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Communication between RNA folding domains revealed by folding of circularly permuted ribozymes

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Abstract

To study the role of sequence and topology in RNA folding, we determined the kinetic folding pathways of two circularly permuted variants of the *Tetrahymena* group I ribozyme, using time-resolved hydroxyl radical footprinting. Circular permutation changes the distance between interacting residues in the primary sequence, without changing the native structure of the RNA. In the natural ribozyme, tertiary interactions in the P4–P6 domain form in 1 second, while interactions in the P3–P9 form in 1–3 min at 42 °C. Permutation of the 5' end to G111 in the P4 helix allowed the stable P4–P6 domain to fold in 200 ms at 30 °C, five times faster than in the wild type RNA, while the other domains folded five times more slowly (5–8 min). By contrast, circular permutation of the 5' end to G303 in J8/7 decreased the folding rate of the P4–P6 domain. In this permuted RNA, regions joining P2, P3 and P4 were were protected in 500 ms, while the P3–P9 domain was 60–80% folded within 30 s. RNase T1 digestion and FMN photocleavage showed that circular permutation of the RNA sequence alters the initial ensemble of secondary structures, thereby changing the tertiary folding pathways. Our results show that the natural 5'-to-3' order of the structural domains in group I ribozymes optimizes structural communication between tertiary domains and promotes self-assembly of the catalytic center.

INTRODUCTION

An unresolved question is the extent to which the linear organization of RNA domains within a gene determines the final structure of the RNA. This question is important for understanding the optimization of genes that code for stable RNAs during molecular evolution. Several studies have shown that RNA encoding genes can be scrambled, without complete loss of function.^{1; 2; 3} However, the 5' to 3' order of structural motifs, and the sequence separation between nucleotides which must base pair with each other, can alter the RNA folding pathway, and thus the fitness of the gene.

The order in which the structural elements of an RNA are encoded in its sequence is important to its folding kinetics, because interactions between neighboring nucleotides are kinetically and thermodynamically favored over interactions between nucleotides distant in sequence. Not

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Author contributions. R.A.L. performed footprinting, RNase T1 and FMN experiments on CP303 RNA (Fig 3, 5–7), analyzed the data and wrote the paper. T.A. performed footprinting, RNase T1 and oligonucleotide hybridization experiments on CP111 (Fig 2–4, 6), and helped edit the paper. S.H.M. did preliminary experiments on CP RNAs (Fig 2). S.W. analyzed the data and wrote the paper.

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only are base pairs between nearby residues formed more quickly, but they cost less in conformational entropy than base pairs between residues far apart in the primary sequence.⁴ On the other hand, long-range interactions between hairpin loops or helices often define the three-dimensional structure of the folded RNA, and are thus critical to forming the correct tertiary structure.⁵

The effects of contact order, secondary structure and native topology on the folding kinetics of small proteins are well documented.^{6; 7; 8; 9} As the polymer becomes longer, the increased probability of incorrect contacts further reduces the folding rate.¹⁰ As described by Go,¹¹ local interactions that are energetically favorable but incompatible with the native tertiary structure result in “topological frustration”, trapping some molecules in metastable misfolded conformations.^{12; 13} In RNA, kinetically trapped intermediates often contain incorrect base pairs and rearrange slowly.^{14; 15; 16} Thus on a biological timescale, the competition between short-range and long-range interactions can determine the functional state of individual RNA molecules.

To understand how the linear organization of a gene influences the folding pathway of the RNA, we compared the folding pathways of two circularly permuted forms of the *Tetrahymena* group I ribozyme by time-resolved hydroxyl radical footprinting. Circular permutation of protein and RNA sequences has been widely used to probe the connection between topology and folding time, because the distance between interacting residues can be altered without grossly perturbing the native structure.^{17; 18; 19} As illustrated in Fig. 1, sequences that form short-range base pairs or tertiary interactions in the wild type RNA are separated by an intervening nick in the polynucleotide chain, while the original 5' and 3' ends are joined. New (non-native) interactions can form at the junction between the original 5' and 3' ends.

We previously found that circular permutation of the *Tetrahymena* intron changed the global folding kinetics of the RNA, compared with the wild type intron and its L-21 ribozyme derivative.²⁰ In the “wild type” L-21 ribozyme, time-resolved hydroxyl radical footprinting showed that the stable P4–P6 domain folds within 1 s at 42 °C, while tertiary interactions with peripheral helices saturate in 3–5 s, and those in the P3–P9 domain saturate in ≥ 1 min.^{21; 22} Mispairing of the P3/P7 pseudoknot (altP3) causes ~90% of the RNA population to become kinetically trapped in intermediates in which the P3–P9 domain is misfolded.²³ Studies on circular permuted RNAs using native gel electrophoresis and anti-sense oligonucleotides showed that the permutation of the 5' and 3' ends of the RNA to J8/7 (nt 303), which places a nick next to altP3, allowed part of the RNA population to globally fold more quickly than the L-21 ribozyme.²⁰ By contrast, permutation of the RNA ends to various positions within the P4–P6 domain caused the entire RNA population to fold more slowly.²⁰

Here, we use time-resolved footprinting to probe the folding pathways of two circular permuted forms of the *Tetrahymena* intron, in order to understand how sequence connectivity affects the formation of RNA secondary and tertiary structure. The equilibrium and kinetic folding pathways of the circularly permuted RNAs were compared by hydroxyl radical footprinting, partial ribonuclease digestion, and flavin mononucleotide (FMN) cleavage. The results show that nicks in the RNA backbone and the junction between the 5' and 3' ends of the intron create large differences in the kinetic tertiary folding pathway. These differences are consistent with localized changes in the population of secondary structure present at the beginning of the folding reaction. The results also suggest that disjoining tertiary structure domains weakens the cooperative interactions necessary for properly regulated folding of the catalytic core.

