Characterization of the Mode of Binding of Substrates to the Active Site of *Tetrahymena* Self-Splicing RNA Using 5-Fluorouracil-Substituted Mini-Exons†

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ABSTRACT: Splice-site selection specificity in *Tetrahymena* self-splicing RNA is thought to be mediated by a base-paired complex between a CUCUCU sequence on the end of the 5′ exon and a GGGAGG guide sequence in the intron. The substitution of uracil (U) in oligonucleotide mini-exons with 5-fluorouracil (U^F), an analogue bearing a much more acidic N-3 proton, allowed us to test the role of hydrogen bonding between complementary bases in the splice-site selection process. The affinities of (U) and (U^F) mini-exons for the ribozyme active site were similar and several orders of magnitude greater than expected from base pairing alone. In contrast to CUCU, the CU^FCU^F mini-exon lost substrate activity with increasing pH, presumably due to ionization of the U^F residues. However, the apparent p*K* values of these residues were several p*K* units above that of free U^F, indicating that the mini-exon is shielded from the solvent by an active site of low polarity. Loss of the pyrimidine N-3 hydrogen bond by selective ionization of the U^F residues decreased the binding of CU^FCU^F to the ribozyme only 3-fold but did prevent its ligation to the 3′ exon. Temperature dependence of substrate activity was identical for both (U) and (U^F) mini-exons, whereas the U^F-substituted ribozyme lost activity at a considerably lower temperature than did the natural (U) ribozyme. These observations indicate that hydrogen-bonded base pairs involving the U residues contribute little to the total binding energy of the 5′ splice site with the active site of the ribozyme, but probably help to align the splice sites properly for ligation.

The discovery of autocatalytic or "self-splicing" RNA several years ago (Kruger et al., 1982) was an exciting event because it irrevocably altered the concept of RNA solely as an informational molecule. Catalytic RNAs (or "ribozymes"), just as their protein enzyme counterparts, are capable of enormously accelerating reaction rates as well as showing remarkable specificity in splice-site selection (Cech, 1987). A number of the catalytic strategies of ribozymes are thought to be similar to those of protein enzymes, including the use of specific binding sites for the "substrates" of the ribozyme—the splice-site sequences. The mode of substrate binding is incompletely understood. In the case of *Tetrahymena* self-splicing RNA, a base-pairing interaction between a GGGAGG sequence [designated the "internal guide sequence" (IGS)[1]] within the intron and a partially complementary CUCUCU sequence on the end of the 5′ exon has been proposed to be critical for splice-site selection (Waring & Davies, 1984). Mutations in the GGGAGG sequence cause either loss of splicing activity or changes in the specificity (Been & Cech, 1985, 1986, 1987; Waring et al., 1986). However, there are also indications that the active site of the ribozyme is capable of more complex interactions than just simple base pairing between two short oligonucleotide sequences. For example, oligonucleotides appear to bind to the IVS of *Tetrahymena* rRNA more tightly than expected solely from the formation of Watson–Crick base pairs (Zaug & Cech, 1986; Sugimoto et al., 1988). It has also been shown that the binding of the 3′ splice site does not appear to be very dependent on the presence of complementary base pairs in the IVS (Been & Cech, 1985). The nature of the active site of ribozymes is of great interest not only for understanding the mechanisms involved in RNA catalysis but also for obtaining insights into the evolution of catalytically active biomolecules.

Besides normal splicing, the *Tetrahymena* self-splicing RNA also catalyzes an intermolecular splicing reaction with short oligonucleotide segments that correspond to a portion of the sequence on the end of the 5′ exon (e.g. CUCUCU) (Inoue et al., 1985; Garriga et al., 1986). These segments become joined to the 5′ end of the 3′ exon, thereby in effect acting as mini-exons that presumably bind to the IVS active site normally occupied by the 5′ exon splice site. Since structural alterations can be made in mini-exons that are not possible to achieve in specific locations of the ribozyme, the trans-splicing reaction provides a means of characterizing the active site of the ribozyme through structure–activity studies similar to those used historically to study the active sites of proteins. One strategy for studying the mode of binding of the mini-exons to the ribozyme would be to substitute fraudulent bases such as U^F for U and to determine the effects of this substitution on binding and trans-splicing activity. The N-3 proton of U^F is much more acidic than that of U (p*K* values of 7.9 and 10.1, respectively) (Cushley et al., 1968), conferring in theory a greater hydrogen-bonding capability to the analogue (Saenger, 1984) as well as making it possible selectively to ionize U^F residues incorporated into oligonucleotides. In terms of size, however, U^F is very similar to U; it is incorporated readily into all species of RNA and in most cases has a minimal perturbing effect on the functioning of the RNA (Heidelberger et al., 1983). The putative duplex between the end of the 5′ exon and the IGS contains a U–A pair in addition to a U–G interaction at the terminus (Figure 1A). Since a hydrogen bond from the N-3 position of U is an important

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[1] Abbreviations: U, uracil; U^F, 5-fluorouracil; IVS, intervening sequence of the *Tetrahymena* ribozyme; IGS, internal guide sequence; CU, pppCpU; CUC, pppCpUpC; CUCU, pppCpUpCpU; CUCUC, pppCpUpCpUpC; CUCUCU, pppCpUpCpUpCpUp; GGCCCUCU, pppGpGpCpCpCpUpCpU; $CUCU_{OX}$, periodate oxidized CUCU; abbreviations for the (U^F) oligonucleotides are analogous.

