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The Self-Splicing RNA of *Tetrahymena* Is Trapped in a Less Active Conformation by Gel Purification

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ABSTRACT: When the circular form of the self-splicing intervening sequence of *Tetrahymena thermophila* was purified by denaturing polyacrylamide gel electrophoresis by standard methods, the rate of its reaction with tetrauridylate decreased 150-fold at 30 °C and at least 1000-fold at 0 °C. The activity of the self-splicing RNA was restored by heating it to high temperature and letting it renature in the presence of Mg^{2+}. The rate of reaction of tetrauridylate with the self-splicing RNA flanked by exons was also greatly decreased by gel purification. the difference in activation energies for the reaction of native and denatured intervening sequences suggests that a substantial conformational rearrangement of the gel-purified RNA occurs prior to reaction.

Like all RNAs, catalytic RNAs require proper folding of the polynucleotide chain to function. Previous evidence for the importance of structure in reactions of the self-splicing intervening sequence (IVS) from the large rRNA precursor (pre-rRNA) of *Tetrahymena thermophila* is its reduced activity at elevated temperatures and in the presence of denaturants (Cech & Bass, 1986).

In the first step of the self-splicing of pre-rRNA, free guanosine nucleotide causes cleavage at the 5′-splice site and becomes attached to the 5′-end of the IVS. In the second step, the 5′-exon causes cleavage at the 3′-splice site and becomes ligated to the 3′-exon (Kruger et al., 1982). Two reactions have been described that resemble the second step of splicing. In a reaction known as intermolecular exon ligation, an oligonucleotide is incubated with pre-rRNA in the absence of guanosine and cleavage occurs at the 3′-splice site with concomitant ligation of the oligonucleotide to the 3′-exon (Inoue et al., 1985). In a similar reaction called reverse circularization, an oligonucleotide cleaves the linkage between A16 and G414 of the circular form of the IVS (C-IVS) and becomes attached to A16 (Sullivan & Cech, 1985).

Pre-rRNA was prepared by in vitro transcription of plasmid DNA (Inoue et al., 1985), and C-IVS was prepared either by allowing pre-rRNA to splice or by allowing a previously purified linear form of the IVS (L-IVS) to circularize (Sullivan & Cech, 1985). We report here that the usual method of purification substantially reduces the reactivity of the catalytic RNAs and describe a protocol that restores activity to the IVS.

Materials and Methods

Pre-rRNA containing the 413-nucleotide IVS of the large ribosomal RNA precursor of *T. thermophila*, 32 nucleotides of the 5′-exon, and 37 nucleotides of the 3′-exon was prepared by run-off transcription of the plasmid pTTT1A3 using T7 RNA polymerase (Zaug et al., 1986). The transcription reaction mixture contained 40 mM Tris-HCl, pH 8.1, 6 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 0.8 mM of each NTP (1 Ci/mmol [α-^{32}P]ATP), and 50 μg/mL T7 RNA polymerase. After incubation for 2 h at 30 °C, pre-rRNA was purified by electrophoresis of the reaction mixture in a 4% polyacrylamide–7 M urea gel; elution from the gel slice into 0.25 M NaOAc, 0.1% (w/v) sodium dodecyl sulfate, 1 mM Na_2EDTA, and 10 mM Tris-HCl, pH 8.0; precipitation with ethanol; chromatography using coarse Sephadex G-50 (Pharmacia) in the same buffer as was used for elution from the gel; two additional precipitations with ethanol; and resuspension in water at about 500 nM RNA. To prepare C-IVS or L-IVS, the RNA in the transcription reaction mixture was precipitated with ethanol, resuspended in H_2O, and incubated in 0.1 mM GDP, 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5, for 15 min at 42 °C to give predominantly C-IVS or 30 min at 30 °C to give predominantly L-IVS. Gel-purified C-IVS and gel-purified L-IVS were obtained from these reaction mixtures by the same procedure used to purify pre-rRNA. Native C-IVS was obtained by incubating gel-purified L-IVS for 15 min at 42 °C in 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5, and used immediately. Renatured pre-rRNA and renatured C-IVS were prepared by incubating the gel-purified RNAs in water for 3 min at 95 °C, adding one-fifth volume 500 mM $(NH_4)_2SO_4$, 50 mM $MgCl_2$, and 150 mM Tris-HCl, pH 7.5, and centrifuging the tube to return condensation to the tube's bottom. Cooling time to room temperature was 1–2 min.

