

Comparison of Binding of Mixed Ribose-Deoxyribose Analogues of CUCU to a Ribozyme and to GGAGAA by Equilibrium Dialysis: Evidence for Ribozyme Specific Interactions with 2' OH Groups[†]

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ABSTRACT: Dissociation constants at 15 °C were measured by equilibrium dialysis for the binding of rCrUrCrU, dCrUrCrU, rCdUrCrU, rCrUdCrU, and rCrUrCdU to the L-21 *ScaI* form of the self-splicing group I LSU intron from *Tetrahymena thermophila*. Substitution of deoxyribose for ribose in each of the middle two positions makes the free energy change for binding 1–2 kcal/mol less favorable, compared to about 0.3 kcal/mol less favorable for each of the terminal positions. Dissociation constants for binding of the same oligomers to rGGAGAA were measured by optical melting methods. Substitution of a single deoxyribose for ribose makes the free energy change for binding less favorable by 0.4–0.9 kcal/mol for this simple duplex formation. Comparison of the effects for binding to ribozyme and to rGGAGAA indicate that ribozyme-specific tertiary interactions dependent on the middle two 2' OH groups of rCrUrCrU add about 2 kcal/mol of favorable free energy for binding to L-21 *ScaI*. Comparisons are made with results from gel retardation studies [Pyle, A. M., McSwiggen, J. A., & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 8187–8191; Pyle, A. M., & Cech, T. R. (1991) *Nature (London)* 350, 628–631].

The advent of rapid sequencing techniques has led to a demand for methods to predict RNA structure from sequence. An important phase in the development of these methods is an understanding of the interactions that determine RNA structure. Nearest-neighbor rules for base pairing (Borer et al., 1974) are able to predict free energies of duplex formation within about 5% for helices containing only AU and GC pairs (Freier et al., 1986a). Kinetic (Sullivan & Cech, 1985; Sugimoto et al., 1988; Herschlag & Cech, 1990a), fluorescence titration (Sugimoto et al., 1989a), and gel shift (Pyle et al., 1990; Pyle & Cech, 1991) studies of oligomers binding to two forms of the self-splicing LSU group I intron from *Tetrahymena thermophila* (Kruger et al., 1982), however, suggest this ribozyme is able to bind oligomer substrates more than 1000 times tighter than expected from nearest-neighbor base pairing rules. Recent advances in methods for synthesizing RNA (Kierzek et al., 1986; Usman et al., 1987) have made it possible to synthesize chimeric RNA–DNA oligomers. Such oligomers have been useful for investigating the role of various 2' OH groups in RNA interactions (Sugimoto et al., 1989b; Perreault et al., 1990, 1991; Yang et al., 1990; Pyle & Cech, 1991). From such studies, it has been suggested that the additional free energy for oligomer binding to the self-splicing intron is at least partially due to interactions of the ribozyme with 2' OH groups of the oligomer (Sugimoto et al., 1989b; Pyle & Cech, 1991). In this paper, binding of rCrUrCrU, dCrUrCrU, rCdUrCrU, rCrUdCrU, and rCrUrCdU to the L-21 *ScaI* shortened form of the LSU intron (Zaug et al., 1988) is studied by equilibrium dialysis (Uhlenbeck, 1972). The results provide the first equilibrium measurements that confirm the special interactions of the 2' OH for binding.

MATERIALS AND METHODS

Oligonucleotides. pdG was obtained from Sigma and its purity confirmed by normal-phase TLC. rUCU, rCrUrCrU,

dCrUrCrU, rCdUrCrU, rCrUdCrU, rCrUrCdU, rGGAGAA, and rUCGA were synthesized on solid support with a phosphoramidite method (Kierzek et al., 1986) and purified by HPLC on a PRP-1 semipreparative column (Ikuta et al., 1984; Longfellow et al., 1990). Final purity was checked by HPLC on a Beckman C-8 analytical column. rCCCUCU and rCUCUCU were generous gifts from Drs. A. M. Pyle and T. R. Cech.

