Slow formation of a pseudoknot structure is rate limiting in the productive co-transcriptional folding of the self-splicing *Candida* intron

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ABSTRACT

Pseudoknots play critical roles in packing the active structure of various functional RNAs. The importance of the P3–P7 pseudoknot in refolding of group I intron ribozymes has been recently appreciated, while little is known about the pseudoknot function in co-transcriptional folding. Here we used the *Candida* group I intron as a model to address the question. We show that co-transcriptional folding of the active self-splicing intron is twice as fast as refolding. The P3–P7 pseudoknot folds slowly during co-transcriptional folding at a rate constant similar to the folding of the active ribozyme, and folding of both P3–P7 and P1–P10 pseudoknots are inhibited by antisense oligonucleotides. We conclude that when RNA folding is coupled with transcription, formation of pseudoknot structures dominates the productive folding pathway and serves as a rate-limiting step in producing the self-splicing competent *Candida* intron.

Keywords: antisense oligonucleotide (AON); *Candida albicans*; pseudoknot; ribozyme

INTRODUCTION

As an important class of RNA motifs, a pseudoknot is minimally composed of two helical segments connected by single-stranded regions or loops (Staple and Butcher 2005). RNA pseudoknots play diverse biological roles. They promote programmed ribosomal frameshifting in many viruses (Shen and Tinoco 1995; Nixon et al. 2002). Such a frameshifting signal has been found in bacterial, yeast, and even human genes recently, and therefore is used to predict –1 ribosomal frameshift events (Belew et al 2008; Theis et al. 2008). The P3–P7 pseudoknot is essential for assembly of the compact structure of group I intron ribozymes (Adams et al. 2004). By folding into a double-pseudo-knot structure, the small HDV ribozyme self-cleaves to produce the single-genome RNA of hepatitis

delta virus (Thill et al. 1993; Ferré-D'Amaré et al. 1998). It has been reported that there is a highly conserved pseudoknot structure at the 5' end of the 451-nucleotide (nt) human telomerase RNA, and this pseudoknot is required for telomerase activity (Theimer et al. 2005).

The importance of the P3–P7 pseudoknot in refolding of the *Tetrahymena* group I ribozyme has been appreciated for over two decades (Laggerbauer et al. 1994; Zarrinkar and Williamson 1994). It has long been recognized that formation of the P3–P7 structure is a rate-limiting step in refolding of the active ribozyme, and the slow kinetics can be converted by mutations destabilizing the alternative P3 base pairing or base triples, or by disconnecting the nonnative interactions (Pan and Woodson 1998; Rook et al. 1998; Treiber et al. 1998; Ohki et al. 2001; Heilman-Miller and Woodson 2003a). Also, it has been reported that the refolding of the genomic HDV ribozyme is dominated by slow formation of the pseudoknot (Chadalavada et al. 2002).

However, single molecule study has shown that the majority of *Tetrahymena* group I ribozyme molecules enter the nonproductive folding pathway (Zhuang et al. 2000), raising questions regarding the relevance of the slow P3–P7

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formation in the productive refolding pathway. Interestingly, we have recently showed that folding of the P3–P7 pseudoknot of the *Candida* ribozyme in the productive refolding pathway is much more rapid and not rate limiting (Zhang et al. 2005).

Folding kinetics of the pseudoknot during transcription remains unclear. In living cells, RNA folding is coupled with the transcription process (referred to as “co-transcriptional folding”), i.e., each RNA chain starts to fold once it emerges from the polymerase, which is markedly different from “refolding” starting from a full-length RNA molecule. Interestingly, coupling of folding with in vitro transcription accelerates the native folding of the *Tetrahymena* ribozyme and an RNase P ribozyme about onefold (Pan et al. 1999; Heilman-Miller and Woodson 2003b).