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Table I: Kinetic Parameters for U_4 Addition to C-IVS and Pre-rRNA and for Their Hydrolysis

	temp (°C)	$10^5 K_m$ (M)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	k_{hyd} (min^{-1})
native C-IVS	30	3	3	1×10^5	0.004
renatured C-IVS	30	2	2	1×10^5	0.003
gel-purified C-IVS	30	2	0.02	1×10^3	0.0009
native C-IVS	0	0.6	0.9	1×10^5	0.001
renatured C-IVS	0	3	0.8	3×10^4	0.004
gel-purified C-IVS	0		<0.0005[a]		
renatured pre-rRNA	30	3	0.4	1×10^4	0.001
gel-purified pre-rRNA	30	9	0.03	3×10^2	0.0009
renatured pre-rRNA	0	3	0.6	2×10^4	0.002
gel-purified pre-rRNA	0		<0.0005[a]		

[a] Rate of reaction with 0.2 mM U_4.

Initial rates for reverse circularization and intermolecular exon ligation were determined at five different U_4 concentrations with about 50 nM C-IVS or with a combination of 50 nM each of pre-rRNA and C-IVS in 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5. Aliquots were withdrawn, quenched with 25 mM EDTA, and analyzed by using 4% polyacrylamide–7 M urea gel electrophoresis. Reactant and product bands were cut out and the extent of each reaction was determined by liquid scintillation counting. Initial rates of reaction were found by a linear least-squares fit of the logarithm of the extent of reaction versus time. The rates of hydrolysis (k_{hyd}) were determined for reactions where U_4 was omitted and were used to correct the rates of U_4 addition. K_m and k_{cat} were obtained by using a linear least-square fit of an Eadie–Hofstee plot. While no turnover occurs in the reaction, Michaelis–Menten kinetics can be used as has been done for an irreversible inhibitor with an enzyme (Kitz & Wilson, 1962; Fahrney & Gold, 1963; Main, 1964).

The Arrhenius activation energy (E_a) for reverse circularization was found by plotting the log of the rate constants against reciprocal temperature. The slope of the least-squares fit of the data equals $-E_a/R$, and its vertical axis intercept equals ln (A). The entropy of activation equals $R[\ln (Ah/k_BT) - 1]$, where k_B is the Boltzmann constant, h is the Planck constant, and R is the gas constant (Chang, 1981).

Results

The reactivities of three different preparations of C-IVS are considered: (1) gel-purified C-IVS prepared by using the standard procedure of Sullivan and Cech (1985); (2) native C-IVS prepared by incubating gel-purified L-IVS in the reaction buffer (although the circularization of L-IVS was not complete, the C-IVS present is assumed to have been in the native conformation since it had just undergone splicing); (3) renatured C-IVS obtained by denaturing the gel-purified RNA and cooling it in the presence of magnesium ion. As shown in Figure 1, the reaction of U_4 with the native and renatured molecules was complete after 0.2 min of incubation while the gel-purified molecule was still largely unreacted after 10 min.

The kinetics of the addition of U_4 to the three different preparations of C-IVS were analyzed by Michaelis–Menten kinetics. As shown in Table I, the differences in k_{cat} were much larger than the differences in K_m among the three preparations of C-IVS. Native C-IVS showed the greatest rate of reaction (k_{cat} = 3 min^{-1} at 30 °C). This k_{cat} is 150-fold greater than the k_{cat} of 0.02 min^{-1} for U_4 addition to gel-purified C-IVS under the same reaction conditions and more than 400-fold greater than the k_{cat} of 0.007 min^{-1} obtained for the addition of CpU to gel-purified C-IVS at 30 °C (Sugimoto et al., 1988). The k_{cat} for U_4 addition to gel-purified C-IVS was restored to 2 min^{-1}, or about 70% of that of native C-IVS, by the renaturation process. The specificity constant (k_{cat}/K_m) was 100-fold less for gel-purified C-IVS than for native C-IVS but was restored by renaturation.

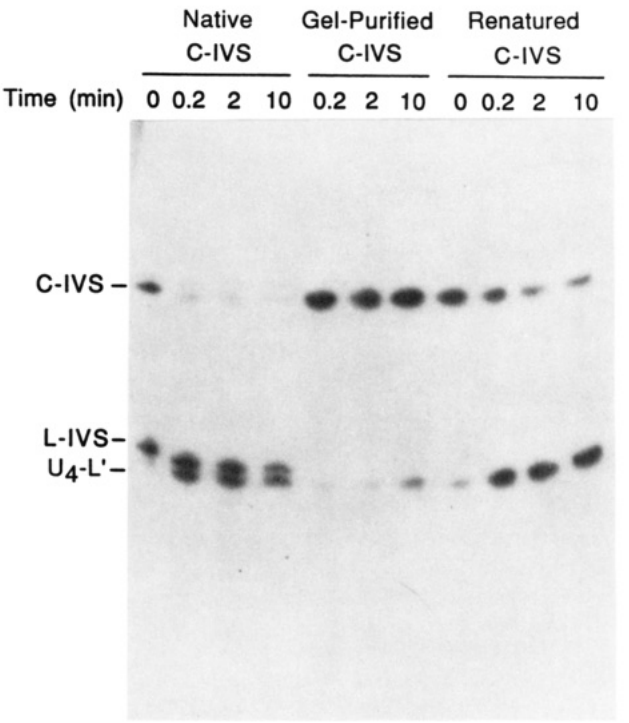

FIGURE 1: Time course of reactions of 0.2 mM U_4 with native C-IVS (lanes 1–4), gel-purified C-IVS (lanes 5–7), and renatured C-IVS (lane 8–11). Reactions were carried out at 30 °C in 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5.