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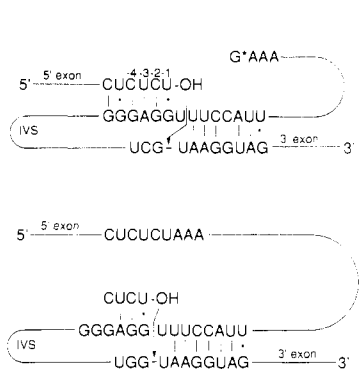

FIGURE 1: Putative base-pairing interactions between the IGS of the active site of the *Tetrahymena* self-splicing RNA and (A) the splice site at the terminus of the 5′ exon and (B) the oligonucleotide CUCU in the intramolecular splicing reaction. G* represents the guanosine nucleotide added to the ribozyme to initiate the normal splicing reaction.

determinant of the stability of Watson–Crick base pairs between U and A, the use of U^F in place of U should constitute a unique tool for assessing the role of base-pairing interactions in the binding of the 5′ exon to the IGS.

In this study, we compared the interaction of the natural and the U^F-containing mini-exons with the ribozyme by determining binding constants and catalytic activity of these substrates under conditions of pH and temperature designed to cause dissociation of U^F–A base pairs. The results show that most of the binding energy of these substrates comes from forces other than base-pairing interactions, but suggest that the latter may be important in splice-site alignment. The active site of the ribozyme resembles that of many protein enzymes in that it protects substrates from the environment and has hydrophobic character.

Experimental Procedures

Plasmid pT7-TT1A3 was a gift of Dr. T. R. Cech, University of Colorado. U^FTP was purchased from Sierra Biochemicals, Tucson, AZ. [^{3}H]CTP and [^{32}P]CTP were obtained from Amersham Inc. All other chemicals were of molecular biology grade.

Synthesis of Precursor RNA. *Tetrahymena* prespliced RNA (the ribozyme) was prepared by transcription of plasmid pT7-TT1A3 according to the procedures described by Price and Cech (1985). So that the concentration of the transcript could be determined more easily during the experiments, 6 μCi of [^{3}H]CTP was added to the transcription mixture, along with CTP, ATP, GTP, and UTP. When the U^F-substituted ribozyme was desired, U^FTP was used instead of UTP.

Preparation of Mini-Exons. Preparation of the oligoribonucleotide substrates for the ribozyme was based on methods described by Martin and Coleman (1987) and Milligan et al. (1987). Complementary oligodeoxyribonucleotide strands were chemically synthesized on an Applied Biosystems DNA synthesizer to contain the primer sequence for T7 polymerase plus a GAGAGA template for the hexanucleotide CUCUCU on the coding strand. The strands were annealed and transcribed with T7 polymerase in the presence of [^{32}P]CTP and either UTP or U^FTP. Electrophoresis on a 20% polyacrylamide gel indicated that the transcription product consisted of five major bands. The sections of the gel containing each band were cut out, and the material was eluted from the gel by shaking in 0.5 M ammonium acetate overnight at 4 °C. The solutions were placed on Sep-Pak columns (Waters Associates) to adsorb the oligoribonucleotides. The columns were washed first with 10 mL of water and then with 3 mL of 60% methanol. The methanol fractions containing the oligoribonucleotides were evaporated to dryness.

Reaction of the Mini-Exons with the Ribozyme. Trans-splicing reactions were carried out by combining unspliced precursor RNA (0.04 μM) with the ^{32}P-labeled oligonucleotide substrates at five different concentrations ranging from 0.1 to 10 μM, 50 mM HEPES buffer at pH 7.5, 10 mM $MgCl_2$, and 200 mM NaCl in a total volume of 5 μL. The reactions were incubated at 30 or 42 °C for 30 min, which was within the initial velocity period for all substrate concentrations. The reactions were stopped by adding 1 μL of a solution containing 57 mM EDTA and 170 mM Tris, pH 7.5, and freezing in dry ice-methanol. In the experiments in which pH was varied, PIPES buffer was used for pH 6.1–6.8, HEPES for pH 7–8.2, Tris for pH 8.4–8.6, and CHES for pH 8.8–9.5. The reaction mixtures were electrophoresed on 8% polyacrylamide gels [20:1 acrylamide–bis(acrylamide)] containing 7 M urea, 90 mM Tris–borate, and 2.5 mM EDTA. The bands migrating at about 90 nt were cut from the dried gels by using the autoradiogram as a guide and quantitated by liquid scintillation counting. Velocities were calculated from the amount of the ^{32}P-labeled 90-nt trans-splicing product formed at 30 min. K_m and V_{max} values were obtained from Lineweaver–Burk plots of reciprocal velocities versus substrate concentration using weighted least-squares linear regression calculations.

Preparation of $CUCU_{OX}$ and $CU^FCU^F{}_{OX}$. CUCU or CU^FCU^F dissolved in water was treated with 23.4 mM sodium periodate for 30 min at 25 °C. The reaction was stopped by the addition of ethylene glycol to 20% v/v. Completeness of reaction was confirmed by lack of substrate activity of the material from the reaction mixture. The inhibitors were used without further isolation.

