Chemistry & Biology, Vol. 11, 237–246, February, 2004, ©2004 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.02.011

New Strategies for Exploring RNA's 2′-OH Expose the Importance of Solvent during Group II Intron Catalysis

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Summary

The 2′-hydroxyl group contributes inextricably to the functional behavior of many RNA molecules, fulfilling numerous essential chemical roles. To assess how hydroxyl groups impart functional behavior to RNA, we developed a series of experimental strategies using an array of nucleoside analogs. These strategies provide the means to investigate whether a hydroxyl group influences function directly (via hydrogen bonding or metal ion coordination), indirectly (via space-filling capacity, inductive effects, and sugar conformation), or through interactions with solvent. The nucleoside analogs span a broad range of chemical diversity, such that quantitative structure activity relationships (QSAR) now become possible in the exploration of RNA biology. We employed these strategies to investigate the spliced exons reopening (SER) reaction of the group II intron. Our results suggest that the cleavage site 2′-hydroxyl may mediate an interaction with a water molecule.

Introduction

To develop a fundamental understanding of the catalytic repertoire and strategies by which RNA enzymes (ribozymes) accelerate chemical reactions, we must identify individual functional groups that contribute to catalysis and establish their chemical roles (for examples, see [1–4]). The 2′-hydroxyl illustrates one such functional group that commonly provides essential energetic contributions to the folding and function of RNA, mediating tertiary interactions via hydrogen bonding or metal ion coordination or serving as a scaffold for the integral hydration network associated with RNA ([5] and references therein). Functional RNAs usually experience deleterious effects in folding and/or function upon 2′-deoxyribonucleotide substitution at positions in which the 2′-hydroxyl groups make important energetic contributions. However, deoxyribose substitution alone provides no information about the chemical basis for the energetic contribution of a particular 2′-OH. In addition to the more obvious direct contributions from hydrogen bonding or metal ion coordination, the 2′-OH also may exert indirect contributions to structure and catalysis that arise as a consequence of its capacity to fill space, withdraw electrons inductively, or engender a ribonucleotide with a preference for the 3′-*endo* sugar conformation. Several commercially available nucleoside analogs provide approaches to probe possible direct contributions, but these approaches remain incomplete. Moreover, they offer no means by which to account for the possible energetic contribution from the indirect factors. Herein we develop new approaches to decipher these contributions, and we apply them to investigate a reaction catalyzed by a group II intron.

Self-splicing group II introns provide an important experimental system by which to investigate the principles of RNA folding and catalysis, intron mobility, and the orchestration of intron excision via lariat formation [6, 7]. Multipartite assays (*trans* assays) that divide the self-splicing group II intron into separate ribozyme and substrate portions have greatly facilitated biochemical analysis and have revealed an extensive repertoire of group II catalytic activities (for examples, see [8–12]). In one *trans* assay that resembles the first step of reverse splicing, the ribozyme binds oligonucleotides containing sequences complimentary to its exon binding sites and catalyzes hydrolytic cleavage immediately downstream of the resulting duplex [13]. Griffin et al. used this assay to show that the group II intron cleaves substrates containing ribose at the splice junction about an order of magnitude faster than those containing deoxyribose [14]. In contrast, group I introns and ribonuclease P, large ribozymes in the same mechanistic class as group II introns, experience 10^3- to 10^4-fold losses in catalytic power upon removal of the cleavage site 2′-hydroxyl group [15–17]. Group II introns therefore cleave DNA substrates relatively well. Nevertheless, even the 10-fold preference for RNA over DNA, though subtle, could have biological and evolutionary significance regarding intron mobility and dispersal, as group II introns may encounter DNA and RNA targets naturally during reverse splicing [7, 18]. To investigate the underlying basis for this modest preference, we developed new approaches for dissection of the chemical role played by the 2′-hydroxyl group.

Results and Discussion

The Spliced Exons Reopening Reaction

In the first step of reverse splicing, the lariat intron nucleophilically attacks the phosphodiester linkage at the boundary between exon 1 and exon 2. The spliced exons reopening reaction (SER) mimics this reaction, whereby a divided group II ribozyme, consisting of domains 1 through 3 (D123) combined with a separate domain 5 (D5), catalyzes the hydrolysis of a short oligonucleotide (27-mer) that mimics spliced exons (E1-E2; Figure 1; [19]). Su et al. showed that the sequence downstream of exon 1 has no consequence in this assay [20], so that the SER reaction should exhibit the same sensitivity to

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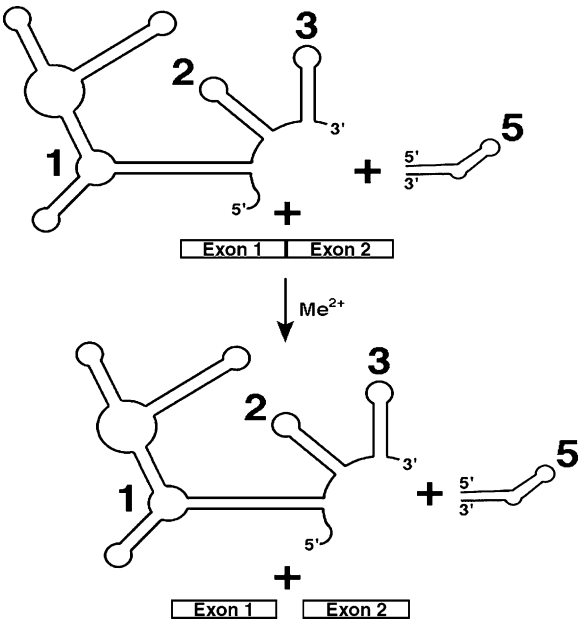

Figure 1. The Spliced Exons Reopening (SER) Reaction

D5 of the group II intron acts in *trans* with D123 to catalyze hydrolysis of an exon 1-exon 2 (E1-E2) substrate in a reaction that mechanistically resembles the first step of reverse splicing [19].

removal of the 2′-hydroxyl group as observed in the *trans* assay of Griffin et al. [14]. To validate this expectation and to provide a reference point for the work described herein, we measured the catalytic contribution of the 2′-OH in the SER reaction.

