Loop Swapping in an Antisense RNA/Target RNA Pair Changes Directionality of Helix Progression*

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Jacoba G. Slagter-Jäger and E. Gerhart H. Wagner‡
From the Institute of Cell and Molecular Biology, Department of Microbiology, Biomedical Center, Uppsala University, Box 596, Husargatan 3, S-75124 Uppsala, Sweden

The binding pathway of the natural antisense RNA CopA to its target CopT proceeds through a hierarchical order of steps. It initiates by reversible loop-loop contacts followed by unidirectional helix progression into the upper stems. This involves extensive breakage of intramolecular base pairs and the subsequent formation of two intermolecular helices, B and B'. Based on the known tRNA anticodon loop structure and on results from the Sok/Hok antisense/target RNA system, it had been suggested that a U-turn (or π-turn) in the loop of CopT might determine the directionality of helix progression. Data presented here show that the putative U-turn is one of the structural elements of antisense/target RNA pairs required to achieve fast binding kinetics.

Swapping of the hypothetical U-turn structure from the target RNA to the antisense RNA retained regulatory performance in vitro but altered the binding pathway by changing the direction in which the initiating helix was extended. In addition, our data indicate that a helical stem immediately adjacent to the target loop sequence is required to provide a scaffold for the U-turn.

Antisense RNAs regulate gene expression in many bacterial systems. A small number of antisense RNAs are known to be encoded by the Escherichia coli chromosome, although recent genome-wide screens have identified many new non-coding RNAs that also may regulate target RNAs by base pairing (1–3). By far, the majority of antisense RNA systems and the most well characterized cases are found in accessory genetic systems for an elucidation of the biology as well as the biochemistry of antisense RNA control. In particular, their antisense and/or structure have been shown to determine binding rate and/or structure have been shown to determine binding rate constants followed by unidirectional helix progression into the upper stems. This involves extensive breakage of intramolecular base pairs and the subsequent formation of two intermolecular helices, B and B'. Based on the known tRNA anticodon loop structure and on results from the Sok/Hok antisense/target RNA system, it had been suggested that a U-turn (or π-turn) in the loop of CopT might determine the directionality of helix progression. Data presented here show that the putative U-turn is one of the structural elements of antisense/target RNA pairs required to achieve fast binding kinetics.

Swapping of the hypothetical U-turn structure from the target RNA to the antisense RNA retained regulatory performance in vitro but altered the binding pathway by changing the direction in which the initiating helix was extended. In addition, our data indicate that a helical stem immediately adjacent to the target loop sequence is required to provide a scaffold for the U-turn.

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‡ To whom correspondence should be addressed: Dept. of Microbiology, Institute of Cell and Molecular Biology, Biomedical Center, Uppsala University, Box 596, Husargatan 3, S-75124 Uppsala, Sweden. Tel.: 46-18-4714866; Fax: 46-18-5309396; E-mail: Gerhart.Wagner@icm.uu.se.

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ers with desired base changes. The mutated copA/copT sequences were transferred to pGW177L (repA-lacZ translational fusion plasmid) (25), resulting in plasmids of the pGW177-mut-III-L series. To construct low copy CopA donor plasmids (pGB2-CopA series), copA was excised from plasmids of the pGW643 series (HindIII and EcoRI) and ligated into HindIII/EcoRI-cleaved pGB2 (pSC101 vector) (27).

In Vivo repA-lacZ Expression Assays—RepA-LacZ fusion protein synthesis was measured in extracts from exponentially growing cultures of *E. coli* strain D95a as described (25).

Preparation of CopT, CopA, and CopI RNA—CopT, CopA, and CopI (only stem loop II of CopA) RNAs were transcribed in vitro from PCR-generated templates by T7 RNA polymerase. Long CopT transcription templates are as follows: primer T7G3 (5′-GAA ATT AAT ACG ACT CAC TAT AGG GGT AAG GTA TTT GGC TGG-3′ with T7 promoter underlined) and primer SeqP/II (5′-GGT CCG GTT CTT TA-3′). Short CopT templates (for lead(II) hydrolysis and ethynylthiouracilessay) are primers T7G3 and GW141M (5′-GCT ATA CGG TTT AAG TGG-3′). Cop templates are primers T7S1 (5′-GAA ATT AAT ACG ACT TAC TAT ACG GCC CCG GTA ATC TTG TCG T-3′) and T7E1 (5′-AAA CCC CGA TAA TCT TCT TCA-3′), and CopA templates are primers T7SA (5′-ATT AAT ACG ACT CAC TAT AGG GTA TTT TTG GGC TGG-3′) and Cop (or CopI) was labeled by [32P]UTP. RNAs were purified as described previously (6, 13) and dissolved in TMN buffer (20 mM Tris-acetate, pH 7.6, 10 mM magnesium acetate, 100 mM NaCl).