Results

Synthesis of Oligoribonucleotide Mini-Exons. T7 polymerase transcription of the synthetic oligonucleotide containing the T7 promoter sequence attached to GAGAGA did not give the longest transcript as the major product, but instead yielded a ladder of five major radiolabeled bands. These products were deduced to be the oligonucleotides CU, CUC, CUCU, CUCUC, and CUCUCU in the approximate molar ratio of 1:3:4:3:2, respectively, on the basis of (a) previous studies describing the use of the T7 promoter to synthesize defined oligoribonucleotides (Coleman & Martin, 1987; Milligan et al., 1987) in which, in addition to the full-length transcript, shorter transcripts also were detected that resulted almost exclusively from initiation at the first nucleotide of the template sequence and then premature termination of transcription and (b) digestion patterns obtained with ribonuclease phy M, which cleaves specifically at U residues (Donis-Keller, 1980). For example, phy M digestion of the band corresponding to a tetramer gave exclusively a dimer, which would be the predicted product from cleavage of CUCU after the U residue. On the other hand, if the tetramer band were UCUC, the product of initiation at position +2 of the template, the expected hydrolysis products would be the monomer pppUp*, the trimers pppUP*CpUp* and CpUp*C, and possibly the dimer CpUp* from hydrolysis of the latter. (The asterisk represents a radiolabeled phosphate.) None of these products were seen in the phy M hydrolysate. The analogous (U^F) oligonucleotides in the same ratios were obtained when U^FTP was used in the transcription mixture instead of UTP except that the overall yield of the products was about 20% less. When purifying the (U) and (U^F) oligonucleotides by polyacrylamide gel electrophoresis, we noted that the (U^F) oligonucleotides migrated faster than the corresponding (U) nu-

Table I: K_m and k_{cat} Values for Various Oligonucleotide Mini-Exons in the Trans-Splicing Reaction Catalyzed by the *Tetrahymena* Self-Splicing RNA

substrate	K_m (μM)	k_{cat} (min^{-1})
CU	7.7 ± 2.2	0.021 ± 0.002
CUC	0.42 ± 0.10	0.013 ± 0.003
CUCU	0.11 ± 0.04	0.027 ± 0.005
CUCUC	0.12 ± 0.03	0.011 ± 0.003
CUCUCU	0.080 ± 0.03	0.028 ± 0.005
CU^F	50 ± 5	0.021 ± 0.002
CU^FCU^F	0.16 ± 0.03	0.021 ± 0.003
CU^FCU^FC	0.25 ± 0.04	0.010 ± 0.002
$CU^FCU^FCU^F$	0.11 ± 0.02	0.020 ± 0.004
$GGCCCU^FCU^F$	0.11 ± 0.02	0.029 ± 0.008
$GGCU^FCU^FCU^F$	0.066 ± 0.015	0.019 ± 0.002

cleotides and that the difference increased with the number of U^F residues in the product. This difference in migration rates is presumabnly due to the greater degree of ionization of the U^F residues at the pH of the gel buffer.

Kinetic Constants of the (U) and (U^F) Mini-Exons. To evaluate the effect of substitution with U^F residues on binding to the active site and on ribozyme activity, the ^{32}P-labeled oligonucleotides CU, CUC, CUCU, CUCUC, and CUCUCU and the U^F-substituted analogues CU^F, CU^FC, CU^FCU^F, and $CU^FCU^FCU^F$ were tested as trans-splicing substrates for the natural (U) ribozyme. With all of these mini-exons, the time-dependent formation of a radiolabeled fragment of about 90 nt was observed. This fragment has previously been shown to consist of the radiolabeled oligonucleotide ligated to the 5′ end of the 3′ exon (Inoue et al., 1985). The time course of the reaction of 42 °C was linear for at least 30 min with all the substrates at the highest concentrations that were used, corresponding to consumption of less than 10% of the initial amount of ribozyme. Thus, we used 30 min as a convenient time point for the subsequent ribozyme kinetics studies. The formation of the 90-nt product as a function of the concentration of all of the oligonucleotide substrates followed a hyperbolic curve, thereby confirming the presence of a saturable binding site for these mini-exons on the self-splicing RNA. The system can therefore be described by the Michaelis–Menten scheme

$$(CU)_n + \text{ribozyme} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (CU)_n\text{·ribozyme} \overset{k_{cat}}{\rightleftharpoons} (CU)_n\text{–IVS–3′ exon}$$

Double-reciprocal plots of the rate data were linear (not shown) and were used to obtain K_m and k_{cat} values for both the (U) and (U^F) oligonucleotide substrates (Table I). The k_{cat} values differed by about 3-fold among these substrates, but no systematic variation of this parameter with substrate structure is discernible. The magnitude of k_{cat} for the trans-splicing reaction appears to be on the average about 50-fold lower than that of the nucleotidyl transferase activity of the *Tetrahymena* IVS, another intermolecular reaction catalyzed by this ribozyme (Cech, 1987). K_m values decreased about 70-fold for the (U) mini-exons and about 300-fold for the (U^F) mini-exons as the length of the substrate increased from two to four nucleotides, but did not decrease appreciably thereafter. There was no significant difference between the kinetic constants of the (U) and the (U^F) mini-exons, except in the case of the dinucleotides.

