Self-Splicing of the *Tetrahymena* Pre-rRNA Is Decreased by Misfolding during Transcription†

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ABSTRACT: RNA processing depends in part on the ability of nascent transcripts to fold into the desired conformation. Self-splicing of the group I intron from *Tetrahymena* was used to assess the folded state of preribosomal RNA transcripts when synthesized in vitro. A simple method for isolating nondenatured RNA from a T_7 RNA polymerase reaction was tested. The intron alone is fully active when transcribed at 30 °C, suggesting that the active structure is both kinetically and thermodynamically favored. Longer precursor RNAs, however, were less than completely active in self-splicing. Full activity, as judged by both the initial rate and the extent of product formation, was restored by brief incubation at 95 °C and rapid cooling in the presence of magnesium ion. This result did not depend on the length of the precursor RNA in any simple way, but correlated loosely with the presence of intact exon domains. When transcribed in the absence of cellular proteins, a significant portion of the pre-RNA appears to be trapped in a conformation that does not readily undergo the first step of splicing.

The relationship between the primary sequence and three-dimensional structure of macromolecules is a basic tenet of biochemistry. The ability of RNA molecules to fold independently was most convincingly shown by the discovery that certain RNAs retain their catalytic function in vitro (Cech et al., 1981; Guerrier-Takada & Altman, 1984). The manner in which a polyribonucleotide chain folds, however, is poorly understood. Furthermore, RNA folding in vivo is likely to be facilitated and regulated by the presence of RNA-binding proteins. Little is known about the process by which very long transcripts achieve their final conformation.

A simple question is whether longer transcripts will fold correctly when synthesized in vitro, and whether the resulting structure is kinetically or thermodynamically favored. We have used self-splicing of the *Tetrahymena* group I intron [reviewed in Cech (1990)] as an indicator for proper folding of the pre-rRNA. The catalytic activity of the intron depends on formation of a unique three-dimensional structure in the presence of divalent metal ions (Grosshans & Cech, 1989; Celander & Cech, 1991). Recognition of the splice sites also depends on specific base-pairing and non-Watson–Crick interactions (Davies et al., 1982; Been & Cech, 1986; Pyle et al., 1990). For the *Tetrahymena* intron, conformational rearrangements are likely to be rate-limiting under most reaction conditions (Herschlag, 1990). Thus, a faster initial rate of self-splicing can be taken to represent a population of molecules where the structure more closely resembles that of the reactive state.

Natural *Tetrahymena* precursor RNAs can adopt two alternative secondary structures within the exon sequences. Formation of a stable and conserved rRNA hairpin immediately upstream of the 5′ splice site inhibits 5′ splice site activation and results in a concomitant loss of splicing (Woodson & Cech, 1991). The equilibrium between these alternative conformers is affected by sequences both upstream and downstream of the splice junction; the minimum length necessary for optimal splicing is 145 nucleotides upstream and 85 nucleotides downstream of the intron (Woodson, 1992). These sequences comprise much of domain IV of the 26S rRNA (Noller, 1984). An interesting problem is whether the pre-rRNA adopts a specific conformation during in vitro synthesis and, if so, which of the alternative structures is preferred. We use T_7 RNA polymerase and self-splicing to examine whether the primary sequence of ribosomal RNA is sufficient to direct folding of the pre-RNA toward the most active structure, in the absence of normal cellular proteins.

In order to assay self-splicing activity immediately following transcription, pre-RNAs were isolated from T_7 RNA polymerase reactions without prior denaturation using rapid gel filtration. This is in contrast to the usual method of purifying transcripts from denaturing polyacrylamide gels. The *Tetrahymena* intron RNA obtained in this manner is fully active, as anticipated. Contrary to our expectations, however, precursors RNAs containing natural ribosomal exon sequences isolated directly from transcription do not splice at the maximum rate. The fraction of highly active RNA at 30 °C is increased following a heat/cool renaturation treatment, as seen previously for the gel-purified precursor (Woodson, 1992). Thus, the predominant form of the pre-RNA after in vitro synthesis does not correspond to the active structure. This outcome does not depend directly on the length of the transcript, nor does the presence of additional rRNA sequence enhance the fraction of active precursor obtained. Our results are most consistent with a model where folding of the RNA is controlled by sequence-dependent interactions within a minimal structural domain. The reduced splicing activity of synthetic transcripts highlights the requirement for additional protein–RNA contacts in vivo.

