Refolding of rRNA exons enhances dissociation of the *Tetrahymena* intron

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ABSTRACT

Self-splicing of the *Tetrahymena* pre-rRNA is inhibited by a conserved rRNA hairpin P(–1) upstream of the 5' splice site. P(–1) inhibits self-splicing by competing with formation of the P1 splice site helix. Here we show that the P(–1) hairpin also enhances dissociation of the spliced products, which was monitored by native gel electrophoresis. Mutations that stabilize the rRNA hairpin increase the rate of dissociation approximately 10-fold, from 0.5 min^{–1} for the wild-type RNA to ~4 min^{–1} at 30 °C. Conversely, mutations or oligonucleotides that inhibit refolding of the exons and that stabilize the P1 helix decrease the rate of product release. The results suggest that refolding of products can be used to stimulate the turnover of ribozyme-catalyzed reactions. In the pre-rRNA, this conformational change helps shift the equilibrium of self-splicing toward the mature rRNA.

Keywords: group I intron; product dissociation; ribozyme; rRNA; self-splicing

INTRODUCTION

In the active sites of enzymes, interactions that enable specific substrate recognition must be tuned so that the products are released efficiently. Many catalytic RNAs recognize their substrates via stable base pairing interactions. As the products are usually bound to the ribozyme by the same base pairs, product dissociation is slow and can limit turnover under steady-state conditions (reviewed in Narlikar & Herschlag, 1997). Poor turnover due to slow product release can diminish the effectiveness of ribozymes in biotechnology applications. However, the known naturally occurring ribozymes (except for RNase P) have been derived from self-cleaving or self-splicing RNAs, which need only react once. Nonetheless, separation of the products is expected to help drive self-splicing reactions toward the mature RNAs (Woodson & Cech, 1989). Similarly, product dissociation has a large effect on the equilibrium between cleavage and ligation by the hairpin ribozyme (Nesbitt et al., 1999).

Here, we find that the group I intron from *Tetrahymena* pre-rRNA provides a natural example of how refolding of RNA can accelerate product release. The 5' splice site is recognized by base pairing between the 5' exon and a guide sequence (IGS) in the intron, which forms the P1 splice site helix (Davies et al., 1982; Been & Cech, 1986; Fig. 1A). Tertiary interactions dock the P1 helix into the active site cleft of the intron where the external G-cofactor is also bound (Pyle & Cech, 1991; Strobel et al., 1998). In multiple turnover reactions, release of oligonucleotide products that mimic the cleaved 5' exon RNA is slower than internal steps (Herschlag & Cech, 1990a,b). However, these stable interactions help ensure that the 5' exon remains bound in the active site after the first step of splicing, and prevents its release until exon ligation is complete (Narlikar & Herschlag, 1997).

Experiments with the *Tetrahymena* ribozyme have suggested that exon ligation stimulates undocking of the P1 splice site helix (Mei & Herschlag, 1996). However, the rRNA is likely to remain base paired with the intron after exon ligation. The natural 3' exon makes 6 bp with the 5' end of the IGS (P10; Fig. 1A). The P10 helix contributes to 3' splice site recognition (Suh & Waring, 1990) and increases the stability of the product complex. If P1 and P10 helices both stabilize the product complex, the dissociation rate would be approximately 10^{–3}–10^{–4} min^{–1} at 30 °C. This es-

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FIGURE 1. Exon secondary structure of the *Tetrahymena* pre-rRNA. **A:** Alternative pairings (P) in the 5' exon affect 5' splice site recognition and product release. Thick line: exon sequence; thin line: intron (IVS); open circle: 5' splice site; closed circle: 3' splice site. **B:** Secondary structure of the 26S rRNA (Noller et al., 1981) showing the position of the intron. Sequences that are involved in alternative pairings are colored as in A. Deletions in P(-2) are indicated schematically. Adapted from Cao & Woodson (1998).

time is based on nearest-neighbor free-energy parameters for helix stability (Freier et al., 1986; Xia et al., 1998) and assumes an association rate of 10^6 – 10^7 $\text{M}^{-1}\text{s}^{-1}$ (Craig et al., 1971; Porschke & Eigen,

1971). However, the rate of product release after splicing of the *Tetrahymena* pre-rRNA was found to be 0.4 – 0.5 min^{-1} at 30°C (Emerick et al., 1996), faster than expected. This suggested that other interactions

in the pre-rRNA lower the stability of the spliced product complex.