We wanted to use the K_m values of the mini-exons to compare their binding to the active site of the ribozyme, but the K_m values will approximate the true dissociation constants only if the binding and release of the substrates are in thermodynamic equilibrium before the ligation reaction takes place (i.e., if k_1 and $k_2 \gg k_{cat}$). Although this is a reasonable assumption considering the slow turnover of the trans-splicing reaction, we nevertheless tested it by determining the K_i values of CUCU and CU^FCU^F that had been oxidized with periodate and thus rendered incapable of undergoing splicing. The reasoning was that oxidation of the terminal ribose moiety should not affect most of the interactions responsible for binding, and thus the binding constant of the oxidized substrate should be comparable to that of the active substrate. As shown in Figure 2, both $CUCU_{OX}$ and $CU^FCU^F{}_{OX}$ were linear competitive inhibitors of trans-splicing with K_i values of 0.2 and 0.5 μM, respectively, which are only slightly more than 2-fold greater than the K_m value of the corresponding substrates.

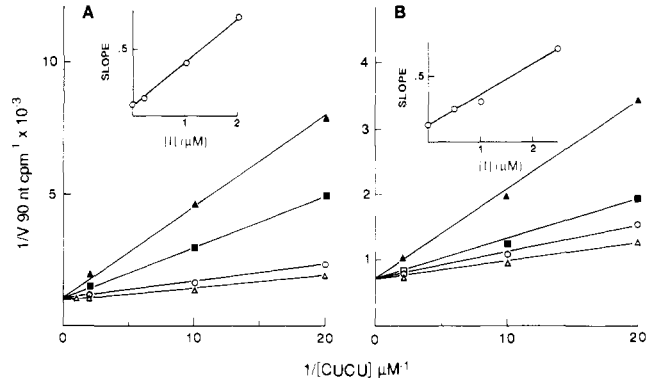

FIGURE 2: Inhibition of trans-splicing by (A) $CUCU_{OX}$ and (B) $CU^FCU^F{}_{OX}$ at concentrations of (△) 0, (○) 0.25, (■) 1.0, and (▲) 2.0 μM. The reaction was carried out as described under Experimental Procedures, except that the concentration of the substrate CUCU was varied as indicated.

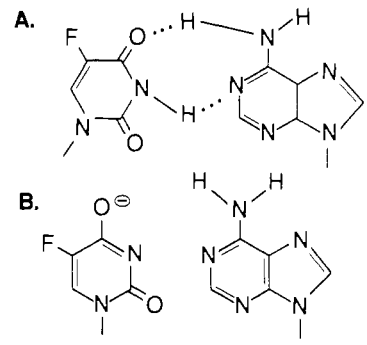

FIGURE 3: (A) Watson–Crick base pairing between U^F and A. (B) Dissociated base pair due to loss of N-3 proton upon ionization of the U^F residue.

Effect of pH on the Catalytic Activity of the Ribozyme and Substrate Activity of Mini-Exons. Previous NMR studies had shown that by increasing solvent pH it was possible to denature selectively U^F–A base pairs due to ionization of the U^F residues and loss of hydrogen bonding between the bases (Kremer et al., 1986) (Figure 3). The activity of the ribozyme requires a specific secondary and tertiary structure that depends on base pairing between complementary segments (Kim & Cech, 1987). Thus, to test the use of U^F as a probe for catalytically essential U–A base pairs, we measured the activity of the fully U^F-substituted ribozyme with the natural analogue CUCU as the substrate as a function of pH. As shown in Figure 4 (solid triangles), the (U^F) ribozyme at low pH gave a 90-nt product at a rate similar to that of the natural (U) ribozyme, thus demonstrating that U^F–A base pairs in the secondary structure of the ribozyme are stable enough at physiological pH to permit a more or less normal folding of the molecule. With increasing pH, however, the formation of the 90-nt product began to decline precipitously (Figure 4). The trans-splicing