MATERIALS AND METHODS

Plasmid Constructions. Plasmids pJK43-T7 and pSW012 have been described previously (Woodson, 1992) and encode 1299-nt and 657-nt *Tetrahymena* precursor RNAs, respectively. pSW012LE (Woodson & Emerick, 1993) contains the ligated exon sequences of pSW012. Construction of pβGST$_7$ (Been & Cech, 1986) and pTZIVSΔ12 (Woodson & Cech, 1991) has been described.

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Plasmids TB43 and pTP43 were constructed by subcloning the *Bam*HI–*Eco*RI or *Pst*I–*Eco*RI rDNA fragments, respectively, from pGY17 (Kiss et al., 1981) into the multiple cloning site of pTZ19U (Bio-Rad). The *Bam*HI site falls at position 6683, *Pst*I at position 5224, and *Eco*RI at position 8047 in the rDNA sequence (Engberg & Nielsen, 1990). The intron sequences (413 nucleotides) begin at position 7010. pTB012 was constructed by ligating the 2.5-kb *Sca*I fragment of pTB43 with the 1.1-kb *Sca*I fragment of pSW012. pTP012 was constructed in a similar manner, using the 4.0-kb *Sca*I fragment of pTP43.

Preparation of Precursor RNA. Plasmid DNA was restricted with *Eco*RI prior to use in transcription reactions. The lengths of the transcription products of newly constructed plasmids are 846 nt (TB012), 2285 nt (pTP012), 1384 nt (pTB43), and 2823 nt (pTP43). Uniformly labeled RNA was transcribed by T_7 RNA polymerase (Davanloo et al., 1984) in the presence of [α-^{32}P]ATP (New England Nuclear), as described previously (Been & Cech, 1987). The reaction was carried out at 30 °C for 30–45 min, to reduce accumulation of spliced products.

Following RNA synthesis, the reaction mixture was immediately passed over a G-100 gel filtration spin column (Clontech) equilibrated in either 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl),[1] pH 7.5, or 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 6 mM $MgCl_2$, at room temperature. Columns were prepared by spinning 3 times for 2 min each at 3500 rpm in a microcentrifuge (Eppendorf) at room temperature. The transcription reaction (40 μL) was loaded onto the column, and the column was spun for 5 min at 3500 rpm.

Riboendonuclease Assays. The substrate rGGCUCUC-UA_5 was prepared as described previously (Zaug et al., 1988), and 5′ end-labeled with T_4 polynucleotide kinase (U.S. Biochemicals) after treatment with calf intestinal phosphatase (Promega) and extraction with phenol and chloroform. The concentration of the unlabeled substrate was determined by its absorbance at 260 nm. The radiolabeled substrate was isolated from a 20% polyacrylamide gel as described (Herschlag & Cech, 1990). L-21 *Sca* ribozyme RNA was transcribed from pT_7 L-21 DNA (Zaug et al., 1988) linearized with *Sca*I. The concentration of the ribozyme was determined spectrophotometrically using an extinction coefficient at 260 nm of 3.2×10^6 M^{-1} cm^{-1} (Zaug et al., 1988).

Reactions were carried out in 100 mM ammonium sulfate, 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 0.1 mM GTP at 30 °C. In some trials, the ribozyme RNA was incubated 10 min at 50 °C in the buffer above, minus GTP, or heated to 95 °C 1 min and cooled in the presence of the buffer (Woodson, 1992). Reactions were initiated with the addition of GTP, following a 2-min preincubation at 30 °C. Reaction products were separated on 20% polyacrylamide gels, and the radioactivity in each lane was quantitated using a PhosphorImager (Molecular Dynamics). Initial rates of product formation (k_{obs}) were determined from linear fits to $\ln(1 - f_{prod})$, where f_{prod} is the fractioon of pGGCUCUCU product in each lane.

Self-Splicing Reactions. Reactions were carried out under the same conditions used for the riboendonuclease reaction above, and as described previously (Woodson, 1992). Prior to the addition of GTP, the RNA was either heated to 95 °C for 1 min and cooled rapidly in the presence of splicing buffer, or used directly. Reaction products were separated on 8 M urea/4% polyacrylamide gels. Initial rates of splicing (k_{obs}) were determined as described previously (Woodson, 1992) from linear fits to $\ln(1 - f_{sp})$, where f_{sp} is the fraction of ligated exon product. Typical reaction profiles are biphasic, with 50–70% of the precursor splicing during the initial burst. In all cases, more than 90% of the precursor had reacted after 2 h. Reported values are based on several (2–5) isolations of each precursor, and vary 20–30%.

