not induce more than 65–70\% termination of repC-mRNA. This is in contrast to comparable experiments with pIP501, in which RNAIII concentrations of 7 \times 10^{-7} \text{ M} resulted in 90–95\% termination. RNAI-induced attenuation occurred equally efficient as shown in Fig. 7 (data not shown).

Calculation of the rate constant of inhibition

The rate constant of inhibition was calculated as described previously (for RNAII of pIP501; Brantl and Wagner, 1994), using the following equation:

\[
k_{\text{inhib}} = \frac{\ln 100 - \ln (N(t))}{[\text{RNAII}] \times t}
\]

where \( t \) is the time window for inhibition (here: 15 s), \( N(t) \) is the percentage of repC-RNA transcripts that escaped inhibition at time \( t \) (F band) determined from gels as in Fig. 7(A) and [RNAII] is the concentration of antisense RNA present. For both RNAI and RNAII, rate constants of inhibition of 4–7 \times 10^{-9} \text{ M}^{-1} \text{ s}^{-1} were determined. At the highest antisense RNA concentrations tested (5–7 \times 10^{-7} \text{ M}), we observed greater variations and calculated lower values for \( k_{\text{inhib}} \). At these high concentrations of RNAI or RNAII, a minor fraction of termination-incompetent repC-RNA transcripts may obscure the measurements. In contrast, at very low concentrations of antisense RNA, the difference between antisense RNA-induced and intrinsic termination is very small, and the degree of inhibition cannot be measured exactly. Therefore, we have based the calculations on experiments in which antisense RNA concentrations ranged from 5 \times 10^{-9} \text{ M} to 1 \times 10^{-7} \text{ M} (5\% to 40\% inhibition).

Structure mapping of attenuation in vitro

The attenuation mechanism predicts alternative folding of sense RNAs dependent on the presence or absence of antisense RNA. Here, single-round transcription reactions were performed in the presence (3 \times 10^{-8} \text{ M}; Fig. 8A, middle) and absence (Fig. 8A, left) of RNAII, samples were phenolized, precipitated and subsequently partially digested by RNases T1 and T2. RNA digests were separated on a 8\% denaturing gel alongside two size markers and partial T1 and T2 digests of 5’-labelled repC-RNA132. In the absence of RNAII, T1 cleavages occurred 3’ of G_{26}, G_{31} and G_{38} in the target loop of repC RNA, and less pronounced in the single-stranded region 3’ of G_{61}. When transcription was performed in the presence of RNAII, these positions were protected. Instead, T1 cuts were visible at lower electrophoretic mobility (3’ of G_{73}, G_{111} and G_{131}). The locations of some cleavages are shown in Fig. 8B. These data are qualitatively consistent with a protection of at least part of the target region (in particular the first target loop) by the antisense RNA and with the induced formation of the terminator stem–loop. As a result of the mixed population of non-terminated/terminated transcripts (e.g. Figs 6 and 7), a more detailed structure mapping was not feasible.
Discussion

In this communication we present an *in vitro* analysis of the replication control system of plasmid pT181. The thorough genetic analysis of the pT181 regulatory system presented by the Novick group had defined the sequence and structure elements that permit the two mutually exclusive foldings of the *repC*-mRNA that are the key to the transcriptional attenuation mechanism. The results presented here are, at a general level, entirely consistent with previous findings, but offer new insights into the details of the structures of the RNAs involved as well as the RNA binding and inhibition kinetics. The first conclusion that can be drawn is that the structures of RNAI and II, the two antisense RNAs encoded by pT181, differ slightly from those predicted previously. The most notable and important difference lies in the size of loop 2 (Fig. 2). Based on probing experiments, loop 2 carries six unpaired bases (instead of 16; Novick *et al.*, 1989) and is located on a stem segment proposed to contain two non-canonical A-G basepairs. Interestingly, this general loop structure is congruent with the emerging pattern seen in other antisense systems; the main antisense or target recognition loops most often carry six or seven unpaired nucleotides on top of relatively unstable upper stem segments. Furthermore, the six-base loop sequence contains the sequence element 5′-UUGG, which conforms to the YUNR motif implicated in the formation of a particular U-turn structure. This structural motif appears to be favourable for rapid recognition, as exemplified in the context of tRNA (Grosjean *et al.*, 1998) and has recently been implicated in antisense/target RNA interaction as well (Asano *et al.*, 1998; Franch *et al.*, 1999). Both antisense RNAs have the same structural elements, except that two additional stem–loops are present in the 3′ portion of RNAII. The sequences and structures at the 3′ ends of RNAI and II suggest that the longer one is generated by read-through of the (relatively ineffective) terminator of RNAI.