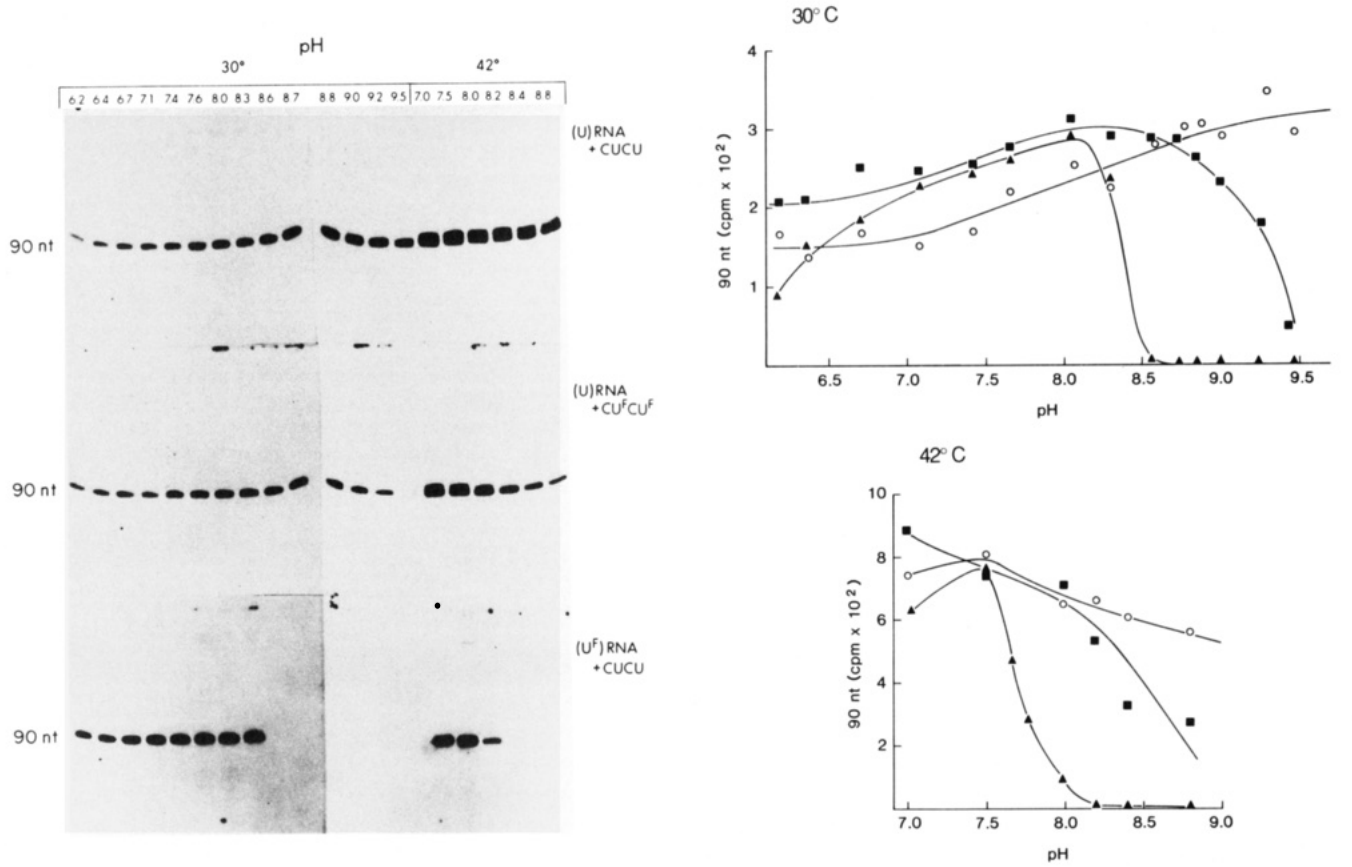

FIGURE 4: Trans-splicing reaction with various combinations of oligoribonucleotide substrates and the *Tetrahymena* ribozyme as a function of pH. The reaction was carried out essentially as described under Experimental Procedures except that [^{32}P]CUCU or [^{32}P]CU^{F}CU^F was used at a single concentration of 2.0 μM and different buffers were used to obtain different pH values. The panels on the left show representative gels of the trans-splicing products at two temperatures (30 and 42 °C). The faint bands at the top of the gels are the products of the oligonucleotides reacting with the closed circular product of splicing to give an opened circle with the radiolabeled oligonucleotide covalently attached (Been & Cech, 1985). The panels on the right are quantitative representations of the amount of the 90 nt product formed at 30 min of incubation. (○) (U) ribozyme plus CUCU; (■) (U) ribozyme CU^{F}CU^F; (△) (U^F) ribozyme plus CUCU. The profile for the (U^F) ribozyme plus CUCU at 42 °C contains data points from several different experiments.

activity of the natural (U) RNA with CUCU as the substrate, which served as the control reaction for this experiment, showed only a slight downward trend at 42 °C, but actually increased somewhat at 30 °C at higher pH. Thus, the midpoints of the downward portions of the curves obtained with (U^F) RNA represent the pK values of U^F residues whose ionization leads to loss of catalytic function. These values are 7.7 at 42 °C and 8.3 at 30 °C, somewhat higher than the pK of free 5-fluorouridine, which is 7.7 at 25 °C. Slight increases in the observed pK of the N-3 proton (up to 7.9–8.3) have been observed when the U^F ring is incorporated into an oligonucleotide (Massoulie et al., 1963; Kremer et al., 1986); in addition, base pairing of U^F within a duplex may also contribute to a change in its pK. These data strongly indicate that pH-dependent loss of catalytic activity is caused by dissociation of Watson–Crick base pairing between U^F and A due to ionization of the U^F residues and a consequent unraveling of some portion of the secondary structure.

To evaluate the role of the U^F–A base pair at position −3 in substrate binding to the active site, we measured the substrate activity of CU^{F}CU^F with the natural (U) ribozyme as a function of pH. As shown in Figure 4, the amount of 90-nt product formed with these reactants declined in a pH-dependent manner. Since product formation by the (U) ribozyme and CUCU was altered only slightly over the same pH range (open circles in Figure 4), the decline of splicing activity with CU^{F}CU^F as the substrate can be considered to be due specifically to ionization of its U^F residues and not to any ionization on the ribozyme molecule. The apparent pK values (ca. 8.5 at 42 °C and 9.4 at 30 °C) of the U^F residues of CU^{F}CU^F, however, were considerably higher than that of free U^F or that of the essential U^F residues in the secondary structure of the (U^F) ribozyme, indicating that the substrate is shielded from the solvent by the active site.

