Self-splicing of a group I intron reveals partitioning of native and misfolded RNA populations in yeast

SCOTT A. JACKSON,^{1,3} SUJATHA KODUVAYUR,^{1,4} and SARAH A. WOODSON²

¹Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, USA

²Department of Biophysics, Johns Hopkins University, Baltimore, Maryland 21218, USA

ABSTRACT

Stable RNAs must form specific three-dimensional structures, yet many RNAs become kinetically trapped in misfolded conformations. To understand the factors that control the accuracy of RNA folding in the cell, the self-splicing activity of the *Tetrahymena* group I intron was compared in different genetic contexts in budding yeast. The extent of splicing was 98% when the intron was placed in its natural rDNA context, but only 3% when the intron was expressed in an exogenous pre-mRNA. Further experiments showed that the probability of forming the active intron structure depends on local sequence context and transcription by Pol I. Pre-rRNAs decayed at similar rates, whether the intron was wild type or inactivated by an internal deletion, suggesting that most of the unreacted pre-rRNA is incompetent to splice. Northern blots and complementation assays showed that mutations that destabilize the intron tertiary structure inhibited self-splicing and processing of internal transcribed spacer 2. The data are consistent with partitioning of pre-rRNAs into active and inactive populations. The misfolded RNAs are sequestered and degraded without refolding to a significant extent. Thus, the initial fidelity of folding can dictate the intracellular fate of transcripts containing this group I intron.

Keywords: RNA folding; RNA stability; metastable structure; pre-rRNA processing

INTRODUCTION

Stable noncoding RNAs must fold into specific three-dimensional (3D) structures to function properly in the cell. Many RNAs misfold when transcribed in vitro, however, and the propensity to misfold is sensitive to sequence, the concentration of Mg²⁺ and other ions, and the rate of transcription elongation (Uhlenbeck 1995; Treiber and Williamson 1999; Woodson 2000; Pan and Sosnick 2006). Thus, maintaining the fidelity of RNA folding under intracellular conditions is critical to RNA function and evolution. An important question is whether structured RNAs must fold correctly within a certain time window after transcription, or whether misfolded RNAs or RNPs have many chances to refold before they are destroyed by nucleases.

We used the self-splicing activity of the *Tetrahymena* group I intron to investigate the fidelity of RNA folding in yeast. Self-splicing depends only on the 3D structure of the RNA and the availability of Mg²⁺ and GTP (Cech 1990). Thus, the level of splicing reflects the amount of correctly folded RNA. In *Tetrahymena thermophila*, the intron is located in the large subunit (26S) rRNA and self-splices early during pre-rRNA processing (Cech and Rio 1979; Din et al. 1979), with an apparent $t_{1/2} \sim 2$ sec (Brehm and Cech 1983). The *Tetrahymena* intron was similarly reactive when inserted into the *Escherichia coli* 23S (Zhang et al. 1995) or yeast 25S rRNA (Lin and Vogt 1998). In contrast, splicing is 20–200 times slower in vitro ($t_{1/2} = 0.7$ –7 min) because up to 95% of the pre-rRNA misfolds when transcribed in vitro (Bass and Cech 1984; Emerick and Woodson 1993). The misfolded intermediates are stable and refold slowly at 30°C, limiting the observed rate of self-splicing (Emerick et al. 1996).

The greater activity of the *Tetrahymena* intron in vivo is probably not due to greater stability of its tertiary structure, as experiments on the *td* group I intron in *E. coli* (Brion et al. 1999) and the hairpin ribozyme in yeast (Donahue et al. 2000) showed that the catalytic function and thermodynamic stability of these ribozymes are similar in the cell and in “physiological” buffers. A more likely explanation is that a larger fraction of the pre-rRNA forms the active conformation in the cell than in typical in vitro

³Present addresses: Center for Food Safety and Applied Nutrition, Food and Drug Administration, 8301 Muirkirk Road, Laurel, MD 20708, USA; e-mail: Scott.Jackson@cfsan.fda.gov. ⁴Department of Physiology and Biophysics, University of Illinois at Chicago, 835 S. Wolcott Avenue, Chicago, IL 60612-7342, USA; e-mail: skoduvay@uic.edu.

Reprint requests to: Sarah A. Woodson, Department of Biophysics, Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218, USA; e-mail: swoodson@jhu.edu; fax: (410) 516-4118.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.184206>.

experiments. For example, mutations that increase misfolding of the *Tetrahymena* pre-rRNA in vitro have a much milder effect on splicing in *E. coli* (Nikolcheva and Woodson 1999). This may be because fewer transcripts misfold initially, or because misfolded RNAs are rapidly refolded by RNA chaperones. A number of RNA-binding proteins and DEAD-box ATPases have the potential to stimulate RNA refolding and strand exchange reactions (Schroeder et al. 2004). For example, overexpression of *E. coli* StpA stimulates splicing of the *td* group I intron in *E. coli* and is able to resolve misfolded RNAs as long as the native structure is thermodynamically stable (Waldsich et al. 2002; Grossberger et al. 2005).

To dissect the factors contributing to the fidelity of RNA folding in the cell, we compared the self-splicing activity of the *Tetrahymena* intron in various genetic contexts in yeast. The results are best explained by the partitioning of newly transcribed pre-rRNAs into active and inactive pools, as we proposed previously for pre-rRNAs expressed in *E. coli* (Koduvayur and Woodson 2004). The probability of forming the active ribozyme is greatest when the intron is integrated into the chromosomal rDNA repeats of yeast, and lowest when it is placed at the 5' end of a pre-mRNA transcribed by RNA polymerase II.