The structure of the shortest target RNA (RNA$_{-12}^{112}$) carries two stem–loops. The 5′-most one has a large loop (Figs 3A,B), supported by many single-strand specific cleavages. The presence of V1 cuts may indicate some tendency to form weak basepairs within the loop. Upon extension to form RNA$_{132}^{132}$, the first stem–loop persists and the second one appears to be slightly changed (Fig. 3A and C). Because the second stem–loop of these two RNA species are of about the same stability, the significance of this difference is doubtful. The 3′-part of RNA$_{132}^{132}$ shows a very stable, long stem topped by a four-base loop. We propose that the stability of this structure module aids in the refolding of the RNA as it is elongated during transcription. The elongation of the nascent chain beyond this structure might permit the refolding of the mRNA into the read-through-proficient structure by invasion of the so-called pre-emptor sequence (Novick *et al.*, 1989). A structure resulting from this event is consistent with probing of RNA$_{184}^{184}$ (Fig. 3A and D). As can be seen, the stable long stem–loop (extending horizontally; Fig. 3D) remains unchanged. The left half of the first target stem of RNA$_{112}^{112}$/RNA$_{132}^{132}$ is proposed to be basepaired to the proximal half of the attenuator sequence. This is predicted to prevent premature termination. The predominant folding of this structure in the absence of antisense RNA is consistent with almost complete read-through in single-round experiments (see below). Even although the details of the structure of RNA$_{184}^{184}$ differ from that predicted by Novick *et al.* (1989), the crucial elements interacting to enable read-through are in agreement. It thus appears that, in the absence of antisense RNA, the read-through conformation is energeticallyfavoured. This is in striking contrast to pIP501, in which *in vitro* longer sense RNA species show indications of the terminator conformation, and in which single-round experiments supported intrinsic propensity for termination (Brantl and Wagner, 1994).

Analysis of RNA binding kinetics showed that the two antisense RNA species were equally effective binders (Figs 4 and 5). Rate constants of stable complex formation with shorter target RNAs were measured to be $\approx 1 \times 10^6$ M$^{-1}$ s$^{-1}$, i.e. in the range of many antisense–target RNA pairs. Binding to the longer, refolded target RNA, RNA$_{184}^{184}$, was fourfold slower. We suggest that the lower binding rates reflect a less accessible target conformation. Figure 3D shows that the target loop sequences at which the binding process initiates are mostly single stranded but not suitably presented. Truncations indicated that both stem–loops that are present in RNAI and RNAII are required for full activity (Fig. 5) and thus may be involved in initial recognition. Clearly, the two additional stem–loops of RNAII are dispensable. In contrast, a similar study showed that stable complex formation occurred at a 10-fold lower rate with RNAII/RNAII of pIP501 and was not affected by removal of the two 5′-proximal stem–loops (Brantl and Wagner, 1994). Also, RNAIII bound to shorter and longer target RNAs (180 and 260 nt, lacking and containing the terminator sequences) at identical rates, indicating favourable target conformations in both species.