Binding of the Ionized Mini-Exon to the Active Site. Previous mutagenesis studies, which had shown that substituting the U with a G at position −3 of the 5′ exon substantially decreased ligated exon formation, were presented as supporting a base-paired duplex between the splice sites and the IGS (Been & Cech, 1986). The finding that CU^F(−)CU^F(−) lacks substrate activity seemed to be consistent with these studies because pH-dependent dissociation of the U^F–A base pair at this position would be expected to produce the same disruption of base pairing as the U to G mutation.[2] Unpaired bases exert a strong destabilizing effect on the duplex regions (Tinoco et al., 1973) so it was a reasonable initial assumption that both mutation and ionization of the substrate result in a substantially weaker affinity between the oligonucleotide substrate and the active site. In an effort to verify this assumption, we

[2] It has been shown that high pH does not dissociate U^F–G base pairs but converts them from their normal "wobble" structure to one resembling Watson–Crick geometry (Sowers et al., 1988). However, this in itself may not cause the ionized substrate to be inactive, since mini-exons ending in a C residue (e.g., CUC and CUCUC, Table I), which would naturally form a Watson–Crick base pair with the G residue of the IGS, still undergo splicing at normal rates.

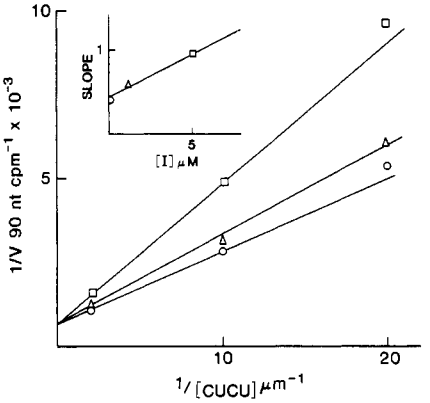

FIGURE 5: Inhibition of trans-splicing by CU^FCU^F at pH 9.5. The trans-splicing reaction was carried out as described under Experimental Procedures except that the concentration of CUCU was varied at constant levels of CU^FCU^F. (○) No inhibitor; (△) 2 μM CU^FCU^F; (□) 5 μM CU^FCU^F.

determined K_m values of some of the (U^F) mini-exons at pH 8.75, expecting that, since the U^F residues of these substrates would be about 90% ionized at this pH, the apparent K_m values of these substrates should be correspondingly higher because of the lower concentration of the active (i.e., protonated) form available. Surprisingly, however, the K_m values for the (U^F) mini-exons at pH 8.75 were 0.31 ± 0.05, 0.13 ± 0.02, 0.15 ± 0.03, and 0.08 ± 0.002 μM for CU^FCU^F, $CU^FCU^FCU^F$, $GGCCCU^FCU^F$, and $GGCU^FCU^FCU^F$, respectively, which are not appreciably different than those at pH 7.8 (Table I). This observation suggested that the ionized substrate still binds with an appreciable affinity to the active site despite the loss of U^F–A base pairing and then, because of its increased basicity when bound to the ribozyme, becomes reprotonated, according to

$$R + CU^FCU^F \rightleftharpoons R{\cdot}CU^FCU^F \rightarrow CU^FCU^F\text{-}3'\ \text{exon}$$
$$pK = 7.7 \qquad pK = 9.3$$
$$R + CU^F(-)CU^F(-) \rightleftharpoons R{\cdot}CU^F(-)CU^F(-) \qquad (R = \text{ribozyme})$$

To test this hypothesis, we measured reaction rates of the natural (U) ribozyme using radiolabeled CUCU as the substrate in the presence of CU^FCU^F at pH 9.5, where the fluorinated mini-exon had little or no substrate activity. As shown in Figure 5, CU^FCU^F, which is a substrate at low pH, becomes a linear competitive inhibitor of the trans-splicing reaction at high pH. This result demonstrates that the dianionic species $CU^F(-)CU^F(-)$ does indeed bind to the active site of the ribozyme, and moreover, the loss of binding specifically attributable to the ionization of the U^F residues of the mini-exon is only about 3-fold.[3]

Effect of Temperature on Ribozyme and Mini-Exon Activity. Temperature-sensitivity experiments provided an independent method for determining the extent to which destabilization of base pairs involving (U^F) would affect the substrate activity of the fluorinated mini-exons. As shown in Figure 6, the (U^F) ribozyme (with CUCUCU as the substrate) began to lose activity at a temperature about 15 °C lower than

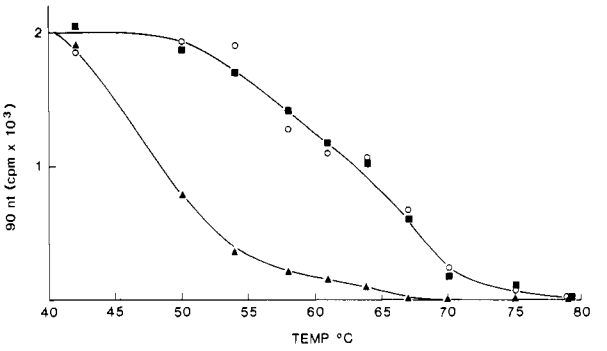

FIGURE 6: Temperature dependence of the trans-splicing reaction of (▲) the (U^F) ribozyme with CUCUCU as substrate, (■) the (U) ribozyme with CUCUCU as substrate, and (○) the (U) ribozyme with $CU^FCU^FCU^F$ as substrate. Reaction conditions were as described under Experimental Procedures. The substrate concentration was 2 μM.

did the (U) ribozyme, confirming that U^F–A base pairs are less stable and thus have a lower "melting temperature" than do U–A base pairs. However, the substrate activity of $CU^FCU^FCU^F$ as a function of temperature was identical with that of the normal analogue CUCUCU.