The P3–P7 pseudoknot is a conserved core structure among all group I ribozymes (Michel and Westhof 1990). Absence of this structure results in inactive ribozyme, and thus ribozyme activity provides a direct readout for the correct formation of the pseudoknot. Here we used the *Candida* group I intron as a model to study the folding kinetics of the P3–P7 pseudoknot during transcription. Interestingly, we demonstrated that both P3–P7 and P1–P10 pseudoknots are effectively captured by antisense oligonucleotides (AONs), suggesting a slow formation of these two pseudoknots. We further showed that formation of the native P3–P7 pseudoknot serves as a rate-limiting step during co-transcriptional folding of the catalytically active *Candida* intron, which dramatically differs from its refolding behavior. This finding suggests that the folding kinetics of the pseudoknot structure is reprogrammed by the transcription process.

RESULTS AND DISCUSSION

Co-transcriptional folding of the self-splicing *Candida* intron is twice as fast as refolding

When the purified transacting *Candida* ribozyme Ca.L-11 that catalyzes the esterification reaction at the 5' splice site is refolded in the presence of $\text{Mg}^{2+} \geq 2$ mM, the major ribozyme population reaches its active structure with a rate constant of 2 min^{-1} via a pathway in which the P3–P7 pseudoknot forms much more rapidly (Xiao et al. 2003; Zhang et al. 2005). Another transacting *Candida* ribozyme catalyzing the exon ligation at 3' splice site also shows a fast folding constant ($\geq 1.8 \text{ min}^{-1}$) (Bao et al. 2008). Interestingly, under the similar refolding condition, <20% of the self-splicing intron molecules folded to the catalytically active structure with a rate constant of 0.09 min^{-1} (Fig. 1A), suggesting that the presence of long exon sequences dramatically decreases both the rate and efficiency in folding of the *Candida* intron (Woodson and Cech 1991).

We next addressed how the transcription process modulates the folding kinetics of the self-splicing *Candida*

FIGURE 1. The kinetics of refolding and single-round co-transcriptional folding of the self-splicing *Candida* group I intron. (A) The observational first-order reaction constant for the ribozyme refolding ($k_{\text{fold}} = 0.094 \pm 0.011 \text{ min}^{-1}$) was determined by plotting the fraction of spliced ribozyme against the splicing time, and then calculated by fitting curves to a single exponential equation. The precursor RNA was purified and denatured as previously described (Zhang et al. 2005), and refolded and spliced in the same transcription condition as for the co-transcriptional folding in the presence of 10 mg/mL heparin (Materials and Methods). (B) Similarly, the observational first-order reaction constant for folding of the self-splicing ribozyme during one-round transcription ($k_{\text{fold}} = 0.19 \pm 0.02 \text{ min}^{-1}$) was obtained. Folding of the active structure of the *Candida* group I intron is much slower than catalysis of each ester-transfer reaction (Jiang et al. 2006; Bao et al. 2008); thus, the observed self-splicing activity in this study reflects the folding kinetics of the ribozyme.

intron. Heparin was used to study the single-round co-transcriptional folding kinetics (Heilman-Miller and Woodson 2003b); and 10 mg/mL heparin ensured a fast single-round transcription from each template (data not shown). As shown in Figure 1B, a folding rate of 0.19 min^{-1} was obtained, demonstrating that co-transcriptional folding of the self-splicing *Candida* ribozyme is about twice as fast as refolding. This increase is similar to those observed for the *Tetrahymena* group I ribozyme (Heilman-Miller and Woodson 2003b) and the RNase P ribozyme (Pan et al. 1999).

Co-transcriptional folding, but not refolding of both pseudoknots, of the self-splicing *Candida* intron is sensitive to AON inhibition

We have recently showed that co-transcriptional formation of the P3–P7 pseudoknot of the *Candida* intron is inhibited

by AONs, suggesting this pseudoknot forms slowly when folding is coupled with transcription (Zhang et al. 2009). The folding kinetics of all different local structures of intron was measured by using 21 AONs covering the whole intron; these AONs were designed to pair with the corresponding target sequence with similar affinity (Fig. 2B). Each AON at 4 μ M was added to either the co-transcriptional folding condition or the refolding condition where the precursor RNA was incubated with transcription buffer and T7 polymerase. Only the slow folding regions should be accessible to the complementary AONs.

