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

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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 vivo and binding rates 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 elements such as plasmids, transposons, and phages (4, 5). Plasmid copy number control systems have served as excellent models for an elucidation of the biology as well as the biochemistry of antisense RNA control. In particular, their antisense and target RNAs provide models for RNA-RNA interactions, folding pathways, and binding kinetics.

The antisense RNA CopA of plasmid R1 controls the replication rate by inhibiting initiator protein (RepA) translation. Previous studies show that 1) binding of CopA to its target CopT, the complementary RNA segment in the repA mRNA leader, occurs at a binding rate constant of ~10⁶ M⁻¹ s⁻¹ (6, 7); 2) binding is on-rate-controlled rather than determined by KD (7); 3) the product of the binding reaction is not a fully paired RNA duplex but a four-helix junction structure (8, 9); and 4) this structure blocks initiation of repA translation (10). The CopA-CopT binding pathway is shown in Fig. 1. Subsequent to loop-loop recognition (“kissing”) (Fig. 1, A and B), the resulting short RNA-RNA helix is extended “unidirectionally” into the upper stem regions to form helix B (Fig. 1, B and C). Helix B must form prior to helix B’ (Fig. 1, C and D) (11) to promote a side-by-side alignment of helices that permits helix C to form (Fig. 1E) (12). Destabilized upper stems of CopA and CopT are crucial for unimpeded helix progression (12–14). Thus, the series of steps that ultimately result in a stable inhibitory complex follows a defined folding pathway that is constrained by topology. Full duplex formation occurs at very low rates and is not required for inhibition (8, 15).

What drives the directionality of the rapid extension of the initial short loop-loop helix? It has been proposed that the target stem loop of plasmid ColIb-P9 (loop sequence identical to that of CopT) might form a U-turn (16), a structural element first described in the anticodon loop of yeast tRNA⁵⁰⁰⁰ (17) where it promotes rapid and specific decoding (18). In tRNA, its structure is characterized by a sharp turn in the phosphodiester backbone 3’ of the invariant U. This conformation is stabilized by intraloop bonds and presents the anticodon in a preformed A-helical conformation predisposed for binding to the codon in a mRNA. Disruption of intraloop hydrogen bonds involving the invariant U33 or substitution of this uridine by a bulky pyrimidine strongly reduced codon-anticodon recognition (19). In the Sok/Hok antisense/target RNA system (20), a U-turn acts as a binding rate enhancer (21). The ubiquitous presence of the characteristic sequence motif YUNR (Y = pyrimidine, N = any nucleotide, R = purine) in antisense or target RNA loops suggests its importance for efficiency of control (22). We speculated that the corresponding motif in CopT (loop sequence 5’-UUGGCG with YUNR underlined) might additionally bias the directionality of helix progression (11).

Here, we addressed this question by exchange of the CopT and CopA loop sequences.

Recognition among antisense-target RNAs (23), tRNA-mRNA (18), or human immunodeficiency virus dimers (24) requires optimal presentation of the nucleotides involved in initiation of binding. Interacting loops are presented as defined three-dimensional structures. Changes in CopA-CopT loop size and/or structure have been shown to determine binding rate (23). In this paper, we report on the importance of the upper stem segments in CopA and CopT for proper presentation of the U-turn loop structure.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Mutations in copA/copT were introduced in plasmids pGW58-III (R1 control region; copA promoter mutation) (25) and pGW643 (pUC vector; copA gene) (26) using a site-directed mutagenesis kit (QuikChange, Stratagene) and PCR primers.

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ers with desired base changes. The mutated copA/copT sequences were transferred to pGW171L (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 were 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* 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 GTT AAG GAA TTT TGT GGC TGG-3' with T7 promoter *underlined*) and primer SeqP/II (5'-GGTT GCG GTT CTT TA-3'). Short CopT templates (for lead(II) hydrolysis and ethylnitrosourea assay) are primers T7G3 and GW141M (5'-GCT ATA CGG TTT-3'). CopI templates are as follows: primer T7SI (5'-GGTT GATT AAT ACG ACT CAC TATAG GCC CCG GTA ATC TTT TCG T-3') and T7EI (5'-AAA CCC CGA TAA TCT TCT TCA-3'), and CopA templates are primers T7SA (5'-GAA ATT AAT ACG ACT CAC TAT AGT AGC TGA ATT GTT GGC TGG-3') and primer SeqP/II (5'-GGTT GCG GTT CTT TA-3').

