Antisense RNA regulation of the par post-segregational killing system: structural analysis and mechanism of binding of the antisense RNA, RNAII and its target, RNAI

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Summary

The par stability determinant of the Enterococcus faecalis plasmid pAD1 is the first antisense RNA regulated post-segregational killing system (PSK) identified in a Gram-positive organism. Par encodes two small, convergently transcribed RNAs, designated RNAI and RNAII, which are the toxin and antitoxin of the par PSK system respectively. RNAI encodes an open reading frame for a 33 amino acid toxin called Fst. Expression of fst is regulated post-transcriptionally by RNAII. RNAII interacts with RNAI by a unique antisense RNA mechanism involving binding at the 5’ and 3’ ends of both RNAs. Par RNA interaction requires a complementary transcriptional terminator stem-loop and a set of direct repeat sequences, DRa and DRb, located at the 5’ end of both RNAs. The secondary structures of RNAI, RNAII and the RNAI–RNAII complex were analysed by partial digestion with Pb(II) and ribonucleases. Probing data for RNAI and RNAII are consistent with previously reported computer generated models, and also confirm that complementary direct repeat and terminator sequences are involved in the formation of the RNAI–RNAII complex. Mutant par RNAs were used to show that the binding reaction occurs in at least two steps. The first step is the formation of an initial kissing interaction between the transcriptional terminator stem-loops of both RNAs. The subsequent step(s) involves an initial pairing of the complementary direct repeat sequences followed by complete hybridization of the 5’ nucleotides to stabilize the RNAI–RNAII complex.

Introduction

Antisense RNAs are small, untranslated transcripts that bind to the mRNA of a target gene, thereby controlling expression of that gene. Typically, antisense RNAs and their target RNAs are transcribed from opposite strands of the same DNA template by opposing promoters. This allows for the antisense RNA to be complementary to its target RNA. However, in some cases, the antisense and target genes are unlinked and complementarity is only partial (Delihas, 1995). Numerous antisense RNA regulated systems have now been identified, and the mode of antisense regulation varies among the different systems (for review see Wagner and Simons, 1994). Some examples of the different types of regulation include translation inhibition (Ma and Simons, 1990; Wagner et al., 1992; Thisted et al., 1994), transcription attenuation (Novick et al., 1989; Brantl and Wagner, 1994), inhibition of primer formation (Masukata and Tomizawa, 1986), and facilitation of mRNA decay (Krinke and Wulff, 1990).

The binding reaction between antisense and target RNAs has been well characterized for several antisense RNA regulated systems. Many are involved in regulating plasmid copy number including RNAI–RNAII of CoIE1 and CopA–CopT of R1 (Tomizawa, 1984; Persson et al., 1990a). Antisense pairing mechanisms have also been determined for RNA-IN/RNA-OUT controlling IS10 transposition (Kittle et al., 1989), as well as, the hok/sok post-segregational killing system (PSK) of plasmid R1 (for review see Gerdes et al., 1997). In all cases, RNA structure has been proposed to play an important role in promoting rapid and efficient pairing of the RNAs. Generally, antisense RNAs contain one or more stem-loop structures that are complementary to similar structures on their target RNAs. Binding initiates between two loops or a loop and a single-stranded segment of the RNA. This initial contact, called a ‘kissing complex’, serves as the precursor for complete duplex formation, which can either proceed from the site of initial interaction (Kittle et al., 1989; Thisted et al., 1994) or initiate at a separate site (Tomizawa, 1984; Persson et al., 1990a; Siemering et al., 1994; Argaman and Altuvia, 2000). However, evidence now suggests that complexes involving a limited number of interstrand basepairs are sufficient to regulate target gene expression (Tomizawa, 1990; Wagner et al., 1992; Wagner and Brantl, 1998). Another common feature in
antisense RNA systems is the presence of a U-turn motif (YUNR) on the loops involved in initial interaction (Franch et al., 1999). The sharp turn at the 3’ phosphate of the invariant U positions the downstream bases into a half A-form Watson–Crick structure. This structure is also present in the anticodon of tRNAs and is crucial for proper translational decoding (Grosjean et al., 1976; Ashrat et al., 1999).

The par stability determinant of the Enterococcus faecalis plasmid pAD1 is the first and only antisense RNA regulated PSK identified in a Gram-positive organism (Weaver et al., 1993; 1996; 1998; Weaver, 1995). The par locus encodes two small transcripts designated RNAI and RNAII (Weaver and Tritle, 1994), encoding the toxin and antitoxin of the PSK system respectively (Weaver et al., 1996). The product of the RNAI transcript is Fst, the faecalis plasmid-stabilizing toxin. Expression of fst is regulated post-translationally by the RNAII transcript (Greenfield et al., 2000a). RNAII is less stable than RNAI and therefore pAD1 must be maintained to prevent Fst toxin expression. If a daughter cell does not receive a copy of the plasmid, RNAI levels decline and Fst can be expressed, killing the cell. Thus, the par system maintains pAD1 in the host population by killing plasmid-free segregants.