Because stable binding rates are not necessarily identical to rates of inhibition, we used a previously developed protocol to measure inhibition kinetics. The single-round transcription experiment shown in Fig. 6 shows that, in the absence of antisense RNA, transcript elongation progresses through a series of paused intermediates and either results in termination or read-through. A comparison between transcription reactions using plasmid pT181 and pIP501 templates, respectively,
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shows that in the absence of antisense RNA repC-mRNA fails to terminate (Fig. 6, left panel), whereas RNAII (of pIP501) yields a substantial fraction of terminated product (Fig. 6, right panel; Brantl and Wagner, 1994). Thus, the propensity for termination (pIP501) is a property of the RNA in question, and not a result of experimental conditions. The time course shown was also used to estimate the time window during which the regulatory decision has to occur (see Results) and was then used in calculations of the inhibition rate constant. To determine inhibition kinetics, we added defined concentrations of antisense RNA to single-round transcription reactions and measured the ratio between terminated and read-through transcripts. This assay is similar to a binding assay in that a second rate constant is measured, however, conceptually different because the antisense RNA will interact with a moving target; a nascent transcript that is able to undergo folding changes as elongation proceeds. Only during a given time interval (corresponding to lengths of nascent target RNAs long enough to contain target sequences, but short enough not to contain the terminator sequence) the antisense RNA can affect the fate of the target RNA. This time interval was estimated to be \( \approx 15 \) s, the same as in the case of pIP501 (see Fig. 6 and Results). Attenuation as a function of [RNAII] was assayed (Fig. 7, upper panel) and the degree of inhibition is indicated in a staple diagram (Fig. 7, lower panel). From these values the rate constant of inhibition \( (k_{\text{inhib}}) \) was calculated to be \( 4-7 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) (see Results). Because the transcription buffer differs in composition from the standard binding buffer, we conclude that the twofold difference between \( k_{\text{app}} \) and \( k_{\text{inhib}} \) is insignificant. Note also that the binding window is estimated conservatively, which also indicates that \( k_{\text{inhib}} \) is probably underestimated. Thus, stable complex formation and inhibition in the pT181 system occur at similar rates, whereas inhibition occurred at least 10 times faster than binding in pIP501 (Brantl and Wagner, 1994). This implies that one cannot infer from these data whether the antisense RNA of pT181 requires full duplex formation for its inhibitory effect (see, for example, Wagner and Brantl, 1998).

As in the binding experiments (Figs 4 and 5), RNAI and RNAII were equally efficient in attenuation (data not shown). This result raises a question related to earlier in vivo work from the Novick group. The analysis of a plasmid mutation (\( \text{cap-622; Carleton et al., 1984} \)) led to the suggestion that RNAII may be a less effective inhibitor than RNAI (Novick et al., 1989). The \( \text{cap-622} \) mutation changed G73 and showed a copy-up phenotype, attributed to a termination defect that would result in a higher RNAII/RNAI ratio. Because RNAII and RNAI show equal efficiency in vitro, an alternative explanation can be suggested. G73 is located in the lower stem 2 of the antisense RNA as part of a G-C basepair. Because alterations in stem regions can have profound effects on the presentation of primary recognition loops (Hjalt and Wagner, 1995), this mutation may simply affect the activity of RNAII.

In Fig. 8, a partial mapping of transcripts is shown, under conditions in which regulation occurs (Fig. 7). Although the resolution of the analysis is relatively poor, the data indicate that, when antisense interacts with target RNA, a folding change in the latter is induced, as expected from the attenuation mechanism. The protection from single-strand-specific cleavages induced by RNAII is indicative of binding across some part, but probably not all, of the complementary region in repC-RNA, which might indicate that complete duplex formation does not occur. Overall, this experiment lends structural support to the mutually exclusive conformations predicted to result in termination and read-through. An additional conclusion from the in vitro experiments in Figs 7 and 8 is that host proteins are not required for attenuation.

For a complete understanding of the replication control circuit it is desirable to know both the kinetic properties of the key molecules involved and their steady-state concentrations in vivo, as determined for plasmid ColEl (Brenner and Tomizawa, 1991) and pIP501 (Brantl and Wagner, 1996; Steinmetzer et al., 1998). The present study reporting on the kinetic properties of the regulatory molecules contains part of this information for pT181. However, for this plasmid only in vivo quantifications of RepC have been published so far; in the absence of antisense RNAs, about \( 10^6 \) molecules of RepC protein per cell were measured and, in the wild-type situation (presence of RNAI and RNAII), only about 300 (Novick et al., 1989). Thus, antisense RNAs abort \( \approx 97\% \) of the primary transcripts. Using an inhibition rate constant of \( \approx 5 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \), an antisense RNA concentration of \( \approx 5 \times 10^{-7} \text{ M} \) would be required to obtain 97\% inhibition, if assay buffer and intracellular conditions were comparable.

Fig. 8. Partial structure mapping of transcriptional attenuation in vitro.