Intermolecular Splicing Reactions. Intermolecular splicing of uniformly labeled precursor RNA was carried out as described previously (Woodson & Cech, 1991), but in the absence of GTP, and with the addition of 30 μM unlabeled oligonucleotide $rGGCUCUCU_{OH}$. The concentration of precursor was typically 0.5 μM. Reaction products were electrophoresed on 8 M urea/6% polyacrylamide gels, and the products were quantitated as above, based on the appearance of oligonucleotide–3′ exon product.

RESULTS

Nondenaturing Purification of in Vitro Transcripts. In order to measure the self-splicing activity of RNAs produced during in vitro transcription, a reliable method of isolating precursor RNAs with minimal denaturation was required. This was complicated by the fact that the *Tetrahymena* intron splices during transcription (Cech et al., 1981). In order to measure the initial rates of splicing, it is helpful to arrest further processing of the intron. Many of the requirements for splicing, such as GTP, magnesium ion, and neutral pH, are shared by T_7 RNA polymerase. The most straightforward means of stopping the splicing reaction without denaturation of the RNA was removal of GTP by gel filtration chromatography.

Following transcription by T_7 RNA polymerase, the reaction mixture containing the desired precursor was immediately passed over a small G-100 gel filtration spin column. Prior to use, the column was equilibrated in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, either with or without 6 mM $MgCl_2$. As shown in Figure 1, this treatment results in complete removal of nucleotide triphosphates and abortive initiation products from the full-length transcript. In a typical experiment, spliced products (linear intron and ligated exon RNAs) that have accumulated during transcription account for approximately 8% of the total transcripts, while about 3% of the precursors have undergone 3′ splice site hydrolysis. About half of the spliced products are lost during gel filtration, bringing the observed fraction of precursor RNA from 89% after transcription to 92% following purification.

The L-21 Intron RNA Is Fully Active following Synthesis by T_7 RNA Polymerase. We next examined whether the intron RNA is folded into its active form under these conditions. The L-21 *Sca* ribozyme form of the intron (Zaug et al., 1988) was transcribed and purified with G-100 resin as above. The activities of the ribozyme preparations were compared by determining the initial rates of cleavage of a small oligonucleotide substrate in the riboendonuclease reaction (Zaug & Cech, 1986), as shown in Figure 2. Preincubation of the column-purified L-21 *Sca* RNA at 50 °C for 10 min or at 95 °C for 1 min had little effect on the initial rate of product formation at 30 °C. These treatments have been previously shown to improve the reactivity of the intron RNA after isolation from denaturing gels (Herschlag & Cech, 1990; Walstrum & Uhlenbeck, 1990). Initial velocities measured here are comparable to those determined previously for the L-21 ribozyme at 30 °C (T. McConnell, personal communication). These results confirm that the intron RNA folds into an active structure during transcription.

Several parameters of the purification protocol were varied, to determine whether they had any effect on transcript activity.

[1] Abbreviations: DNase I, deoxyribonuclease I; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