We synthesized an SER substrate containing a 2′-deoxycytidine residue at the cleavage site ($E1_{2'H}E2$) and measured the reactivities of $E1_{2'OH}E2$ and $E1_{2'H}E2$ in the presence of saturating D123•D5 and 100 mM $MgCl_2$ (pH 7.0). Figure 2A shows that $E1_{2'H}E2$ reacts ~10-fold slower than $E1_{2'OH}E2$. To address whether the 2′-OH contributes to the binding of substrate to the D5•D123 complex, we determined dissociation constants for $E1_{2'OH}E2$ and $E1_{2'H}E2$ by examining the rate of product formation over a range of D5•D123 concentrations (data not shown; see Supplemental Data at http://www.chembiol.com/cgi/content/full/11/2/237/DC1). The data were fit according to a simple binding isotherm and gave a dissociation constant for $E1_{2'H}E2$ that was in the low nanomolar range ($K_d = 50$ nM), similar to the dissociation constant for $E1_{2'OH}E2$ ($K_d = 60$ nM). These data confirm the findings of Griffin et al. [14], that the 2′-hydroxyl makes no contribution to the stability of the ground state E1-E2•D5•D123 complex but exhibits an approximately 10-fold stimulatory effect on the transition state.

Functional Groups Lacking Lone Pair Electrons Maintain a Catalytic Contribution

The oxygen atom of the 2′-hydroxyl group contains two lone pairs of electrons that can accept a hydrogen bond or coordinate a divalent metal ion. To investigate whether the 2′-OH accepts a hydrogen bond, we examined the effect of removing these lone pairs of electrons. Substitution of the 2′-hydroxyl group with an amino

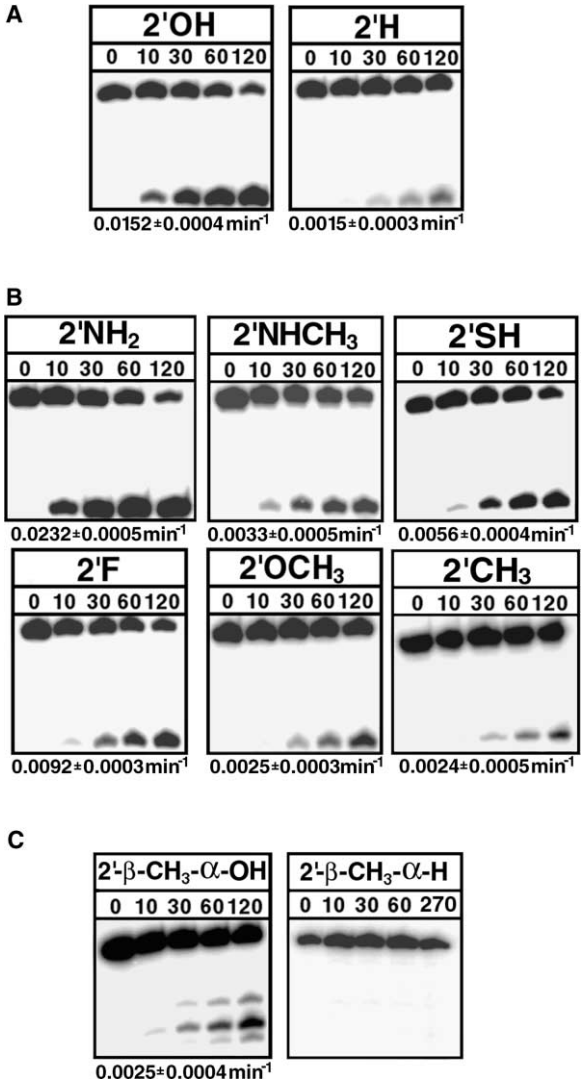

Figure 2. The 2′-Hydroxyl Group at the E1-E2 Splice Junction Stimulates the SER Reaction

Reactions contained 5 μM D5, 200 nM D123, trace radiolabeled substrate, 100 mM $MgCl_2$, and 40 mM NaMOPS (pH 7.0). The E1-E2 substrates used in the SER reaction contain the modifications at the cleavage site as indicated in (A)–(C). Samples were incubated at 42°C for times (minutes) indicated at the top of the gel. The numbers below each gel panel indicate the first order rate constant for the reaction.

group removes one lone pair of electrons, but at low pH values ($< pK_a$ ~6.1–6.2; [9, 23, 24]) the amino group is protonated ($-NH_3^+$) and therefore lacks any lone pair electrons with which to accept a hydrogen bond (Figure 3A). We measured the pH dependence of the reactivity of $E1_{2'NH_2}E2$ with both subsaturating (data not shown) and saturating concentrations of D123•D5 (Figure 3B). At pH 7.0, $E1_{2'NH_2}E2$ reacts slightly faster than $E1_{2'OH}E2$ (Figures 2A and 2B). The pH-rate profile of $E1_{2'NH_2}E2$ exhibits a log-linear dependence between pH 5.25 and 7.0, identical to $E1_{2'OH}E2$ throughout the entire pH range (Figure 3B; compare squares and circles). To confirm that the 2′-amino group ionizes at low pH values, we independently monitored its protonation state by follow-

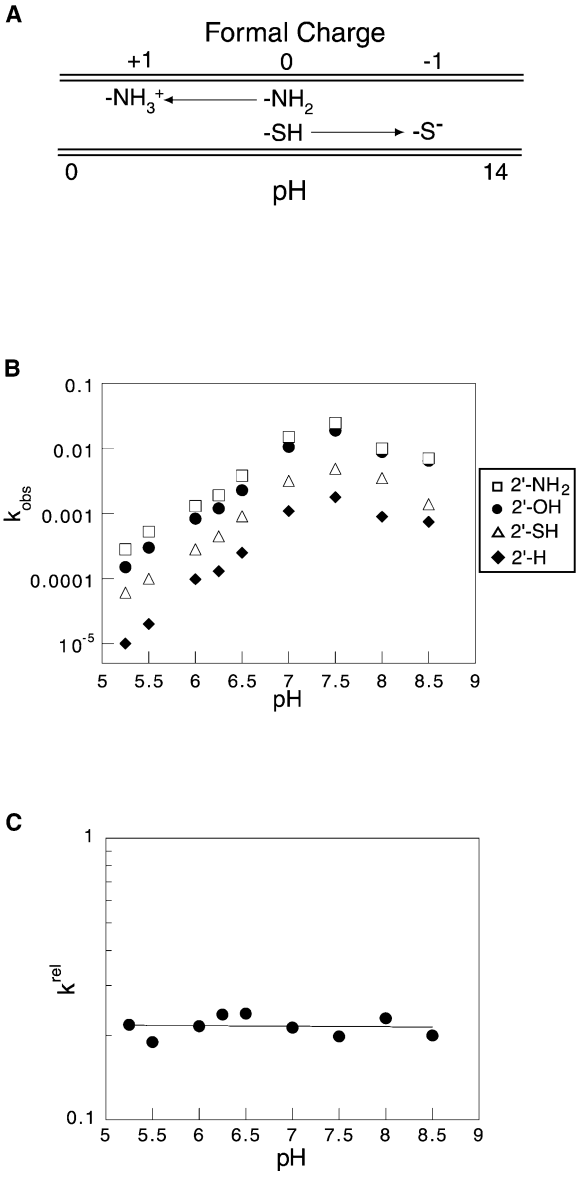

Figure 3. 2′-Mercaptonucleosides Complement 2′-Aminonucleosides as Probes of Electrostatic Environment