When reverse circularization was carried out at 0 °C instead of 30 °C, the differences in rates obtained for differently prepared C-IVS were even greater. The k_{cat} for native C-IVS was 0.9 min^{-1}, whereas the reaction rate with gel-purified C-IVS was too slow to obtain accurate kinetics at subsaturating U_4 concentrations. At 0.2 mM U_4, which should be saturating, k_{cat} can be estimated from the initial rate of reaction to be <0.0005 min^{-1}, or at least 1000-times slower than for native C-IVS. The specificity constant at 0 °C for renatured C-IVS was nearly restored to that of native C-IVS, but the renaturation procedure was apparently not able to convert all of the C-IVS molecules to the proper conformation. Extremely slow reaction rates of gel-purified forms of the IVS at 0 °C have been reported previously (Sugimoto et al., 1988; Cech et al., 1983; Tanner & Cech, 1985). For C-IVS, it is clear now that this results from the RNA being trapped in an inactive conformation, since reaction of native or renatured molecules is quite rapid at 0 °C.

The possibility that inactive conformations might affect the rate of other reactions of the IVS was investigated. The kinetics of U_4 addition to gel-purified pre-rRNA was compared to pre-rRNA renatured by the same protocol as was used for C-IVS (Table I). Native pre-rRNA could not be assayed, since pre-rRNA in the transcription reaction is mixed with a variety of RNA molecules. At 30 °C the effect of renaturation on pre-rRNA (13-fold increase in k_{cat}; 30-fold increase in k_{cat}/K_m) was not as large as for C-IVS, but at 0 °C at least a 1000-fold difference was observed between k_{cat} for renatured pre-rRNA and k_{obs} for 0.2 mM U_4 addition to gel-purified pre-rRNA. The slight increase in k_{cat} for renatured pre-rRNA upon reducing the temperature from 30 to 0 °C could reflect some irreproducibility in the renaturation process, since separate preparations of pre-rRNA were used for the two temperatures. The effect of renaturation on the entire self-splicing

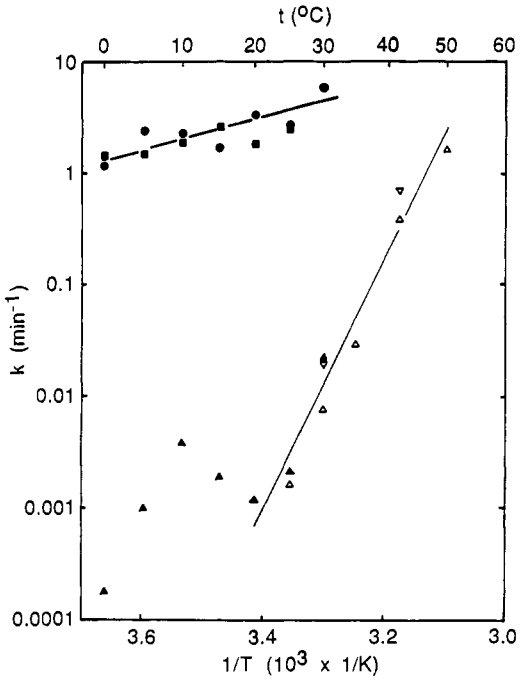

FIGURE 2: Arrhenius plots of rate constants for 0.2 mM U_4 addition to C-IVS (closed circles), renatured C-IVS (closed squares), and gel-purified C-IVS (closed triangles); CU addition to gel-purified C-IVS (open triangles) (Sugimoto et al., 1988); and U_3 addition at 42 °C (Sullivan & Cech, 1985) and CU addition at 30 °C (Sullivan, 1986) to gel-purified C-IVS (inverted open triangles).

reaction was also briefly investigated. The rate of appearance of intron and ligated exons upon the addition of 0.1 mM GDP to pre-rRNA was determined at 30 °C in 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5. The rate of reaction was 4–5-fold faster for renatured pre-rRNA than for gel-purified pre-rRNA. This is a much smaller effect than the 100-fold and 30-fold effects on k_{cat}/K_m seen at 30 °C for the two U_4 addition reactions. One possible explanation of this difference is that the overall splicing reaction may have a different rate-limiting step after renaturation. With gel-purified pre-rRNA, the second step limits the rate of overall splicing, while the first step may become rate limiting when pre-RNA is renatured.