Discussion

In this study, we used mini-exons and the intermolecular splicing reaction as a model system to obtain information about the mode of binding of the 5′ splice site to the active site of the *Tetrahymena* ribozyme. The salient idea behind these experiments was that substitution with U^F in the mini-exons would make it possible to (a) alter hydrogen-bonding interactions normally involving U residues and (b) selectively dissociate the putative base pair at position −3 of the complex between the 5′ splice site and the active site of the ribozyme and thus better define the role of base pairing in splice-site selection.

The competitive inhibition patterns obtained by using the periodate oxidized mini-exons with CUCU as the trans-splicing substrate further substantiate the existence of an enzyme-like binding site on the *Tetrahymena* ribozyme and thus strengthen the analogy between the action of protein enzymes and ribozymes. The fact that both $CUCU_{ox}$ and $CU^FCU^F_{ox}$ are competitive with CUCU confirms that the natural (U) mini-exons and the (U^F) mini-exons bind to the same site on the ribozyme. The similarity between the K_i values of the oxidized mini-exons and the K_m values of the analogous substrates shows that the hydroxyl groups of the ribose moiety of the pyrimidine base at the terminus of the 5′ exon do not contribute appreciably to the binding of the exon. In contrast, removal of the hydroxyl groups of the guanosine nucleotide substrate leads to a substantial loss of binding (Bass & Cech, 1986).

From an examination of the K_m values for the (U) and (U^F) mini-exons in Table I, several conclusions can be made regarding the interaction of the mini-exons with the binding site of the *Tetrahymena* ribozyme:

(1) Because the binding of the mini-exons progressively increased up through the tetramer CUCU, with no appreciable change thereafter, most of the interaction of the 5′ exon with the binding site appears to occur through the last four nucleotides. This conclusion is further supported by the fact that, in the U^F oligonucleotide series, changing the sequence of the mini-exon from $GGCU^FCU^FCU^F$ to $GGCCCU^FCU^F$ resulted in a small decrease in binding, despite the possibility of a more favorable C–G interaction at position −5. Most of the binding energy of the mini-exons seems to be gained from interaction

[3] Although the K_i value of 5 μM for $CU^F(-)CU^F(-)$ is 30-fold greater than the K_m of CU^FCU^F at pH 7.5, the relative loss of binding specifically attributable to the ionization of the U^F residues of CU^FCU^F is only slightly more than 3-fold because the K_m of the variable substrate CUCU was also greater at pH 9.5 than at pH 7.5, by about 10-fold. That is, the ratio of the K_m values of CU^FCU^F and CUCU is 1.5 at pH 7.5, whereas the ratio of the K_i of $CU^F(-)CU^F(-)$ to the K_m of CUCU at pH 9.5 is 5.0. Thus, the ratio of the ratios, representing the change in binding due to ionization, is 3.3.

of the nucleotides at positions −2 and −3 with the active site (C and U, respectively). These data support the assumption that the trans-splicing reaction with mini-exons and the normal splicing reaction are analogous processes because Price et al. (1985) also found that a pre-RNA with a short 5′ exon truncated to the four terminal nucleotides (i.e. CUCU) still was capable of accurate splicing, although with somewhat decreased rate efficiency.

(2) The simple base-pairing model shown in Figure 1 is not adequate to account for the binding of the mini-exons. First, on the basis of previous NMR studies which showed that the stability of a U^F–A base pair is lower than that of a T–A base pair when located in a short DNA helix (Kremer et al., 1986; Sowers et al., 1987), substitution with U^F would be expected to weaken base-paired duplexes between the substrate oligonucleotides and the active site. However, except for the dinucleotides CU and CU^F, where about a 6-fold difference in binding was observed, neither the binding nor the substrate activity of the (U^F) mini-exons was noticeably different from those of the natural (U) analogues. Second, from the magnitudes of the K_m values in Table I it is clear that the binding of the oligonucleotide mini-exons to the pre-RNA is much stronger than would result from base-pairing alone. On the basis of its K_m value of 0.1 μM, the tetranucleotide CUCU can be calculated to bind to the ribozyme with a free energy of −9.7 kcal/mol. By comparison, the free energy of duplex formation between CUCU and GAGG, predicted according to the nearest-neighbor parameters of Freier et al. (1986), is only about −3 kcal/mol. A more dramatic example is that of the binding of CU. The predicted free energy of a CU–GG interaction is +0.4 kcal/mol, indicating that this dinucleotide pair should not form a stable complex. Yet CU binds to the ribozyme with $\Delta G = -7$ kcal/mol ($K_m = 8$ μM), about the same as that of the unusually stable complexes of tetranucleotides with their complementary segments in exposed anticondon loops of tRNA (Uhlenbeck, 1972). Thus, the ribozyme active site increases the binding free energy of the oligonucleotides by as much as 8 kcal/mol over that expected from the formation of conventional hydrogen-bonded base-paired structures such as shown in Figure 1. A similar conclusion was reached in a recent study of the binding of oligonucleotides to the circular form of the IVS (Sugimoto et al., 1988).

The temperature-sensitivity study shown in Figure 6 supports the contention that base-pair formation between the substrates and the IGS constitutes only a fraction of the total binding interaction between the mini-exons and the active site. The trans-splicing activity was lost at a temperature much higher than the predicted melting temperature of a base-paired duplex between CUCUCU and GGGAGG (which we estimate to be only about 28 °C at a concentration of 2 μM, assuming the stability of a G–U base pair to be the same as that of an A–U base pair), indicating the presence of additional binding forces between the corresponding two segments of the ribozyme. Additionally, the (U^F) mini-exon lost no substrate activity at a temperature that was shown to denature essential U^F–A base pairs in the secondary structure of the (U^F) ribozyme (Figure 6) (although it should be noted that a binding loss up to 10-fold may not be detected by measuring the reaction velocity, since the substrate was at a saturating level about 20 times the K_m value).