RESULTS

Circularly permuted RNAs with different folding kinetics

Circularly permuted (CP) variants of the *Tetrahymena* intron that retain ribozyme activity were previously identified from a pool of randomly nicked circular intron RNA.²⁰ In the CP RNAs, the phosphodiester bond between G414 at the 3' end of the intron and U20 near the 5' end can be formed by a natural autocyclization reaction of the intron (cyclization junction; Fig. 2B).^{24; 25}

We characterized the folding pathway of two circular permutants whose folding kinetics differed most from that of the L-21Sca ribozyme (Fig. 2B). The first permutant, CP111, begins at G111 and ends at G110, and was chosen because it refolds very slowly. This permutation creates a nick in the RNA backbone within a conserved region of the P4 helix that participates in a critical triple helix within the intron core.²⁶ The CP111 ribozyme was about half as reactive as the circular C-19 intron toward site-specific hydrolysis of the cyclization junction, and refolded more slowly than the linear L-21 ribozyme when assayed by native gel electrophoretic mobility (0.02 min^{-1} vs. 0.4 min^{-1} at 37°C ; Fig. 2B).²⁰ This slow folding was partially rescued when CP111 RNA was folded sequentially during transcription.²⁷

We also probed the folding pathway of CP303 RNA, which begins with G303 and ends with A302 (Fig. 2B). This permutation places the 5' and 3' ends of the RNA in J8/7, a conserved sequence element that mediates tertiary interactions between the P4–P6 and P3–P9 domains and the P1 splice site helix.^{28; 29; 30} We previously found that CP303 RNA is reactive toward hydrolysis of the cyclization junction but not transesterification with a 5' exon RNA,²⁰ perhaps because the nick in J8/7 compromises P1 docking. This permutant was chosen for further study because about 50% of the CP303 population folded more rapidly than the wild type ribozyme in splicing buffer containing 200 mM NH_4^+ (3.2 min^{-1}), while the remaining RNA folded more slowly (0.07 min^{-1} ; Fig. 2B, below).²⁰ A nick in J8/7 may improve the formation of native-like structures by destabilizing the non-native alt P3 helix or by lowering the energy barrier to refolding of the core.²⁷

Global analysis of kinetic folding intermediates

We initially probed the structure of the CP introns by comparing the accessibility of the RNA domains to antisense oligonucleotide probes (Fig. 2B–C). Using this strategy to probe formation of the P3/P7 pseudoknot, we previously found that an oligonucleotide complementary to the 5' side of P3 hybridized more strongly with CP111 RNA than with either CP303 or the L-21 ribozyme, consistent with mispairing of P3 in CP111.²⁰

To probe other regions, L-21 ribozyme, CP111 and CP303 RNAs were refolded in 10 mM MgCl_2 , 25 mM Hepes , $100 \text{ mM (NH}_4)_2\text{SO}_4$, 1 mM EDTA at 37°C for 0–30 min, with trace ^{32}P -end-labeled oligodeoxynucleotide complementary to P2.1, P7 or P9 (Fig. 2). We chose these regions of the ribozyme because they are involved in tertiary interactions that stabilize the ribozyme core. Hybridization of labeled antisense oligomer with the RNA was detected by a native gel mobility shift (Fig. 2C). Radiolabeled RNA in the absence of the probe was run in an adjacent lane for comparison (*RNA; Fig. 2C).

The variation in the accessibility of each RNA to the labeled antisense oligomers indicated that these three RNAs fold through distinct intermediates rather than a common folding pathway. For example, the 3' side of P2.1 (nt 81–95) remained inaccessible in the L-21 ribozyme throughout the folding reaction, suggesting that P2.1 is stably formed. In CP111, the 3' side of P2.1 was initially accessible but less so after 30 min of folding in MgCl_2 (Fig. 2C), while the 3' side of P2.1 hybridized strongly with the probe in CP303. Thus, permutation of the ribozyme sequence alters the stability of the P2.1 helix.

An antisense probe complementary to P7 hybridized with CP303 but not CP111 or L-21 RNA, suggesting that the nick in J8/7 makes the intron more accessible. The P9 probe was able to hybridize with L-21 RNA after 30 min, but not CP111 and CP303 RNA. As discussed below, these differences can be explained by an alternative secondary structure in the P9 domain of CP111 and CP303, which is favored by the junction between G414 and U20.²⁷ In CP303, hybridization of the P7 probe with a slower migrating intermediate at 30 min is consistent with the formation of structures containing altP9, which are not accessible to the P9 probe.

Tertiary folding of permuted RNAs

We next used hydroxyl radical footprinting to probe the formation of tertiary interactions within the circularly permuted RNAs (Fig. S1). The cleavage patterns of the folded CP RNAs are similar to that of the L-21 ribozyme in 10 mM MgCl₂, suggesting that they have similar structures as the wild type RNA. In CP111, however, we observed an additional protection of nt 350–357 in P9.1a (Fig. S2, B–C), while the expected protection of the RNA backbone in P2.1 (nt 80–83) was weak or absent. These results suggested that the overall fold of CP111 is correct, but that the P13 kissing-loop interaction between P9.1 and P2.1 is perturbed (Fig. S2, B–C). In CP303, we observed additional weak protection of G147–148 in P5b.