CopA-CopT Binding Rate Constants—The binding of uniformly labeled Cop (0.13 nm) to an excess of unlabeled CopT (2 nm wild type, Flip, or Mut2; 20 nm Rev, Mut1, or Mut3) was performed at 37 °C. Aliquots were withdrawn at different times, stopped by addition of formamide dye, and separated on 6% denaturing polyacrylamide gels. Bands corresponding to the CopA-CopT complex and free CopA and CopT were quantified by PhosphorImager. The initial rates of binding were calculated for each concentration of inhibitor present. From graphs of ∆A/∆t, the initial rate in absence/initial rate in presence of inhibitor) against the inhibitor concentration, the inhibition constant 

Structure Probing—*Pb2+*-induced hydrolysis was carried out on endlabeled CopT or CopA, free or in complex (8). Reactions contained TMN buffer, 1 μg of carrier tRNA, and 8 mM Pb2+ acetate. Samples were incubated for 2.5 min at 37 °C, stopped by addition of loading buffer, and run on 15% sequencing gels. Limited RNase T1 (Pharmacia) cleavages on 3′-end-labeled CopT or 5′-end-labeled CopA, free or in complex were performed in 10 μl of TMN for 7.5 min at 37 °C in the presence of 1 μg of carrier tRNA and 0.1 unit of the enzyme. RNase V1 (Pharmacia) cleavages followed the same protocol, but reactions were stopped after 4 min and 0.0025 units of enzyme were used. Ethynylthiouracilessay was performed on 3′-end-labeled CopT was done as described previously (21).

RESULTS

The Loop-swapping Mutant Retains Efficiency of Control in Vivo—To assess regulatory activity of CopA in vivo, RepA-LacZ fusion protein synthesis was measured in extracts from exponentially growing cultures. Fusion plasmids (pGW177-III-L series) carried a mutated copA promoter (28), and CopA was provided in trans from a compatible plasmid (pGB-CopA series). Two mutations were created to change the loop sequences. In Flip, the loops of CopA and CopT were swapped, transferring the putative U-turn motif from CopT to CopA. In Rev, the upper eight nucleotides were inverted so that a YUNR sequence is absent from either RNA. As a control, we measured relative intracellular RNA levels by Northern blot analysis. None of the mutations significantly affected the abundance of either the antisense or the target RNAs (data not shown).

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**Fig. 1.** Binding pathway of CopA and CopT. A, free CopA and CopT interact at bases within their hexanucleotide loop sequences to form a reversible intermediate. B, complex formation proceeds by helix progression into the upper stems, resulting in the formation of helix B (C) and subsequently helix B′ (D) and helix C (E).

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1 The abbreviations used are: Mut, mutant; WT, wild type.
patterns of Pb2, below), was also probed. They disrupt the upper (Mut1), lower to investigate the effect on loop structure presentation (see right panel (Fig. 2), loops. Binding rates require initiation to be confined to the recognition loops were located adjacent to two upper stem segments bordered by bulges (Fig. 2). Secondary structures of 3’-end-labeled Flip and Rev mutant CopT RNAs were analyzed by limited Pb2-induced hydrolysis and RNase T1 cleavage (Fig. 2, right panel). Another set of three mutations, designed to test whether the presentation of the secondary structures of Wild-type CopA/CopT pairs behaved identically. The Rev mutant pair was severely impaired (Flip/Rev, WT/WT). Impairment of wild type and mutant CopA/CopT pairs is given in Fig. 3 indicate that the U-turn in affecting the directional preference of helix progression.