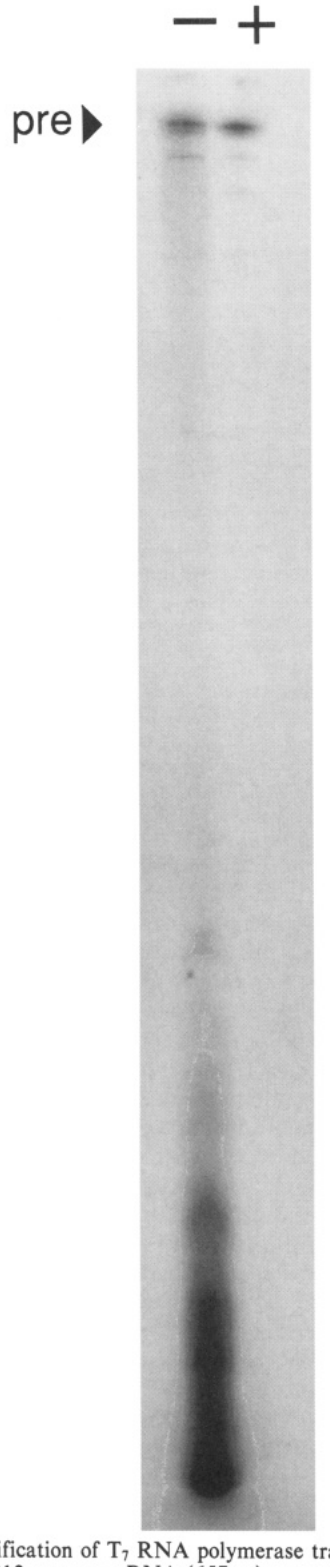

FIGURE 1: Purification of T_7 RNA polymerase transcripts by gel filtration. SW012 precursor RNA (657 nt) was transcribed by T_7 RNA polymerase in the presence of [γ-^{32}P]GTP. Transcripts initiated with radioactive GTP retain a label at the 5′ end. The precursor RNA was passed through a G-100 column equilibrated with 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Aliquots were removed before and after gel filtration (−/+ column) and analyzed on an 8 M urea/20% polyacrylamide gel. Experiments (not shown) with columns equilibrated in buffer additionally containing 6 mM $MgCl_2$ gave similar results. The position of the precursor RNA is indicated. The free intron is radiolabeled by addition of [^{32}P]GTP to the 5′ splice site. Nucleotide triphosphates and products of abortive initiation are toward the bottom of the figure.

The concentration of magnesium ion in the gel filtration column was varied from 0 to 5 mM. This made surprisingly little difference to subsequent activity of the RNA. From this, we

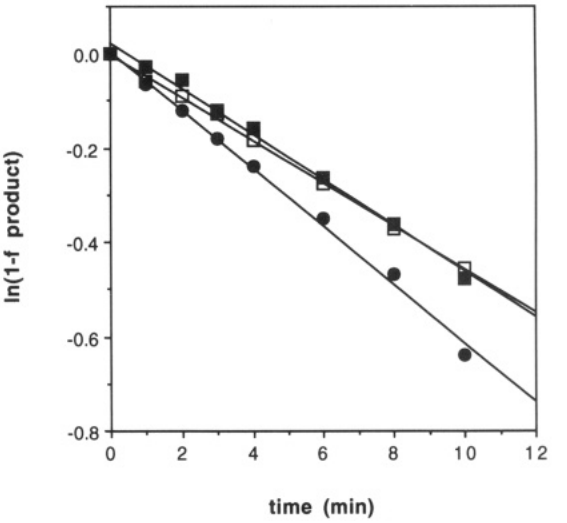

FIGURE 2: Activity of column-purified L-21 *Sca* ribozyme in the riboendonuclease reaction. 50 nM 5′ end-labeled wild-type RNA substrate, $^{5'}$pGGCUCUCUA$_5$, was incubated with 10 nM L-21 *Sca* ribozyme under splicing conditions at 30 °C. Ribozyme RNA was purified from a G-100 column as described under Materials and Methods. (■) No renaturation treatment; (□) incubation at 50 °C for 10 min in the presence of 5 mM $MgCl_2$; (●) incubation at 95 °C for 1 min followed by rapid cooling in the presence of 5 mM $MgCl_2$. $\ln(1 - f_{product})$ is plotted versus time, where $f_{product}$ is the fraction of $^{5'}$GGCUCUCU$_{OH}$. Lines represent a least-squares fit to the data. Rate constants for the experiments shown were determined to be 0.048, 0.046, and 0.062 min^{-1}, respectively.

presume that some ions must remain associated with the RNA during chromatography. The presence of additional $MgCl_2$ in the buffer, however, is desirable to ensure the stability of the folded RNA structure. Transcription reaction mixtures were treated with DNase I, pyrophosphatase, and proteinase K, to test whether the presence of plasmid DNA, pyrophosphate, or RNA polymerase was in any way deleterious to subsequent RNA-catalyzed reactions. These procedures also appeared to have no detectable effect on activity (data not shown). Likewise, each component of the T_7 RNA polymerase reaction buffer was tested independently. None of these compounds appeared to either stimulate or inhibit self-splicing of the RNA following gel filtration (data not shown).