RESULTS

In vivo splicing is linked to rRNA

To determine which factors in eukaryotic cells favor folding of a highly structured RNA, we compared the self-splicing activity of the *T. thermophila* intron in different precursors in yeast (Fig. 1A). The 413 nucleotides (nt) intron is inserted after helix 69 in the large subunit rRNA (position 1925, *E. coli* numbering), a conserved region of the large subunit rRNA that directly contacts the small ribosomal subunit (Cate et al. 1999).

Lin and Vogt (1998) found that the *Tetrahymena* intron spliced efficiently in the yeast *Saccharomyces cerevisiae*, when transposed into the 25S rRNA gene at the position homologous to its natural splice junction in *Tetrahymena*. Cells remained viable when the intron was integrated into all of the chromosomal rDNA repeats, demonstrating that the spliced 25S rRNA was functional. Northern analysis of total RNA with a probe complementary to the intron revealed a large accumulation of spliced intron RNA and <1% unspliced 25S and 27S pre-rRNA (Fig. 1B). When the intron sequences were cloned into a yeast plasmid expressing the full-length 35S pre-rRNA from the *GAL7* promoter (Fig. 1A; Nogi et al. 1991), we again observed little unspliced pre-rRNA on Northern blots (Fig. 1B).

The extent of splicing was measured with standard curves comparing the intensity of the intron and pre-rRNA bands with the amount of actin mRNA (see

Materials and Methods). The ratio of intron to pre-rRNA at steady state was 260:1 for the chromosomal 35S pre-rRNA transcribed by Pol I, and 15:1 for plasmid-encoded pre-rRNA transcribed by Pol II (Table 1). Thus, splicing is efficient in yeast when the intron sequences are inserted in the 25S rRNA gene, although the extent of splicing is greater when the pre-rRNA is expressed from the natural rDNA repeats.

As group I introns are often found in rRNA genes (Cannone et al. 2002), we next asked whether the pre-rRNA context is necessary to ensure a high level of self-splicing in yeast, by placing the intron cassette within a Pol II-expressed pre-mRNA. The intron was fused to the 5' end of a GFP coding sequence under the control of a galactose-inducible promoter (Fig. 1A), so that splicing restores the GFP reading frame. Compared with the pre-rRNAs, the GFP pre-mRNA spliced poorly, as judged by the amount of free intron on Northern blots (Fig. 1C) and weak expression of GFP protein (data not shown). These data indicated that the intron sequences are less likely to fold correctly in the GFP pre-mRNA than in the pre-rRNA. The *Tetrahymena* intron was reported to also splice poorly when inserted in mammalian pre-mRNAs (Hagen and Cech 1999; Long and Sullenger 1999).

rRNA improves fidelity of folding

The rRNA exons influence 5'-splice site recognition and the folding pathway of the intron core in vitro (Pan and Woodson 1998). The pre-rRNAs may splice better than the GAL-IVS-GFP mRNA, simply because the local sequence context of the intron is more favorable. To determine whether flanking rRNA exon sequences are sufficient for efficient splicing in yeast, we expressed minimal pre-rRNAs from a *GAL* promoter in yeast (Fig. 2A). These "mini-pre-rRNAs" contained 146 nt of rRNA upstream of the 5'-splice site and 86 nt downstream from the 3'-splice site, corresponding to the minimal rRNA sequences necessary for optimal self-splicing activity in vitro (Woodson 1992). The mini-pre-rRNAs spliced three times more than the IVS-GFP pre-mRNA, when the flanking rRNA was taken from the *Tetrahymena* 26S gene, and about the same when the rRNA was from yeast (Fig. 2B; Table 1). However, the mini-pre-rRNAs were three to five times less active than the full 35S pre-rRNA expressed from the *GAL* promoter (Fig. 2B; Table 1). Thus, the sequence or structure of the rRNA surrounding the intron is important, but factors involved in rRNA transcription and ribosome biogenesis likely also increase the propensity of the intron RNA to fold and self-splice.

A pool of inactive pre-rRNA

The amount of each RNA at steady state depends on turnover of the pre-RNA and intron, as well as the rate of

FIGURE 1. Splicing of the *Tetrahymena* intron in yeast. (A, top) Chromosomal rDNA from yeast strain INVSc-TtLSU1 (Nogi et al. 1991) transcribed by RNA Pol I; pre-rRNA in pNOY102-IVS, transcribed from the GAL7 promoter (Lin and Vogt 1998). The 413-nt *Tetrahymena* intron was inserted in the 25S rRNA at the position homologous to the natural splice junction. The rRNA sequences flanking the intron are identical in *Tetrahymena* and yeast, preserving the splice sites. (A, bottom) GAL1-IVS-GFP expression cassette in pSJ831. (B) Northern blot of total RNA isolated from yeast expressing the *Tetrahymena* intron in 35S pre-rRNA from a Pol I promoter (INVSc-TtLSU1) or a Pol II promoter (pNOY102-IVS). Results for two independent yeast transformants are shown for each RNA. Membranes were hybridized with 32 P-labeled probes specific for the intron and yeast ACT1. (IVS) Spliced intron; (5'25S-IVS) product of 3'-splice site hydrolysis. (C) Northern blot of yeast total RNA from two strains transformed with pSJ831 and expressing IVS-GFP mRNA, with an intron probe.