### Structural Probing of Wild-type and Flip CopA/CopT Complexes—Because loop swapping maintained wild-type-like activity in vitro and binding rates in vitro despite a changed binding pathway, we asked whether the secondary structures of the inhibitory complexes were similar. Complexes were subjected to Pb2-induced hydrolysis or limited cleavage by RNase T1 (specific for unpaired G residues), or RNase V1 (double-stranded/stacked regions). Probing results indicated that the overall four-helix junction structure of the Flip mutant RNA pair was similar to that of the wild type (Fig. 4). Differences between mutant and wild-type complexes were confined to the lengths of helices B and B’ and the connecting loop; helix B’ of the Flip RNA pair appears to have increased (from 6 to 8 base pairs) and helix B to have decreased (from 9 to 8 base pairs).

### Proper Loop Presentation Requires the Upper Stem Segment—Mutations Mut1-3 (disrupting the upper, lower, or both stem segments located above the lower bulges in CopA and CopT) were designed to test whether the presentation of the primary recognition sequences within the loop requires a structural scaffold. Thus, the mutant RNAs carry identical initiating loop sequences but are presented in different structural contexts. Probing results supported the expected accessibility
changes in the stem segments (Fig. 2). RepA-LacZ fusion protein synthesis was measured to study the regulatory performance of these mutants in vivo (Table I). In the absence of CopA, all of the plasmids gave similar high specific activities (pSP64 + pGW177-mut-L series). Strikingly, a supply of cognate CopA in trans resulted in wild-type-like inhibition for Mut2 but poor inhibition for Mut1 and Mut3, indicating the importance of the stem segment immediately adjacent to the recognition loop. Disruption of this stem segment (in Mut1 and Mut3) may prevent the formation of a scaffold for the U-turn loop structure.

Apparent binding rate constants, determined in vitro, were congruent with the in vivo results. The $k_{\text{app}}$ value of the Mut2 RNA pair was close to that of the wild type, whereas Mut1 and Mut3 were severely impaired (14- and 76-fold lower than wild type, respectively) (see Table II).

**DISCUSSION**

For regulation of plasmid copy number, fast binding kinetics is crucial for the regulatory efficiency of antisense RNAs. Formation of inhibitory complexes is different from simple hybridization reactions among complementary strands. In particular, most antisense RNAs fail to form full duplexes on a biologically relevant time scale but proceed through defined stages of topologically permitted folding pathways to arrive at very complex structures (9, 15, 32, 33). In plasmid R1, the steps through the CopA-CopT binding pathway (Fig. 1) have been elucidated by mutational, structural, and kinetic analyses. One noteworthy feature is that unidirectional helix progression into the upper stem, forming helix B, precedes the formation of the opposing intermolecular helix, B', resulting in a stable complex (11, 12). This paper addresses the effect of the loop structure in CopT and its proper presentation on the ordered folding pathway.

In almost all of the antisense/target RNA systems encoded by bacterial accessory elements, one of the interactant loops carries a sequence motif, UVR or YUNR, that is predicted to form a U-turn (21, 22). Such structures in anticodon loops of tRNAs favor rapid and accurate decoding of mRNA (18, 19). Specific interactions between antisense and target RNAs also require high rate and specificity for maximal regulatory efficiency. The rate-enhancing properties of a putative U-turn have been supported by studies of the Hok/Sok system. Mutation of the invariant uracil had a dramatic effect on the binding rate (21). Strongly reduced association rates were also obtained for the loop-loop complexes between correspondingly mutated RNAI/ RNAII of ColE1 (34), Incl/RepZ of ColIb-P9 (16), and RNA- OUT/RNA-IN of IS10 (35). In line with this finding, the inversion of CopA-CopT loop sequences (Rev lacking YUNR motif) resulted in a 13-fold lower binding rate and impaired in vivo control (Tables I and II). By contrast, swapped loops (Flip)

![Fig. 2. Secondary structures of CopT mutant RNAs.](image2)

![Fig. 3. Competitive inhibition assay.](image3)
retained wild-type-like performance, indicating that the presence of the beneficial loop structure was required, but that it may be located either in CopT (wild type) or CopA (Flip). The Flip mutation addresses an additional point. The U-turn in CopT would present the GGC sequence immediately following the invariant U in a helical conformation available for interaction with the complementary sequences in CopA. This sequence is known to be the site at which binding initiates (16, 36). Extension of this helix from the uridine into the upper stem generates helix B. If so, the presence of a U-turn in CopA instead of CopT (Flip mutant) should reverse the overall direction of helix progression so that helix B' is formed first. The competition experiment in Fig. 3 indicates that this was the case. Thus, the folding pathway was altered in the Flip mutant, although the rate of stable complex formation was the same as that in the wild type. Complexes formed were similar in wild-type and Flip RNA pairs (Fig. 4) and conformed to the same overall structure as deduced for a number of related plasmids (see Ref. 37).