Nondenatured Ribosomal Precursors Still Require a High-Temperature Incubation for Maximal Splicing. As a next step, the ability of precursor RNAs to self-splice following in vitro synthesis was tested. As shown in Figure 3A, *Tetrahymena* pre-RNAs were purified using G-100 columns, and the rates of self-splicing were measured at 30 °C under standard conditions, as listed in Table 1. These rates were compared with those obtained when the RNA was first renatured by heating to 95 °C and cooled in the presence of 5 mM $MgCl_2$. The observed activity of nascent precursors containing either very short (49 nt) exons, such as TZIVSΔ12, or nonribosomal exons, such as βGST$_7$, corresponded closely to the rates of splicing measured previously for gel-purified and thermally renatured RNAs (Woodson & Cech, 1991; Flor et al., 1989). Heat renaturation of column-purified transcripts similarly resulted in very little change in the observed rate of splicing.

In contrast, precursor RNAs containing longer exons of ribosomal sequence, such as SW012 with 244-nt exons, spliced slowly unless the RNA was heated to 95 °C prior to the assay. In earlier experiments using gel-purified transcripts, precursor SW012 was shown to be the shortest ribosomal pre-RNA that still self-spliced rapidly (Woodson, 1992). We now observe that a heat/cool renaturation treatment is required for the highest initial rates even when the precursors have not

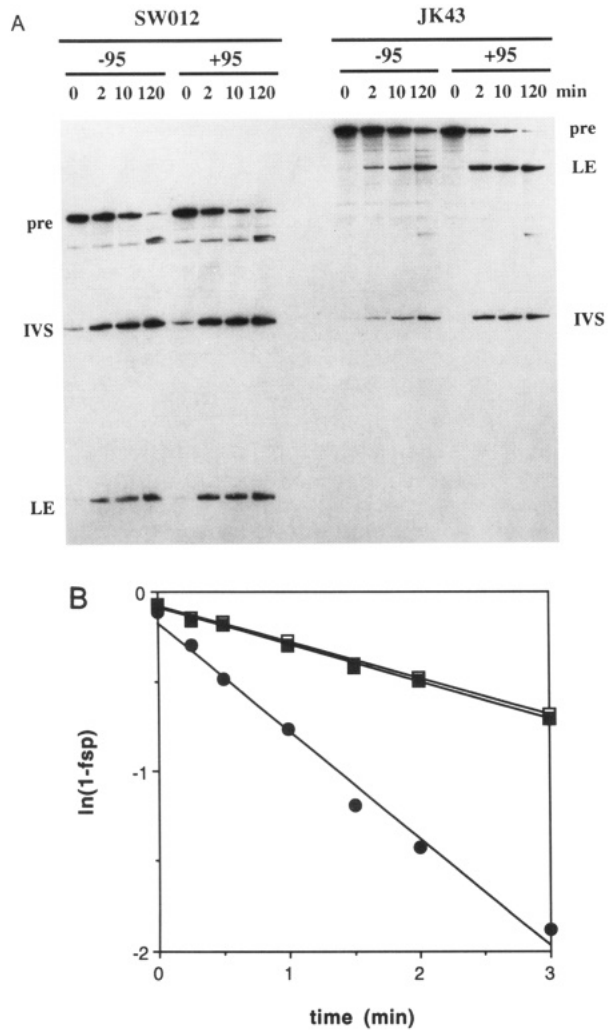

FIGURE 3: Self-splicing of column-purified *Tetrahymena* pre-RNAs. (A) Uniformly radiolabeled SW012 and JK43 precursor RNAs were isolated from transcription reactions by G-100 gel filtration, and incubated under standard splicing conditions. Precursors were either used directly (–95) or renatured (+95) prior to initiation of splicing by the addition of 0.1 mM GTP. Aliquots were removed at the times (minutes) indicated above the lanes and electrophoresed on 4% polyacrylamide gels containing 8 M urea. Abbreviations: pre, precursor; IVS, linear intron; LE, ligated exon product. (B) The fraction on SW012 ligated exon product (f_{sp}) was quantitated for splicing reactions carried out as in part A, and $\ln(1 - f_{sp})$ was plotted versus time (minutes). Lines represent the best linear fit to the first 3 min of the reaction. (■) The G-100 column was equilibrated with 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA (TE); (□) column was in TE with 3 mM $MgCl_2$; (●) the column contained TE buffer, and RNA was heat/cool-renatured prior to splicing.

been otherwise denatured following transcription. Renaturation does not appear to substantially alter the fraction of reactive precursor; approximately 55% of the RNA splices during the initial linear phase of product formation. (This is in addition to the 10% that has reacted during transcription.) While the intron sequences alone are able to fold during in vitro transcription, longer ribosomal precursor RNAs are not able to splice at the maximum rate, and thus presumably undergo an additional slow conformational rearrangement.