We previously found that the folded conformation of CP111 RNA is ~3 kcal/mol less stable than that of the L-21 ribozyme in splicing buffer, while CP303 has a stability similar to the L-21 ribozyme.²⁰ To compare the relative stability of individual tertiary domains in CP111, 5'-[³²P]-labeled RNA was equilibrated in 0–10 mM MgCl₂ before hydroxyl radical footprinting with Fe(II)-EDTA (Figs. S1A and S2A). As in the L-21 ribozyme, contacts in the P4–P6 domain were saturated at lower Mg²⁺ concentrations than those in the P3–P9 domains, although all the folding midpoints for CP111 were increased relative to those in the L-21 ribozyme, consistent with a less stable structure overall. Nucleotides in the P3–P9 domain were 50% protected in ~1.3 mM MgCl₂ in CP111 at 30 °C (Fig. S2; Table S3), and ~0.9 mM in the L-21 ribozyme at 42 °C.³¹ Thus, circular permutation of the RNA destabilized the global fold but did not noticeably change the relative stabilities of the P4–P6 and P3–P9 domains.

Kinetic folding pathway of CP111

We next asked whether circular permutation of the 5' and 3' ends to the P4–P6 domain changes the dominant kinetic folding pathways of the intron, either by stabilizing new intermediates or by changing the flux through pathways that are normally accessible to the L-21 RNA. The formation of tertiary structure in CP111 in 10 mM Na-cacodylate, 10 mM MgCl₂ at 30 °C was mapped using time-resolved hydroxyl radical footprinting. The hydroxyl radical was generated either with a synchrotron X-ray beam,³² which allowed us to probe folding times of 20 ms – 180 s, or via the Fenton reaction using Fe(II)-EDTA, to monitor folding events between 0.5 – 120 min.³³ The extent of protection from hydroxyl radical cleavage at each region of the RNA backbone was quantified (Fig. 3; Fig. S1B and Table S2) and the rates of protection determined as described previously.^{34; 35}

As depicted on the secondary structure schematic in Fig. 4, the P4–P6 domain surprisingly folded five times faster in CP111 than in the L-21 ribozyme. Nucleotides in CP111 were protected with apparent rate constants of 5–9 s⁻¹ at 30 °C (blue; Fig. 4A). In the L-21 ribozyme, residues in the P4–P6 domain are protected from hydroxyl radical at 1–2 s⁻¹ at 42 °C (green and orange; Fig. 4B).³² By contrast, the other domains of CP111 folded 5–10 times more slowly, at rate constants of 0.001–0.004 s⁻¹ (dark pink; Fig. 4A) compared with 0.01–0.4 s⁻¹ in the L-21 ribozyme (yellow; Fig. 4B). This difference is not due to temperature, as initial experiments showed that the P3–P9 domain of the L-21 also folds at an apparent rate of 0.01 s⁻¹ at 30 °C, while the P4–P6 folds in less than 15 seconds (data not shown). The slow formation

of tertiary interactions in the P3–P9 domain of CP111 explains why this permuted RNA folds more slowly overall than the L-21 ribozyme.

Kinetic folding pathway of CP303

Our previous experiments showed that a subpopulation of CP303 folds more rapidly than the L-21 ribozyme when the RNA is refolded in 100–200 mM monovalent salt plus 10 mM MgCl_2 .²⁰ To determine whether this fast-folding population correlates with more rapid formation of tertiary interactions in the P3–P9 domain, we mapped the folding pathway of CP303 RNA by time-resolved hydroxyl radical footprinting at 42 °C in 10 mM MgCl_2 (Figs. 3, 5, and S1B), in the absence or presence of 100 mM NaCl (Fig. 5AB). To control for the effect of NaCl on the folding kinetics, we also measured the folding kinetics of the L-21 ribozyme in 10 mM MgCl_2 plus 100 mM NaCl (Fig. 5D).

When 100 mM NaCl was present in the reaction, tertiary interactions in both RNAs formed more quickly than when no NaCl was added to the reaction (Fig. 5). These results are consistent with previous work showing that the ribozyme folds faster in mixtures of monovalent and divalent metal ions or in monovalent ions alone.^{36; 37; 38} For the L-21 RNA, the folding progression was similar in the presence and absence of 100 mM NaCl, with interactions in the P4–P6 domain saturating first ($4\text{--}7\text{ s}^{-1}$), followed by P14 and the central triple helix ($\sim 1\text{ s}^{-1}$), and lastly the P3–P9 domain ($\sim 0.3\text{ s}^{-1}$; Fig. 5 C–D). The overall folding rate of the P3–P9 domain is 10–20-fold faster in 10 mM MgCl_2 plus 100 mM NaCl than in MgCl_2 alone ($0.01\text{--}0.09\text{ min}^{-1}$; ²¹).

In contrast to the L-21 ribozyme, the folding profile of CP303 RNA was sensitive to salt concentration. Not only did the overall rate of folding increase in 100 mM NaCl, but the relative order in which various domains of the RNA folded was changed. In the absence of salt, a few contacts in the P4–P6 domain formed at 1 s^{-1} , such as P5c and the P14 pairing between L5c and L2, as well as the 5' side of P3 (orange in Fig. 5A). Other interactions in P4–P6, including the A-rich bulge (nt 183–186), however, folded much more slowly in CP303 than in the L-21 ribozyme, as did the entire P9 domain (yellow, $0.05\text{--}0.2\text{ s}^{-1}$). Thus, when CP303 is refolded in 10 mM MgCl_2 and no NaCl, the P4–P6 domain is only partly formed or (more probably) partially misfolded in early folding intermediates.