Here, we show that release of the spliced intron is accelerated by refolding of the rRNA. Earlier work demonstrated that a conserved rRNA hairpin in the 5' exon of the natural *Tetrahymena* pre-rRNA, which we call P(−1), competes with the P1 splice site helix (Fig. 1A; Woodson & Cech, 1991). Another helix, PX, competes with P(−1) and shifts the secondary-structure equilibrium toward P1 (Fig. 1B; Woodson & Emerick, 1993). PX is stabilized by a long stem-loop in the rRNA, which we designate P(−2) (Cao & Woodson, 1998). Deletion of P(−2) disfavors formation of PX, and leads to poor self-splicing activity. The role of rRNA structure in intron release was tested by measuring changes in the rate of product dissociation caused by mutations that alter the equilibrium between alternative secondary structures.

RESULTS

Dissociation of spliced products is slower than self-splicing

Under conditions of saturating GTP, self-splicing can be treated as a pseudo-first-order transformation of the pre-rRNA (Bass & Cech, 1984). The mechanism of splicing and dissociation of the spliced products can be described by the following scheme (Cech, 1990):

where 5'ex and G-IVS-3'ex are intermediates, G-IVS is the linear intron, and LE is the ligated exons. For the *Tetrahymena* intron, the second step of splicing is faster than the first step, so that the observed rate of splicing, k_{sp} , is approximately equal to k_1 when the reaction is saturated with GTP. The spliced products are typically separated by denaturing polyacrylamide gel electrophoresis, so that dissociation of the products (k_d) does not contribute to k_{sp} .

Native gel electrophoresis was used to monitor the appearance of dissociated intron and ligated exon RNAs over time. The product complex of a 657-nt pre-rRNA is stable enough to be trapped on a native polyacrylamide gel if GTP is added to the pre-rRNA before loading (Emerick & Woodson, 1994). The product complex has an electrophoretic mobility that is similar to the correctly folded pre-rRNA, whereas the free intron and ligated exon RNAs migrate much more rapidly in the gel. Splicing reactions were initiated by the addition of 0.1 or 0.5 mM GTP at 30°C and the products were analyzed on a native 4% polyacrylamide gel at 10–12°C (Fig. 2). The rates at which the free intron appeared on native gels, which we will call k_{prod} , were

FIGURE 2. Dissociation of spliced products. Splicing reactions of uniformly [^{32}P]-labeled wild-type (pSW012) or $\Delta\text{P}(-2)$ pre-rRNA were initiated with the addition of 0.5 mM GTP at 30°C. Aliquots were loaded on a native 4% polyacrylamide gel at the times indicated above each lane (Emerick & Woodson, 1994). The gel was run continuously during the experiment. Pre: pre-rRNA and spliced product complex; IVS: linear intron; LE: ligated exons. The $\Delta\text{P}(-2)$ pre-rRNA appears as both the active (4) and inactive (2b) conformations.

0.40 and 0.52 min^{-1} at 30°C in the presence of 0.1 and 0.5 mM GTP, respectively (Table 1). These results are the same, within error, as product dissociation rates obtained in previous experiments (Emerick et al., 1996).

Reactions carried out under the same conditions but without glycerol were also analyzed on denaturing 4% polyacrylamide gels (data not shown). The rates of self-splicing in the presence of 0.1 and 0.5 mM GTP were 2.3 and 4.2 min^{-1} , respectively (Table 1), again consistent with previous work (Emerick et al., 1996; Cao & Woodson, 1998). Thus, for the wild-type pre-rRNA, k_{prod} was five to eight times slower than the rate of self-splicing, and reflects the net rate of dissociation ($k_{prod} \approx k_d > k_{sp}$). Therefore, the overall rate for releasing spliced

TABLE 1. Rates of self-splicing and product dissociation (min^{-1}).