To confirm that the isolation procedure itself does not reduce the activity of precursor RNA, an aliquot of a T_7 RNA polymerase reaction was diluted directly into splicing buffer, while the remainder was column-purified as usual. The rate and extent of splicing in the two samples were the same (data not shown). The effect of RNA concentration was also examined. The amount of plasmid DNA template was varied, such that the concentration of SW012 pre-RNA synthesized

Table 1: Self-Splicing of Precursors Purified by Gel Filtration Chromatography

pre-RNA	5′ exon[a]	3′ exon[a]	k_{obs} (min^{-1})[b] +95 °C[c]	k_{obs} (min^{-1})[b] –95 °C[d]
TZIVSΔ12	20	29	0.02	0.02
TZIVSΔ12:–16C,–18C	20	29	fast[e]	fast[e]
SW010	120	70	0.09	0.07
SW012	163[a]	86	0.61	0.22
JK43	262	624	0.21	0.02
βGST_7	204[a]	29	fast[e]	fast[e]

[a] The length of the exon in nucleotides. The 5′ exon of precursor SW012 includes 146 nucleotides of ribosomal sequence and 17 nucleotides of vector sequences. The 5′ exon sequences of precursor βGST_7 (204 nt) are derived from lac Z′, except for 6 nt preceding the 5′ splice site. [b] Observed rate constants for self-splicing at initial times, as described under Materials and Methods. Conditions were 50 mM HEPES, pH 7.5, 100 mM $(NH_4)_2SO_4$, 6 mM MgCl, 1 mM EDTA, and 0.5 mM GTP, 30 °C. [c] Precursor RNAs were heated to 95 °C and cooled in the presence of Mg^{2+} before addition of GTP, as described under Materials and Methods. [d] Precursor RNAs were used in the splicing assay without any further manipulation following gel filtration. [e] Extensive formation of spliced products during transcription prevented an accurate determination of the splicing rate of the remaining population of precursor RNA. This presumably reflects very rapid processing of these transcripts.

ranged from 5 pM to 0.65 μM. For this precursor, there was no change in the splicing activity, although a RNA concentration dependence has been observed for precursor JK43 which is somewhat longer (see Table I). Analysis of transcription products by nondenaturing gel electrophoresis showed little or no dimerization or aggregation (V. L. Emerick and S. A. Woodson, unpublished results). Thus, the outcome of these experiments does not seem to be an artifact of the RNA isolation procedure.

Trans Splicing of Nondenatured RNA Precursors. Earlier results demonstrated that conformational changes in the ribosomal exons can affect splice site recognition (Woodson & Emerick, 1993; Woodson & Cech, 1991). Poor splicing might result from complete misfolding of the RNA. It could also arise from adoption of an alternative secondary structure that cannot undergo addition of GTP at the 5′ splice site. To distinguish between these possibilities, the rate of the second step of splicing, exon ligation, was measured using an intermolecular (trans) splicing assay (Inoue et al., 1985). As shown in Figure 4, column-purified SW012 pre-RNA is able to undergo intermolecular splicing, and the rate and extent of splicing are the same whether or not the RNA is first heated to 95 °C. Formation of the alternative secondary structure in the exons only results in inactivation of the 5′ splice site, and not of the 3′ splice site (Woodson & Cech, 1991). Trans splicing does not depend on activation of the intramolecular 5′ splice site, but still requires folding of the intron catalytic core. Thus, the data suggest that the lower rates of intramolecular splicing for nondenatured SW012 precursor result from adoption of the inactive alternative secondary structure in the ribosomal exon sequences, rather than from gross misfolding of the entire transcript.

Self-Splicing Is Not Further Improved in Very Long Precursor RNAs. One explanation for the lower reactivity of synthetic precursors might be that correct folding of the RNA during transcription requires even longer ribosomal exons, if upstream sequences direct the choice of folding pathways during RNA synthesis. On the other hand, the number of available conformational states increases dramatically with length [for example, see Gautheret and Cedergren (1993)]. Thus, longer transcripts might be predicted to have a lower probability of achieving the desired structure, resulting in lower rates of splicing.