RNAI–RNAII interaction appears to be unique among antisense RNA systems in that the RNAs interact at multiple dispersed sites within the 5’ and 3’ ends of both RNAs. RNAI and RNAII are convergently transcribed from promoters located at each end of par to a common bidirectional terminator (Fig. 1). Additionally, both RNAs are transcribed across a pair of direct repeat sequences, which on RNAI flank the translation initiation signals for Fst. RNAII interacts with RNAI at the terminator stem-loop and at these direct repeat sequences to inhibit ribosome binding to the Shine–Dalgarno sequence of fst (Greenfield et al., 2000a,b). In this study, the secondary structures of the par RNAs were determined, and the interaction between RNAI and RNAII was followed using Pb(II) and RNase probing experiments. Using multiple in vitro interaction assays, the binding reaction was shown to consist of multiple steps with an initial interaction between the terminator loops and a second interaction between the direct repeats at the 5’ end of the RNAs that progresses into neighbouring sequences to stabilize the RNAI–RNAII complex.

**Results**

**Secondary structure analysis of RNAI and RNAII**

The pairing process between antisense and target RNAs can be greatly affected by the structures of both RNAs. Therefore, a complete understanding of the binding mechanism requires knowledge of the individual RNA structures. To determine the par RNA secondary structures, chemical and enzymatic structure probing experiments were performed on RNAI and RNAII transcripts produced in vitro. End-labelled RNAI and RNAII transcripts were subjected to partial Pb(II) cleavage and partial nuclelease digestion with RNaseV1. Pb(II) cleaves single-strand regions of RNA with little sequence specificity (Gornicki et al., 1989), and RNaseV1 cleaves paired and stacked nucleotides. Reactions were run alongside a cleavage reaction using RNaseT1 (cleaves single-stranded G residues) and an alkaline ladder produced by partial hydrolysis of the RNA. The cleavage patterns for RNAI and RNAII are shown in Fig. 2, and the positions of Pb(II) and RNaseV1 cuts are marked schematically on the secondary structures of each RNA in Fig. 3. For RNAI, cleavage sites marked beyond nt 150 are not easily seen on Fig. 2B, but were confirmed by extending the time of electrophoresis (data not shown).

The predicted model for RNAII consists of two stem-loops in which the complementary regions involved in pairing are exposed in single-strand regions (Fig. 3). Nuclease and lead probing data are consistent with a molecule consisting of a small 5’ stem-loop and a larger 3’ transcriptional terminator stem-loop separated by a short single-stranded region (Figs 2 and 3). As predicted, the regions used for antisense RNA interaction are exposed on single-stranded regions of RNAI. Lead cleavages

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**Fst:** MKDLMLVAIPIFVGLVLEMSRVLDEEDDSRK

**Fig. 1.** Features of the pAD1 par stability determinant. Promoters for RNAI and RNAII are indicated by black arrows on each end. The open reading frame, fst, encodes a 33 amino acid peptide. The two RNAs read in opposite directions across direct repeats labelled Dra and DRB (arrows) to a bidirectional terminator (converging arrows). The SpeI site used to create RNAI terminator deletion mutant is marked. The amino acid sequence for Fst is also shown.

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Fig. 2. Pb(II) and RNaseV1 probing of par RNAI (B) and RNAII (A). Par RNA transcripts produced in vitro were end-labelled and subjected to partial digestion with Pb(II) and RNaseV1. Digestion reactions were run on 8–12% polyacrylamide gels. An autoradiograph of the cleavage pattern is shown. The concentration of Pb(II) (lanes 3–8) and the time of incubation with RNaseV1 (lanes 9–14) used to probe reactions is indicated above each lane. A+ above lanes 6–8 and 12–14 indicates the addition of an excess of the other par RNA. T (lane 1) is an RNaseT1 cleavage reaction of the RNA, and L (lane 2) is an alkaline hydrolysis ladder. The positions of DRa, DRb, and the terminator loop sequences are as indicated.

Fig. 3. Proposed structures of par RNAI and RNAII. Arrowheads mark RNaseV1 cuts, and filled circles mark Pb(II) cuts. Asterisks on the stems of the arrows denote that the cleavage pattern of that nucleotide was altered when the other par RNA was added to the probing reaction. Cleavage sites marked beyond nt 120 in RNAI were confirmed by extending the time of electrophoresis (data not shown). The terminator stem-loop of RNAI was determined by computer modelling, but because of the thermodynamically stable nature of this structure and its complementary to the terminator stem-loop in RNAII, it almost certainly forms in RNAI. DRa, DRb, and the terminator loop are shaded on both RNAs. The nucleotides that are mutated in the par RNA mutants are circled. The ribosome binding site and start codon for fst are also indicated on RNAI.