In the presence of 100 mM NaCl, the various regions of CP303 were protected in a roughly similar order as the wild type ribozyme ($\text{P5abc} > \text{P4–P6}, \text{P2}, \text{P9–P9.2} > \text{P3–P8}$). The P5abc subdomain, however, formed slightly more slowly in CP303 than in the L-21 ribozyme ($2\text{--}3\text{ s}^{-1}$ vs. $4\text{--}7\text{ s}^{-1}$; Figure 5 and Table S2). The central triple helix, which is formed by J3/4 and J6/7, was protected more rapidly in CP303 than in the L-21 ribozyme, while the P9 domain was protected at 1 s^{-1} . The junction between P2.1 and P3 (J2/3; nt 93–97) was protected in two kinetic phases, with apparent rate constants of 5 s^{-1} and 0.1 s^{-1} (blue/yellow; Fig 5B). These results suggest that a fraction of the CP303 population rapidly forms intermediates in which the center of the RNA is protected from solvent. A 3' ^{32}P -label and Fe-EDTA cleavage was used to monitor the backbone protection of nucleotides in P3–P8, which are at the 3' end of CP303 RNA. Nucleotides in P3–P8 were 60–80% protected relative to the folded RNA within the first 30 s, which corresponds to a folding rate of at least 0.04 s^{-1} or 3 min^{-1} (yellow; Fig 5B). The remaining 20–40% of the RNA folded very slowly, at $\geq 0.1\text{ min}^{-1}$. These results are consistent with our previous measurements of global folding rates in splicing buffer containing 100 mM ammonium sulfate, in which 60% of CP303 RNA folded at 3 min^{-1} and the remainder folded at 0.07 min^{-1} .²⁰

Circular permutation changes RNA secondary structure

The time-resolved footprinting results on CP111 and CP303 RNAs show that permutation of the RNA sequence can dramatically change the tertiary folding pathway of the RNA. A nick in P4 allows the P4–P6 domain to fold very rapidly; a nick in J8/7 lowers the folding rate of the P4–P6 domain but allows some interactions within the core to form more quickly. These changes could derive solely from altered connections between domains. Alternatively (or in addition), folding may be perturbed by changes in the RNA secondary structure.

To address this question, the secondary structures of CP111 and CP303 were probed by partial digestion with ribonuclease (RNase) T1, which cleaves after unpaired guanosine residues (G's). The extent of cleavage for each ^{32}P -end-labeled RNA was compared in the absence of Mg^{2+} in either cacodylate buffer or 6 M urea ("G ladder"; Fig. 6A). The consensus of several experiments are summarized on the secondary structure diagrams (Fig. 6, B–C).

The digestion pattern of the L-21 Sca ribozyme was qualitatively consistent with previous footprinting studies.^{39; 40} In the absence of Mg^{2+} , G's in stable helices, such as P2, P6b and P8 were protected from nuclease digestion, while those in less stable helices such as P2.1 and P7 were cleaved (Fig. 6B–C, black circles). In CP111, G's within P2.1 and P5c appeared more double-stranded than in the L-21 ribozyme, while G's in P3, P8 and P9.1a were more single-stranded (Fig. 6B, triangles). Destabilization of the P3–P9 secondary structure is consistent with the much slower tertiary folding rate of this domain in CP111. The cleavage pattern of P5c in the absence of Mg^{2+} agrees with an extended conformation seen in NMR studies.⁴¹

In CP303, G's in P3, P7 and P9.0 were more double-stranded than in the L-21 ribozyme, while many G's in the P4–P6 domain and P5a were more single-stranded (Fig. 6C, triangles). Thus one explanation for the slower and more heterogeneous folding of the P4–P6 domain (Fig. 5) is that the secondary structure of the unfolded RNA is more disordered. The resistance of the 5' side of P3 (G100) to nuclease digestion is particularly significant, as these residues are predicted to be single-stranded if P3 is replaced by altP3. Base pairing of P3 and P7 is consistent with a greater probability of correctly folding the ribozyme core, relative to the L-21 ribozyme under the same conditions. One caveat is that our gels only resolved the 5' side of P3. Moreover, protection from RNase T1 indicates that bases in P3 and P7 are paired, but not whether they form the correct base pairs. Nonetheless, the T1 digestion results suggest that one effect of sequence permutation is to change the distribution of secondary structures present at the start of the refolding reactions, and that this results in a different 3D folding pathway.

Long-range pairing of peripheral loops

When Mg^{2+} was added to the L-21 ribozyme, all of the G's were protected from RNase T1 within the first 30 seconds, as expected (open circles, Fig. S3, B–C). Excepted were G's in the loops L2, L2.1, L5c and L9.1a, which were protected more slowly ($\sim 0.2\text{--}0.3\text{ min}^{-1}$; Fig. S3; Table S3). These G's participate in the long-range P13 and P14 loop-loop interactions in the native ribozyme.⁴² RNase T1 cleavage of G288 in L8 increased over time (data not shown). In CP111, G's in these peripheral loops were protected from RNase digestion tenfold more slowly than in the L-21 ribozyme (Table S3), consistent with slower formation of these long-range tertiary contacts. In CP303, G73 and G77 in L2.1 were protected with biphasic kinetics, consistent with partitioning of the RNA population into different folding intermediates.