**CopT CopT Binding Rate Constants**—The binding of uniformly labeled CopA (<0.1 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 were quantified using a PhosphorImager (Amersham Biosciences). From a graph depicting the percentage of free CopA as a function of incubation time, the second-order binding rate constant, \( k_{app} \), was calculated as described previously (6, 23).

**Equilibrium Dissociation Constants of CopI-CopT Pairs—**Unlike CopA-CopT complexes that are stable in denaturing gels, CopI-CopT-kissing complexes require non-denaturing gels. Binding reactions of uniformly labeled CopI (0.13 nM) and different concentrations of unlabeled CopT (0, 2, 3, 4, 7, 9, 13, 18, 75, and 37.5 nM) were performed at 37 °C for 10 min. Samples were analyzed on 7% non-denaturing polyacrylamide gels run in TMN buffer, and bands subsequently were monitored by PhosphorImager as above. From these experiments, equilibrium dissociation constants (\( K_d \)) were determined (23).
patterns of Pb2, or both stem segments (Mut3). The observed cleavage below), was also probed. They disrupt the upper (Mut1), lower
binding rates require initiation to be confined to the recognition
3. Secondary structures of loops.
Not directly proportional to the stability of kissing complexes.
control is reflected by CopA whereas the rate constant of the Rev RNA pair was decreased
by limited Pb2 segments bordered by bulges (Fig. 2). Secondary structures of
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3. Mutations resulted in the predicted accessibility changes
b)

\[ \text{TABLE I} \]

| Plasmids \(^a\) | CopA/CopT pairs | Specific \(\beta\)-gal activity \(^b\) | Relative \(\beta\)-gal activity \(^b\) |
|----------------|----------------|--------------------------------|---------------------------------|
| pSP64         | WT/WT          | 1856 ± 397                    | 0.29                            |
| pSP64 + pGW177-L \(^b\) | WT/WT          | 1856 ± 397                    | 0.29                            |
| pSP64 + pGW177-III-L | WT/WT          | 1624 ± 2309                   | 1.00                            |
| pSP64 + pGW177-Mut1-III-L | Mut1          | 6224 ± 1949                   | 0.98                            |
| pSP64 + pGW177-Mut2-III-L | Mut2          | 6206 ± 1679                   | 0.98                            |
| pSP64 + pGW177-Mut3-III-L | Mut3          | 5782 ± 848                    | 0.91                            |
| pSP64 + pGW177-Flip-III-L | Flip          | 5292 ± 1079                   | 0.84                            |
| pSP64 + pGW177-Rev-III-L | Rev           | 6034 ± 1156                   | 0.95                            |
| pGB2-CopA     | WT/WT          | 190 ± 47                      | 0.03                            |
| pGB2-CopAflip + pGW177-Flip-III-L | Flip/Flip | 150 ± 20                      | 0.02                            |
| pGB2-CopArev + pGW177-Rev-III-L | Rev/Rev | 4200 ± 688                    | 0.06                            |
| pGB2-CopAmut1 + pGW177-Mut1-III-L | Mut1/Mut1 | 2706 ± 328                    | 0.45                            |
| pGB2-CopAmut2 + pGW177-Mut2-III-L | Mut2/Mut2 | 258 ± 43                      | 0.04                            |
| pGB2-CopAmut3 + pGW177-Mut3-III-L | Mut3/Mut3 | 4648 ± 107                    | 0.74                            |

\(^a\) Fusion plasmids and CopA-donor plasmids. pGB2-CopA carries the copA gene. The pGW177-III-L series of plasmids carry the control region of plasmid R1 (45). Mutation III inactivates the copA promoter (25).
\(^b\) Plasmid pGW177-L carries the wild-type copA/copT region, i.e. produces CopA even in absence of donor plasmid.
\(^c\) Miller units (46).