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occurred at nts 7–14 and nts 18–29 marking the loop of the 5′ stem-loop and the single-stranded region between the two stem-loops respectively. Minor lead cleavages were observed at nts 15–19 suggesting that the short 5′ stem-loop is unstable or that subpopulations exist in which this stem-loop is not formed. Lead cleavage is also shown at nts 43, 46, and 48 marking the loop of the transcriptional terminator stem-loop. Strong RNaseT1 cleavages at G44, G45, and G47 support the single-stranded nature of this region. Consistent with the existence of two stem-loops, the 5′ helix is defined by RNase V1 cleavage at nts 3–6 and 15–17. The terminator helix is marked by RNaseV1 cuts at nts 36–39 and 54–57. Additional lead cleavages at nts 29–32 indicate that either the single-stranded stretch between the two stem-loops extends past the predicted sequence or more likely that there is extensive breathing at the A-U basepairs at the end of the helix. Continuous RNaseV1 cleavages are also seen at nts 24–30. Because RNaseV1 will cleave a stacked base whether it is basepaired or not, the observed cleavage may imply that nucleotides in the RNAII DRb region are helically oriented.

This procedure also confirmed the points of interaction between RNAI and RNAII. Unlabelled RNAI was added to the reaction mixture and allowed to bind RNAII before chemical and nuclease probing. RNAI binding was shown to significantly change the cleavage pattern for RNAII (Fig. 2). Nucleotides of the DRa sequence which lie in the loop of the 5′ stem-loop, the DRb repeat, and the transcriptional terminator loop were no longer sensitive to Pb(II) cleavage, indicating interaction at these repeats. Furthermore, some of these nucleotides were now sensitive to cleavage with RNaseV1, indicating that these nucleotides were now paired. RNaseV1 cleavage of the 5′ stem-loop of RNAII diminished suggesting that this stem-loop may dissociate to aid interactions at the direct repeats. In fact, the absence of any lead cleavage from nts 7–29 suggests that interactions at the 5′ end may extend to nucleotides other than those of the DRa and DRb regions. Even though the sequence between the direct repeats is not completely complementary, the presence of several potential G:U pairs could permit the extension of the interactions at the direct repeats.

Computer modelling of RNAI using either Mfold or Star (van Batenburg et al., 1995) generates several possible secondary structures. Pb(II) and RNaseV1 probing data showed RNAI to be highly structured consisting of multiple stem-loops (Fig. 2). This data also showed that the complementary sequences are exposed on single-stranded regions of the RNA molecule. This would be expected because these regions are involved in RNA interaction (Greenfield et al., 2000b). This data was used to determine which predicted structure was correct. Nucleotides determined to be single stranded or double stranded were included in the computer modelling programs. Using these parameters only one model was predicted (Fig. 3).

Pb(II) was shown to cleave nucleotides in the region from the complementary DRb repeat through the DRa repeat. Addition of RNAII before probing induced changes in the cleavage pattern of this region that were similar to those observed in the probing of RNAII. RNAII binding was able to protect the nucleotides in the DRa and DRb regions from Pb(II) cleavage, indicating interaction at these repeats. Additionally, some of the nucleotides in these repeats were now sensitive to RNaseV1 cleavage. As was observed with RNAII, the regions protected from Pb(II) cleavage in the RNAI–RNAII complex extend into sequences between the direct repeats. As noted above, the potential for G:U pairing could allow for complete hybridization of the sequence between DRa and DRb. Cleavage sites within the terminator stem-loop ran too close to full length RNAI to distinguish, but this is a thermodynamically stable structure predicted by various modelling programs that is complementary to the transcriptional terminator shown for RNAII and so almost certainly forms.

Strong RNaseV1 cleavage sites were present starting at the 5′ end of RNAI through to the SDfst region indicating that this region is double stranded. This data along with computer modelling shows that two helices exist at the 5′ end of RNAI. The first is formed by interaction of the nts 3–9 with nts 135–141. The second is a stem-loop formed by interaction of nts 10–15 with nts 21–26. Previous results suggested that sequences or structures at the 5′ end of RNAI might suppress translation of fst (Greenfield et al., 2000a). The mechanism by which this structure interferes with fst translation is currently under investigation (see below).

Structural analysis of par RNA binding mechanism

The binding pathway for several antisense-target RNA pairs has been determined and in some instances the interaction was shown to be a stepwise process (Siemerig et al., 1994; Persson et al., 1990a,b; Tomizawa, 1984; 1986). Because par RNAI and RNAII interact at multiple sites, it was presumed that binding might also occur in multiple steps. RNase probing analysis was used to follow the binding reaction between RNAI and RNAII. A time course experiment was performed in which end-labelled RNAII was incubated with unlabelled RNAI. Samples were withdrawn from the binding reaction at various time points and subjected to cleavage by RNaseT1. There are three strong RNaseT1 sites in the terminator loop (G44, G45 and G47) and one in DRa (G5). There is also a weak RNaseT1 site near DRb (G21). Binding at each site was followed by the disappearance of the cleavage product in that region. As seen in Fig. 4, band intensity at all three sites decreases equally with time. Densitometric analysis of the bands in DRa and the
terminator loop provided a binding rate constant of \( \approx 2 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) approximately one order of magnitude higher than that previously determined using traditional gel-shift assay (Greenfield et al., 2000b).

