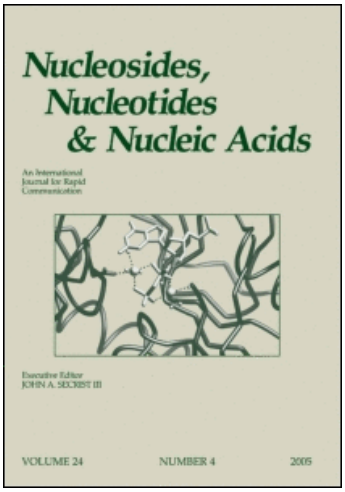

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ANTISENSE RNA IN REPLICATION CONTROL OF THE INC FII PLASMID R1

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Abstract: This paper gives a short summary of some approaches to investigate antisense RNA control in general, exemplified by studies of a particular model system - replication control of plasmid R1.

Introduction. Antisense RNA has been shown to be involved in a variety of control mechanisms in prokaryotes[1]. Even though there are great differences between the functions that are controlled as well as the mode of regulation, we would for this paper emphasize the similarities in order to approach the question of why countertranscripts are used at all. Clearly, in the cases studied in some detail, the genes whose expression is controlled by antisense RNAs are inhibited severely[2,3,4,5], indicating that these RNAs work efficiently. Half lifes for these molecules are generally short, which at least in plasmid replication control is a prerequisite for quick responses to copy number changes. Furthermore, the inhibition is very specific, a point to which we will return below.

Structurally, antisense RNAs are very similar. All of these RNAs are small (mostly 70-120 nt in length) and have a high degree of secondary structure, often with one long stem and a 6-7 base loop as the major specificity determinant. We have been interested in the question of what features are essential for a functional antisense RNA.

The CopA/CopT replication control system of plasmid R1. The model system we have chosen to work with is that of replication control in the IncFII plasmid R1[6]. Here, the main replication/copy number control is exerted by a ≈91 nt RNA, CopA RNA. Replication of this plasmid requires a rate-limiting plasmid-encoded function, the RepA protein. The synthesis of RepA is regulated negatively by CopA RNA. This RNA is synthesized constitutively from the DNA strand opposite to that encoding the leader region of the RepA mRNA such that both transcripts are completely complementary. CopA RNA's target on the mRNA is denoted CopT. Interaction between inhibitor and target affects translation initiation at the RepA ribosome binding site

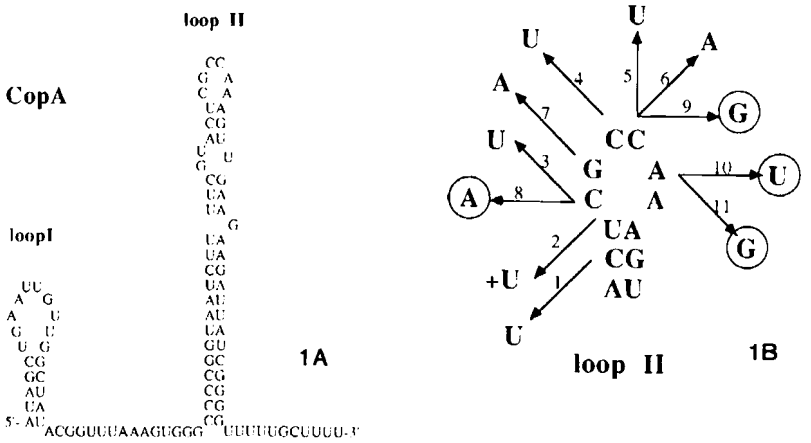

FIG. 1- Secondary structure of CopA RNA and the location of various mutations in loop II. Figure 1A shows the secondary structure of CopA RNA[8]. Figure 1B shows the base changes found in copy number mutants in several laboratories (references in 9). The circled bases were constructed using site-directed mutagenesis (see text, and Wagner, unpublished).

located some 80 nucleotides downstream. The mechanism for this indirect translational inhibition is so far unknown.

For studies of the properties of antisense RNAs one needs an assay system that permits qualitative and quantitative measurements of the two critical parameters of the reaction, effectiveness (rate) and specificity. Plasmid R1 offers these possibilities, since we can assay for CopA RNA's effect on the copy number of the plasmid[7]; we can measure repA expression as the activity of a repA-lacZ translational fusion under the control of the CopA/CopT system[3]. Finally we can investigate structure and function relationships of the inhibitor/target molecules with in vitro techniques.