(A) pH induced ionization of the amino and mercapto groups site-specifically introduces formal charge within RNA. (B) The pH dependence of the SER reaction. Reactions contained saturating D123•D5, 100 mM MgCl2, and $E1_{2'OH}E2$ (circles), $E1_{2'H}E2$ (diamonds), $E1_{2'NH_2}E2$ (squares), or $E1_{2'SH}E2$ (triangles) substrate. Linear fits to the data between pH 5.25 and 7.0 gave slopes of 1.0 ($E1_{2'OH}E2$), 1.1 ($E1_{2'H}E2$), 0.95 ($E1_{2'NH_2}E2$), and 0.97 ($E1_{2'SH}E2$), respectively. (C) The rate of $E1_{2'SH}E2$ plotted relative to $E1_{2'NH_2}E2$ ($k^{rel} = k^{E1_{2'SH}E2}/k^{E1_{2'NH_2}E2}$) at each pH value.

ing the pH dependence of an amine modifying reaction under SER reaction conditions [22]. We observed a pK_a of ~6.0 for the 2′-amino group, which agrees well with literature values [9, 21, 22]. These results indicate that a free lone pair of electrons does not contribute to the reactivity of $E1_{2'NH_2}E2$ and raise the possibility that the 2′-OH donates rather than accepts a hydrogen bond.

$E1_{2'NH_2}E2$ also provides a means to test whether the 2′-OH coordinates to an important metal ion, as nitrogen interacts with softer metals such as Mn^{2+} more favorably than Mg^{2+} [9, 23, 24]. The unperturbed reactivity of $E1_{2'NH_2}E2$ relative to $E1_{2'OH}E2$ in 100 mM Mg^{2+} (Figures 2A and 2B) and the lack of a $E1_{2'NH_2}E2$-specific enhancement upon the addition of 10 mM Mn^{2+} (data not shown) suggest the absence of a 2′-OH-metal ion interaction, consistent with the apparent ambivalence of the SER reaction to the presence of lone pair electrons on the cleavage site 2′-OH.

Functional Groups Lacking a Hydrogen Bond Donor Maintain a Catalytic Contribution

The lack of evidence for metal ion coordination and hydrogen bond acceptance led us to investigate whether the catalytic contribution of the 2′-OH arises from its ability to donate a hydrogen bond. Using standard methods, we substituted the 2′-OH with a fluorine atom ($E1_{2'F}E2$) or a methoxy group ($E1_{2'OCH_3}E2$), as neither can donate a hydrogen bond. The reactivity of each substrate was determined with saturating D123•D5 in 100 mM $MgCl_2$ at pH 7.0. $E1_{2'F}E2$ reacts only slightly less efficiently than $E1_{2'OH}E2$ (Figure 2B), suggesting that hydrogen bond donation by the 2′-OH makes little contribution to catalysis.

$E1_{2'OCH_3}E2$ reacts 6-fold slower than $E1_{2'OH}E2$ under the same conditions (Figures 2A and 2B). However, comparison of 2′-OH to 2′-OCH_3 cannot by itself reveal a role for hydrogen bond donation, as deleterious effects could arise from addition of the methyl group, removal of the hydrogen atom, or both. To delineate these possibilities and thereby clarify the interpretation of the 2′-OH→2′-OCH_3 modification, we synthesized an E1-E2 substrate containing a 2′-$NHCH_3$ modification (see the Supplemental Data at http://www.chembiol.com/cgi/content/full/11/2/237/DC1). According to the cycle in Figure 4A, this new analog allows quantitation of 2′-OH to 2′-OCH_3 in terms of contributions from methyl addition and hydrogen removal. Comparison of 2′-NH_2 to 2′-$NHCH_3$ reveals the cost of installing the methyl while maintaining capacity for hydrogen bond donation. Similarly, comparison of 2′-OCH_3 to 2′-$NHCH_3$ provides a measure of the energetics of installing hydrogen bond donating capacity within a similar structural context. $E1_{2'NHCH_3}E2$ reacts slower than $E1_{2'NH_2}E2$ but with the same efficiency as $E1_{2'OCH_3}E2$ (Figure 2B), indicating that the reduced reactivity of $E1_{2'OCH_3}E2$ relative to $E1_{2'OH}E2$ arises from the methyl group rather than the lack of hydrogen bond donating ability at the cleavage site. These results, together with the implications from the 2′-F substitution, suggest that functional groups lacking a hydrogen bond donor maintain a catalytic contribution.

2′-Mercapto (-SH) substitution provides another approach to test the role of the 2′-OH as an H bond donor, as the mercapto group occasionally may supplant the 2′-OH in this capacity [25]. Ionization of the mercapto group (pK_a ~8.0) to give the thiolate anion abolishes the potential to donate a hydrogen bond (Figure 3A). $E1_{2'SH}E2$ reacts faster than $E1_{2'H}E2$ (Figure 2B), indicating that the 2′-SH group stimulates the SER reaction relative to a hydrogen atom. However, the reactivity of $E1_{2'SH}E2$