Alternative structure in the P9 domain by FMN cleavage

To obtain additional information about the secondary structure of the permuted RNAs, we mapped the location of stacked G:U base pairs by cleavage with flavin mononucleotide (FMN; Fig. 7 and S4). FMN binds and intercalates into RNA helices containing G•U pairs⁴³.

Irradiation of the FMN-RNA complex with visible light in the presence of magnesium specifically cleaves the RNA phosphodiester backbone 3' of uridine residues (U).

L-21, CP111 and CP303 RNAs were treated with FMN under conditions that either generate the native-like state (N) or folding intermediates (lane I; Fig. 7A).²⁰ The cleavage pattern of the three renatured (N) RNAs were consistent with their predicted secondary structure, except that U247 and U253 were not cleaved (Fig. S4). The lack of cleavage at these two residues may be due to unfavorable stacking interactions with the isoalloxazine.⁴³ Unexpectedly, U59 was cleaved in both CP introns but not in the L-21 ribozyme, although U59 was predicted to pair with G92 (Fig. S4). We also observed cleavage at U71 in all three RNAs, which is not predicted by current structural models. A predicted wobble pair between U305 and G272 in the non-native altP3 helix was cleaved weakly in intermediates of CP111, but not detectably in L-21 or CP303 RNAs.

Both of the CP RNAs produced additional products resulting from FMN cleavage at U403, 404, 409 and 412, under annealing conditions which favor the folding intermediates (Fig. 7A, lane I, numbers with dots). These cleavages are not found in the L-21 ribozyme (Fig. S4; gel not shown). Therefore, the P9 domain folds differently in the intermediates of the circularly permuted RNAs than of the L-21 ribozyme.

The FMN cleavage pattern of the CP RNAs can be explained by an alternative secondary structure which extends P9.2 to include nts 403–404 (compare Fig. 7A lanes N and I, nt 333 and nt 403–412; Fig. 7B and C). The FMN cleavage results are also consistent with a previously suggested alternative pairing between P9 (nt 316–333) and the cyclization junction (nt 407–27), except that products at U317 and U326 predicted by the previous model were not observed.²⁷ It is likely that this alternative structure is stabilized by the link between U20 and G414, as a novel RNA that contains just the P9 domain residues shown in Fig. 7C yielded the same FMN cleavage pattern (data not shown). Both alternative P9 conformations are predicted to disrupt P13 and tertiary interactions between the P9 domain and the intron core. Because the P9 domain stabilizes the intron core⁴⁴ and promotes folding of P7 and P3,⁴⁵ it is likely that misfolding of this domain also contributes to the slow folding and decreased activity of the catalytic center in the circularly permuted RNAs.

DISCUSSION

RNAs with structural or catalytic roles in the cell must fold to a specific three-dimensional structure to obtain their function. Because local interactions within RNA domains initially form within milliseconds, which is much faster than the rate of transcription, the organization of structural domains within the primary sequence of the RNA dictates the propensity to form metastable folding intermediates.⁴⁶ The 5' to 3' order of structural domains may be particularly significant in the evolution of genes that encode stable RNAs, as RNA stem-loops are stable even in the absence of 3D structure.

Here, we show that circular permutation of the *Tetrahymena* group I ribozyme results in large differences in the kinetic tertiary folding pathway of the RNA. Two permutations resulted in completely different folding kinetics, demonstrating that these differences are not solely due to the cyclization junction between G414 and U20, but also depend on the location of the new 5' and 3' ends. Not only are the structures of the intermediates changed by sequence permutation, but the kinetic partitioning among competing folding pathways is also changed. Once the RNA is folded, the penalty for introducing a nick into the center of a large RNA is apparently not too great, as previous studies on several RNAs showed that many circular permutations can be tolerated without total loss of function.^{1; 2; 20; 47}

Comparison of folding pathways

The folding pathways of the (wild type) L-21 ribozyme have been characterized by a number of experimental methods, including oligonucleotide hybridization, native PAGE, time resolved footprinting and single-molecule FRET.^{48; 49} The P4–P6 domain, which is independently stable, folds within 1–3 s,²¹ while native tertiary interactions with the peripheral P2/P2.1 and P9.1/P9.2 domains appear at intermediate times and those in the P3–P9 domain require 1–3 minutes to form completely (Fig. 8).⁵⁰ This discrepancy in the apparent folding times of the individual domains arises from the presence of intermediates in which only one or two domains are in the native conformation.⁵¹ Backbone protections in the P3–P9 domain appear slowly, because many RNA molecules become trapped in intermediates in which the P3–P9 domain is misfolded.²³ It was recently shown that the tertiary folding revealed by time-resolved hydroxyl radical footprinting is consistent with the simultaneous folding of the L-21 RNA population along different pathways,²² as originally proposed⁵² and shown in single-molecule FRET studies.⁵³

CP111—Relocation of the RNA ends to P4 in CP111 greatly increases the difference in the folding rates of the major tertiary domains (Fig. 4). Tertiary interactions within the P4–P6 domain form in 100–300 ms, about 5 times faster than in the wild type RNA, while the rest of the CP111 RNA folds in 10–20 min. Our data suggest two explanations for this difference. First, the introduction of a nick within P4 likely destabilizes the central triple helix, weakening an important structural link between the P4–P6 and P3–P9 domains.^{26; 54; 55} We previously found that the P4–P6 domain folds faster in isolation than in the intact ribozyme, suggesting its folding is normally inhibited by interactions with other regions of the RNA.^{36; 56} Experiments with a pyrene-tagged P4–P6 RNA showed that the isolated domain folds even more rapidly at 30 °C than at 42 °C.⁵⁶ The loss of an interdomain connection may allow the independently stable P4–P6 domain to fold more rapidly than it does in the L-21 ribozyme. At the same time, the central triple helix was shown to play an important role in refolding of the P3–P9 domain, which explains why other regions of the CP111 permutant fold more slowly.⁵⁴