Figure 1A shows the structure of CopA RNA as determined by nuclease mapping in solution[8]. Similar studies have been started to analyze the secondary structure of CopT RNA. Nuclease techniques and the localization of single-stranded regions by short deoxyribonucleotides complementary to target sequences followed by RNAse H cleavage have unambiguously identified both CopT loops (data not shown). This means that both inhibitor and target loops are exposed and single-stranded.

We have also used a gel shift assay (Figure 2) to measure the apparent second-order rate constant of binding between CopA and CopT RNAs in vitro[9,10] ; it was found to be $\approx 10^6\ M^{-1}s^{-1}$ at 37°. Copy number differences and incompatibility (inability of two plasmid species to coexist in a cell) between wild type and mutant plasmids in vivo are reflected faithfully in corresponding changes in the binding parameters measured in vitro (data not shown).

Structural features of CopA RNA and its function. High copy number mutants map almost exclusively in four of the six single-stranded bases of the major loop (II) or alter bases such that

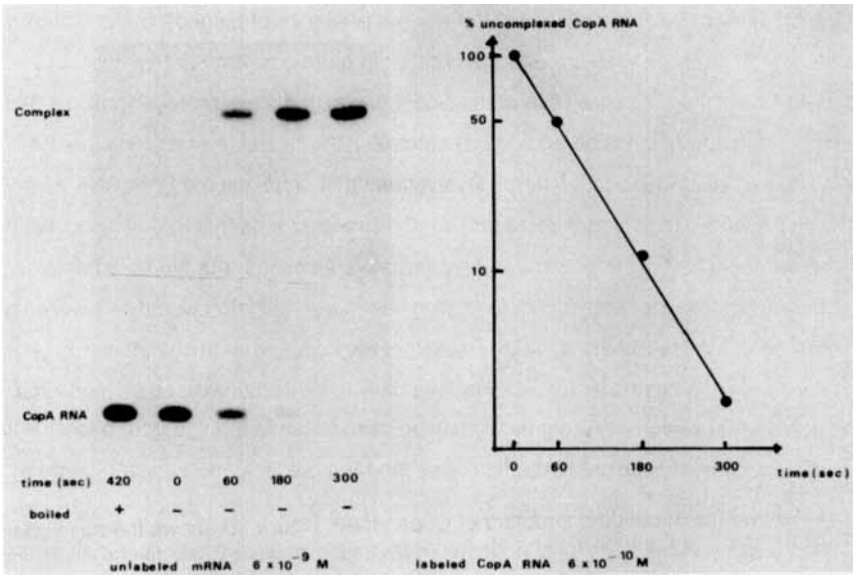

FIG. 2 - Kinetics of binding between CopA RNA and CopT RNA. Binding between radiolabelled CopA RNA and unlabelled CopT RNA was assayed as described[10]. The left-hand panel shows an autoradiogram of the binding reaction.The data are displayed on a lin-log scale in the right-hand panel. From the slope a pseudo-first order rate constant is derived. From this constant and the [CopT RNA], we calculate k_2 (the second order binding rate constant).

the loop size is affected (Figure 1B). From these mutants we conclude that it is both the primary sequence (at least the CGCC sequence) and the size of the loop that determines the rate of the interaction. In general, mutations changing a potential G-C base pair between inhibitor and target loops to an A-U base pair are assumed to decrease the binding rate. Two mutants with altered loop sizes have C-U base changes in the first C of the CopA loop II or in the C in the upper stem (Figure 1B, mutants 3 and 1). In the one case, a four base loop is created, in the other case the loop becomes very large (>12 nucleotides)[8]. Both of these alterations lead to higher copy numbers. This is especially interesting in the case of the second mutant which has the upper 8 nucleotides identical to the wild type. We therefore conclude that an appropriate presentation of the recognition sequences requires a loop size of (in this system) six nucleotides. We are at present testing these assumptions by analyzing novel mutants. These mutants are, unlike all previous mutants found in IncFII plasmids, not selected as high copy number mutants. Instead we have chosen to alter specific bases in the recognition loop using site-directed mutagenesis. In this way, phenotypically silent or very low-copy number mutants can be made and analyzed. Similarly we can insert single or multiple nucleotides in the inhibitor/target loops to increase the loop size.