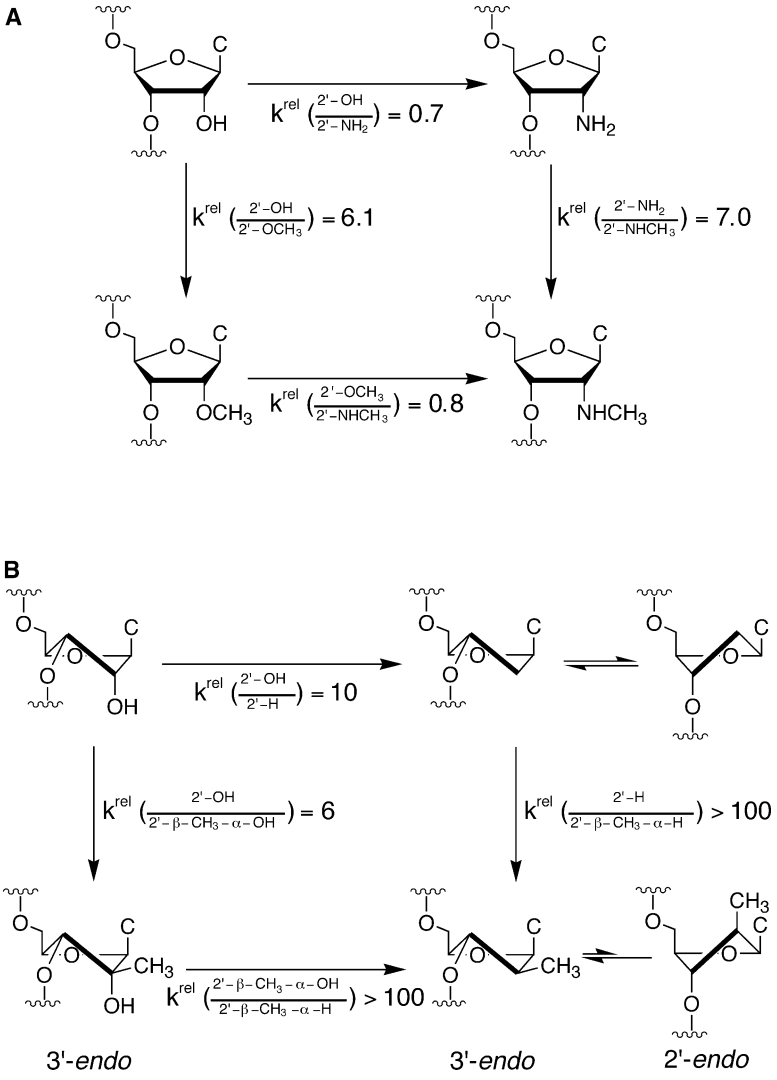

Figure 4. Atomic Perturbation Cycles

(A) 2′-Methylamino nucleosides resolve the ambiguity inherent to experiments using 2′-*O*-methyl nucleosides. These new analogs present the same structural context as 2′-*O*-methyl nucleosides but maintain the capacity to donate a hydrogen bond. The cycle accounts for the energetics of the 2′-*O*-methyl modification in terms of contributions from methyl installation (2′-NH_2→2′-$NHCH_3$) and hydrogen removal (2′-$NHCH_3$→2′-OCH_3).

(B) 2′-*C*-β-methyl nucleosides maintain sugar pucker in the absence of the 2′-hydroxyl group. The cycle allows quantitation of the effects from the 2′-OH→2′-H modification without a concomitant change in sugar pucker preference.

relative to $E1_{2'H}E2$ remains constant over the pH range 5–8.5 (Figure 3B), suggesting that loss of the proton from the mercapto group has no effect on reactivity. To confirm that the 2′-SH group ionizes at high pH values, we independently monitored its protonation state by following the pH dependence of a mercaptan modification reaction under SER reaction conditions (J.P.S. and J.A.P, unpublished data). We observed a pK_a ~8.3 for the 2′-SH group, which agrees well with literature values [26]. These observations reinforce the conclusion that the 2′-functional group can exert a stimulatory effect without the capacity to donate a hydrogen bond.

Formal Charge on the 2′-Functional Group Exerts Little Influence on Catalysis

pH-induced ionization of the 2′-SH and 2′-NH_2 groups generates charge at the 2′ position. The pH dependence of the reactivity of $E1_{2'NH_2}E2$ and $E1_{2'SH}E2$ relative to $E1_{2'OH}E2$ should therefore reveal the effects of charge (+1, 0, or −1) adjacent to the scissile phosphate, providing a means to probe the electrostatic environment at the active site. $E1_{2'NH_2}E2$, $E1_{2'SH}E2$, $E1_{2'OH}E2$, and $E1_{2'H}E2$ exhibit identical pH dependencies (Figure 3B) such that a plot of the rate of reaction of $E1_{2'SH}E2$ relative to $E1_{2'NH_2}E2$ ($k^{rel} = k^{E1_{2'SH}E2}/k^{E1_{2'NH_2}E2}$) exhibits no dependence on pH (Figure 3C). Formal charge at the 2′ position therefore appears to exert little effect on the SER reaction.

The *Tetrahymena* group I ribozyme active site exhibits a very different response when probed with protonated 2′-aminoguanosine ($G_{2'NH_3^+}$), in which an -NH_3^+ group replaces the 2′-OH of the guanosine nucleophile [21]. $G_{2'NH_3^+}$ binds ~200-fold more tightly to the ribozyme than G or $G_{2'NH_2}$, suggesting that the positively charged -NH_3^+ group may interact with a negatively charged phosphoryl group in the active site [21]. Additionally, $G_{2'NH_3^+}$ appears to compete with a catalytic divalent metal ion for binding in the active site, either as a result of electrostatic repulsion or possibly from competition for interaction with an active site phosphoryl group. The response of the group I active site to 2′-mercaptoguanosine and its corresponding anion remains unexplored, however.

Accounting for Indirect Contributions to Catalysis by the 2′-OH

The preceding approaches provide no evidence to attribute the catalytic contribution of the 2′-OH (relative to 2′-H) to H bond donating or H bond accepting capability. Consequently, we considered mechanisms by which the 2′-OH could exert a catalytic effect indirectly. Concomitant with the loss of hydrogen bonding capacity, the 2′-OH to 2′-H modification changes other properties of the cleavage site nucleotide, including (1) space filling capacity, (2) conformation, and (3) the leaving ability of the 3′-oxygen.

Space Filling Capacity

A hydroxyl group occupies a larger volume than a hydrogen atom, such that the 2′-OH→2′-H modification creates a cavity at the active site (assuming the D123•D5•E1-E2 ternary complex undergoes no relaxation to reduce the cavity volume). To investigate whether such space filling capacity accounts for the greater reactivity of $E1_{2'OH}E2$ relative to $E1_{2'H}E2$, we synthesized a suitably protected phosphoramidite derivative for 2′-deoxy-2′-*C*-α-methylcytidine ($E1_{2'\text{-}\alpha\text{-}CH_3}E2$; see the Supplemental Data at http://www.chembiol.com/cgi/content/full/11/2/237/DC1) and incorporated it at the E1-E2 cleavage site. The methyl group occupies a greater volume than a hydrogen atom and still lacks hydrogen bonding and metal ion coordination capabilities. With saturating D123•D5 in 100 mM $MgCl_2$ at pH 7.0, $E1_{2'\text{-}\alpha\text{-}CH_3}E2$ reacts only as fast as $E1_{2'H}E2$ (Figure 2B), providing no evidence to attribute the stimulatory effect of the 2′-OH solely to its capacity to fill space.