In the light of the currently accepted stepwise mechanism of splicing in which the 5′ exon is cleaved form the IVS prior to ligation (Cech, 1987), we interpret the tighter than expected binding of the substrates to mean that the binding energy derivable from base pairing alone is insufficient to prevent substantial dissociation of the 5′ exon from the active site before ligation can occur. Thus, the ribozyme uses the strategy of minimizing the rate constant for dissociation of the exon (k_2) with respect ot the catalytic rate constant k_{cat} to achieve a "stickier" substrate, i.e., one more committed to reacting rather than dissociating (Cleland, 1973).

The inactivation of the (U^F) ribozyme with increasing pH (solid triangles, Figure 4) shows that substitution with U^F can be used to establish or confirm the presence of functionally important U–A base pairs in RNA molecules. The absolute extent of denaturation of the molecule concomitant with loss of catalytic activity cannot be estimated from these experiments, but we have observed that base hydrolysis of U^F-substituted C-IVS [which occurs at a specific site that is rendered exceedingly labile by proper folding of the molecule (Cech, 1987)] is only about 4-fold slower at pH 9.5 than at pH 7.5, suggesting that unraveling of the (U^F) ribozyme even at this high pH is not very extensive (P. V. Danenberg, unpublished results). An NMR study of U^F-substituted tRNA showed the presence of a substantial percentage of hidden U^F residues that could not readily be dissociated at higher pH (Hardin et al., 1986). The apparent pK values of those U^F residues whose ionization leads to loss of activity are close to that of free U^F, suggesting that these residues are reasonably well exposed to solvent. Thus, the loss of catalytic activity of the (U^F) ribozyme could be due to dissociation of a relatively small number of U^F–A base pairs in a local critical region of the molecule, such as the highly conserved P7 stem, where disruption of base pairing by insertion of noncomplementary bases has been shown to destroy splicing activity (Burke et al., 1986).

The observation that CU^FCU^F lost activity with increasing pH, just as did the (U^F) ribozyme, seemingly is consistent with the need for base-pairing interactions between the splice site and the IGS in the splicing reaction. However, the surprising ability of the dianionic species $CU^F(-)CU^F(-)$ to form a strong nonproductive complex with the ribozyme shows that the N-3 protons of the U residues that normally would participate in base pairing are not essential for binding of the mini-exons. Because the effect of their removal is mainly to lower k_{cat} for the overall splicing reaction, one or more of these protons appear to be necessary for promoting the ligation reaction. The N-3 proton of the terminal U of the 5′ exon may have a catalytic function, perhaps acting as a general acid–base catalyst or possibly forming a hydrogen bond that helps to align the 5′ and the 3′ splice sites properly for ligation. A similar phenomenon was noted in the case of the guanosine nucleotide substrate. Deletion of the ribose hydroxyl groups of this substrate still permits binding but not splicing activity, leading to the proposal that hydrogen bonds formed by these hydroxyl groups are necessary for catalysis (Bass & Cech, 1987). Another possible reason for the lack of substrate activity of $CU^F(-)CU^F(-)$ is that electrostatic repulsion or attraction due to the presence of the negative charge on the pyrimidine ring simply causes the ionized mini-exon to assume an abnormal geometry in the active site, thereby preventing ligation. In any case, the data suggest that even though base pairing does not account for most of the binding of the splice site to the active site, it still has a critical role in splicing, perhaps later on in the reaction pathway.

If, as the data presented here indicate, base-pairing interactions are not the sole or even the major mode of binding of the 5′ exon to the active site of the ribozyme, the question is naturally raised as to what sort of additional binding forces might be involved. The upward shift in the apparent pK of

ribozyme-bound CU^FCU^F molecules (Figure 4) indicates a hydrophobic active site that can shield the substrates from the pH environment of the medium (Fersht, 1985), so the binding of the exon to the active site might well include tertiary hydrophobic interactions between the bases at the splice sites and those belonging to other segments of the ribozyme. In fact, a model for the structure for the IVS proposed by Kim and Cech (1987) shows the 5′ splice site–IGS duplex binding in a cylindrical pocket comprised of stacked, single-stranded segments. A possible analogue of the 5′ splice site-active site complex might be found in certain tertiary interactions within tRNA, where intercalation of bases from one secondary structural element between neighboring bases in another has been observed (Tinoco et al., 1987).

With the discovery of autocatalytic RNA, there has been considerable discussion about RNA as the primeval enzyme that preceded protein enzymes in the course of the evolution of life (Orgel, 1986). The possibility that ribozymes could have developed specifically shaped 3-dimensional hydrophobic binding sites would strengthen the basis for postulating such a role, since a ribozyme then might not be limited to catalyzing just oligonucleotide cleavages and ligations. In this regard, it is of interest to point out the recent discovery of Yarus (1988) that the *Tetrahymena* ribozyme specifically binds to L-arginine. This finding does nothing to discourage the speculation that ribozymes might have functioned at one time as protein synthesis catalysts.

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