From the data in Table II, it is also clear that the stability ($K_D$) of kissing complexes is not proportional to binding rates or in vivo control. This is in agreement with results from earlier studies in which CopA:CopT loop size mutants were analyzed (23) and with studies in the ColE1 plasmid system where inversion of a wild-type loop sequence in RNAI:RNAII resulted in 7000 times more stable complexes but strongly decreased binding rate (34). Hence, rapid transient interaction among properly presented loop structures is a prerequisite for maximal binding rates.

Presentation of a U-turn structure should be dependent on base pairing of the adjacent upper stem nucleotides. Mutational disruption of the upper three base pairs (Fig. 2, Mut1) resulting in large unstructured loops indeed has a profound effect on binding rate (Table II) and in vivo control (Table I), even though the stability of a kissing complex is not affected significantly (Table II). Mutation 3, breaking both helices, had an even more severe phenotype. By contrast, disruption of a more distal helical segment (Mut2) resulted in wild-type-like activity (Tables I and II). From this observation, we conclude that a structural scaffold is required to present a U-turn loop structure for rapid interaction. Interestingly, the upper stem elements represent a compromise. They have to be sufficiently destabilized not to impede the invasion of the upper stem following initial binding, yet they must provide a base-paired stacked stem for the presentation of the U-turn.

Are these features common to other plasmid systems? It has recently been proposed that antisense and target RNAs of plasmids of the IncI, IncB, IncZ family, as well as those related to R1, form structurally equivalent complexes (37). All of the target RNAs carry identical loops (including the UUGG motif) and have upper stems that are predicted or have been demonstrated to be unstable because of bulges and internal loops. On the other hand, the sequences in these stem regions vary significantly, giving rise to plasmid compatibility. This implies that the formation of inhibitory complexes must be preceded by base pairing throughout the upper stem regions. Ultimately, interstrand helix progression becomes arrested because of the topological stress that stems from the restrictions imposed by the connecting loops. In CopA:CopT, the four-helix
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The presence of U-turn structures in antisense or target RNAs is so far circumstantial but can be inferred from similarities to tRNAs (17). Experimental support has only been obtained for the hook mRNA target stem loop by probing with ethylisourea (21). Using the same approach, CopT RNA failed to yield a corresponding signature (data not shown), which may not be surprising since also some tRNAs failed to show the expected modification pattern (38, 39). However, several observations suggest the presence of U-turns in antisense or target RNAs: 1) the similarity in preferred loop sizes to those of tRNA anticodon loops along with similar functional requirements (rapid and accurate recognition of a complementary sequence) (22); 2) YUGG sequence motifs are ubiquitously present in equivalent positions in antisense/target recognition loops (21); 3) the UUGG motif is invariant in the target (or antisense (40)) loop sequences of many plasmids (41) although neighboring sequences vary; and 4) mutations predicted to abolish the U-turn result in impaired regulation (16, 21, 34, 35). Additionally, the nucleotide 5′ of the invariant U forms a U-G wobble pair in CopT (23), restricting the size of the target loop to four nucleotides (Fig. 2). Two hexaloop U-turn structures from ribosomal RNAs have been determined by NMR. One of these carries a U-U, and the other carries a G:A closing base pair at the equivalent position (42, 43). In the anticodon loop of tRNAs, the first base is usually involved in a non-canonical base pair with the seventh nucleotide of the loop (44). These similarities strongly argue for a U-turn structure in the CopT loop.

In conclusion, rapid interaction among complementary folded RNAs is dependent on properly presented loop structures. U-turns provide a restricted set of bases for initiation and a nucleation site for subsequent progression to more stable and inhibitory structures. The almost invariant UUGGNN loop sequences of antisense RNAs, presented on scaffolds provided by upper stem segments (of variable sequence), indicate that U-turn structure motifs are evolutionarily selected (22, 41). Structural studies are under way to gain insights into the exact geometry of U-turn structures in CopT and other antisense/target RNAs.

REFERENCES