splicing. To determine whether the differences we observed are due to a difference in the splicing rate, we measured the half-lives of the spliced intron RNA and each pre-rRNA after inhibition of GAL transcription by glucose at 30°C (Parker et al. 1991). Addition of glucose inhibits the expression of GAL genes within 1–2 min (Ronen and Botstein 2006). The amount of intron or pre-rRNA remaining at various intervals after glucose shutoff was fit to a first-order rate equation (Fig. 3). The free intron had a half-life of 46–50 min ($k_{d,IVS} = 0.015 \text{ min}^{-1}$) (Figs. 2C, 3C). Electrophoresis on polyacrylamide gels showed that nearly all of the free intron RNA was linear and full length (data not shown).

The observed half-life of the pre-rRNA depends on the rate constants for splicing and nucleolytic degradation, $k_{d,pre}(\text{obs}) = k_{sp} + k_{d,pre}$. Thus, highly active pre-RNAs should have a shorter half-life than inactive pre-RNAs, and the rate of splicing can be determined by comparing the half-lives of active and “dead” mutant pre-rRNAs (Donahue and Fedor 1997). The observed half-life of the 25S–27S pre-rRNA containing the wild-type intron was ~10

min ($k_{d,pre} = 0.07\text{--}0.1 \text{ min}^{-1}$) (Fig. 3C). To measure the rate of degradation ($k_{d,pre}$), we deleted 45 nt from the catalytic core of the intron (Δcore) to block splicing (Fig. 3B). Surprisingly, the “dead” Δcore pre-25S rRNA disappeared at nearly the same rate as pre-25S rRNA containing the wild-type intron, within the error of the experiment (Fig. 3C). This result was inconsistent with rapid splicing of the pre-rRNA at steady state. Instead, we concluded that the residual unspliced pre-rRNA represents a population that is incompetent to splice and that is primarily turned over by cellular nucleases.

The half-lives of mini-pre-rRNAs containing the wild-type intron or an inactive G-site mutant (G264A) were also similar to each other (0.2 versus 0.015 min^{-1}) (Fig. 2C). The difference in the decay rates of the wild-type and mutant pre-rRNAs yield an apparent splicing rate of 0.05 min^{-1} , if one assumes that all of the wild-type transcripts have the same potential to self-splice. This apparent splicing rate, however, is slower than refolding

TABLE 1. Splicing of *Tetrahymena* intron in yeast

pre-RNA	[IVS]/[pre]	f_{IVS}	[pre]/[actin]	$k_{d,pre} (\text{min}^{-1})$	Φ
35S (rDNA) ^a	258	1.00	1.14	— ^c	0.98
GAL-35S rRNA	14.6	0.94	0.81	0.07–0.1	0.76
Δcore	0	0	14.6	0.08	0
G100C	0.39	0.28	5.5	—	0.08
C260G	0.01	0.01	16.2	—	0
G264A	0	0	19.1	—	0
tri mutant ^b	0.32	0.24	3.0	—	0.06
mini-rRNA; <i>Tth</i>	3.0	0.75	1.5	0.18	0.23
mini rRNA; <i>Sce</i>	1.4	0.59	2.7	0.20	0.13
G100C	0	0	8.7	0.15	0
C260G	0	0	11.1	0.15	0
G264A	0	0	8.7	0.15	0
tri mutant ^c	0	0	8.8	0.15	0
GAL-IVS-GFP	0.53	0.35	—	—	0.03
Δcore	0	0	—	0.25	0

The splicing parameters were measured as described in Materials and Methods: [IVS]/[pre], steady-state ratio of spliced intron to pre-rRNA; fraction spliced intron, $f_{IVS} = (\text{counts IVS}) / ((\text{counts IVS}) + (\text{counts pre}))$; unspliced RNA relative to actin mRNA at steady-state, $[\text{pre}]/[\text{actin}] = (\text{counts pre}) / (\text{counts actin})$; $k_{d,pre}$, observed pre-rRNA decay rate. The partition factor Φ was calculated as described in Materials and Methods, with $k_{d,IVS} = 0.015 \text{ min}^{-1}$ and $k_{d,pre} = 0.07 \text{ min}^{-1}$ for GAL-35S pre-rRNAs, 0.15 min^{-1} for mini-pre-rRNAs, and 0.25 min^{-1} for GAL-IVS-GFP. The precision is 20% for [IVS]/[pre], 10%–15% for the decay rates, and 30% for Φ . Results are the average of two to four trials. Assuming a 1–2-min delay in glucose shutoff would double decay rates for the mini-rRNAs, with $\Phi = 0.13$ and 0.07 , respectively. This assumption would negligibly affect the splicing parameters of the other pre-RNAs.

^aIntron integrated into chromosomal rDNA.

^bC78G, G77C, U273A.

^cNot determined.