A property of several antisense RNAs is the presence of bulged-out nucleotides in the upper stem structures. Bulges are often implicated in protein binding to highly structured RNAs[11]. In RNAI (the antisense RNA of the ColE1 plasmid) a small protein binds to a stem containing a bulged-out cytosine and helps to increase the rate of interaction with RNAII[12]. In plasmid R1, we have no indication for an involvement of a protein in the RNA/RNA interaction. In fact, the RepA leader region encodes a protein of a similar size as that in ColE1, but we have ruled out a comparable role for this protein[13]. An alternative function for bulged-out nucleotides could be that during binding (perfect) duplex formation with the target RNA would be favored over refolding of the (imperfect) helix in CopA RNA, thereby driving the reaction towards completion, once helix nucleation has taken place. Experimentally this question can be tackled in the same way as above. Site-directed mutagenesis may be used to remove bulged-out bases or to insert complementary bases opposite to the first ones, and the resulting mutants can be analyzed.

Are stem-loops really necessary? So far we have argued that sequence and structure of CopA RNA are important for its function as an inhibitor. However, we lack data that permit us to answer the question whether a high secondary structure in general is favorable for rate and/or specificity. We have to remind ourselves that the existence of stable hairpin loops in all antisense RNAs might simply reflect the necessity to terminate transcription correctly. In principle, therefore, it is conceivable that binding between a largely unstructured antisense RNA and a target RNA is equally effective or even faster than for CopA/CopT. Here, because of the need for a transcription termination structure, in vivo experiments are not feasible. Using the gel shift assay in vitro we will be able to test the binding between two complementary RNAs from the RepA coding region (which is not tailored for high secondary structure) which we subcloned in opposite orientation in two SP6-vector plasmids[14]. In the same way, we can test very highly structured RNAs such as tRNA molecules and their ability to form a duplex with an in vitro transcribed "anti-tRNA".

Specificity. The interaction between the recognition loops of CopA and CopT RNA is of high specificity. We know that many mutations in these bases not only affect the copy number but also create new incompatibility groups, meaning that a wild type and a mutant replicon do not interfere with each other's replication inside the bacterial cell. This means that one base change (out of ≈91 bases) is sufficient to prevent hybridization to the heterologous target. Such a specificity hints at a small number of bases being involved in the primary (recognition) reaction. We have proposed[9] that the initial binding might involve triplets of bases in CopA and CopT RNA's loops II.

Mechanism of CopA/CopT binding. The kinetics of binding has been studied for both wild type and mutant combinations of CopA/CopT RNA[9,10]. We lack, however, an understanding for the details of the reaction pathway. The recognition reaction takes place at the main loop II, but the site for the nucleation step in helix formation is unknown. Antisense RNAI and RNAII of ColE1

form a recognition contact between loops, followed by nucleation starting from the free 5'-end of RNAI[15]. Such a pathway is unlikely for CopA RNA, since the 5'-end is buried in a stem. In vitro binding assays using fragments of CopA RNA obtained after oligodeoxyribonucleotide-directed RNAse H cleavages will help us to decide what parts of the molecule are required.

Translation inhibition by CopA RNA. Apart from the general properties of antisense RNAs that can be investigated studying the CopA/CopT system as discussed above, we would like to emphasize the specific interest of this system for the understanding of indirect translational inhibition in control of replication. The presumed site of inhibition, the ribosome binding site of the repA gene, is located some 80 nucleotides downstream of the part of the leader RNA that is involved in binding of CopA RNA. How then can an RNA/RNA interaction in one region of a molecule affect a distal event? So far, no experimental data are available to answer this question. The only detailed hypothesis has been proposed by Rownd et al.[16]. Based on RNA folding predictions, they suggested that RNA-E (identical to CopA RNA) binding in the CopT region would result in an RNA folding change in the region of the ribosome binding site of the repA gene. Experiments are needed to test this hypothesis .

We believe that antisense RNA control systems are of increasing importance in prokaryotic gene expression. We suggest, that the thorough study of a well-defined system of the type described above, will yield results that contribute to an understanding of the general aspects of antisense RNA control as well as of the particular role these RNAs play in the function they regulate.

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