\[ \text{TABLE II} \]

| CopT     | CopA/\(^b\) | \(k_{\text{app}}\) \(^b\) | Impairment relative to wild type | \(K_d\) \(^b\) | Increase relative to wild type |
|----------|-------------|----------------|-------------------------------|--------------|-------------------------------|
|          | (\(\text{s}^{-1}\)) \times 10^6 | \(\text{nm}\)                                      |                                        |              |                                |
| WT       | WT          | 106 ± 11       | 1\(\times\)                   | 2.5 ± 0.5    | 1\(\times\)                   |
| Mut1     | Mut1        | 7.4 ± 1.9      | 14\(\times\)                  | 2.5 ± 0.5    | 1\(\times\)                   |
| Mut2     | Mut2        | 58 ± 9.1       | 2\(\times\)                   | 2.5 ± 0.5    | 1\(\times\)                   |
| Mut3     | Mut3        | 1.4 ± 0.1      | 76\(\times\)                  | 4.5 ± 0.5    | 1.5\(\times\)                 |
| Flip     | Flip        | 103 ± 12       | 13\(\times\)                  | 3 ± 0.5      | 1\(\times\)                   |
| Rev      | Rev         | 8 ± 0.7        | 13\(\times\)                  | 8.5 ± 1.5    | 3\(\times\)                   |

\(^b\) CopA was used for binding rate measurements, CopT for \(K_d\) determinations.

\(^c\) Values for \(k_{\text{app}}\) were based on four measurements, and values for \(K_d\) were from two experiments.

The Putative U-turn in CopT Determines the Directionality of Helix Progression—Previous studies had shown that the initial loop-loop helix progresses rapidly toward the 5’ side of the upper stem in CopA (11, 12). If the 5’ to 3’ direction of the YUNR U-turn motif drives the direction of helix progression toward helix B (Fig. 1) in the wild type, one would expect helix B’ to be formed first in the Flip mutant RNA pair (loops swapped). This was tested by a competitive inhibition experiment (11, 31, 32). Two oligoribonucleotides, R5 and R6, complementary to either side of the upper stem of CopT-Flip were designed as competitive inhibitors (Fig. 3). Initial rates of the binding of CopA-Flip to CopT-Flip were determined in the presence of varying concentrations of R5 or R6. \(K_d\) values (calculated as described under “Experimental Procedures” and Ref. 11) were determined to be \(\sim \text{13 nm}\) (R5) and \(>\text{150 nm}\) (R6). Thus, competitive binding to the ascending side of CopT-Flip interferes more strongly with CopA-CopT binding, whereas the relative inhibitory effect is opposite for CopT-WT (R4, 7 nM; R3, >0.2 \(\mu\)M), i.e. the relative efficiency of competitors targeting the left (forming helix B’) and right side (helix B) was reversed between Flip and wild-type RNAs (arrows in Fig. 3 indicate direction of first helix formed). This is consistent with a role for 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 vivo 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, UNR 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), IncI/RepZ of ColIb-P9 (16), and RNAOUT/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)
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

![Fig. 4. Structural probing of Flip and wild-type CopA-CopT complexes. Right, autoradiogram of probing experiment. C, control; P, lead(II) acetate; T, RNase T1 cleavages; V, RNase V1 cleavages; L, hydrolysis ladder. Limited chemical or enzymatic cleavages were performed on 5'-labeled CopA, free or in complex with CopT, as indicated. Nucleotides around the respective loop regions are indicated to the left and right of the autoradiogram. Left, secondary structure models. Red, CopA sequences; blue, CopT sequences. Circled nucleotides indicate mutational changes in Flip.](image.png)
junction is situated at the position of the lower bulge in CopA (Fig. 4), as in the case of the Inc-RepZ complex (37).

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 hok mRNA target stem loop by probing with ethylnitrosourea (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) YUNG 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 disrupt 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 UUGGGN 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.

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