For CUCU analogues, absence or presence of a ribose at positions other than the 3' end was confirmed by partial hydrolysis of 5'-³²P-labeled oligomers. Oligomers were boiled in aqueous NH₄OH at pH 9.0 for 10 min, dried, incubated at pH 3 for 3 h at 37 °C to open cyclic phosphates, and run on a 20% polyacrylamide sequencing gel. Bands were present at positions of ribose nucleotides and absent at positions of deoxyribose nucleotides. The presence of a 3' deoxyribose in rCrUrCdU was confirmed by its resistance to oxidation by NaIO₄ (Wells & Cantor, 1977; Agrawal et al., 1986). CUCU analogues were incubated at 4 °C for 30 min in 4 mM NaIO₄ (freshly made in water and filtered) and 0.1 M NaOAc, pH 5.0. HPLC on a Beckman C-8 column was used to detect reaction. CUCU analogues with a 3' terminal ribose gave broad peaks with retention times shorter than observed before treatment. This is expected for conversion of the 3' terminal ribose to the dialdehyde. rCrUrCdU gave a sharp peak with mobility identical to that of unreacted material, as expected for a 3' terminal deoxyribose.

Oligomer concentrations were determined optically at 260 nm with the following extinction coefficients in units of 10⁴ M⁻¹ cm⁻¹ derived from a nearest-neighbor model (Borer, 1975; Richards, 1975): UCU, 2.66; CUCU and its analogues, 3.33; UCGA, 4.11; GGAGAA, 6.92; pdG, 1.14.

Oligomers were labeled with [³²P]ATP from New England Nuclear and T4 polynucleotide kinase from New England Biolabs and purified by polyacrylamide gel electrophoresis (PAGE) on a 20% acrylamide sequencing gel. Bands were cut out, eluted, dialyzed against water, dried down, and redissolved in water. Concentrations were determined by scintillation counting of aliquots in Ecoscint A.

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L-21 *ScaI* Ribozyme. L-21 *ScaI* was prepared from plasmid PT7L-21 (Zaug et al., 1988) cut with *ScaI*, by transcription with T7 RNA polymerase (Davanloo et al., 1984). The transcript was purified on a Qiagen anion-exchange column, precipitated with 2-propanol, redissolved in water, and stored at -20°C . Purity was confirmed by running aliquots on 4% polyacrylamide/8 M urea denaturing gels and staining with ethidium bromide. Concentrations were determined optically with an extinction coefficient of $3.2 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm (Zaug et al., 1988). For experiments with trimer and tetramers L-21 *ScaI* was renatured before each experiment by heating at 90°C for 3 min in 0.1 mM Na_2EDTA /10 mM Tris, pH 7.5, centrifuging at room temperature for 10 s, and allowing it to stand for 15 min at room temperature and for 15 min on ice before simultaneous addition of the final MgCl_2 , NaCl, and HEPES buffer. For experiments with hexamers, L-21 *ScaI* was renatured as described by Pyle et al. (1990).

Equilibrium Dialysis. Equilibrium dialysis was done in a constant-temperature room at 15 or 25°C with a Hoefster Scientific Instruments EMD 101 apparatus. For trimer and tetramers, the buffer was 50 mM HEPES, pH 7.4 (at 15°C), made from 25 mM Na^+ salt and 25 mM free acid. Solutions also contained either 50 mM Mg^{2+} , or 5 mM Mg^{2+} with 135 mM NaCl to maintain ionic strength. For hexamers, the buffer was 50 mM Tris, pH 7.5, 0.1 mM EDTA, 10 mM NaCl, and either 5 or 10 mM MgCl_2 (Pyle et al., 1990). Membranes had molecular weight cutoffs of about 13 000. In a typical experiment, 48 μL of a sample containing L-21 *ScaI* was placed on one side of the membrane, and 48 μL of radiolabeled oligomer was placed on the other side. The approach to equilibrium was followed by taking aliquots of 5–10 μL from each chamber and quantifying radioactivity by liquid scintillation counting with Ecoscint A. Equilibrium was reached in 3 days since the ratio of radioactivity in the two chambers was the same after 3- and 4-day incubations. Experiments with hexamers were initiated by placing premixed L-21 *ScaI* and oligomer on one side of the membrane and buffer on the other side. Aliquots run on a 4% polyacrylamide denaturing gel and stained with ethidium bromide showed no degradation and no migration between chambers for L-21 *ScaI*. Aliquots run on a 20% polyacrylamide denaturing gel showed more than 20% covalent coupling of CUCU to L-21 *ScaI* after 1 day. This could reflect reaction between CUCU and L-21 *ScaI* catalyzed by a second L-21 *ScaI* molecule. Except for rCdUrCrU at greater than 0.2 μM L-21 *ScaI* in 50 mM Mg^{2+} /25 mM Na^+ , this reaction was essentially eliminated by including 5 mM pdG on each side of the membrane. For rCdUrCrU, the extent of coupling was determined by cutting and scintillation counting of bands and was less than 10%. The concentration of rCdUrCrU on the L-21 *ScaI* side of the dialysis chamber was corrected for this covalent coupling. This was only necessary for R-1 plots. rCdUrCrU has the lowest binding constant and therefore required the highest L-21 *ScaI* concentrations.