Nucleotide Conformation at the Cleavage Site

Ribonucleotides prefer the 3′-*endo* conformation, but 2′-deoxy nucleotides populate the 3′-*endo* and 2′-*endo* conformations equally well [27]. Indeed, the preference of the 2′-F nucleotide for the 3′-*endo* conformation [28, 29] could explain its effectiveness as a substrate in the SER reaction. To probe the effect of the 2′-OH→2′-H modification without changing the inherent sugar pucker preference of the nucleotide, we installed a methyl group in place of the 2′-β-hydrogen. 2′-β-methylnucleotides maintain a strong preference for the 3′-*endo* sugar conformation even in the absence of the 2′-OH ([30] and references therein). We synthesized E1-E2 substrates containing at the cleavage site 2′-*C*-β-methylcytidine ($E1_{2'\text{-}\beta\text{-}CH_3\text{-}\alpha\text{-}OH}E2$; [31]) and 2′-*C*-β-methyl-2′-deoxycytidine ($E1_{2'\text{-}\beta\text{-}CH_3\text{-}\alpha\text{-}H}E2$; see the Supplemental Data at http://www.chembiol.com/cgi/content/full/11/2/237/DC1) and compared the reactivity of these substrates to $E1_{2'OH}E2$ and $E1_{2'H}E2$ according to the cycle in Figure 4B. The reactivity of each substrate was determined with saturating D123•D5 in 100 mM $MgCl_2$ at pH 7.0 (Figure 2C). The substrate with 2′-*C*-β-methylcytidine at the cleavage site ($E1_{2'\text{-}\beta\text{-}CH_3\text{-}\alpha\text{-}OH}E2$) reacts ~6-fold slower than $E1_{2'OH}E2$ and experiences miscleavage, perhaps due to steric and/or hydrophobic effects from the β-methyl group (Figure 2C). However, $E1_{2'\text{-}\beta\text{-}CH_3\text{-}\alpha\text{-}H}E2$ reacts more than 100-fold slower than $E1_{2'\text{-}\beta\text{-}CH_3\text{-}\alpha\text{-}OH}E2$, suggesting that the 2′-OH plays a role beyond that of maintaining sugar pucker. The smaller effect of 2′-OH removal in the absence of the -CH_3 group and the greater effect of β-CH_3 installation in the 2′-deoxy background could arise if the greater flexibility of the 2′-deoxy nucleotide partially compensates for the loss of the 2′-OH. The 2′-OH→2′-H comparison therefore might reflect a lower limit for the true catalytic contribution of the 2′-OH. Nevertheless, some factor(s) other than the preference for the 3′-*endo* sugar conformation must account for the stimulatory effect of the 2′-OH.

Inductive Effects

A hydroxyl group withdraws electrons more strongly than a hydrogen atom and could provide the 10-fold rate enhancement by an inductive effect that enables the 3′-oxyanion leaving group to accommodate better the developing negative charge in the transition state. If the 2′-OH contribution arises solely from such an inductive effect, the reactivity of the $E1_{2'\text{-}X}E2$ substrates should correlate with the electron-withdrawing ability of the 2′-functional group and follow the order 2′-F > 2′-OH > 2′-NH_2 > 2′-SH > 2′-H > 2′-CH_3. However, the observed order, 2′-NH_2 > 2′-OH > 2′-F > 2′-SH > 2′-CH_3 > 2′-H and 2′-NCH_3 > 2′-OCH_3 does not support an inductive effect as the sole mode of stimulation by the 2′-OH. Moreover, ionization of the 2′-NH_2 and 2′-SH groups should enhance (2′-NH_3^+ > 2′-NH_2) and diminish (2′-SH > 2′-S^-) electron withdrawal relative to the corresponding neutral form, respectively. The observation that the reaction experiences little change upon ionization provides further evidence against the inductive model as the sole reason for the stimulatory effect of the 2′-hydroxyl group.

A Quantitative Structure Activity Relationship

None of the preceding model-based approaches account for the group II intron's preference for ribose at the splice junction. We sought therefore to analyze further the data in Figures 2A and 2B using a holistic approach that to our knowledge has not been applied previously in the field of RNA catalysis. Quantitative structure activity relationships (QSAR), pioneered by Hansch and coworkers in the field of drug design [32–34], attempt to correlate the measured activities for a series of compounds with their actual chemical properties. We analyzed our SER data in this manner with the hope of exposing what governs the role of the 2′-hydroxyl group.

The first and most critical step of the QSAR approach involves choosing the parameters against which to correlate the kinetic data. Given the significant steric bulk associated with some of the 2′ substituents and the observed sensitivity of the SER reaction to steric factors, we included a molecular volume parameter as calculated by Chem 3D (version 5.0, CambridgeSoft). To parameterize the inductive effect of the substituents, we used Chem Sketch (version 5.0, Advanced Chemistry Development) to calculate the pK_as of model compounds (see the Supplemental Data at http://www.chembiol.com/cgi/content/full/11/2/237/DC1) containing each substituent located vicinal to a secondary alcohol. As a measure of the inductive effect, we defined the parameter σ as the pK_a of the model compound relative to that for the corresponding compound in which a hydrogen replaces the substituent. As the final parameter, we included the octanol/water partition coefficient

(P) as a measure of hydrophobicity. P describes the ratio of the concentration of a compound in octanol to its concentration in water at equilibrium at a specified temperature. P values for model compounds can be routinely and accurately calculated by a variety of computer programs. We used Chem Sketch to calculate the P values for the functional groups used herein. We defined the parameter π as the log P_X − log P_H, where the former term represents the log P value of a model compound containing the functional group of interest and the latter term represents the log P value of the model compound containing a hydrogen at that same position.

In light of these chosen parameters, we constructed two new substrates, one containing a 2′-chlorine and one containing a 2′-ethyl group, and measured their reactivity in the SER reaction (data not shown). The chlorine atom occupies approximately the same volume as a methyl group but possesses significantly greater electron withdrawing capacity. The ethyl group expands the series of two atom substituents (excluding hydrogen atoms) that extend the 2′ substituent by a methyl group (-OH → -OCH_3, -NH_2 → -$NHCH_3$, -CH_3 → -CH_2CH_3).

We fit the kinetic data to combinations of the three parameters in different functional forms, including their logarithms and squares, in search of the best correlation. Using this standard QSAR procedure, the data were fit best with the following function (statistics in the Supplemental Data):

$$\log k_{obs} = -2.1 - 0.21\pi - (3.37 \times 10^{-4}) \times Vol^2.$$

Strikingly, our analysis shows that the π and molecular volume parameters account for 95% of the data. Including σ in a variety of functional forms does not improve the correlation and in many cases worsens it. The dependence of log k_{obs} on the square of volume suggests a steric threshold below which the size of the group has no significant effect and above which increasing size of the group strongly compromises activity. With this insight, we separated the analogs into two "steric groups," one that contains single atom substituents and one that contains two atom substituents (-$NHCH_3$, -OCH_3, and -CH_2CH_3). As the substituents within each group occupy similar volumes, the log k_{obs} values correlate well using only the partition coefficient parameter. Figure 5 shows these correlations, with log k_{obs} and π expressed as free energies ($\Delta\Delta G^{\ddagger}$ and $\Delta\Delta G_{transfer}$, respectively). The two linear fits give similar slopes ($n \cong 0.34$) for each size group. This analysis quantitatively accounts for all the data in Figures 2A and 2B.