**Inhibition of duplex formation by mutant par RNAs**

The data presented above suggests that if binding occurs in multiple steps, subsequent steps occur so rapidly after the initial rate-limiting step that they are indistinguishable from it. Such rapid conversion from initial interaction to extended kissing complex formation has been observed in the antisense RNA regulated systems of plasmids R1 (Malmgren et al., 1997; Kolb et al., 2000), pMU720 (Siemering et al., 1994) and Coll-P9 (Asano et al., 1998). Nevertheless, three lines of evidence suggest that the initial interaction occurs between the terminator stem-loops of RNAI and RNAII to facilitate the interaction of the 5' direct repeats. First, mutations introduced into the loop of the terminator stem-loop severely reduced the rate constant for par RNA interaction in vitro. Second, these same mutations prevented RNAII from suppressing fst translation in vivo most likely because of the slow rate of binding (Greenfield et al., 2000b). Third, the YUNR motif (Franch et al., 1999) is present in the loop of the terminator stem-loop of RNAI. If the initial interaction is at the terminator stem-loop, a par RNA mutant that is incapable of stable binding should be able to inhibit stable complex formation between WT RNAI and RNAII as long as the mutant RNA contains a WT terminator stem-loop. The WT terminator stem-loop of this mutant RNA would compete with WT par RNAs for the initial step of kissing complex formation which, in turn, would interfere with full RNAI–RNAII interaction. RNAI mutants containing two nt changes in either DrA, DrB or the terminator loop were included in a binding reaction between labelled RNAI (WT) and unlabelled RNAI (WT), and RNA complex formation was analysed using gel shift analysis. These mutants were shown previously to bind complementary RNAII mutants at rates similar to those observed for WT par RNAs, indicating no major structural differences. As shown in Fig. 5, the RNAI DrB mutant, containing WT DrA and terminator loop sequences, is able to inhibit binding between WT par RNAs. The RNAI terminator loop mutant is not able to prevent RNAI–RNAII interaction suggesting that the terminator stem-loop is involved in the initial interaction between par RNAI and RNAII. Interestingly, the RNAI DrA mutant was also unable to inhibit RNAI–RNAII interaction even though it contains a WT terminator stem-loop. These results exclude DrB from a role in initial interaction, but do not distinguish between the importance of DrA and the terminator stem-loop.

**Binding of mutant par RNAs and structural analyses of their complexes**

The results presented above indicate that both a WT DrA (Franch et al., 1999) is present in the loop of the terminator stem-loop of RNAI. If the initial interaction is at the terminator stem-loop, a par RNA mutant that is incapable of stable binding should be able to inhibit stable complex formation between WT RNAI and RNAII as long as the mutant RNA contains a WT terminator stem-loop. The WT terminator stem-loop of this mutant RNA would compete with WT par RNAs for the initial step of kissing complex formation which, in turn, would interfere with full RNAI–RNAII interaction. RNAI mutants containing two nt changes in either DrA, DrB or the terminator loop were included in a binding reaction between labelled RNAI (WT) and unlabelled RNAI (WT), and RNA complex formation was analysed using gel shift analysis. These mutants were shown previously to bind complementary RNAII mutants at rates similar to those observed for WT par RNAs, indicating no major structural differences. As shown in Fig. 5, the RNAI DrB mutant, containing WT DrA and terminator loop sequences, is able to inhibit binding between WT par RNAs. The RNAI terminator loop mutant is not able to prevent RNAI–RNAII interaction suggesting that the terminator stem-loop is involved in the initial interaction between par RNAI and RNAII. Interestingly, the RNAI DrA mutant was also unable to inhibit RNAI–RNAII interaction even though it contains a WT terminator stem-loop. These results exclude DrB from a role in initial interaction, but do not distinguish between the importance of DrA and the terminator stem-loop.

![Fig. 4. Analysis of the binding process between par RNAs by partial RNaseT1 digestion. 5’ End-labelled RNAII (10\(^{-9}\)M) was incubated with unlabelled RNAI (10\(^{-8}\)M) in binding buffer at 37°C. Samples were withdrawn at 0 min (lane 3), 1 min (lane 4), 2 min (lane 5), and 4 min (lane 6), and partially digested with RNaseT1. C (lane 1) indicates at no RNase control, and L (lane 2) is the alkaline hydrolysis ladder. DrA, DrB, and terminator loop regions are as indicated.](image)