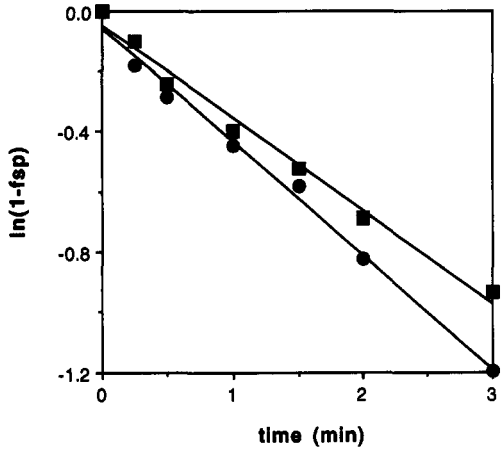

FIGURE 4: Intermolecular splicing of column-purified SW012 pre-RNA. Uniformly ^{32}P-labeled SW012 precursor RNA isolated from a G-100 column in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA was incubated in splicing buffer at 30 °C as above, but without GTP. Trans splicing reactions were initiated with the addition of 30 μM 5′ exon RNA. The intermolecular ligated exon product (f_{sp}) was quantitated, and $\ln(1-f_{sp})$ was plotted vs time. (■) No renaturation; (●) RNA was renatured (95 °C) prior to trans splicing.

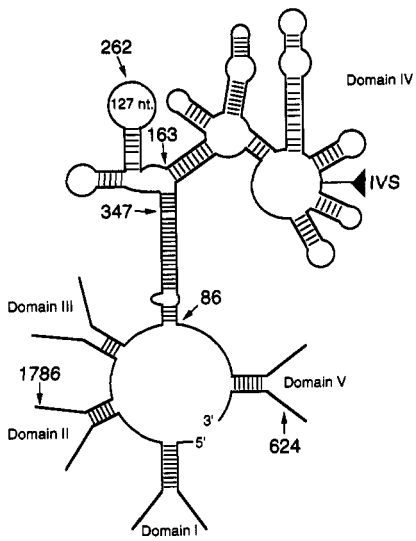

FIGURE 5: Pre-RNAs derived from *Tetrahymena thermophila* rDNA. The 5′ and 3′ limits of the precursor RNAs listed in Table II are depicted relative to the predicted secondary structure for domain IV of the mature 26S rRNA [adapted from Gutell et al. (1990)]. For brevity, other domains of the large rRNA are indicated schematically. Lengths of the 5′ and 3′ exons are shown next to the corresponding arrowhead. The position of the splice junction is indicated by "IVS".

In order to test these possibilities, a series of pre-rRNAs with much longer exons of ribosomal sequence were prepared. The 5′ and 3′ ends of the precursors are diagrammed in Figure 5 relative to the predicted secondary structure of the *Tetrahymena* 26S rRNA (Noller, 1984; Gutell et al., 1990). Initial rates of splicing with and without renaturation are listed in Table 2. Once again, the rate of splicing for even very long transcripts was increased by preincubation at 95 °C. These rates (0.2–0.6 min^{-1}) are similar to that of precursor SW012 (0.6 min^{-1}). Thus, additional ribosomal sequence, either upstream or downstream of the splice junction, does not eliminate the dependence of splicing rates on the heat treatment, although they can still react. For these precursors, preincubation at 95 °C also improved the fraction of molecules reacting in the initial burst, from an average of 30% without renaturation to an average of 70% with renaturation. The additional length may in fact increase the tendency to misfold during synthesis. On the other hand, even a 2820-nucleotide transcript (TP43) completely regains its activity when briefly heated to 95 °C and cooled to room temperature over a period of 2 min.

Table 2: Self-Splicing of Long *Tetrahymena* Precursor RNAs

pre-RNA	5′ exon[a]	3′ exon	k_{obs} (min^{-1})[b] +95 °C	k_{obs} (min^{-1})[b] −95 °C
SW012	163[a]	86	0.61	0.22
JK3021	262	86	0.34	0.04
TB012	347	86	0.64	0.19
TP012	1783	86	0.46	0.12
JK43	262	624	0.21	0.02
TB43	347	624	0.26	0.11
TP43	1783	624	0.20	0.11

[a] The length of the ribosomal exon sequences in nucleotides. All sequences are derived from *T. thermophila* rDNA, except as noted in the legend to Table I. [b] Observed rates of self-splicing at initial times, with or without prior heat/cool renaturation, as in the legend to Table 1.