The inverse correlation of rate with hydrophobicity suggests that, as the D123•D5•E1-E2 complex proceeds to the rate-limiting transition state, the local electrostatic environment surrounding the 2′-hydroxyl group becomes more polar. A conformational switch or the buildup of negative charge on the adjacent leaving group and nonbridging oxygens during the chemical step might trigger this environmental change. In the most literal sense, the strong correlation of rate with $\Delta\Delta G_{transfer}$ indicates that the stimulatory effect of the 2′-substituent depends on its affinity for water. The splice junction 2′-hydroxyl group therefore could exert its stimulatory effect in the SER reaction via one or more water molecule

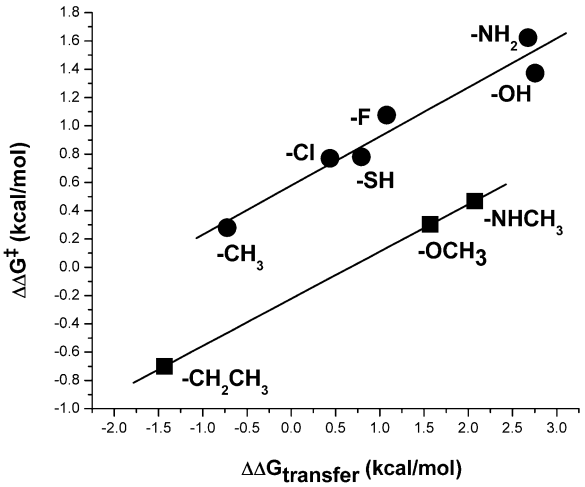

Figure 5. A Quantitative Structure Activity Relationship

Both single atom substituents (circles) and two atom substituents (squares) react with a similar inverse dependence on hydrophobicity. $\Delta\Delta G^{\ddagger} = RT\ln(k_X/k_H)$, where k_X represents the rate constant for the reaction of $E1_{2'X}E2$, and k_H represents that for $E1_{2'H}E2$. $\Delta\Delta G_{transfer} = RT\ln \pi_X$, where π_X represents the change in octanol:water partition coefficient of a model compound containing substituent X relative to the same compound with a hydrogen at that position (see the Supplemental Data at http://www.chembiol.com/cgi/content/full/11/2/237/DC1).

interactions that either form or become stronger as the ternary complex proceeds from the ground state to the transition state.

Crystal structures of RNA duplexes show more extensive hydration than the corresponding DNA duplexes of the identical sequence [35]. In the $[r(C_4G_4)]_2$ crystal structure, the 16 2′-hydroxyl groups per duplex provide 33 hydrogen bonds to water molecules, indicating that the 2′-hydroxyl groups act as a scaffold in a highly ordered water network that spans the minor groove. Solvent organization also occurs within the interior of large folded RNAs [36]. The structure of a mutant form of P4-P6, a 160 nucleotide domain from the *Tetrahymena* intron, reveals that the 2′-OH and other functional groups provide a solvent scaffold for an intricate core of ordered water molecules that may have integral significance for RNA structure and stability [37]. In the group II intron, the cleavage site 2′-hydroxyl may participate in a water network that plays a role in catalysis.

Chemical Rescue of the 2′-H Substitution by Ethanol

As a possible strategy to test whether the splice junction 2′-OH interacts with a water molecule, we investigated whether small alcohols could stimulate the reactivity of $E1_{2'H}E2$ relative to $E1_{2'OH}E2$. The space vacated by the hydroxyl group might allow an alcohol to bind to the D123•D5•E1-E2 ternary complex and position its hydroxyl group via hydrophobic interactions with the deoxyribose ring so as to localize or replace an active site water molecule (Figure 6A). In the presence of saturating D5•D123 in 100 mM $MgCl_2$ (pH 7.0), we measured the effect of added alcohols on the reactivity of $E1_{2'H}E2$ relative to $E1_{2'OH}E2$ (Figure 6B). No rescue occurred upon

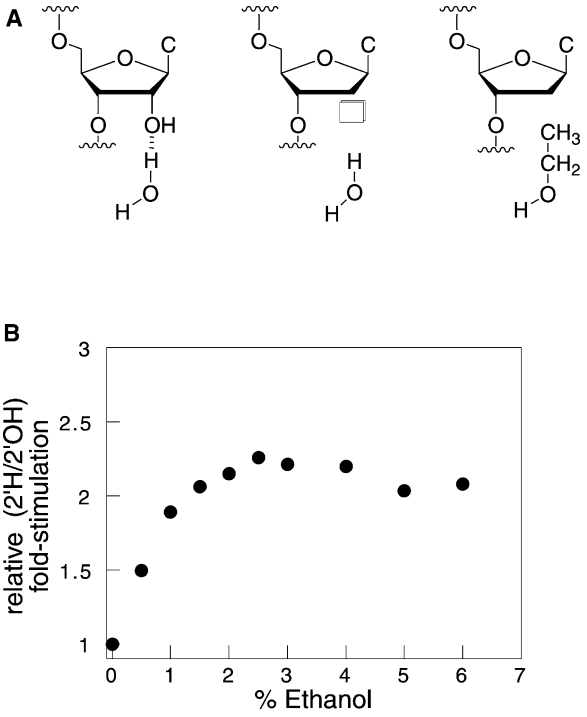

Figure 6. Ethanol Stimulates the SER Reaction of $E1_{2'H}E2$

(A) Hypothetical model showing how ethanol could stimulate the reactivity of $E1_{2'H}E2$. Removal of the splice junction 2′-OH might disrupt solvent organization and allow binding of ethanol.

(B) Ethanol partially rescues the reactivity of $E1_{2'H}E2$ relative to $E1_{2'OH}E2$. Reactions contained $E1_{2'H}E2$ and $E1_{2'OH}E2$, saturating D123•D5, 100 mM $MgCl_2$, and ethanol as indicated. To account for nonspecific effects of ethanol, each point reflects the stimulation of $E1_{2'H}E2$ relative to that of $E1_{2'OH}E2$ at the indicated ethanol concentration. To reduce further any nonspecific effects and experimental uncertainty, both substrates ($E1_{2'OH}E2$ and $E1_{2'H}E2$) were reacted in the same tube (see Experimental Procedures).

addition of isopropanol, *tert*-butanol, or methanol (data not shown). Remarkably, ethanol stimulates the rate of $E1_{2'H}E2$ modestly (~2- to 2.5-fold, Figure 6B) but reproducibly, and this rescue shows apparent saturation. No other substrate in Figure 2B exhibits this behavior.