![Fig. 5. Inhibition of RNAI–RNAII interaction by par RNA binding mutants. Radiolabelled RNAI (10\(^{-8}\)M) was incubated with unlabelled RNAI (10\(^{-8}\)M) in the presence or absence of a competitor RNA (10\(^{-8}\)M). The type of RNA added to each reaction is indicated by a + below each lane. WT in lane 3 indicates the addition of WT RNAI competitor RNA; the B in lane 4 indicates the addition of the RNAI DrB mutant; the T in lane 5 indicates the addition of the RNAI terminator loop mutant; and the A in lane 6 indicates the addition of the RNAI DrA mutant.](image)
and terminator stem-loop are important for interference with stable complex formation between WT par RNAs. It is possible that binding of DRa occurs rapidly after the initial interaction between the terminator stem-loops to make an extended kissing complex, and that this extended kissing complex is required to prevent par RNA interaction because of the highly reversible nature of the initial kissing complex. To further distinguish the roles of DRa and the terminator stem-loop, we determined the ability of various RNAI constructs to form initial kissing complexes with RNAII. If the terminator stem-loop is the initial point of interaction, it should be necessary and sufficient for the formation of the initial kissing complex. Kissing complex formation was evaluated using an approach described by Persson et al. (1990a) which identifies kissing complexes by native gel electrophoresis in RNA binding buffer. Figure 6 shows kissing complex formation as a shift in the migration of labelled RNA. Under these conditions, an RNA containing just the terminator stem-loop attached to ~60 nt of heterologous RNA was shown to interact with RNAII (Fig. 6, lane 6). An RNAI mutant that lacked the terminator stem-loop was unable to bind RNAII (lane 5) unless RNA concentrations and binding time were substantially increased (data not shown). This demonstrates that the terminator stem-loop alone can form the initial interaction between par RNAs.

Significantly, RNAs with mutations in either DRa or DRb were capable of forming complexes under these conditions (Fig. 6, lanes 3 and 4). These same mutations were shown previously to prevent stable RNAI–RNAII interaction *in vitro* using standard gel shift analysis but, paradoxically, these mutant RNAs were still capable of inhibiting Fst translation *in vivo* (Greenfield et al., 2000b). This suggests that these RNAs can form a functional interaction capable of inhibiting Fst translation even though they cannot progress to stable complex formation. Similar unstable binding intermediates have been shown sufficient to control target gene expression in other antisense RNA regulated systems (Tomizawa, 1990; Wagner et al., 1992; Wagner and Brantl, 1998).

Structural analysis of complexes formed with the direct repeat mutants was carried out to determine why they form less stable complexes than the terminator mutants or WT par RNAs. End-labelled RNAII (WT) was incubated with RNAI containing mutations in DRa, DRb or the terminator

![Fig. 6. Analysis of kissing complex formation by native gel electrophoresis. Radiolabelled RNAII was incubated with various RNAI derivatives in binding buffer at 37°C. The type of RNAI added to each reaction is indicated below each lane. Reactions were terminated by dilution in loading buffer at 0°C and run on native 6% polyacrylamide gel in binding buffer at 4°C (See Experimental procedures).](image)

![Fig. 7. Structural analysis of complexes formed by mutant par RNAs. End-labelled RNAII was incubated with each of the RNAI mutants indicated above each lane. Lane 1 represents an RNaseT1 cleavage reaction of RNAII alone. Lane 2 is an alkaline ladder. Lane 3 is an uncleaved control lane, lane 4 shows Pb(II) cleavage of WT RNAII. Lanes 5–8 show Pb(II) cleavage of WT RNAII with various RNAI mutants. Lane 5 shows RNAII bound to WT RNAII. Lane 6 shows RNAII bound to RNAI DRb mutant. Lane 7 shows RNAII bound to RNAI terminator mutant. Lane 8 shows RNAII bound to RNAI DRa mutant. Partial digestion of each reaction was done using 40 mM Pb(II). Samples were run on an 8% polyacrylamide sequencing gel containing 9 M urea. The complementary DRa, DRb, and terminator loop regions are as indicated.](image)
loop, and these complexes were subjected to partial Pb(II) cleavage. Binding time was extended to permit binding of the terminator stem-loop mutant. Each of the mutants was shown to interact with RNAII using just two of the three complementary regions (Fig. 7). In all cases, cleavage protection was still observed at the WT complementary sequences but not at the mutated sequence. Even though only two nucleotides were altered in each complementary region, that entire region remained accessible to Pb(II) cleavage indicating that no binding occurred at that site. Additionally, mutations in either direct repeat permitted cleavage of the non-complementary nucleotides located between the DRa and DRb. As shown above, these nucleotides were protected from Pb(II) cleavage when WT par RNAs were used. Therefore, interactions at both 5' direct repeats are necessary to promote stable RNAI–RNAII complex formation. Interactions with the complementary nucleotides in the region between the DRa and DRb sequences might also contribute to the stability of the 5’ interactions.