Interestingly, none of these AONs significantly inhibits refolding of the active self-splicing ribozyme (Figs. 2C,E), suggesting a faster ribozyme refolding than the AON binding. This is consistent with the fast refolding of the active *trans*-acting *Candida* ribozymes (Xiao et al. 2003;

Bao et al. 2008). Strikingly, the co-transcriptional ribozyme folding was dramatically slowed by AONs targeting two pseudoknot structures, including AONs 239–260R, 239–262R, 242–260R, and 242–262R that target the P3–P7 pseudoknot, as well as AON 1–26R targeting the P1–P10 pseudoknot (Fig. 2). Both pseudoknot structures are known to be essential for the self-splicing activity: P3–P7 pseudoknot constitutes the catalytic active site of the ribozyme, while P1–P10 pseudoknot contains both the 5' and 3' splice sites (Li and Zhang. 2005). These results suggested that the transcription process reprograms the folding of these pseudoknots to kinetics slow enough to be bound and interrupted by pseudoknot-targeted AONs.

Ribonuclease H experiments showed that all AONs precisely bound to the predicted sites and produced the corresponding cleaved RNA segments (Supplemental Fig.

FIGURE 2. Identification of the functionally important substructures of the *Candida* group I intron whose formation is interrupted by AONs. (A) The secondary structure of the intron including two short exons. The two arrows indicate the 5' and 3' sites of the self-splicing intron. The regions most responsive to AON inhibition during co-transcriptional folding are shaded in red, and the moderately responsive regions are in yellow. The bold italic nucleotides have the most significant contributions to the AON inhibition. (B) The primary sequence of the *Candida* group I ribozyme, with the complementary region of the 21 AONs being underlined. For the name of each AON, the numbers indicate the ribozyme region with which it base pairs, and "R" stands for the antisense sequence. (C) self-splicing assay of the refolding of the intron substructures. The purified precursor rRNA was refolded in the absence (control) or presence of 4 μ M of each indicated AON for 1 h. (D) Co-transcriptional self-splicing assay. The precursor rRNA was transcribed in vitro in the absence (control) or presence of 4 μ M of each indicated AON for 1 h. (E) Quantitative analysis and plotting of the results in C and D. Fraction of self-splicing indicates the ratio of the spliced precursor RNA to the total precursor RNA. Red asterisks indicate AONs strongly inhibit the co-transcriptional ribozyme folding, while the blue ones indicate those showing moderate inhibition.

S1), validating the hypothesis that AON-directed inhibition of intron splicing is due to the specific AON binding.

P3–P7 pseudoknot formation becomes rate limiting during the co-transcriptional folding of the self-splicing *Candida* ribozyme

An RNase H-cleavage experiment was then conducted to monitor the P3–P7 folding rate in the whole ribozyme precursor population (Zarrinkar and Williamson 1994). As shown in Figure 3A, a folding rate of about 0.04 min^{-1} was obtained for one-round transcription. A similar folding rate constant 0.07 min^{-1} was obtained for multiple-round transcribed precursor RNA (Fig. 3B). This rate is obviously slower than the co-transcriptional folding of the active ribozyme (Fig. 1B), and may reflect what occurs in a nonproductive folding pathway in which RNA molecules are trapped in misfolded structures.

The rate of co-transcriptional folding of the P3–P7 pseudoknot in the productive folding pathway was then measured based on the fact that disruption of the native pseudoknot formation by AONs compromises the ribozyme activity. We folded single-round transcribed self-splicing *Candida* ribozyme for the indicated time, then added 240–261R (final concentration $10 \mu\text{M}$) to each folding reaction and kept the total folding time (including prior to and after the addition of 240–261R oligonucleotide) as 1 h. Figure 3C demonstrates that the productive P3–P7 pseudoknot folded at a rate constant of 0.2 min^{-1} , which is similar to the rate of folding the active self-splicing ribozyme (see Figure 1B). Therefore, formation of the native P3–P7 pseudoknot dominates the native co-transcriptional folding pathway and serves as the rate-limiting step in the folding of the self-splicing *Candida* ribozyme.