Melting Experiments. Melting curves were measured at 280 nm in 50 mM MgCl_2 /50 mM HEPES, pH 7.5, as previously described (Petersheim & Turner, 1983). Duplexes were prepared by mixing concentrated solutions of strands and buffers to the proper final concentrations and volume without drying to a pellet. Solutions were heated for 10 min at 60°C and centrifuged for 1 s to degas. The total concentration of oligomer, C_T , was varied over a 4–8-fold range limited on the high end by solubility and on the low end by duplex stability. Melting curves for individual strands had broad transitions characteristic of single strands. Melting curves for 1:1 mix-

tures of complementary strands had cooperative transitions as expected for duplex melting. Thermodynamic parameters for duplexes were obtained by two methods (Petersheim & Turner, 1983; Turner et al., 1988): (1) fitting individual melting curves to a two-state non-self-complementary model (Longfellow et al., 1990) and (2) plotting T_m^{-1} versus $\log C_T/4$ according to (Borer et al., 1974)

$$T_m^{-1} = (2.3R/\Delta H^{\circ}) \log (C_T/4) + \Delta S^{\circ}/\Delta H^{\circ} \quad (1)$$

Values of ΔH° obtained from the two methods agree within 7%, consistent with the two-state model.

Kinetic Experiments. Reactions were run in 50 mM MgCl_2 /50 mM HEPES, pH 7.5, at 15°C in a Lauda RC6 constant-temperature bath. After renaturation, L-21 *ScaI* was preincubated for 12–16 h at 15°C in reaction buffer. A 10- μL volume of 5 μM L-21 *ScaI* in reaction buffer was then mixed with 5 μL of a 1–2 nM solution of 5'- ^{32}P -labeled CUCU analogue in reaction buffer and incubated for 10 min at 15°C . Reactions were initiated by adding 5 μL of 800 μM UCGA equilibrated at 15°C in reaction buffer. Reactions were quenched by removing 2–3 μL , mixing with an equal volume of 90% formamide/10% aqueous 0.09 M Tris-borate/0.002 M Na_2EDTA /0.05% bromophenol blue/0.05% xylene cyanol, and freezing in a dry ice/ethanol bath. If this stop solution was added to one reactant before addition of the second, no reaction was observed. Products and reactants were separated by electrophoresis on a 20% polyacrylamide denaturing gel. Bands were visualized by autoradiography, cut out, and quantified by scintillation counting in the presence of 5 mL of Ecoscint A.

RESULTS

Equilibrium Dialysis: rCrUrCrU, dCrUrCrU, rCdUrCrU, rCrUdCrU, and rCrUrCdU. As described under Materials and Methods, dissociation constants for oligomers binding to L-21 *ScaI* were obtained by equilibrium dialysis in the presence of 5 mM pdG to suppress covalent coupling between L-21 *ScaI* and oligomer. When $[\text{L-21 } ScaI] \gg [\text{CUCU}]$, the ratio, R, of concentration of oligomer on the side with L-21 *ScaI* to the side without L-21 *ScaI* is related to the molar dissociation constant, K_{-1} , by (Uhlenbeck, 1972)

$$R-1 = [\text{L-21 } ScaI]/K_{-1} \quad (2)$$

In these experiments, $[\text{CUCU}]$ is constant at about 1–2 nM and $[\text{L-21 } ScaI]$ is varied. R-1 plots are shown in Figure 1. They are linear, as expected from eq 2.