Discussion

Experimental probing data along with computer analysis were used to determine the secondary structures of the par RNAs. Pb(II) and RNaseV1 cleavage sites in RNAII matched the regions that were predicted by computer analysis to be single stranded and double stranded. Although computer modelling of RNAI predicted several related structures, only one matched the experimental data. In both RNA structures, the complementary direct repeats, DRa and DRb, are exposed on single-stranded regions, and the complementary terminators form stem-loop structures. This would be necessary because these regions are required for par RNA interaction (Greenfield et al., 2000b). Interestingly, several computer models for RNAI secondary structure predicted basepairing of various nucleotides in DRa and DRb with sequence elsewhere in RNAI. The presence of continuous Pb(II) cleavage at every nucleotide within the sequence from DRb through DRa shows that this region is indeed single stranded and available for interaction with RNAII. It is also noteworthy that the 5’ end of RNAI was shown to be almost entirely double stranded. Previous studies suggested that structures at the 5’ end of RNAI might be involved in the translational regulation of the RNAI-encoded Fst (Greenfield et al., 2000a). The ribosome binding site for Fst is within a stem-loop. Although this stem-loop may be too weak to interfere with translation, helical stacking with the neighbouring helix might be sufficient to interfere with Fst translation. This possibility is currently under investigation.

Structure probing experiments were also performed on complexed par RNAs. The results of these experiments confirmed previous work showing that the 5’ direct repeat sequences and the 3’ terminator stem-loop were used in RNAI–RNAII complex formation (Greenfield et al., 2000b). It is of interest that the sequence between DRa and DRb also became paired in complexed par RNAs, suggesting that interaction at the 5’ end may extend inwards from each direct repeat, such that complete pairing occurs from the 5’ end of DRa through to the 3’ end of DRb. Even though this sequence is not completely complementary between RNAI and RNAII by standard Watson–Crick pairing, the presence of several potential G:U pairs will allow extension of interstrand pairing. Interestingly, this region is three nucleotides longer in RNAI than RNAII. In complexed RNAII, all of the nucleotides in this region are protected from Pb(II) cleavage, but Pb(II) probing of complexed RNAI shows pairing for all but nucleotides A38, A39, and A40. The fact that Pb(II) can still cleave these three nucleotides indicates that the lack of Pb(II) cuts in this region is not simply caused by inaccessibility of the site, but that complete pairing is occurring within this region with the exception of a three nucleotide bulge.

Complete pairing at the 5’ end of the RNAs probably contributes to the stability of the RNAI–RNAII complex. Previous studies indicated that par RNAs containing mutations in either direct repeat were unable to form a complex that could be visualized using standard gel shift analysis, yet these mutants were able to suppress fst translation in vivo (Greenfield et al., 2000b). Because unstable duplexes have been shown to inhibit target gene expression (Tomizawa, 1990; Wagner et al., 1992; Wagner and Brantl, 1998), it was believed that the direct repeat mutants formed an RNA complex that is stable enough to inhibit fst translation but too unstable to be resolved on a gel. Here, we demonstrate that these mutants do bind through interactions at the terminator stem-loop and the unaltered direct repeat (Figs 6 and 7). This RNA complex is probably a trapped binding intermediate in the par RNA binding pathway. Although this RNA complex cannot proceed to the final stable complex, it is a functional RNA complex capable of inhibiting Fst translation.

RNase probing was also used to follow the binding reaction between par RNAI and RNAII. By this method, the binding rate constant for par RNAs was determined to be \( k_1 \approx 1 \times 10^6 \text{M}^{-1}\text{s}^{-1} \). This falls within the range of association constants reported for other natural antisense RNA systems (reviewed in Wagner and Simons, 1994; Zeiler and Simons, 1998). Previous studies indicated that the binding rate constant for par RNAI–RNAII interaction was \( k_2 \approx 1 \times 10^5 \text{M}^{-1}\text{s}^{-1} \) (Greenfield et al., 2000b). The rate constant in these earlier studies was determined using traditional gel shift assays that rely on a complex stable enough to survive electrophoresis. Because the interaction between the par RNAs uses multiple dispersed sites, the functional complex may be less stable than other
antisense-target RNA pairs resulting in an underestimate of the effective binding rate constant by gel shift analysis. Therefore, the binding rate constant determined by RNase probing probably reflects more closely that required for a functional interaction of the par RNAs.

Investigations into a few antisense-target RNA binding pathways have shown that the interaction occurs in at least two distinct steps (Siemerig et al., 1994; Persson et al., 1990a,b; Tomizawa, 1984; 1986). The first step involves interaction between complementary stem-loops of the antisense and target RNAs. This initial interaction is stabilized by a second interaction between antisense and target RNAs. The second step is facilitated by the initial kissing interaction and leads to complete duplex formation. Here, we conclude that par RNAI and RNAII interaction also requires at least two steps. In the case of par, the terminator stem-loop is required for proper interaction of the two RNAs (Greenfield et al., 2000b) but is clearly not directly involved in inhibiting fst translation. Previous results also indicate that it is not required for the stability of the RNAI–II complex (Greenfield et al., 2000b). Therefore, it seems likely that it is essential for initiating the RNAI–RNAII interaction, particularly because RNAI contains a YUNR sequence within its terminator loop. The results reported here support this role for the terminator stem-loop. Competition analysis indicates that RNAs without the DRb repeat are capable of interfering with RNA complex formation, ruling out a role for DRb in initiation of binding. In addition, the terminator stem-loop was shown to be necessary and sufficient for kissing complex formation.