Second, RNase T1 footprinting shows that the secondary structure of P3, P7 and P8 is less stable (or more disordered) in the initial “unfolded” CP111 population than in the L-21 ribozyme under the same conditions. In addition, ligation of G414 with U20 stabilizes an alternative non-native conformation within the P9 domain of both CP RNAs (Fig. 7). These changes in secondary structure are likely to delay the formation of tertiary interactions within the P3–P9 domain, especially as the P9 domain was shown to promote folding of P3 and P7.⁴⁵ As a result, nearly all the CP111 population becomes kinetically trapped in intermediates in which only the P4–P6 domain is folded, and the transition to the native RNA is very slow (Fig. 8).

CP303—Relocation of the ribozyme ends to J8/7 caused the P4–P6 domain to fold more slowly than in the L-21 ribozyme, but resulted in more rapid protection of the linker joining P2.1 and P3 (J2/3) and the central triple helix (Fig. 5). This suggests that these linkers are internalized in compact intermediates in 200–500 ms, and this is facilitated by circular permutation of the RNA sequence and by the addition of 100 mM NaCl to the folding reaction. The remaining interactions within the P4–P6 and P9 domains saturate at an apparent rate of 1 s^{−1}, except for P2.1 and backbone protections in P3, P7 and P8, which were 60–80% complete in ≤ 30 s. Because the central triple helix and the peripheral helices are protected within a second, it is possible that the catalytic core folds rapidly in part of the CP303 population. A burst of complete folded RNA would not have been detected in our Fe-EDTA assays on 3′ end-labeled RNA. It seems likely that CP303 RNA can fold via multiple pathways, based on the

global biphasic folding kinetics measured by native gel electrophoresis at 30 °C (3 min⁻¹ and 0.1 min⁻¹).²⁰

The footprinting, native gel, RNase T1 and oligonucleotide hybridization assays together suggest that some of the CP303 folding intermediates contain the native P3 pseudoknot, either because the P3 helix is formed early in the folding process, or because the nick in J8/7 facilitates reorganization of the core. By contrast, very little of the L-21 population folds through intermediates in which the P3 pseudoknot is correctly base paired.^{22; 57; 58} Early formation of P3 is expected to favor partitioning of the RNA along pathways leading rapidly to the native structure (Fig. 8C; upper path). More facile reorganization of the core would accelerate the rate at which intermediates refold (Fig 8C; lower path). On the other hand, the nick in J8/7 destabilizes core tertiary interactions, while mispairing of P9, P6a and L2.1 likely inhibit the formation of tertiary contacts by P2.1 and the proper orientation of the extended P9 domain. These factors may explain why part of the CP303 population folds at 0.1 to 0.2 min⁻¹, even in the presence of 100 mM NaCl, despite the greater propensity to form the native P3 helix.

Primary sequence and structural hierarchy

Our results suggest two ways in which circular permutation of the RNA sequence changes the folding kinetics. First, nicking the backbone at sites that are usually in the middle of the sequence disrupts the normal connections between tertiary domains, perturbing the hierarchy of tertiary interactions (Fig. 1). Single-stranded linkers joining helical domains play a critical role in mediating tertiary interactions within the core of group I ribozymes.^{30; 59} For example, joining segments that form the central triple helix were necessary for reconstitution of the *Tetrahymena* ribozyme from separate RNAs containing the P4–P6 and P3–P9 domains,⁵⁵ and mutations that destabilize the triple helix were previously shown to decrease the ribozyme folding rate.⁶⁰ In CP111, the nick in P4 weakens one connection between the P4–P6 and P3–P9 domains, widening the disparity in the folding times of these domains even further.

The results of our RNase T1 and FMN footprinting experiments reveal a second effect of circular permutation, which is to alter the ensemble of secondary structures present at the beginning of the refolding reaction. Different secondary structures in turn produce different tertiary folding intermediates, as some domains have a greater or lesser probability of becoming kinetically trapped in partially misfolded conformations. The increased probability of forming the P3 helix in CP303 may explain why this permuted RNA can fold more rapidly than the L-21 ribozyme.²⁷ The increased preference for P3 is supported by nuclease digestion (Fig. 6) and stochastic folding simulations of the *Tetrahymena* group I intron.⁶¹ Both CP RNAs are also predicted to form non-native helices that could disrupt folding of peripheral domains. These include the alt P9 conformation supported by FMN footprinting (Fig. 7) and a mispairing between L2.1 and the 3' side of P6a/P6 predicted by the Kinefold algorithm^{62; 63} (H. Isambert, personal communication).

The effect of circular permutation on the RNA secondary structure can be understood in terms of the entropic cost of hairpin loop formation. Experiments on DNA and RNA hairpins demonstrated that helices become less stable and hairpin closing times lengthen as the size of the hairpin loop increases.^{64; 65; 66; 67} Pairing between two nearby segments of the RNA chain forms a small loop, exacting a small entropic penalty for conformationally restraining the nucleotides in the loop. If the paired segments are separated by a permutation of the RNA sequence, this entropic penalty will increase, reducing the probability of forming the native secondary structure.