Pre-rRNA ^a	k_{sp} ^b	k_{prod} ^c	k_d ^d
Wild type	4.2 ± 1.1	0.52 ± 0.18	0.75
$\Delta\text{P}(-2)$	1.7 ± 0.1	1.7 ± 0.2	~4
G-75C	0.92 ± 0.04	0.85 ± 0.16	
C-21G	0.15 ± 0.01	0.20 ± 0.04	
G-75;-21G	3.1 ± 0.2	0.68 ± 0.06	
Short	1.1 ± 0.2	1.3 ± 0.1	~5
Short + 50 μM PX9	6.0 ± 1.6	0.75 ± 0.1	0.80

^aThe wild-type pre-rRNA transcribed from pSW012 contains a 146-nt 5' exon and 86 nt of rRNA sequence in the 3' exon. The shorter pre-rRNA transcribed from pSW031 contains a 23-nt 5' exon. Except for this difference, the pre-rRNA sequences are the same.

^bObserved rate of self-splicing obtained from denaturing gel electrophoresis. Reactions contained 0.5 mM GTP and were conducted at 30°C. Uncertainties are given as the standard deviation of values obtained from fits to two to five independent experiments.

^cObserved rate constant for forming free intron obtained from native gel electrophoresis. Reactions conditions as above.

^dRate of product dissociation obtained from pulse-chase experiments (Fig. 5).

RNA is limited by dissociation of the intron and the rRNA exons.

rRNA secondary structure modulates product dissociation

We expected the rate of product dissociation to be directly related to the stability of the P1 and P10 base-pairing interactions. Base mismatches in P1, for example, accelerate release of oligonucleotide products from the *Tetrahymena* ribozyme (Herschlag & Cech, 1990b). Hence, the equilibrium between the P1 splice site helix and the P(-1) hairpin in the 5' exon should play a role in dissociation of the intron and rRNA exons after splicing. We previously found that deletion of an upstream stem-loop in the rRNA, P(-2), destabilizes PX so that the inhibitory P(-1) hairpin is favored over P1 and the rate of self-splicing is reduced (Cao & Woodson, 1998). A partial 17-nt deletion in the P(-2) stem ($\Delta 17$; Fig. 1B) lowers self-splicing activity slightly compared to the wild-type pre-rRNA, whereas deletion of 41 nt ($\Delta 41$) stabilizes PX and P1 and increases the rate of splicing. These mutations were used to modulate the equilibrium between P1 and P(-1) without altering the sequence of the P1 splice site helix.

The apparent rates of product dissociation and self-splicing were compared among pre-rRNAs with deletions in P(-2) and the wild type. As shown in Fig. 3B, pre-rRNAs with partial deletions of P(-2) ($\Delta 17$ and $\Delta 41$) yielded free intron RNA at rates similar to the wild-type pre-rRNA ($k_{prod} = 0.5 \text{ min}^{-1}$). For these RNAs, k_{prod} was 6- to 10-fold slower than the rate of self-splicing. In contrast, although the $\Delta P(-2)$ pre-rRNA spliced two to three times more slowly than the wild-type RNA as expected, the intron and ligated exon RNAs accumulated more rapidly in native gels (Fig. 2). For $\Delta P(-2)$ RNA, the observed rates of product formation on native and denaturing gels were the same ($k_{prod} \approx k_{sp} = 1.7 \text{ min}^{-1}$; Fig. 3B and Table 1), suggesting that product dissociation is no longer rate limiting with respect to splicing. This was confirmed by conducting experiments at various concentrations of GTP (0–1 mM) (Fig. 4). The rate constants k_{prod} and k_{sp} were the same, within error, under all conditions tested and exhibited the same apparent K_M for GTP. Because the deletion of P(-2) promotes the formation of P(-1) instead of PX and P1, these results suggested that the ability of the exons to form P(-1) enhances release of the intron from the spliced rRNA.