Although the percent of rescue is modest, it is specific to the 2′-H substitution and is comparable in magnitude to that observed with chemical rescue experiments involving a variety of protein enzymes. In those experiments, the activity of enzymes with Lys→Ala, Arg→Ala, Glu→Ala mutations were partially restored upon the addition of alkylamines, guanidinium, and formate, respectively [38–42]. Our observation that ethanol partially but specifically compensates for the absence of the hydroxyl group in the 2′-H background (Figures 6A and 6B) supports the hypothesis that the cleavage site 2′-OH may participate in solvent organization within the active site.

A Hydroxyl Group Maintains a Stimulatory Effect when Extended Away from the Ribose Ring

If ethanol stimulates reactivity of $E1_{2'H}E2$ by localizing to a cavity created at the 2′ position and donating its hydroxyl group to a solvent network, we might also observe stimulation of the SER reaction by attaching an alcohol moiety covalently to the 2′ carbon. To investigate this possibility, we synthetically constructed a series of substrates containing hydroxyalkyl groups [$(CH_2)_nOH$; n = 1–4] at the 2′ position and tested them in the SER reaction. As the SER reaction exhibits sensitivity to substituent volume, we also synthesized the corresponding deoxygenated substrates containing straight chain alkyl groups [$(CH_2)_nH$; n = 1–4]. These analogs allow us to quantitate the effect of extending the alcohol functionality away from the sugar ring in one carbon steps successively.

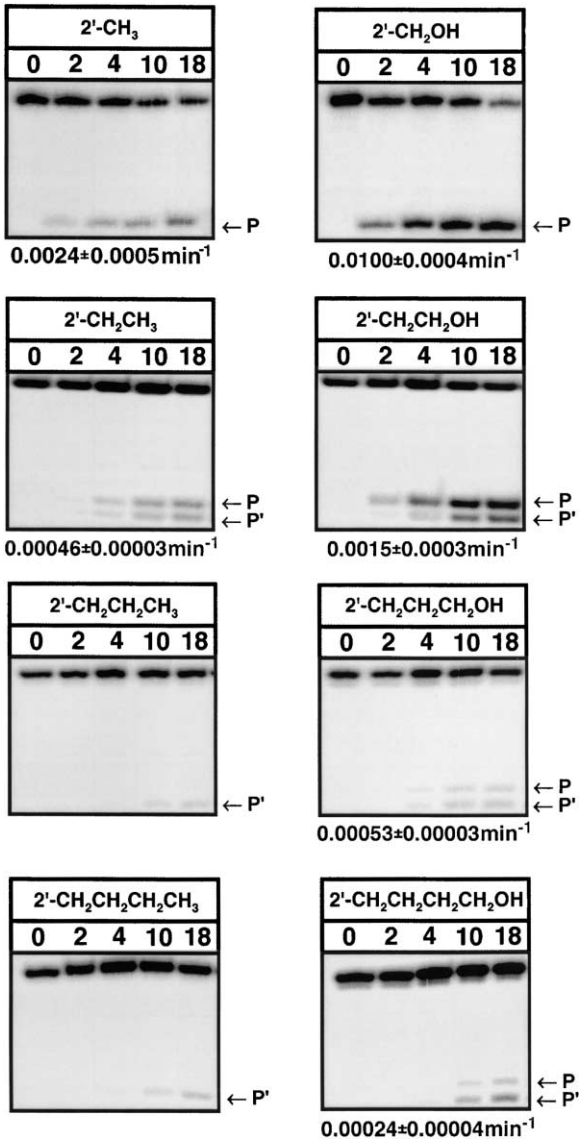

Figure 7. 2′-Hydroxyalkyl Groups at the Splice Junction Stimulate SER Relative to the Corresponding 2′-Alkyl Group

The product of correct cleavage is labeled P, while the miscleavage product, when present, is labeled P′. The data were fit to a parallel-sequential kinetic model, and the rates shown represent those for correct cleavage. Reaction times are given in hours.

Figure 7 shows the results of this analysis, with the reaction of each hydroxyalkyl analog alongside that of

the corresponding alkyl control containing the same number of methylene groups. For the substrates containing two or more methylene groups (n = 2), a significant miscleavage product (P′) appears at a rate that is independent of both the number of methylene units and the presence of the hydroxyl group. To determine the rate constant for the formation of the correct product (P), we fit the kinetic data to a parallel-sequential model [20]. Strikingly, in every case the hydroxyalkyl analog cleaves significantly faster than its alkyl counterpart. Moreover, the apparent stimulation by the hydroxyl group may be even more pronounced as the alkyl analog containing one additional carbon atom may more accurately control for the steric effect. Thus, the hydroxymethyl substrate reacts 20-fold faster than the ethyl substrate. The hydroxyethyl and hydroxypropyl substrates both cleave with measurable rates, whereas the propyl and butyl substrates show no detectable cleavage at the correct site. As the intervening methylene unit(s) substantially diminish the ability of a hydroxyl group to exert an inductive effect on the 3′-oxygen leaving group pK_a, these results demonstrate a mode of stimulation for the hydroxyl group that must occur through space rather then through the sigma bond framework.

Of the analogs tested in this series, the hydroxymethyl substrate reacts the fastest, with almost the wild-type rate. The greater reactivity of the hydroxymethyl substrate compared to the hydroxyethyl substrate apparently contradicts the results from the exogenous alcohol addition, in which ethanol but not methanol stimulates the SER reaction. Covalently attaching the alcohol enforces significant restrictions on its orientational freedom compared to the corresponding noncovalently positioned alcohol, however. Presumably the hydroxymethyl analog can better position its hydroxyl group to maintain the putative solvation network than can the hydroxyethyl analog given the conformational restrictions of the latter. The longer propanol and butanol analogs maintain a stimulatory effect on cleavage relative to their alkyl counterparts. While it seems unlikely that the hydroxyl groups of these longer analogs could be positioned to replace a specific water molecule important for cleavage, they might participate in a solvent network that stimulates cleavage at the splice junction relative to the corresponding alkyl control. These results support a model in which the 2′ hydroxyl mediates an interaction with solvent and seem to discount the role of the inductive effect as a major contributor to the stimulating effect of the splice junction 2′-hydroxyl group.