FIGURE 2. Flanking rRNA exons enhance splicing of the *Tetrahymena* intron in yeast. (A) Plasmids for expression of minimal pre-rRNAs in yeast, with exons derived from yeast (*Scd*) 25S rRNA (pSJ015) or *T. thermophila* (*Tth*) 26S rRNA (pSW015). (B) Northern blot showing mini-pre-rRNA and spliced intron (IVS) at steady state (SS) and at various times after glucose inhibition of GAL transcription. (C) Decay of intron and mini-pre-rRNAs. The RNA remaining was normalized to the steady-state level prior to glucose repression. (●) *Tth* intron ($k_{d,obs} = 0.014 \text{ min}^{-1}$); (▲) *Scd* intron ($k_{d,obs} = 0.014 \text{ min}^{-1}$); (■) *Tth* pre-rRNA ($k_{d,obs} = 0.18 \text{ min}^{-1}$); (▼) *Scd* pre-rRNA ($k_{d,obs} = 0.21 \text{ min}^{-1}$).

of comparable mini-pre-rRNAs in vitro ($0.14\text{--}0.6 \text{ min}^{-1}$) (Pan et al. 1999a) and too small to account for the accumulation of spliced introns in yeast. Thus, we again conclude that RNAs that remain unspliced a few minutes after transcription in yeast have very little (if any) probability of refolding.

Partitioning of transcripts into active and inactive pools

If newly transcribed pre-rRNAs form a single pool with a uniform probability of splicing, then the rate of splicing can be obtained by the intron decay rate and the ratio of intron to pre-rRNA at steady state, $k_{sp} = k_{d,IVS}([IVS]/[pre])_{ss}$ (Brehm and Cech 1983). This “single-pool” model yields apparent splicing rates ranging from 0.008 min^{-1} for IVS-GFP to 4 min^{-1} for chromosomally encoded pre-35S rRNA. As described above, however, this model cannot explain the very similar half-lives of the wild-type and inactivated pre-rRNAs. Instead, the data are better explained by partitioning of nascent transcripts into active and inactive pools, as we proposed for similar experiments in *E. coli* (Koduvayur and Woodson 2004).

In the partitioning model, the active pre-rRNA (pre_N) splices rapidly to produce mature rRNA and free intron, while the inactive pre-rRNA (pre_I) is degraded and only occasionally refolds into the active form. If we assume for simplicity that nearly all of the active pre-rRNA splices, and that splicing is much faster than refolding ($k_{sp} \gg k_f$), then the ratio of intron to pre-rRNA at steady state is

$$\frac{[IVS]}{[pre_N] + [pre_I]} \approx \frac{[IVS]}{[pre_I]} \approx \frac{k_{d,pre}\Phi}{k_{d,IVS}(1 - \Phi)} \quad (1)$$

in which Φ is the fraction of transcripts that fold correctly, $k_{d,IVS}$ is the intron decay rate, and $k_{d,pre}$ is the decay rate of the inactive pre-rRNA (Koduvayur and Woodson 2004). In this model, the yield of spliced 25S rRNA depends on the

proportion (Φ) of transcripts that initially fold correctly, as misfolded transcripts have little chance to refold and self-splice. Using these assumptions, we found that $\Phi = 0.98$ when the intron was integrated into the chromosomal rDNA (Table 1). In contrast, few of the short Pol II pre-rRNAs become active; $\Phi = 0.26$ and 0.13 for mini-pre-rRNAs and $\Phi = 0.03$ for GFP pre-mRNA (Table 1).

Partial suppression of misfolding mutations in pre-rRNA

The results above showed that the likelihood of the intron folding into its active conformation is 76%–98% when it is placed within the full-length pre-rRNA, but only 3%–26% when inserted in a short pre-mRNA transcribed by RNA Pol II. To test whether factors associated with synthesis and maturation of 35S pre-rRNA enhance the fidelity of folding, we compared the phenotypes of mutations known to increase misfolding of the intron in vitro without abolishing catalytic activity (Fig. 4). Mutation of G100 to C, which stabilizes a misfolded secondary structure in the intron core (Pan and Woodson 1998), reduced the fraction of spliced pre-rRNA ~ 10 -fold ($\Phi = 0.08$). A triple mutant (U273A, G77C, C78G), which is less stable than the wild type but folds more rapidly in vitro (Pan and Woodson 1999; Russell et al. 2002), also had reduced activity in yeast ($\Phi = 0.06$). Destabilization of the triple helical scaffold (C260G), which severely reduced the in vitro folding rate (Zarrinkar and Williamson 1996), almost eliminated splicing of the pre-rRNA in yeast.

FIGURE 3. Intron and pre-rRNA stability. Northern blots of yeast total RNA with intron and actin mRNA probes, as in Figure 2. (A) Cells transformed with pNOY102 expressing GAL-35S pre-rRNA at steady state (SS) and at various times after glucose repression. (B) Stability of unspliced 35S and 25S pre-rRNA. Splicing is blocked by a 45-nt deletion in the intron core (Δ core). Cells were transformed with pNOY102-TAG-IVS- Δ P7, which contains a sequence tag in the 25S rRNA (Peculis and Greer 1998). (C) Decay of intron and pre-rRNAs, as in Figure 2. (●) Intron ($k_{d,obs} = 0.017 \text{ min}^{-1}$); (■) Δ core pre-rRNA ($k_{d,obs} = 0.065 \text{ min}^{-1}$); (▼) wild-type pre-rRNA ($k_{d,obs} = 0.098 \text{ min}^{-1}$).