In nature, these effects of primary sequence on the folding kinetics will also depend on the rate of transcription and the strategic pausing of RNA polymerase at sites that suppress the formation of alternative base pairs.^{68; 69} We previously observed that misfolding of CP111

was partly rescued when the RNA was allowed to fold during transcription.²⁷ Circular permutation has also been observed to alter the co-transcriptional folding kinetics of the RNase P RNA⁷⁰ and the hairpin ribozyme.⁷¹ Thus, the evolution of RNA encoding genes must not only preserve the architecture of the active site, but may also involve the optimization of folding during RNA synthesis.

MATERIALS AND METHODS

Preparation of RNA

Specific CP RNA sequences are generated from PCR templates created from a double (tandem) IVS DNA master template plasmid. Circularly permuted RNAs were prepared by in vitro transcription of PCR-DNA templates as previously described²⁰. For footprinting reactions, RNAs were labeled with ³²P at the 5' end and gel purified using standard methods. All RNA footprinting reactions were performed in 1X CE buffer (10 mM sodium cacodylate (pH 7.0), 0.1 mM EDTA). The RNAs were stored at pH 7.0 to reduce hydrolysis of the cyclization junction. Before folding, L-21 and CP303 RNAs were pre-annealed at 70°C for 1 min in 1X CE (pH 7.0) and allowed to cool to room temperature. Folding reactions were initiated by adding 10 mM MgCl₂ or 10 mM MgCl₂ plus 100 mM NaCl to RNA pre-equilibrated in 1X CE at 30°C or 42°C. For equilibrium Mg²⁺ titrations, RNA was incubated 30 min in 0–20 mM MgCl₂. Removal of the 5' triphosphate and replacement with a 5' monophosphate was previously found to increase the reactivity of the CP303 RNA, but had only a small effect on the folding kinetics of the permuted RNAs²⁰.

Oligonucleotide hybridization and native gel electrophoresis

RNA folding reactions and native gel electrophoresis were carried out as described previously,^{20; 23} except that unlabeled RNA (1 μM) was incubated with 5'-end labelled ³²P-DNA oligonucleotide (0.1 pmol, ca. 1 × 10⁶ cpm) in a 20 μl reaction volume (50 mM Na-HEPES (pH 7.5), 100 mM (NH₄)₂SO₄, 1 mM EDTA, 10 mM MgCl₂) at 37°C. Zero time samples were taken immediately after the addition of RNA to the folding reaction. Aliquots (2 μL) were removed at various times and loaded on a non-denaturing 8% polyacrylamide gel in THEM buffer²³. Control reactions with uniformly ³²P-labeled RNA were done as described elsewhere.²³ Antisense oligonucleotides were as follows: anti-P7, 5'ATTTAGTCTG, anti-P9, 5'CCCATTAAGGAGAGG, anti-3'P2.1, 5'TTGCCTTTTAAACCG, and anti-5'P2.1, 5'GCAATCTATTGGTTT.

Hydroxyl radical footprinting

Fenton hydroxyl radical footprinting reactions with Fe(II)-EDTA were performed as described elsewhere.⁷² Synchrotron X-ray hydroxyl radical footprinting was performed as previously described^{35; 73} at beamline X28C at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory. Cleavage products were analyzed on 6–8% polyacrylamide (19:1 or 29:1 mono:bis) sequencing gels and quantified using a Phosphorimager (Amersham Pharmacia Biotech). The fractional saturation of individual protected regions, $\bar{Y}$, was determined as previously described.⁷⁴ For Mg²⁺ titrations, the data were fit to $\bar{Y} = C^n / (C_m^n + C^n)$, C is the Mg²⁺ concentration and C_m is the midpoint of the transition. Time-resolved footprinting data were fit to rate equations with one or two exponential terms, $\bar{Y}(t) = A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t})$

FMN cleavage

RNA cleavage with FMN (flavin mononucleotide; riboflavin) was performed as previously described,⁴³ except that 10⁶ cpm of 5'-end labeled RNA was used in each reaction. FMN was obtained from Sigma. The native RNA was prepared by preheating the RNA to 70° or 90°C

and recooling in the presence of Mg^{2+} . The folding intermediates were prepared by incubation with Mg^{2+} at room temperature for 5–10 min.

Ribonuclease digestion

RNase T₁ structure probing was performed as previously described⁷⁵ in a 10 μ l sample volume containing $5\text{--}10 \times 10^5$ cpm of 5'-labeled RNA in 1X CE plus either 10 mM $MgCl_2$, 10 mM $MgCl_2/100$ mM NaCl, or water (no- $MgCl_2$). RNA was preincubated 5 min at 42°C (L-21, CP303) or 30°C (CP111) before digestion. RNase T₁ (USB) was used at a final concentration of 0.25 U/ μ l. The relative extents of cleavage as a function of time were analyzed as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Effects of RNA sequence on contact order and folding

Circular permutation of the RNA sequence changes the sequence distance between secondary (2°) and tertiary (3°) contacts.