SMUG1, a uracil-DNA glycosylase, provides an interesting biological precedent for our experimental strategy [43]. The enzyme recognizes and excises uracil and 5-hydroxymethyluracil (HMU) but is inactive against thymine. It achieves this discrimination by a "displacement/replacement" mechanism involving a "well-ordered water molecule" at the active site. In the presence of bound uracil, this water molecule is observed crystallographically to be positioned by hydrogen bonds to peptide-NH groups in the active site as well as the O4 carbonyl oxygen of the pyrimidine. When thymine is bound, its C5-methyl group displaces this water, resulting in an energetic penalty that discriminates against its excision. When HMU is bound, the hydroxyl group on its C5-methyl occupies the same pocket as the active site water molecule in the presence of uracil. This hydroxyl group recapitulates the hydrogen bonding interactions of the active site water molecule, thus accounting for the ability of SMUG1 to excise HMU. Likewise, a similar replacement mechanism may account for the stimulatory effect of the hydroxyalkyl analogs in the SER reaction.

Significance

A satisfactory understanding of the relationship between RNA structure and its biological function must include a description of the role played by individual 2′-hydroxyl groups. Deoxynucleotide substitution reliably identifies the important 2′-hydroxyl groups within RNA but gives little information about their functional role. We have established an array of approaches that decouple the modes by which hydroxyl groups impart functional behavior. The nucleoside analogs used span a broad range of chemical diversity that allows the use of quantitative structure activity relationships (QSAR) in the exploration of RNA biology. We used these approaches to elucidate the role of the cleavage site 2′-hydroxyl group in the spliced exons reopening reaction catalyzed by the group II intron. Our results suggest that the cleavage site 2′-hydroxyl group may interact with a water molecule. Hydroxyl-mediated solvent interactions occur ubiquitously in RNA, and this work provides several approaches by which to establish their biological significance.

Experimental Procedures

Oligonucleotides

All oligoribonucleotides were synthesized on a Millipore solid-phase DNA/RNA synthesizer or obtained from Dharmacon Research (Lafayette, CO). The following oligoribonucleotides were used in this study (where subscript $_X$ denotes a 2′-OH, 2′-H, 2′-OCH_3, 2′-NH_2, 2′-$NHCH_3$, 2′-F, 2′-Cl, 2′-SH, 2′-deoxy-2′-*C*-α-CH_3, 2′-deoxy-2′-*C*-β-CH_3, 2′-OH-*C*-β-CH_3, 2′-$[(CH_2)_nH$; n = 1-4], and 2′-$[(CH_2)_nOH$; n = 1–4] modification): (1) D5 (5′-CGUGAGCCGUAUGCGAUGAAAGUC GCACGUACGGUUCUUAC-3′); (2) SER (5′-ACGUGGUGGGACAUU UUC$_X$ACUAUGUAU-3′); (3) SER-32 (5′UUACUCGUGGUGGGACAU UUUCACUAU-3′).

2′-Mercaptocytidine and 2′-*C*-β-methylcytidine phosphoramidites were synthesized and coupled as previously described [30, 44]. Procedures for the synthesis of 2′-deoxy-2′-aminomethyl, 2′-deoxy-2′-*C*-β-methyl, 2′-deoxy-2′-*C*-α-methylcytidine phosphoramidites and for the preparation of oligonucleotides containing 2′-chlorocytidine are described in the Supplemental Data at http://www.chembiol.com/cgi/content/full/11/2/237/DC1. The synthesis of the 2′-hydroxyalkyl and 2′-alkylcytidine phosphoramidites will be described elsewhere. The TBAF deprotection of $E1_{2'\text{-}\beta\text{-}CH_3\text{-}\alpha\text{-}OH}E2$ was increased from 24 to 72 hr in order to remove ~60%–70% of the TBDMS protecting groups [30], and after gel purification the oligonucleotide was further purified by reverse-phase HPLC. All other oligoribonucleotides were deprotected according to standard procedures and purified by denaturing gel electrophoresis. Oligoribonucleotides were 5′-^{32}P-phosphorylated with [γ-^{32}P]-ATP and T4 polynucleotide kinase according to manufacturer's instructions (Amersham Pharmacia). After gel purification and 5′-^{32}P-phosphorylation, the $E1_{2'SH}E2$ oligoribonucleotide was treated with 1.5 mM $AgNO_3$ followed by 2 mM dithiothreitol (DTT) to remove the trityl protecting group [44].

Plasmids and Transcription

Plasmid pG2.D123, when linearized with HindIII and transcribed with T7 RNA polymerase, generates an RNA that begins with nucleotide 1 of the intron and ends after domain 3. pG2.D123 was constructed by subcloning a fragment of pG2.5+10D123 [45], generated by a digest with SnaBI and EcoRI, into a similarly digested pKC.D1-6 (a gift from K. Chin and A. Pyle, Columbia University, New York, NY). Transcriptions were performed as previously described [45], and the RNA was purified by 5% denaturing polyacrylamide gel electrophoresis.

General Kinetic Methods

Reactions were at 42°C and contained D123, saturating D5 (5 μM; K_d = 270 nM, [12]), trace 5′-^{32}P-labeled substrate, 100 mM $MgCl_2$, and 40 mM buffer at the pH indicated (sodium acetate, pH 5–6; NaMES, pH 6.0–6.5; NaMOPS, pH 6.5–7.5; NaTAPS, pH 7.7–8.5). D5, D123, and the substrate were heated separately at 90°C for 1 min and then allowed to cool to 42°C. After cooling, $MgCl_2$ was added and D123 and the substrate were combined and allowed to fold for 20 min at 42°C. Reactions were initiated by the addition of D5, and 1 μl aliquots of reaction mixture were removed at specified times and quenched by the addition of 5 μl of stop solution (8 M Urea/0.5X TBE/0.04% xylene cyanol/0.04% bromophenol blue). Substrates and products were fractionated by denaturing polyacrylamide gel electrophoresis, and their ratio at each time point was quantitated with a Molecular Dynamics phosphorimager.

To reduce experimental uncertainty in the ethanol rescue experiments a 32 nt $E1_{2'OH}E2$ substrate was used as an internal control in the same reaction tube with the 27 nt $E1_{2'H}E2$ substrate. The addition of five nucleotides at the 5′ terminus of the substrate enabled the separation of the both substrates and products by gel electrophoresis.

Acknowledgments

We thank Professors Scott Silverman and Stephen Kent for comments on the manuscript. We also thank members of the Piccirilli lab for helpful discussions and comments on the manuscript. P.M.G. and J.-D.Y. are Research Associates; N.-S.L. is a Research Specialist; and J.A.P. is an Associate Investigator of the Howard Hughes Medical Institute. P.M.G. and R.F. were supported in part by the Medical Science Training Program (5 T32 GM07281) at the University of Chicago.

Received: May 31, 2002
Revised: October 28, 2003
Accepted: November 24, 2003
Published: February 20, 2004

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