To confirm that the stability of P(-2) and the exon secondary structure plays a role in product dissociation, the first base pair of P(-2) was disrupted by point mutations, as shown in Figure 1A. These mutations disturb stacking between P(-2) and PX and result in 5- to 50-fold lower rates of self-splicing (Cao & Woodson, 1998). Because self-splicing is rate determining for these

FIGURE 3. Comparison of k_{sp} and k_{prod} for wild-type and mutant pre-rRNAs. **A:** The fraction of excised intron (f_{ivs}) was quantitated using denaturing gels (●) or native gels (○) as in Figure 2. Data points were fit to a double or single exponential equation, respectively. Reactions were carried out the same way except that the self-splicing reactions do not contain glycerol. **B:** Mean and standard deviation of observed rate constants (bars) for wild-type and mutant pre-rRNAs. Open box: k_{prod} (native gel); hatched box: k_{sp} (denaturing gel).

mutants, $k_{prod} \approx k_{sp}$ (Table 1). The compensatory double mutation that restores the base pairing in P(-2) also restored self-splicing activity, but k_{prod} was much smaller than k_{sp} , like the wild-type pre-rRNA. Therefore, the stability of P(-2) correlates with the rate of product dissociation, consistent with our hypothesis that formation of the P(-1) hairpin helps destabilize base pairing between the 5' exon and the intron.

Pulse-chase experiments

In the experiments above, the spliced product complex cannot be distinguished from the unspliced pre-rRNA in the native gel, and the observed rate k_{prod} reflects both splicing and product dissociation. To obtain a more

FIGURE 4. GTP dependence of splicing of $\Delta P(-2)$ pre-rRNA. Observed rates for the appearance of free intron were measured at various GTP concentrations as in Figure 3. ●: k_{prod} ; ○: k_{sp} . Data were fit to $k_{obs} = k_{max} \times [GTP] / (K_M + [GTP])$, where $k_{max} = 2.1 \pm 0.2 \text{ min}^{-1}$ or $2.2 \pm 0.1 \text{ min}^{-1}$, respectively, and $K_M(G) = 0.16 \pm 0.04 \text{ mM}$ or $0.18 \pm 0.03 \text{ mM}$.

direct measurement of the rate of product dissociation, pulse-chase experiments were carried out to specifically label the product complex. Unlabeled pre-rRNA was incubated with α -[^{32}P]-GTP, which becomes covalently ligated to the 5' end of the intron during the first step of splicing (Zaug & Cech, 1982). After 1 min, the reaction was chased with 500 μM of unlabeled GTP. Noncovalently bound GTP exchanges rapidly with unbound GTP ($k_{off} \approx 0.6 \text{ s}^{-1}$; Profenno et al., 1997) during electrophoresis (V. Emerick & S. Woodson, unpubl.). As a result, labeled GTP is released from the unreacted pre-rRNA, and only the product complex ($G^* \cdot \text{IVS} \cdot \text{LE}$) appears radiolabeled.

As shown in Figure 5, the wild-type spliced product complex disappeared much more slowly than the $\Delta P(-2)$ product complex, after pulse labeling with GTP. The apparent rate of product dissociation for the wild-type RNA was 0.75 min^{-1} , only slightly higher than the value obtained using uniformly labeled pre-rRNA (Table 1). In contrast, about 80% of the complexes formed from $\Delta P(-2)$ pre-rRNA were dissociated about 15 s after the cold GTP chase was added to samples, the time required for loading the sample into the gel. This provides a lower limit on the rate of product dissociation of $\sim 4 \text{ min}^{-1}$. These results show that for pre-rRNAs in which P(-1) is more stable than P1, the intron and ligated exons dissociate rapidly after splicing is complete. This explains why the $\Delta P(-2)$ pre-rRNA yields free intron more rapidly than the wild type, even though the mutant splices more slowly.

FIGURE 5. Pulse-chase method for measuring the rate of product dissociation. Splicing products were labeled with 0.3 μM α -[^{32}P]-GTP at 30°C for 1 min, then chased with 500 μM cold GTP. Aliquots were loaded on a native polyacrylamide gel at various times. **A:** Native gel electrophoresis. SP: spliced product complex; IVS: free intron that was covalently labeled with ^{32}P -GTP. **B:** Fraction of free intron (f_{IVS}) versus time (after the chase) was fit to a first-order rate equation. ●: wild type; ○: $\Delta P(-2)$; ▲: wild type before chase; △: $\Delta P(-2)$ before chase.