Thus, splicing in yeast correlates with the stability of the RNA tertiary structure and the propensity of the intron to fold correctly. The amount of spliced product is probably not due to rapid degradation of the mutant intron RNA, as the half-lives of the wild-type and mutant introns ($k_{d,IVS}$) were very similar in *E. coli* at 30°C (Nikolcheva and Woodson 1999). We were unable to detect any spliced intron when these same mutations were introduced into the yeast mini-pre-rRNA (data not shown), although 10–20-fold less intron RNA would have been detectable in our Northern blots. The misfolded intron may be more able to recover in the full-length pre-rRNA than in mini-pre-rRNAs transcribed by Pol II, because of upstream sequences or the recruitment of ribosome assembly proteins to the 35S pre-rRNA.

Complementation assay for spliced 25S rRNA

Because the intron is inserted in a conserved region of the 25S rRNA, unspliced 25S rRNA is not expected to be functional. To evaluate whether misfolded mutations reduce the production of active 60S ribosomes, we determined whether plasmids expressing the 35S pre-rRNA from a Pol II promoter (Fig. 1A) could complement a temperature-sensitive mutation in the large subunit of RNA Pol I (Nogi et al. 1993). At the nonpermissive temperature (37°C),

synthesis of the endogenous pre-rRNA is inhibited, and cells depend on plasmid-encoded rRNA for growth.

Transformants expressing 35S pre-rRNA containing the wild-type intron from a GAL promoter grew as well as an intronless strain at 37°C (Fig. 4C), confirming that the spliced 25S rRNA is functional. Plasmids containing mutations that only partially inhibited splicing, such as G100C or the triple (tri) mutant, were also able to complement the Pol I *ts* phenotype (Fig. 4C). Plasmids containing misfolding mutations that severely inhibited splicing (C260G), however, were unable to support growth at 37°C, as were plasmids with mutations that disrupted the intron catalytic site (Δ core and G264A). Thus, the results of the complementation assay corroborated the extent of splicing visible on Northern blots.

Unspliced pre-25S rRNA is degraded in yeast

We next asked whether misfolding of the intron affects only splicing, or whether it affects other steps in processing of pre-rRNA. To follow the fate of the plasmid-encoded pre-rRNA, we used a unique 18-nt sequence tag at the 5' end of the 25S rRNA (Peculis and Greer 1998). The tag does not affect pre-rRNA processing (Peculis and Greer 1998) or splicing of the *Tetrahymena* intron (data not shown).

Hybridization with a probe complementary to the 25S tag or the intron revealed that the unspliced RNAs accumulated as 27S and 25S intermediates rather than

FIGURE 4. Intron misfolding inhibits splicing in yeast. (A) Schematic of intron mutations. (B) Steady-state expression of GAL-35S pre-rRNA, containing mutations shown in A. (—) No intron; (tri) triple mutant C78G, G77C, U273A. RNA from two independent transformants was analyzed for each mutant. The Northern was hybridized with intron and actin probes. Bands indicate unspliced 35S and 25S pre-rRNAs, 5'-25S-intron hydrolysis product, actin mRNA, and spliced intron (IVS). (C) Complementation of Pol I *ts* mutation *rpa190-3* by plasmids expressing GAL-35S pre-rRNA (pNOY-TAG-IVS or pNOY-TAG). Yeast cells were spotted onto SC-galactose and replicate plates were grown at the nonpermissive temperature (37°C). (Images of individual colonies were cropped from a single photograph and arranged in the figure.)

FIGURE 5. Defective splicing inhibits processing of ITS 2. Steady-state expression of GAL-35S pre-rRNA with mutations as in Figure 4A. (35S-IVS) unspliced full-length 35S pre-rRNA; (27S-IVS) unspliced 27SB pre-rRNAs containing ITS 2; (25S-IVS) unspliced 25S rRNA, 25S, mature rRNA, and 3' 25S-IVS intron–3'-exon splicing intermediate. (A) Intron probe. (B) Probe specific for sequence tag in plasmid-encoded 25S rRNA. (C) Pre-rRNA processing in yeast leading to mature 25S rRNA. Intermediate steps and alternative processing pathways are not shown.

35S pre-rRNA, even when splicing was completely blocked by deletion of the intron core (Δ core) (Fig. 5). Thus, failure to excise the intron does not inhibit early steps in pre-rRNA processing, but may inhibit processing of internal transcribed spacer 2 (ITS 2). Hybridization with a probe against the 25S tag showed that destabilization of the intron tertiary structure (C260G) or disruption of the active site (G264A and Δ core) resulted in no detectable mature 25S rRNA (Fig. 5B), consistent with the inability of these mutants to complement growth of a Pol I temperature-sensitive strain (Fig. 4C).

The unspliced pre-25S and 27S rRNAs were much less abundant than the plasmid-encoded 25S rRNA from an intron-minus control, suggesting that they are rapidly degraded. Inhibition of ITS 2 processing has been observed in response to various defects in 60S ribosome assembly (e.g., de la Cruz et al. 1998; Peculis and Greer 1998), and such defects can lead to rapid turnover of the rRNA (Allmang et al. 2000). Thus, misfolding of the intron may cause 60S ribosomes to fail an important quality-control checkpoint.