A. Single-round transcription with a plPT2-derived 420 bp fragment. Left: in the absence of antisense RNA. Middle: in the presence of antisense RNA (3 \( \times \) \( 10^{-8} \) M RNAII). After 10 min of transcription, samples were phenolised, precipitated, suspended in TMN buffer and subjected to partial digestions with RNases T1 or T2. Digests were separated on an 8\% denaturing gel. Enzyme dilutions are indicated. Buffer, no enzyme added; M, pBR322, MspI marker; L, alkaline ladder. The positions of the major target loop and the region of antisense RNA complementarity are indicated. F, full-length transcript (365 nt); T, terminated transcript (190 nt). Right: partial digestions of \( 5^\prime \)-labelled repC-RNA\(_{132}\) with T1 and T2, as reference. H: 100 bp ladder

B. Schematic representation of the attenuated repC-RNA based on the T1/T2 digestions shown in A.

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Clearly, a determination of the concentrations of RNAI, RNAII and repC-mRNA in living cells would be useful to model the regulatory circuit of plasmid pT181.

It is of interest to compare the similarities and differences of two plasmid systems, both regulated via antisense RNA-mediated transcriptional attenuation yet different in other properties. Plasmid pT181 replicates via the rolling circle mode, pIP501 by theta replication. In both systems, attenuation has been demonstrated both in vivo and in vitro (Brantl and Wagner, 1994, 1996; Novick et al., 1989; this work). In vitro, inhibition kinetics are similar, whereas RNAIII of pIP501, in contrast to RNAII of pT181, shows slow rates of stable complex formation. The target RNA of pIP501 shows intrinsic termination, whereas the target RNA of pT181 terminates only in the presence of antisense RNA. A peculiarity of pIP501 is that its antisense RNA is unusually stable (Brantl and Wagner, 1996). This potentially deleterious property is compensated for by the use of a second regulator, the CopR protein (Brantl and Wagner, 1997). Preliminary half-life determinations for pT181 RNAI and RNAII in Bacillus subtilis (S. Brantl, unpublished observations) revealed short half-lives of 3–5 min, in good agreement with preliminary determinations performed in Staphylococcus aureus (R. P. Novick, personal communication). Plasmid pT181 does not encode a CopR equivalent. In contrast to plasmids in Gram-negative bacteria, both pT181 and pIP501 fail to show a runaway replication phenotype when the essential copy number control functions are deleted. This surprising and unexpected feature is under investigation.

**Experimental procedures**

**Enzymes and chemicals**

Chemicals used were of the highest purity available. Taq DNA polymerase was purchased from Perkin–Elmer. Ribonucleases T1, T2, V1 and U2 were purchased from Pharmacia and RNase A from Sigma. T7 RNA polymerase nuclease T1, T2, V1 and U2 were purchased from New England Biolabs. Sequencing gels were obtained from New England Biolabs. RNase A from Sigma. T7 RNA polymerase was obtained from Pharmacia and RNase A from Sigma. Digestions were performed according to Sanger et al. (1977) using a Sequenase Kit from Amersham Pharmacia Biotech.

**Construction of plasmid pIPT2**

A single PCR step was performed on plasmid pT181 as template using oligonucleotides SB91 (5′-GAA TTC GGA TCC TTG CTT ATT TTT TTA AAA AGC GAT ATA CTA GAT ATA ACA AAA TAA AAA GGA G) and SB54 (5′-GAA TTC GTC GAC GTC CAA CCG GCT ATT AG) as primers. The resulting DNA fragment was digested with BamHI and SalI and inserted into the polylinker of pUC19 cleaved with the same pair of enzymes, yielding plasmid pIPT2. The sequence of the 318 bp insert contains the pIP501 sense promoter pII, immediately followed by the sequence of the pT181 repC-mRNA.