P(-1) stimulates product dissociation

We tested whether the stability of P(-1) and thus the rate of product dissociation may be modulated by binding of external oligonucleotides to the pre-rRNA. A pre-rRNA with a 23-nt 5' exon was used to evaluate the role of the P(-1) hairpin in the absence of extended rRNA sequences. Base pairing between the first 9 nt of the short 5' exon and a complementary DNA oligomer, PX9, mimics the PX helix in the natural pre-rRNA (Fig. 6A). Digestion of 5' end-labeled pre-rRNA with RNase H released the expected small fragment but no other products, suggesting that the oligonucleotide binds predominantly to the intended site (data not shown).

As expected from earlier work (Woodson & Cech, 1991), the short pre-rRNA spliced slowly in the absence of PX9 ($k_{sp} = 1.1 \text{ min}^{-1}$ and $k_{prod} = 1.3 \text{ min}^{-1}$), because P(-1) competes effectively with the P1 splice site helix when PX cannot form (Fig. 6B,C). In the presence of 50 μM PX9, the rate of self-splicing increased to $\sim 6 \text{ min}^{-1}$ and k_{prod} was decreased to 0.75 min^{-1} . These data were consistent with the results of pulse-chase experiments, which yielded a dissociation rate of

FIGURE 6. Inhibition of product release by a DNA oligomer. **A:** Base pairing between a pre-rRNA with a 23-nt 5' exon and PX9 DNA (dACTCCCGCC) mimics the PX helix in the full-length pre-rRNA. Formation of PX stabilizes P1 and inhibits product release. **B:** Product dissociation assays with labeled short pre-rRNA were carried out as described in Figure 2. -PX9: no PX9 DNA; +PX9: with 50 μ M PX9. Pre/SP: pre-rRNA and spliced product complex; IVS: free intron; LE: ligated exons. **C:** f_{IVS} versus time was fit to a first-order rate equation. $\bullet$: -PX9 ($k_{prod} = 1.37 \pm 0.06 \text{ min}^{-1}$); $\circ$: +PX9 ($k_{prod} = 0.75 \pm 0.12 \text{ min}^{-1}$).

0.8 min^{-1} in the presence of PX9 and $\sim 5 \text{ min}^{-1}$ in the absence of PX9 (Table 1). Therefore, the PX9 oligonucleotide suppresses product dissociation by shifting the secondary-structure equilibrium toward P1. Conversely, conditions that favor P(-1) enhance dissociation of the spliced products.

DISCUSSION

Mechanism of product dissociation

Competition between alternative hairpins strongly modulates the self-splicing activity of the *Tetrahymena* pre-rRNA (Woodson & Cech, 1991). Here, we show that the propensity to form the P(-1) stem-loop in the 5'

exon also correlates with rapid dissociation of the spliced products. Refolding of the spliced rRNA can enhance dissociation in two ways: First, the free energy of the dissociated products relative to the intron•rRNA complex is lowered if P(-1) compensates for base pairs lost when P1 and P10 are broken. Besides stabilizing the products, this could increase the net dissociation rate by preventing rebinding. Under the conditions of our experiments (10–50 nM RNA), product association could theoretically approach 6 min^{-1} as splicing nears completion. However, we are unable to form a product complex on native gels from the intron and rRNA exons, suggesting that even the wild-type rRNA folds into a conformation that cannot be recognized by the intron (V. Emerick & S. Woodson, unpubl.). Moreover, RNAs

containing a stable P(−1) helix are poor substrates for reverse splicing (Woodson & Cech, 1991), indicating that P(−1) inhibits reassociation of the spliced products.

Second, the rate of dissociation is increased if formation of P(−1) populates an intermediate in which the rRNA is bound to the intron only through P10. Because the free energy of opening P10 is 6–9 kcal/mol less than dissociation of P1/P10 (depending on whether P1 and P10 are coaxially stacked) (Freier et al., 1986; Xia et al., 1998), the lifetime of the P10 helix is estimated to be 5×10^3 – 10^6 times shorter than the lifetime of a complex containing both P1 and P10 ($k_d(\text{P10}) \approx 5$ – $1,000 \text{ min}^{-1}$ at 30°C). Displacement of P1 by the P(−1) hairpin is sufficient to account for the dissociation rate that we obtained for the $\Delta\text{P}(-2)$ RNA ($\sim 4 \text{ min}^{-1}$).