Temperature dependence of splicing

Misfolded RNAs can often be rescued by refolding at higher temperatures, while mutations that favor an alternative conformation often have a cold-sensitive phenotype (e.g., Dammel and Noller 1993; Zavanelli et al. 1994). We previously found that mutations that increased misfolding of the *Tetrahymena* intron were less deleterious when expressed in *E. coli* at 42°C than at 25°C (Nikolcheva and Woodson 1999). In yeast, we observed only a modest

increase in the fraction of spliced 35S pre-rRNA at 37°C, for the wild-type intron, G100C, and triple mutant (Fig. 6). Very little total RNA was recovered from yeast grown at 20°C, making low-temperature effects difficult to quantify. Nonetheless, the inability of the G100C or C260G intron to be rescued at 37°C suggested that more rapid refolding of misfolded intron RNA is not sufficient to ensure that the pre-rRNA is spliced in yeast. However, these results are consistent with the idea that transcripts that initially misfold have a high probability of becoming sequestered in an inactive state. The stability of the intron tertiary structure may be important for *in vivo* activity, as C260G is more destabilizing than the other base substitutions tested here (Pan and Woodson 1998, 1999) and is least active at all temperatures.

DISCUSSION

RNA partitioning in vivo

Many RNA sequences can fold into more than one stable structure, and the extent of misfolding is sensitive even to

FIGURE 6. Temperature dependence of 35S pre-rRNA splicing. (A) Transformed yeast were grown in SC-galactose at the temperatures shown above each lane, before Northern analysis as in Figure 4B. (B) The fraction of spliced intron in A was obtained from (counts IVS)/[(counts IVS+counts pre)], as described in Materials and Methods.

single base substitutions. In vitro, RNA molecules partition among competing folding pathways, which lead either to the native or misfolded structures (Thirumalai and Woodson 1996). A number of experiments have shown that the *Tetrahymena* ribozyme folds through multiple pathways (Pan et al. 1997, 2000; Zhuang et al. 2000; Laederach et al. 2006). In vitro, kinetic partitioning may occur early in the folding process, perhaps when the secondary structure is formed. The results presented here suggest that transcripts also partition into active and inactive populations in yeast within a short period after they are synthesized. Because inactive pre-rRNAs are degraded without producing mature 25S rRNA, the fidelity of intron folding determines the fate of the pre-rRNA.

A key observation is that pre-RNAs that should be competent to self-splice disappear at nearly the same rate as pre-RNAs in which the intron active site is crippled or dead. These results, and the amount of spliced RNA at steady state, are best explained by assuming that most of the unspliced RNA is degraded without having a chance to refold and splice. As expected, the proportion of inactive pre-rRNA increases with intron mutations known to promote misfolding. Assuming that all pre-RNAs splice at a single rate or that misfolded RNAs are able to refold at the in vitro rate overestimates the amount of spliced RNA produced by the mutants and the mini-pre-rRNAs, as we observed previously (Koduvayur and Woodson 2004). Although our data suggested that the unspliced RNA is ultimately sequestered in an inactive state, our data do not exclude refolding of RNA domains during or just after synthesis.

Cotranscriptional folding

Early partitioning of ribozyme transcripts into functional and nonfunctional pools implies that folding, and quality control, is closely linked with RNA synthesis. This is consistent with the abundant evidence for physical and functional links between transcription and RNA splicing, polyadenylation, decay, processing, and nuclear export (Neugebauer 2002; Bentley 2005). For ribozymes and certain regulatory RNAs, the order in which RNA sequences are transcribed and the rate of elongation can determine the type of folding intermediates that are formed (e.g., Poot et al. 1997; Pan et al. 1999b; Diegelman-Parente and Bevilacqua 2002; Heilman-Miller and Woodson 2003; Granneman and Baserga 2005; Mahen et al. 2005; Wickiser et al. 2005). Therefore, one effect of transcription is to directly influence the folding pathway of the RNA.

A second effect of transcription is to deliver a specific subset of RNA-binding proteins to the nascent transcript. Many of the yeast and mammalian proteins that have been

implicated in RNA folding are associated with a class of transcripts. For example, there is considerable evidence for cotranscriptional assembly and processing of pre-rRNA (Granneman and Baserga 2005), and these assembly factors are recruited to the pre-rRNA in part via Pol I (Oakes et al. 1993). The yeast La protein binds Pol III transcripts and facilitates assembly and maturation of pre-tRNAs and U6 snRNA (Fan et al. 1998; Pannone et al. 1998; Chakshumathi et al. 2003), while Lsm 2–8 complexes associate with mRNAs (Tharun et al. 2000).

For both of these reasons, it is interesting to consider whether transcription in the cell creates a time window in which RNA domains must fold. Our data show that the probability of folding correctly in yeast (this study) and *E. coli* (Koduvayur and Woodson 2004) depends on which polymerase transcribed the pre-rRNA, suggesting that folding occurs cotranscriptionally. Recent experiments on the hairpin ribozyme, however, suggest that cotranscriptional folding is less important in yeast than thermodynamic stability of the active conformation (Mahen et al. 2005). A possible explanation for this difference is that the smaller hairpin ribozyme refolds more easily within an mRNA than the larger *Tetrahymena* ribozyme.

Factors that facilitate splicing of rRNA introns

Several factors account for the vigorous splicing of the *Tetrahymena* group I intron in its native context. First, the surrounding rRNA appears to favor the active conformation of the intron. Group I introns are often found in dynamic regions of the rRNA (Jackson et al. 2002), and this flexibility may allow the intron to bind the splice sites more easily. Second, factors associated with Pol I transcription and ribosome biogenesis also contribute to the propensity of the intron RNA to fold, as mini-pre-rRNAs that are not assembled into 60S ribosomes splice much more poorly than full-length pre-rRNAs. In addition to ribosomal proteins, which stabilize the folded rRNA, ribosome biogenesis involves a large number of *trans*-acting factors, including many ATP-dependent RNA helicases that are capable of actively unwinding the rRNA (Kressler et al. 1999; Fatica and Tollervey 2002). Thus, in its natural context, the *Tetrahymena* group I intron may hitchhike on a system devoted to the folding and assembly of rRNA.