Rates of hydrolysis of native C-IVS, renatured C-IVS, and renatured pre-rRNA were also measured (Table I). The rates of hydrolysis showed little dependence on either the method of preparation on the RNA or the reaction temperature. The values are consistent with the previously obtained value of 0.0016 min^{-1} for gel-purified C-IVS hydrolysis at 42 °C in 10 mM $MgCl_2$ buffered at pH 7.5 (Zaug et al., 1985).

The rate of oligonucleotide addition to gel-purified C-IVS has been previously reported to have a large temperature dependence, and thus there is a large activation energy for reaction (Sugimoto et al., 1988; Sullivan, 1986). The rates of U_4 addition to the different preparations of C-IVS at a variety of temperatures are plotted in Figure 2, along with rate data obtained by others for addition of oligonucleotides to gel-purified C-IVS (Sullivan & Cech, 1985; Sugimoto et al., 1988; Sullivan, 1986). The reaction rate for U_4 addition to native C-IVS or renatured C-IVS was too rapid above 30 °C to be accurately measured by the conventional pipetting methods used in this study. The accuracy of reaction rates for U_4 addition to gel-purified C-IVS below 25 °C is less than the accuracy of rates measured at higher temperatures, because hydrolysis is the dominant reaction at low temperature. Nevertheless, it is clear that the temperature dependence of the reaction rate for gel-purified RNA is much greater than for native C-IVS and renatured C-IVS. The Arrhenius activation energy (E_a) found in this study for native C-IVS was 6 kcal/mol, while Sugimoto et al. (1988) report an E_a of 50 kcal/mol for gel-purified C-IVS at 25-35 °C. As a result, only above 50 °C does the reaction rate of gel-purified C-IVS approach that of native C-IVS. This suggests that the large activation energy for the reaction of gel-purified C-IVS is required to convert gel-purified C-IVS from an inactive conformation to the active conformation needed for reaction.

Several different renaturation protocols were investigated with gel-purified C-IVS. When the RNA was heated for 1–2 min in the presence of 10 mM Mg^{2+} at different temperatures, only a small improvement in the subsequent reaction rate at 30 °C was observed until 70 °C was reached. While renaturation at 80 °C was reasonably complete, substantial degradation of the circular RNA occurred. This led to protocols involving the denaturation of the RNA in water, then adding 10 mM $MgCl_2$ and buffer, and allowing the sample to cool to room temperature over 1–2 min. This procedure avoided the heat-induced degradation. A survey of denaturation temperatures revealed the greatest rate of reaction at 30 °C required heating to 95 °C prior to the addition of $MgCl_2$.

Discussion

Purifying the self-splicing RNA from *Tetrahymena* in denaturing polyacrylamide gels greatly reduces its rate of reaction with oligonucleotides, although the molecule remains active. Since the rate can be restored by a renaturation procedure, it appears that gel purification causes a conformational rearrangement.

How large of a conformational rearrangement is required to account for the data? Since E_a is quite small for reaction of native C-IVS (6 kcal/mol), it is reasonable to assume that the entire E_a determined for reaction of gel-purified C-IVS (50 kcal/mol; Sugimoto et al., 1988) is due to disrupting an inactive conformation. Similarly, the large positive activation entropy (86 eu/mol.; Sugimoto et al., 1988) for gel-purified C-IVS can be interpreted to be the result of disrupting four to five base pairs. We have calculated an activation entropy for U_4 addition to native C-IVS of −45 eu/mol. (Figure 2), which indicates a transition state that is more ordered than the oligonucleotide–IVS complex by about the entropy change involved in the formation of two base pairs.

The ability to adopt a less active conformation is a relatively common feature of RNA molecules. For example, the catalytic M1 RNA from *Escherichia coli* RNase P is in an inactive state when thawed and a rearrangement with an E_a of 36 kcal/mol is required before it is active (Altman & Guerrier-Takada, 1986). Yeast $tRNA_3^{Leu}$ has a well-defined alternative conformer, which can be converted to the active form with an E_a of 69 kcal/mol (Hawkins et al., 1977). Alternative conformers of other RNAs have been reported including col E1 primer RNA (Tomizawa, 1984; Wong & Polisky, 1985), *E. coli* 5S rRNA (Kao & Crothers, 1980), and the leader sequence of the trp operon mRNA of *E. coli* (Lee & Yanofsky, 1977). These data emphasize that RNA molecules, like proteins, are conformationally complex and do not generally renature easily. This should be noted by those interested in the structure or function of RNA molecules purified with denaturants.

Registry No. Mg, 7439-95-4; tetrauridylate, 47921-53-9.

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