DISCUSSION

Tetrahymena Pre-RNAs Are Not Fully Active When Transcribed in Vitro. A description of how long RNA transcripts achieve their active structure is a first step toward understanding the forces that regulate RNA conformation. This will ultimately contribute to the prediction of the three-dimensional structure from the primary sequence. We have used self-splicing of the *Tetrahymena* group I intron as a probe for the conformational state of pre-rRNA transcripts, as it requires formation of a precise set of RNA interactions. The intron alone is fully active when synthesized in vitro. This result is in agreement with the many experiments characterizing the catalytic and structural properties of group I introns (Cech, 1990).

When natural precursor RNA were transcribed in vitro, however, their ability to self-splice was lower than anticipated. The rates of splicing are increased by heat/cool renaturation, and become similar to that determined for the full-length preribosomal RNA when synthesized and purified from isolated *Tetrahymena* nuclei (Bass & Cech, 1984). The data are consistent with the preferential adoption of an inactive alternative secondary structure during transcription at 30 °C. The population of precursors is redistributed toward the active state by a brief incubation at high temperature. At least for the pre-RNAs tested under these conditions, primary sequence alone is not enough to ensure 5′ splice site activation. For very long pre-rRNAs, a greater fraction are completely inactive, suggesting that these transcripts are structurally heterogeneous when synthesized in vitro at 30 °C.

Models for the Folding of Long RNA Transcripts. Models of RNA folding can be roughly divided into sequential and local interaction models. In the latter case, interactions between nucleotides along the RNA chain nucleate the formation of local structural units, and cause progressively greater organization of the entire molecule. Formation of secondary structure would be likely to precede organization of extrahelical interactions. There is already experimental evidence that the equilibrium structure of tRNA (Crothers et al., 1974; Hilbers et al., 1976) and group I introns (Banerjee et al., 1993; Celander & Cech, 1991) obey such a hierarchy. There is also precedence for the folding of independent RNA domains (Murphy & Cech, 1993; van der Horst et al., 1991).

Sequential models, on the other hand, maintain that the correct folding pathway for a given RNA sequence depends on the order in which structural elements are synthesized. In this case, an RNA would not refold if denatured after transcription. This prediction is not borne out by our results

with self-splicing of ribosomal precursor RNAs. The small difference in the ability of a 0.6-kb and a 2.8-kb transcript to refold after transcription is most consistent with local interactions dominating the formation of the final structure. In this initial survey, the best predictor of self-splicing activity is whether the 5′ and 3′ ends complete a domain of secondary structure.

There is a possibility that the pre-rRNA does fold sequentially, but into a conformation that promotes 50S subunit assembly and not self-splicing. This could explain why a high-temperature incubation is required for optimal splicing rates, but still presents the problem of how to facilitate intron excision before ribosome maturation. Another possibility is that long RNAs cannot fold when transcribed by more rapid phage polymerases. There is recent evidence that 23S rRNA synthesized in *Escherichia coli* by T_7 RNA polymerase is not incorporated into polysomes (Lewicki et al., 1993). In our experiments, transcription at 25 °C (and thus at a slower rate) did not increase the rate of splicing or the fraction of active precursor. Furthermore, chemical and nuclease protection studies of the shorter rRNA fragments used here (precursor SW012) are consistent with the predicted structure for the 26S rRNA (S. Woodson, unpublished results).

Protein Facilitation of Pre-rRNA Splicing. There is abundant evidence, primarily from yeast and bacteriophage genetics, that proteins are generally required to assist splicing of group I and group II introns in vivo [reviewed in Burke (1988) and Lambowitz and Perlman (1990)]. A few group I maturases have been identified and characterized in more detail (Garriga & Lambowitz, 1986; Gampel et al., 1989). They appear to stabilize the structure of the intron core, but do not directly participate in catalysis (Gampel & Cech, 1991; Guo & Lambowitz, 1992). In *Tetrahymena*, estimates of in vivo splicing rates are as much as 50-fold faster than in vitro rates; excision of the intron is one of the earliest discernible steps in rRNA processing (Cech & Rio, 1979; Din & Engberg, 1979). This has lead to the suggestion that one or more proteins facilitate processing of the pre-38S RNA (Cech, 1990). Our results indicate that conformers where the 5′ splice site is activated toward addition of GTP are not preferred when the RNA is transcribed in the absence of cellular proteins. This in turn suggests that splicing of this group I intron is facilitated by one or more proteins bound to the ribosomal RNA, perhaps in addition to those that are specific for the intron itself.

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