Finally, proteins that associate with Pol II transcripts, such as hnRNP proteins, may antagonize intron folding. This is supported by the fact that the *Tetrahymena* intron is more active in mammalian cells when transcribed by T7 RNA polymerase in the cytoplasm, than when expressed from an endogenous Pol II promoter (Byun et al. 2003). Many abundant RNP proteins contain structural motifs that preferentially bind single-stranded RNA (Dreyfuss et al. 2002), and such proteins could invade RNA domains

that are not stably folded. In the future, it will be important to understand how intracellular proteins associated with various transcription complexes modulate RNA folding pathways.

MATERIALS AND METHODS

Yeast strains

S. cerevisiae strain INVSc2/TtLSU1 (MATa *his3-Δ200 ura3-167 25S::TtLSU1*) has the *Tetrahymena* group I intron integrated into every copy of the 25S rDNA on Chromosome XII (Lin and Vogt 1998), and was the gift of V. Vogt. For splicing assays, plasmids expressing the intron were transformed into strain BY4733 (MATa *his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0*). For complementation assays, plasmids derived from pNOY102 were transformed into strain NOY505, which carries the *rpa190-3* temperature-sensitive allele of the large subunit of RNA Pol I (Nogi et al. 1993). Transformants were isolated at 22°C, then shifted to the non-permissive temperature (37°C) on galactose-containing media to induce expression of the plasmid-encoded 35S rRNA from the *GAL7* promoter.

Plasmid construction

Plasmid pSJ831 contains the *Tetrahymena* group I intron immediately upstream and in frame with the GFP coding sequence in the vector p423-GAL1 (Mumberg et al. 1995). Correct splicing restores the GFP reading frame and can be monitored by GFP expression. The sequence of GFP mut3.1 (Andersen et al. 1998) was amplified by PCR with primers UP-GFP (5'-GGCAAGCTTAGCGCCCAATACGCAAACCG-3') and DP-GFP (5'-CGCCTCGAGCTTCCGCTTCCTCGCTCACT-3') and ligated into the EcoRI and XhoI sites of p423-GAL1 to create pSJ431. The sequences of the *Tetrahymena* intron plus 15 nt of upstream and 12 nt of downstream rRNA were amplified by PCR primers UP-IVS (5'-GCGTCTAGATGACTCTCTAAATAGCAATATTTAC-3') and DP-IVS (5'-CCGAGATCTACCTTACGAGTACTCCAAAACCTAA T-3'), and cloned into pSJ431 as an XbaI-EcoRI fragment. Expression of the intron-GFP mRNA is controlled by the *GAL1-GAL10* promoter and ends downstream from GFP with a *CYC1* termination sequence. Inactive variants were prepared by deletion of the 45-bp BglII-NheI fragment in the intron core (Δ core).

Plasmid pSW015 contains the *Tetrahymena* intron plus 146 nt of natural 26S rRNA upstream from the 5'-splice site and 86 nt of rRNA downstream from the 3'-splice site from pSW012 (Woodson 1992). These sequences were amplified by PCR and subcloned into the BamHI and XhoI sites of p425-GAL1. Plasmid pSJ015 is the same as pSW015 except the flanking rRNA exon sequence is derived from the yeast 25S rRNA, amplified from the rDNA of INVSc-TtLSU1 (Lin and Vogt 1998). Point mutations were introduced into pSJ015 by exchanging the SphI-NheI intron fragment with variants of pSW012 carrying the desired mutation (Pan and Woodson 1998).

Full-length 35S pre-rRNA was expressed from pNOY102, which contains the *GAL7* promoter fused to a single copy of the 35S rRNA operon (6922 bp) from *S. cerevisiae* (Nogi et al. 1991) and was the gift from M. Nomura. pNOY102-Tag was the

gift of B. Peculis and contains a unique 18-nt sequence tag in the 5' end of the 25S rRNA (Peculis and Greer 1998). Intron sequences were inserted into pNOY102 and pNOY102-Tag by linearizing the plasmid DNA with I-PpoI. The digested DNA was treated with T4 DNA polymerase and calf intestinal phosphatase before ligation with a PCR product containing the intron plus 1 nt of 5' exon and 3 nt of 3' exon (upstream, 5'-TAAATAGCAATATTTACCTTTGGAGG; downstream, 5'-TTACGAGTACTCCAAAACCTAATCAATAT).

Cell growth and RNA isolation

Splicing of pre-35S rRNA transcribed by Pol I was measured using *S. cerevisiae* strain INVSc2/TtLSU1, grown in YPD medium. Galactose-inducible plasmids were transformed into BY4733 by the LiOAc method (Gietz and Schiestl 1991) and selected at 30°C on synthetic complete medium lacking leucine (p425-GAL1 derivative) or uracil (pNOY102 derivatives), and containing 2% glucose. Liquid cultures of selective media containing 2% galactose were inoculated with isolated colonies and grown for 30 h at 30°C (OD₆₀₀ = 1.0) to achieve stable plasmid expression. Cells were harvested at 4°C and stored at -80°C. Total yeast RNA was purified by two to three extractions with hot acidic phenol/chloroform (Rose et al. 1990). Extracted RNA was precipitated by the addition of 0.3 M sodium acetate (pH 5.0) and 1 volume of 100% isopropanol. Yield was determined by UV absorption at 260 nm, assuming 40 mg/mL = 1 OD₂₆₀.