Figure 2. Circular permutation changes the structures of folding intermediates

(A) Structure of the *Tetrahymena* ribozyme. Ribbon diagram of P4–P6 (green) and P3–P9 domains (blue) from a crystal structure of the intron fragment.⁷⁶ The approximate location of the P2/P2.1 (pink) and P9.1/P9.2 helices (grey) are shown as cylinders.⁴² Nucleotides 111 and 303 are indicated as pink and yellow spheres, respectively. (B) Secondary structure of the C-19 circular intron colored as in (A). Circularly permuted (CP) RNAs were selected from a pool of nicked C-19 circles.²⁰ The cyclization junction (CJ) is between G414 and U20. Transcription of CP111 and CP303 begins at G111 and G303, respectively, and ends at the preceding nucleotide. Paired (P) helices are numbered; dotted lines indicate P13 and P14 loop-loop interactions; IGS, internal guide sequence; TH, triple helix. Antisense DNA probes are depicted as thick black arrows. (C) Four ³²P-labeled antisense deoxyoligonucleotides (5'P2.1, 3'P2.1, P7, P9) were mixed with excess unlabeled intron RNA as refolding was induced with 10 mM MgCl₂ (L-21, CP111) or 10 mM MgCl₂, 100 mM NaCl (CP303). Samples were incubated 0–30 min before native PAGE; lane (–), antisense oligomers were annealed with unfolded RNA at 95 °C. Radiolabeled bands represent antisense probe bound to ribozyme RNA. Lanes *RNA, ³²P-labeled RNA alone was loaded before (I) or after (N) refolding in 10 mM MgCl₂.

Figure 3. RNA folding kinetics by time-resolved hydroxyl radical footprinting

^{32}P -end-labeled RNAs were cleaved in the presence of hydroxyl radical as described in Methods. The fractional saturation of groups of protected nucleotides were fit to first order or biphasic rate equations. (A) CP111 (P5a, nt 180–181; J8/7, nt. 300–306; P9.1a, nt 352–357) in 10 mM Na-cacodylate, 1 mM EDTA (CE), 10 mM MgCl_2 , 30°C. (B) CP303 (P5a, nt 180–181; J2/3, nt 93–96; P9.1a, nt 342–347) in CE plus 10 mM MgCl_2 , 100 mM NaCl, 42°C. Fits for other positions are in Figure S2; rate constants are listed in Tables S2 and S3.

Figure 4. Tertiary folding pathway of CP111

Folding kinetics of (A) CP111 at 30 °C in CE plus 10 mM MgCl₂, compared to (B) the L-21 ribozyme at 42 °C under the same conditions taken from Ref. 21. Protected regions were colored according to the observed rate of protection from hydroxyl radical cleavage (see legend). Rate constants varied $\pm 20\%$ between 2–3 trials except as noted in Table S2.

Figure 5. Tertiary folding pathway of CP303

Summary of time-resolved hydroxyl radical footprinting at 42 °C, as in Figs. 3–4. (A) CP303 in 10 mM MgCl₂, (B) CP303 RNA in 10 mM MgCl₂ + 100 mM NaCl, (C) L-21 in 10 mM MgCl₂ ²¹, (D) L-21 in 10 mM MgCl₂ + 100 mM NaCl. Protected regions were colored by rate constant (see legend). Rate constants varied ± 20% between 2–3 trials except as noted in Table S2. Protections in the P9 domain (P9.1/P9.2, nt 366–369; P9.2, nt 389–397) were easily detected because these residues are closer to the 5' end of the RNA, while protection of J8/7 was not quantified because these residues are very close to the 5' and 3' ends of the RNA.

Figure 6. Secondary structure of circularly permuted RNAs

(A) Partial digestion of ^{32}P -end-labeled RNA with RNase T_1 under denaturing or non-denaturing conditions. Lanes (G), sequencing ladder in 6 M urea; (–), untreated control; (No Mg), RNA digested in 1X CE. Sequencing ladders are numbered according to the L-21 intron. Because the RNAs have different 5' ends, the sequencing gels cannot be directly compared. (B–C) Summary of RNase T_1 digestion in no Mg^{2+} . Circles, L-21; circle with bar, not determined; triangles, CP111 or CP303 RNA. A circle but no triangle indicates a position lacking data on the CP RNA. Open red or blue symbol, not cleaved; black filled symbol, cleaved; green symbol, ~50% cleaved.

Figure 7. Misfolded P9 in CP303 intermediates

FMN cleavage of G•U pairs. (A) End-labeled CP303 RNA was mixed with 10 mM MgCl₂+100 mM NaCl at room temperature to trap folding intermediates (I), or renatured in 10 mM MgCl₂, 100 mM NaCl (N). Controls (C1, C2) omit magnesium. The U residues of G•U base pairs were cleaved by irradiation in the presence of FMN. G-specific sequencing ladder (G) is shown on the right. Bands marked (<), uridines cleaved only in the native RNA; (*), cleaved in the intermediate only. The cyclization junction hydrolyzed slightly during renaturation and is indicated with an asterisk. (B) Cleavage of G•U pairs in the native RNA (N) are consistent with the predicted secondary structure of the P9 domain,⁴² based on 2 trials. (C) FMN cleavage of folding intermediates (I) is consistent with a non-native secondary

structure for the P9 domain. Filled triangles, cleaved in I only; open triangles, cleaved in N only; gray, cleaved in I and N. Numbers indicate the position of the U directly upstream of the cleavage site.

Fig. 8. Folding pathways of circularly permuted ribozymes

The progression of tertiary folding is cartooned for each RNA to illustrate the major folding intermediates. Helices (cylinders) are colored as in Figure 2. The L-21 ribozyme (top) can fold through additional pathways involving different intermediates.^{22; 52} It is likely that the CP RNAs also fold through more than one pathway. In addition to P3, helices P6 and P9 also have the potential to mispair in the CP RNAs. See text for details.