The transcription process may reprogram the folding pathway of group I introns by altering the folding kinetics of pseudoknots

The slow folding nature of the P3–P7 pseudoknot structure when the purified *Tetrahymena* group I ribozyme RNA is subjected to refolding has been appreciated since 1994 (Zarrinkar and Williamson 1994); but a single molecule study (Zhuang et al. 2000) suggests that slow P3–P7 formation is a hallmark of the nonproductive folding pathway. Consistently, our study of the *Candida* group I ribozyme shows that refolding of the P3–P7 pseudoknot in the productive folding pathway is fast and not rate limiting, and the failure of rapid P3–P7 formation leads the ribozyme to a nonproductive folding pathway (Xiao et al. 2003; Zhang et al. 2005; this study). A study from the Woodson group also shows that refolding of the P3–P7 of the *Azoarcus* ribozyme is much more rapid than folding of the catalytically active ribozyme (Rangan et al. 2003). Therefore, there is no previous strong evidence to show

that P3–P7 formation is rate limiting in the productive refolding of group I introns.

This report clearly demonstrates that formation of both P3–P7 and P1–P10 pseudoknots of the *Candida* group I intron is significantly slowed down during co-transcriptional folding. Formation of P3–P7 pseudoknot becomes rate

FIGURE 3. (Legend on next page)

limiting in co-transcriptional folding of the catalytically active ribozyme. This may not be surprising when we consider that during in vitro transcription, after the first strand of the P3 helix emerges from the RNA polymerase, it has to wait nearly 1 sec for the second strand to be released. During this time delay, the naked first strand has many opportunities to form alternative stable structures with RNA sequences synthesized prior to the correct complementary strand (Fig. 4). Because of the high thermodynamic stability and slow dissociation kinetics of these mispaired structures (Uhlenbeck, 1995), formation of the correct P3–P7 pseudoknot is thus delayed. Consistent with this concept, this study suggests that another long-range base-pairing structure P4–P5 also forms slowly during co-transcriptional folding (Figs. 2C, E). Thus, despite co-transcriptional folding being faster than refolding, its rate-limiting pseudoknot folding step in the pathway to the active ribozymes is slower and rate limiting.

This report suggests that the transcription process may reprogram the folding pathway of group I introns and other structured RNAs by altering the folding kinetics of long-range base paired structures. In this regard, it is likely that a critical pseudoknot in a highly structured RNA could act as a sensor of the transcription rate to regulate the folding of the active structure of the host RNA, and therefore could couple the functional RNA folding with the cell growth condition.

Implications for RNA-targeted chemotherapy

Functional RNAs represent new therapeutic targets, and extensive studies have been carried out to develop anti-RNA therapeutic approaches. It has previously been demonstrated that inhibitors of group I introns that alter the ribozyme folding can act as antimicrobial agents in *Candida* strains in which the function of such ribozymes is essential

for viability (Miletti and Leibowitz 2000; Zhang et al. 2002). This report shows that the splicing activity of the *C. albicans* group I ribozyme is effectively compromised by AONs interrupting the folding of functionally important pseudoknot structures. We have recently shown that AONs targeting the P3–P7 pseudoknot specifically and effectively kill the intron-containing *C. albicans* strains (Zhang et al. 2009). Therefore, oligonucleotides blocking the co-transcriptional formation of the pseudoknot structure of the target RNA represent a new class of potential therapeutic agents.

MATERIALS AND METHODS

The template DNA for in vitro transcription of the precursor RNA (658 nt) contained the *Candida* intron, as well as 70 nt upstream and 209 nt downstream adjacent host rRNA sequence (Zhang et al. 2009). All of the in vitro reactions were analyzed on 5% polyacrylamide–8 M urea gels that were then exposed onto PhosphorImager screens for visualization and quantitative analysis using the variable scanner Typhoon 9200 (Amersham Pharmacia Biotech). The data were then plotted using the GraphPad Prism 4.0 program (www.graphpad.com).