Northern blots

Total RNA (10 μ g) was denatured with glyoxal and resolved on a 1.4% agarose gel (1:1 Seakem:NuSieve; FMC). Glyoxal modification reactions (50 μ L) contained 50% dimethyl sulfoxide, 10 mM sodium phosphate (pH 7.0), and 8% deionized glyoxal, and were incubated for 1 h at 50°C. Prior to loading, 5 μ L of 50% glycerol/0.25% bromophenol blue was added, and the reactions were stored on ice. Following electrophoresis, RNA was transferred to a Nytran nylon membrane (Schleicher & Schuell) by capillary wicking in 10 $\times$ SSPE overnight at room temperature. RNA was immobilized on the membrane by UV cross-linking (120 mJ; UV Stratalinker). Following cross-linking, the membrane was washed twice in 40 mM Tris-HCl (pH 8.0) to remove the glyoxal modifications from the RNA.

Oligonucleotide probes complementary to the *Tetrahymena* group I intron (5'-GGCTGTTGACCCCTTTCCCGCAATTTGACGGTCTTGCCCTTTAAACCGATGCAATCTATTGGTTTA), yeast actin mRNA (5'-GGCAATACCTGGGAACATGGTGGTAC), or 25S rRNA "Tag" (5'-ACTCGAGAGCTTCAGTAC) (Peculis and Greer 1998) were labeled with [γ -³²P]ATP and T4 polynucleotide kinase (NEB) and passed through a TE-10 Chromaspin column (Clontech). Probes were hybridized with the membrane overnight at 45°C in 1 $\times$ hybridization buffer (Images kit; USB), washed twice for 5 min each at room temperature in 2 $\times$ SSC/0.1% SDS, once for 15 min at room temperature in 2 $\times$ SSC/0.5% SDS, twice for 15 min at 55°C in 0.2 $\times$ SSC/0.1% SDS, and twice for 5 min at room temperature in 2 $\times$ SSC. The membrane was exposed to a PhosphorImager screen for 14 h and quantified using ImageQuant software.

Quantitation of intron RNA at steady state

The intensities of the intron, pre-rRNA, and rRNA bands in each lane of Northern blots were normalized to an actin mRNA control. The ratio of spliced intron to pre-rRNA at steady-state,

$$\frac{[IVS]_{SS}}{[pre]_{SS}} = \frac{\text{counts IVS}}{\text{counts pre}},$$

was taken from the average of RNA samples from two or more transformants, as described above. Because the spliced intron is abundant in INVSc2/TtLSU1 and BY4733/pNOY102-IVS, the amounts of intron and pre-rRNA in these samples were obtained from standard curves as follows: 2.5–20 µg of each total yeast RNA sample was loaded on agarose gels and analyzed as described above. For each RNA species, the best least-squares fit to counts versus micrograms of total RNA over the concentration range giving a linear change in intensity was used to calculate the intensity (counts) corresponding to 10 µg of RNA. These corrected intensities were then used to calculate the ratio of intron to pre-rRNA. For GAL-IVS-GFP, actin mRNA was not used as a control because it co-migrates with the pre-rRNA.

RNA decay rates

Transformed yeast were grown in 150 mL of selective galactose medium for 30 h as described above. The cells were harvested and resuspended in 15 mL of minimal medium with 2% glucose at 30°C to prevent further transcription from the *GAL* promoter (Parker et al. 1991). Aliquots (1 mL) were harvested at various times by spinning in a microcentrifuge for 10 sec at 14,000g. Cell pellets were immediately frozen in aluminum racks pre-chilled to –76°C (dry ice). Total RNA was isolated and analyzed by Northern hybridization as described above. The fraction of spliced intron or pre-rRNA relative to actin mRNA at each time was normalized to the level of steady-state expression before the addition of glucose and fit to a first-order rate equation,

$$[RNA](t) = [RNA]_{SS} \exp(-k_{obs}t).$$

Splicing partition factor

The partition factor or fraction of active RNA, Φ , was calculated from Equation 1, rewritten as

$$\Phi = \alpha/(1 + \alpha),$$

in which

$$\alpha = \frac{[IVS]_{SS}}{[pre]_{SS}} \cdot \frac{k_{d,IVS}}{k_{d,pre}}$$

and $k_{d,pre}$ and $k_{d,IVS}$ are the decay rates for the unspliced inactive precursor and spliced intron RNA, respectively (Koduvayur and Woodson 2004). The average decay rate of the free intron was 0.015 min^{–1}.

ACKNOWLEDGMENTS

We thank M. Nomura, B. Peculis, and J. Boeke for reagents and helpful advice; and R. Moss for technical assistance. This work was supported by a grant from the NIH (GM46886).

Received June 8, 2006; accepted September 14, 2006.

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Scott A. Jackson, Sujatha Koduvayur and Sarah A. Woodson

RNA 2006 12: 2149-2159 originally published online October 24, 2006

Access the most recent version at doi:[10.1261/rna.184206](https://doi.org/10.1261/rna.184206)

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