From this data, we propose the following model describing the binding reaction between par RNAI and RNAII (Fig. 8). The first step is the formation of the initial kissing complex between the sequences in the complementary loops of the 3' terminator stem-loops of RNAI and RNAII. The next step(s) involves conversion to an extended kissing complex by pairing between one or both direct repeat sequences located on the 5' ends of both RNAs. Whether DRa or DRb binds first is uncertain. The finding that DRa also contains the YUNR motif and that an RNA molecule containing just WT terminator loop and DRa sequences effectively inhibits par RNA interaction but an RNA with just WT terminator loop and DRb sequences does not (Fig. 5) suggests that DRa binding may be the first step to an extended kissing complex. This step most likely occurs very rapidly after the initial interaction between terminator stem-loops because the rate of protection from RNaseT1 cleavage appears to be similar for all three complementary regions (Fig. 4). Even though the extended kissing complex is not stable, it is apparently sufficient to inhibit fst translation. Finally, after the initial pairing between the direct repeats, intrastrand melting occurs within the 5' stem-loop of RNAII permitting complete interstrand pairing of the 5' sequences.

![Fig. 8](image-url)
The final structure of the RNAI–RNAII complex results in a side-by-side alignment of the terminator stems, which may explain the disappearance of the RNaseV1 cuts at nts 36–39 in the terminator stem-loop of RNAII (Fig. 2). It should be noted that the conclusions presented here are derived primarily from experiments performed in vitro. Therefore, it is possible that there are slight differences in the binding mechanism between par RNAI and RNAII in vivo. Nevertheless, our in vivo observations showing that an RNAII terminator mutant is unable to suppress fst translation but an RNAII direct repeat mutant is still functional is consistent with the overall binding mechanism in which the initial rate-limiting interaction occurring between the terminator stem-loops is followed by a second extended interaction at the 5′ direct repeats.

It is interesting that a system would require 3′ binding for the initial interaction because previous studies indicate that interaction at the 5′ end is necessary to inhibit fst translation (Greenfield et al., 2000b). Because interaction between the terminator stem-loops alone is not sufficient to inhibit fst translation, binding at the direct repeats has to occur immediately after initial kissing complex formation to effectively inhibit fst translation (Fig. 4). Rapid conversion from kissing to stable interaction has been shown for many antisense-regulated systems (Siemerig et al., 1994; Malmgren et al., 1997; Asano et al., 1998; Kolb et al., 2000). Unfortunately, this does not provide an explanation for the use of the 3′ terminator sequence to initiate binding. Because the function of the par PSK system is to kill plasmid-free daughter cells, a pool of RNAI must be present in each daughter cell. If RNAII immediately forms a stable complex with RNAI, presumably leading to degradation of the RNA complex, there would be no RNAI available for fst translation in plasmid-free cells. However, within the pool some form of translational regulation of fst must exist to prevent cell killing. As noted above, structures at the 5′ end of RNAI might provide this type of regulation. Because these structures are adjacent to DRb, they may provide a way of temporarily suppressing fst translation and impeding complete par–RNA interaction. A similar form of regulation has been described for the hok/sok system of plasmid R1 (Thisted et al., 1995; Nagel et al., 1999). Therefore, it is possible that a constant on–off pairing might occur between the terminator stem-loops of RNAI and RNAII, but complete pairing would be slowed by the presence of the 5′ structures. After processing or rearrangement of these structures, RNAI can either form a complex in the presence of RNAII or be translated and kill the cell in the absence of RNAII. In this way, the binding of the 3′ terminator stem-loop provides a ready supply of RNAII in plasmid-containing cells while maintaining the pool of RNAI necessary for par to function as a PSK system in plasmid-free cells.

Experimental procedures

In vitro transcription of par RNAs:

Transcripts were synthesized using T7 RNA polymerase (New England BioLabs) according to manufacturers directions. DNA templates for transcription of par RNAs are as described in (Greenfield et al., 2000a, b). pDAK901 and pDAK902 were used for the synthesis of WT RNAI and RNAII transcripts respectively. The RNAI DRa mutant was transcribed from pDAK913; the RNAI DRb mutant from pDAK911; and RNAI terminator mutant from pDAK912 plasmid DNA. The terminator deletion mutant was created by cutting pDAK901 with SpeI before transcription to remove the 3′ end of RNAI. pDAK904 was used to transcribe the heterologous RNA containing just the RNAI terminator (Greenfield et al., 2000b). Transcription reactions were run on 6%, polyacrylamide 6 M urea gels for 45 min at 350 V. Transcripts were gel purified by eluting with 0.5 ml of elution buffer (0.1 M sodium oxyacetate, pH 5.7, 10 mM EDTA, 0.5% SDS) at room temperature for 4 h. Gel debris was removed by centrifugation. The supernatant was extracted with phenol–chloroform, ethanol precipitated, and resuspended in RNase-free H2O (Diethyl pyrocarbonate-treated, Sigma).