**Generation of DNA templates for in vitro transcription of different RNA species**

DNA templates were generated by PCR from plasmid pT181. For the PCR fragment encoding wild-type RNAI, oligodeoxynucleotides SB57 (5′-GAA ATT ATA CGA CTC ACT thinsp;ATA GGA TAC AAG ATT ATA AAA ACA ACT C) and SB58 (5′-AAG GAG TCG CTC ACC CCC TGA) were used. For the PCR fragments encoding the repC-mRNA species of different lengths, SB59 (5′-GAA ATT AAT ACG ACT CAC TAT AGG AAC AAA ATA AAAG AGT CCG) was used either in combination with SB60 (5′-TCG TTT AAT ACG TATA TAA ATA TAC; repC-RNA112), SB90 (5′-TCT TGA TGT ATA TT AAA; repC-RNA142), or SB61 (5′-ATG ATC GCC TTA ATG TCA A; repC-RNA184). Templates for transcription of truncated RNAI species were generated with SB57 and SB79 (5′-CAT TCA AAG AAA AAA ACA CGT; RNA143), SB57 and SB83 (5′-CAA AGT TTG TGA ACG ACA TCA; RNA153), SB80 (5′GAA ATT AAT ACG ACT CAC TAT AG G TCT TTG AAT GAC TGT CAC) and SB58 for RNAI, SB57 and SB81 (5′-AAA AAA GAC TAC TTA TCA G) for RNAII. PCRs with these primer sets yielded fragments carrying a T7 RNA polymerase promoter in front of the sequences to be transcribed. For better RNA transcription, two additional G residues were encoded at the transcription start sites. The 420 bp pIPT2 DNA fragment used as template in the single-round transcription assay was generated by PCR using universal (5′-GTT TTC CCA CCA ACG AC) and reverse (5′-AGC GGA TAA CAA TTT CAC ACA GGA) sequencing primers. PCR fragments were purified from agarose gels.

**In vitro transcription**

RNAI, RNAII and repC-mRNA were synthesized in vitro by T7 RNA polymerase from PCR-generated DNA templates. For structural analyses, RNAs were either 5′-end-labelled with [γ-32P]-ATP (Hartmann Analytic) as described previously (Brantl and Wagner, 1994) or 3′-end-labelled with RNA ligase and [32P]-pCp (ICN). For binding experiments, antisense RNAs were synthesized in the presence of 10 μCi of [α-35S]-UTP. ‘Unlabelled’ RNA species were synthesized in the presence of 100 μM [3H, α-35S]-UTP to allow for accurate determination of the RNA concentration. The RNAs were gel purified before use.

**Secondary structure analysis**

Partial RNase digestions were carried out as described previously (Brantl and Wagner, 1994) except that tRNA was included at 0.08 mg ml⁻¹ and RNase concentrations required for appropriate partial digestions were determined empirically (see figure legends). Digestions were performed for 5 min in a total volume of 5 μl of TMN buffer (Persson et al., 1988), followed by addition of 5 μl of formamide loading dye, boiling and separation of aliquots on 8% sequencing gels. Dried gels were exposed to X-ray film. Alkaline ladders were generated as described (Brantl and Wagner, 1994).
In vitro binding assays

For determinations of binding kinetics, [3H]-UTP-labelled repC-mRNA and either [α-32P]-UTP-labelled RNAI or RNAll species were used at the concentrations indicated. Binding assays and subsequent gel electrophoresis were performed as described previously (Brantl and Wagner, 1994). Visualization and quantification of the bands were performed on a Fuji Phosphorimager and autoradiograms for Fig. 4 were made from wet, frozen gels. Binding rate constants (kobs) were calculated as described previously (Persson et al., 1988).

Single-round transcription assays

Single-round transcription assays were performed using a 420 bp DNA fragment generated by PCR from plasmid pIP2 as template. The transcription buffer contained 60 mM Tris-Cl pH 7.8, 12.5 mM MgCl2 and 1 mM dithiothreitol (DTT). In the preincubation step, initiation complexes were allowed to form by the inclusion of 100 μM of the dinucleotide ApA (Sigma), 20 μCi of [α-32P]-ATP, an NTP mix containing 5 μM ATP and 20 μM CTP, template DNA (≈ 10−9 M) and Bacillus subtilis αδRNA polymerase at n = 79 μg ml−1 (prepared by J. M. Sogo, Madrid, Spain). After 5 min at 30°C, solution 2 was added to start elongation of the preformed, short RNA chains. Solution 2 contained, in transcription buffer, rifampicin (100 μg ml−1) and all four NTPs to yield final concentrations of 100 μM. This allowed for elongation of initiated transcripts and prevented new initiations. Aliquots withdrawn at appropriate times were phenol extracted, precipitated, dissolved in formamide loading dye, boiled and separated on 6% sequencing gels. Gels were dried, autoradiographed and quantified as above.

The protocol of the attenuation experiments was as follows. Tubes containing 3H-labelled antisense RNAs appropriately diluted in transcription buffer (to yield the final concentrations indicated in the figures) were prewarmed at 30°C, aliquots of the preincubation mix were added and elongation of repC-RNA transcription was started by addition of solution 2. For gel analysis, the reactions were stopped as before (but at 10 min).

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