Tertiary interactions between the rRNA and the intron also are likely to influence the observed rate of product dissociation. If a high proportion of the P1–P10 helix in the product complex remains docked in the active site of the intron, product release would be slower than predicted from the free energy of base pairing alone (Pyle & Cech, 1991; Herschlag, 1992). However, there is evidence that exon ligation triggers undocking of P1. First, dissociation of the products after the second step of splicing is ~ 100 -fold faster than release of the 5' exon after the first step of splicing (Emerick et al., 1996; Mei & Herschlag, 1996), despite the fact that the 5' exon makes fewer base pairs with the IGS. Second, an oligonucleotide that imitates the ligated exons dissociates from the ribozyme about ~ 200 -fold faster than a 5' exon oligonucleotide (Mei & Herschlag, 1996). If P1 is primarily undocked in the product complex, exchange between P(−1) and P1 could promote release of the ligated exons. This helix exchange occurs much more rapidly than self-splicing (Y. Cao & S. Woodson, unpubl. data).

Implications for the equilibrium of self-splicing

Conformational flexibility provides a basis for functional regulation of RNA. A classic example is the mechanism of translational attenuation in the *Escherichia coli* trp operon (Lee & Yanofsky, 1977). Our results show that in the *Tetrahymena* pre-rRNA, the P1 and P(−1) hairpins have opposing effects on the forward and reverse splicing rates. They suggest that the relative stability of the P1 splice site helix should remain within a narrow window for optimal intron excision. On the one hand, P1 must be sufficiently stable to ensure that the 5' splice site is recognized within the context of the pre-rRNA and to minimize premature dissociation of the 5' exon after the first step of splicing. At the same time, failure to release the intron after exon ligation would increase the rate of the reverse reaction (Woodson & Cech, 1989). The reverse of the second step of splicing is as efficient as the forward reaction, with an equilib-

rium constant ~ 1 for oligomer substrates (Mei & Herschlag, 1996). However, P(−1) inhibits reassociation of the intron with its spliced junction in the rRNA (Woodson & Cech, 1991).

The free intron RNA is rapidly degraded in *Tetrahymena* cells ($t_{1/2} \sim 6 \text{ s}$; Brehm & Cech, 1983), and this is expected to play an important role in driving the splicing reaction forward. When overexpressed in *E. coli*, however, the intron accumulates. It associates with 50S ribosomal complexes (Nikolcheva & Woodson, 1997), and can reintegrate into *E. coli* rRNA by reverse splicing (Roman & Woodson, 1998). Interestingly, mutations in a mitochondrial exonuclease complex result in accumulation of the omega group I intron in yeast, and this leads to cleavage of the 21 S rRNA and loss of mitochondrial function (Margossian et al., 1996). These observations suggest that dissociation and degradation of the excised intron RNA is essential for tolerance of group I introns by the host cell.

Finally, it is important to note that release of the intron is necessary for proper folding of the large subunit rRNA. The P(−1) hairpin corresponds to the 1920 stem-loop (helix 69) in the *E. coli* 23S rRNA (Noller et al., 1981). This stem-loop is conserved across all phyla and is an important element in the interface between the large and small subunits of the ribosome (Joseph et al., 1997; Cate et al., 1999). Although the rRNA influences folding of the intron, the intron reciprocally alters the conformation of the rRNA. Thus, there is selective pressure to ensure that the intron does not remain bound too tightly to the rRNA. The requirements of 5' splice site recognition must be balanced against those of intron release.

Implications for ribozyme design

The idea that refolding of one or both partners can stimulate dissociation of an RNA complex suggests ways in which the rate of product release and turnover of natural or artificial ribozymes can be improved. In some sense, this is the converse of the complex annealing mechanisms observed in many natural antisense RNAs (Nellen & Sczakiel, 1996). In both instances, the overall change in free energy associated with forming or disrupting an intermolecular helix is compensated by corresponding base pairing changes of the two partners. A strategy for stimulating product release is likely to be particularly useful for RNA ligases, in which the products typically make at least as many, if not more, base pairs with the ribozyme than the substrates.