5′ End-labelling of RNA

RNA (10 pmol) was dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs) in a total reaction volume of 10 μl. The reaction was incubated at 37°C for 15 min. The reaction was stopped by phenol extraction followed by ethanol precipitation. The pellet was resuspended in 6 μl of water, and 2 μl of [γ-32P]-ATP was added to the RNA along with 1 μl of polynucleotide kinase (PNK) and 1 μl of PNK buffer (New England Biolabs). Total reaction volume was 10 μl. The reaction was incubated at 37°C for 15 min. The reaction was subsequently gel purified as described above.

Pb(II) and RNase V1 probing of par RNAs

32P-end labelled RNA (50,000 c.p.m.) was partially digested with RNase V1 (Pharmacia) or lead acetate (Sigma) in TMN buffer (20 mM Tris OAc pH 7.5, 10 mM Mg(OAc)2, 100 mM NaCl) containing 5 μg carrier tRNA. Total reaction volume was 10 μl. For Pb(II) cleavage, 2 μl of lead acetate (either 4 mM or 40 mM) was added and the reaction was incubated at room temperature for 5 min. The reaction was stopped by adding 5 μl of 0.1 M EDTA followed by ethanol precipitation. For RNaseV1 digestion, 2 μl of RNase V1 (0.08 units μl−1) was added. The reaction was incubated at 37°C for the time indicated above each lane (either 3 or 15 min; Fig. 2). The digestion reaction was stopped by phenol–chloroform extraction followed by ethanol precipitation. The same procedure was used for RNase T1 (0.1 U μl−1) digestion. Pellets were resuspended in 10 μl of FD buffer (92% formamide, 17 mM Na2EDTA, 0.025% xylene cyanol). All samples were denatured at 90°C for 3 min before running on 12% (for RNAI) or 8% (for RNAII) polyacrylamide sequencing gels containing 9 M urea. Electrophoresis was carried out in TBE buffer at a constant 45 W for 1.5–2 h. To determine

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structures near the 3' end of RNAI, running time was extended to 4–5 h. After electrophoresis, gels were dried and exposed to Kodak Biomax X-ray film (Fisher Scientific) at −70°C. The alkali ladder was obtained by incubation of the end-labelled RNA in 0.5 M NaOH, 10 mM EDTA at 100°C for 15 s. The reaction was stopped by the addition of an equal volume of alkaline stop buffer (9.5 M urea, 85 M NaOAc, 1% glacial acetic acid) and a half volume of FD loading buffer. For probing experiments of the RNAI–RNAII duplex, a 10-fold molar excess of unlabelled RNAI or RNAII was added to reactions containing labelled RNAII or labelled RNAI respectively. The binding reaction was allowed to proceed for 15 min at room temperature before addition of Pb(II) or RNaseV1.

**Binding rate assay by RNase cleavage/protection**

5' End-labelled RNAII (10⁻⁹M) was incubated with a 10-fold molar excess of unlabelled RNAI in TMN binding buffer. Samples were taken at the time points indicated in Fig. 4 and immediately subjected to cleavage by 45 units of RNaseT1. Reactions were stopped by phenol–chloroform extraction and subsequently precipitated, washed with 80% ethanol, and resuspended in FD loading buffer. Samples were run on an 8% polyacrylamide sequencing gels as described above. Band intensities were determined by phospho-imager. Calculation of binding rate constants for the decay of cleavage products was carried out according to Persson et al. (1988).

**Inhibition of binding by mutant par RNAs**

Radiolabelled RNAI (10⁻⁹M) was incubated with unlabelled RNAII (10⁻⁶M) in the absence or presence of one of the competitor RNAI molecules (10⁻⁶M) listed in Fig. 5. Reactions were carried out in TMN binding buffer at 37°C for 15 min. Total reaction volume was 20 µl. Reactions were stopped by diluting with an equal volume of FD loading buffer on ice. Reactions were run on 5% polyacrylamide gels as described in Greenfield et al. (2000b). After electrophoresis, the gel was dried and autoradiography was performed overnight at −70°C.

**Demonstration of kissing complex formation**

End-labelled RNAII (10⁻⁶M) was incubated with unlabelled WT or mutant RNAI molecules (10⁻⁷M) in TMN binding buffer at 37°C. The total reaction volume was 10 µl because the binding rate constant for par RNA interaction was known. RNA concentrations and binding time were limited to identify kissing complexes that formed in a time frame similar to that observed for WT par RNAs. After binding for 1 min, the samples were diluted with 0.5 volumes of loading buffer (TMN buffer containing 30% glycerol, 0.05% bromophenol blue and 10 µg of RNA ml⁻¹) at 0°C and immediately loaded onto a native 6% polyacrylamide gel in TMN buffer. Electrophoresis was performed for 6 h (50 V) at 4°C with continuous recycling of running buffer to eliminate changes in pH. Autoradiography was performed overnight at −70°C.

**Computer prediction of RNA secondary structure**

RNA secondary structures were predicted using the Structure Analysis of RNA (STAR) software package (Van Batenburg et al., 1995) and drawn using the LOOPdLOOP drawing package (Gilbert, 1992).

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**References**


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