Assay of the in vitro folding of the self-splicing *Candida* ribozyme

To study the ribozyme folding during multiple-round transcription, the template DNA was transcribed and spliced in 10 μ L reactions containing six units of T7 polymerase, 500 μ M of rATP, rCTP, and rUTP, 100 μ M rGTP, and 0.01 μ Ci [α - 32 P] GTP (3000 Ci/mmol) in 10 μ L of 1 $\times$ T7 transcription buffer at 37°C for 60 min, in the presence or absence of AONs. Because 6 mM of Mg^{2+} and sufficient GTP were present, the ribozyme self-splicing occurred during transcription. The reactions were stopped by EDTA-containing loading buffer, and analyzed on denature PAGE gels. To study the active ribozyme refolding, the purified *Candida* intron precursor was incubated at the same condition as for the co-transcriptional folding.

To study the ribozyme folding during single-round transcription, 1 μ g of the same template DNA was mixed with 20 μ Ci [α - 32 P] GTP (3000 Ci/mmol) (Perkin-Elmer–NEN) and 200 units T7 RNA polymerase (MBI Fermentas) in T7 transcription buffer. This mixture was incubated for 2 min at 37°C. Then a mixture of four nucleotides (Roche) and heparin (Sigma) was added to bring the reaction volume to 40 μ L and final concentrations of rATP, rCTP, and rUTP to 0.5 mM, rGTP to 0.125 mM, and heparin to 10 mg/mL. To measure the rate constant for folding of the active self-splicing *Candida* ribozyme, a 2 μ L aliquot was removed immediately after the addition of four nucleotides and heparin for the zero time point, which was actually about a 5–10-sec reaction due to the time required for the manual operation. At various time points, 2 μ L aliquots were removed and mixed with loading dyes containing EDTA.

To measure the rate constant for folding of the native P3–P7 pseudoknot, the 2 μ L aliquots were removed at various time points and placed into an 8 μ L reaction mix containing 1 $\times$ T7 buffer (final concentration) and unmodified 240–261R oligonucleotide (AON, final concentration of 10 μ M). The mixture continued to react at 37°C and the total reaction time including

FIGURE 3. Measuring the kinetics of co-transcriptional folding of the P3–P7 pseudoknot using AON 240–261R. (A) Detection of the formation of the P3–P7 pseudoknot structure by RNase H cleavage during single-round transcription. A representative gel is shown in the upper panel, and the results of two independent experiments were quantified. The fraction of the intact RNA was plotted against the transcription time by fitting curves to a single exponential, resulting in an observed folding constant ($k_{f(P3-P7)}$) of $0.039 \pm 0.003 \text{ min}^{-1}$ (lower panel). (B) Detection of the formation of the P3–P7 pseudoknot structure by RNase H cleavage during multiple-round transcription. $S(t)$ indicated the amount of total precursor synthesized at each time point and $N(t)$ indicated the amount of the ribozyme with the folded P3–P7 pseudoknot (inaccessible to RNase H) at each time point. The folding rate of the P3–P7 pseudoknot in the nascent RNA transcript was described as $N(t)/S(t) = 1 - (1 - e^{-k_f t})/k_f t$ (Pan et al. 1999), resulting in a $k_{f(P3-P7)}$ of $0.068 \pm 0.006 \text{ min}^{-1}$. (C) Detection of the formation of the native P3–P7 pseudoknot during single-round transcription. Self-splicing was conducted for 1 h for each reaction and the AON 240–261R was added at each indicated time during the reaction. The splicing fraction was plotted as in Figure 1, resulting in an $k_{fN(P3-P7)}$ of $0.20 \pm 0.02 \text{ min}^{-1}$.

Transcription-coupled folding *in vitro*:

Rate-limiting formation of P3-P7 pseudoknot and other long-range base-pairing

FIGURE 4. A model of the group I intron RNA folding scheme during *in vitro* transcription (see the text for details).

prior to and after the addition of T7 buffer and 240–261R oligonucleotide was kept as 1 h for each reaction.

To measure the mixed folding rate of the P3–P7 structure, the 2 μ L aliquots removed at each time point were immediately subjected to a 0.5-min RNase H cleavage assay (Zarrinkar and Williamson 1994). The reaction concentrations of AONs (SaiBaiSheng) and RNase H (Takara) were 10 μ M and 0.4 U/ μ L, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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