MATERIALS AND METHODS

Plasmids

pSW012 and its derivatives have been described previously (Woodson, 1992; Cao & Woodson, 1998). Plasmid pSW012

encodes a pre-rRNA with 146 nt of natural rRNA sequence in the 5' exon and an 86-nt 3' exon (Woodson, 1992). Plasmid pSW031, which contains a 23-nt 5' exon (rRNA sequence), was constructed by site-directed mutagenesis of pSW012 (Kunkel et al., 1987).

Preparation of pre-rRNA

Plasmids were linearized with *EcoR* I or *Hind* III for in vitro run-off transcription of pre-rRNAs. Pre-rRNAs were transcribed using T7 RNA polymerase and isolated from 4% polyacrylamide gels containing 8 M urea as described previously (Zaug et al., 1988; Woodson & Cech, 1991; Cao & Woodson, 1998). RNA concentrations were determined from the absorbance at 260 nm as described previously (Cao & Woodson, 1998).

5'-end-labeled pre-rRNAs were prepared from calf intestinal phosphatase- (Promega) treated RNA using T4 polynucleotide kinase (U.S. Biochemical) and γ -[32 P]-ATP (New England Nuclear), and gel purified as above. Pre-rRNAs used for splicing assays and for native gel electrophoresis were uniformly labeled with α -[32 P]-ATP during transcription by T7 polymerase, and purified with TE-100 gel filtration spin columns (Clontech) preequilibrated in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA (Emerick & Woodson, 1993). Transcription reactions were carried out at 30 °C for 20–30 min to minimize splicing.

Self-splicing assays

Uniformly labeled pre-rRNAs were heated to 95 °C and cooled rapidly in the presence of splicing buffer (100 mM (NH₄)₂SO₄, 50 mM HEPES, 5 mM MgCl₂, pH 7.5) as previously described (Walstrum & Uhlenbeck, 1990; Emerick et al., 1996). Splicing reactions were carried out at 30 °C. Pre-rRNA (10–50 nM) was preincubated at 30 °C for 2–5 min before beginning reactions with the addition of 0.5 mM GTP (unless otherwise specified). Where present, oligodeoxynucleotides were added after the pre-rRNA was renatured. Spliced products were separated on 4 or 6% polyacrylamide gels, and the radioactivity was quantified with a Molecular Dynamics PhosphorImager. The fraction of spliced products at each time point, f_{sp} , was fit to a first-order rate equation (Emerick et al., 1996). The precision of individual fits ($\pm 5\%$) was typically better than the variation between independent trials (± 10 –25%).

Product dissociation assays

Uniformly labeled pre-rRNAs were annealed at 95 °C as for splicing reactions, then mixed with 10% glycerol (v/v). The mixture was preincubated at 30 °C for 2–5 min before the addition of 0.5 mM GTP, unless otherwise specified. Aliquots were taken at various times (the first time point was taken before the GTP was added) and loaded on a 4% polyacrylamide gel containing 34 mM Tris, 66 mM HEPES, 0.1 mM EDTA, and 10 mM MgCl₂, pH 7.5, at 10–12 °C as previously described (Pyle et al., 1990; Emerick & Woodson, 1994). The dissociated products (linear intron or ligated exons) were quantified with a Molecular Dynamics PhosphorImager. The fraction of dissociated spliced products (linear intron or ligated exons) at each time point was normalized to its specific ac-

tivity and fit to a first-order rate equation (Emerick et al., 1996).

Pulse-chase dissociation assay

Unlabeled pre-rRNA (1 μ M) was annealed as above, then mixed with 10% glycerol (v/v). Where stated, oligodeoxynucleotide PX9 (5'-dACTCCCGCC) was added after renaturation of the pre-rRNA. The mixture was incubated at 30 °C for 5 min before the addition of 0.3 μ M α -[32 P]-GTP (0.24 μ Ci/ μ L) (New England Nuclear), in a total reaction volume of 20 μ L. An aliquot was removed right after α -[32 P]-GTP was added (time 0 $\approx$ 5 s), and loaded on a 4% nondenaturing polyacrylamide gel at 10–12 °C. After 1 min, 500 μ M unlabeled GTP was added to the reaction, and additional aliquots were loaded on the gel at various times. The fraction of free linear intron relative to the total radioactivity in each lane was fit to a first-order rate equation. The amount of 32 P in each lane remained relatively constant ($\pm 20\%$) over the course of the reaction.

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