Dissociation constants can also be derived from Scatchard plots (Scatchard, 1949; van Holde, 1985). For a set of independent binding sites, each with an identical K_{-1} , the Scatchard plot follows the equation

$$\nu/[\text{CUCU}] = (n/K_{-1}) - (\nu/K_{-1}) \quad (3)$$

Here ν is the average number of bound ligand molecules per L-21 *ScaI*, $[\text{CUCU}]$ is the concentration of CUCU on the side of the membrane without L-21 *ScaI*, and n is the number of CUCU molecules bound per L-21 *ScaI* at infinite $[\text{CUCU}]$. In these experiments, $[\text{L-21 } ScaI]$ is constant and $[\text{CUCU}]$ is varied from about 0.1 $[\text{L-21 } ScaI]$ to 10 $[\text{L-21 } ScaI]$. Scatchard plots are shown in Figure 2 and in the supplementary material (see paragraph at end of paper regarding supplementary material).

At 5 mM Mg^{2+} /160 mM Na^+ , Scatchard plots were measured for rCrUrCrU and rCrUdCrU. They are linear with x-intercepts of 0.9 and 1.1, respectively, consistent with one strong binding site per L-21 *ScaI* (see supplementary mate-

Table I: Thermodynamic Parameters for Binding to L-21 *ScaI*

15 °C in 50 mM HEPES/25 mM Na ⁺ , pH 7.4								
5 mM MgCl ₂ /135 mM NaCl				50 mM MgCl ₂				
oligomer	$K_{-1}(\text{sc})^a$ (nM)	$n(\text{sc})^b$	$K_{-1}(\text{R-1})^c$ (nM)	$-\Delta G_{15}^d$ (kcal/mol)	$K_{-1}(\text{sc})^a$ (nM)	$n(\text{sc})^b$	$K_{-1}(\text{R-1})^c$ (nM)	$-\Delta G_{15}^d$ (kcal/mol)
rCrUrCrU	23 ± 1	0.88 ± 0.06	27 ± 4	9.98 ± 0.08	5.9 ± 0.6	0.28 ± 0.04	4.9 ± 0.3	10.96 ± 0.04
dCrUrCrU			79 ± 7	9.36 ± 0.05	10.0 ± 0.6	0.31 ± 0.02	7.6 ± 1	10.70 ± 0.09
rCdUrCrU			1500 ± 150	7.68 ± 0.06	120 ^f ± 12 (2300) ^f	0.30 ± 0.03 (0.70)	130 ± 8	9.08 ± 0.03
rCrUdCrU	240 ± 13	1.1 ± 0.08	370 ± 59	8.48 ± 0.09	36 ± 2	0.32 ± 0.02	35 ± 2	9.83 ± 0.03
rCrUrCdU			98 ± 10	9.24 ± 0.06	6.9 ± 0.3	0.27 ± 0.02	9.0 ± 0.5	10.61 ± 0.03
rUrCrU							950 ^e ± 110	7.94 ± 0.06

			25 °C in 50 mM Tris, pH 7.5	
			$K_{-1}(\text{R-1})$ (nM)	
			5 mM MgCl ₂ /10 mM NaCl	10 mM MgCl ₂ /10 mM NaCl
oligomer				
rCCCUCU			0.004 (0.004) ^h	0.004 ^h
rCUCUCU			0.1 (0.2) ^h	0.04 ^h

^a $K_{-1}(\text{sc})$ are derived from Scatchard plots. ^b $n(\text{sc})$ is the number of binding sites from Scatchard plots. ^c $K_{-1}(\text{R-1})$ are derived from R-1 plots, assuming $n = 1$. The R-1 plots were forced through the origin as per eq 2. ^d ΔG_{15} derived from $K_{-1}(\text{R-1})$ values. ^e Values calculated from R-1 plots assuming $n = 0.3$ for the tight binding site. The R-1 plots were forced through the origin as per eq 2. ^f These parameters assume two independent binding modes. ^g Calculated by a competition experiment as indicated in the text. ^h Errors are estimated as a factor of 2. Values in parentheses were obtained in the absence of dG.

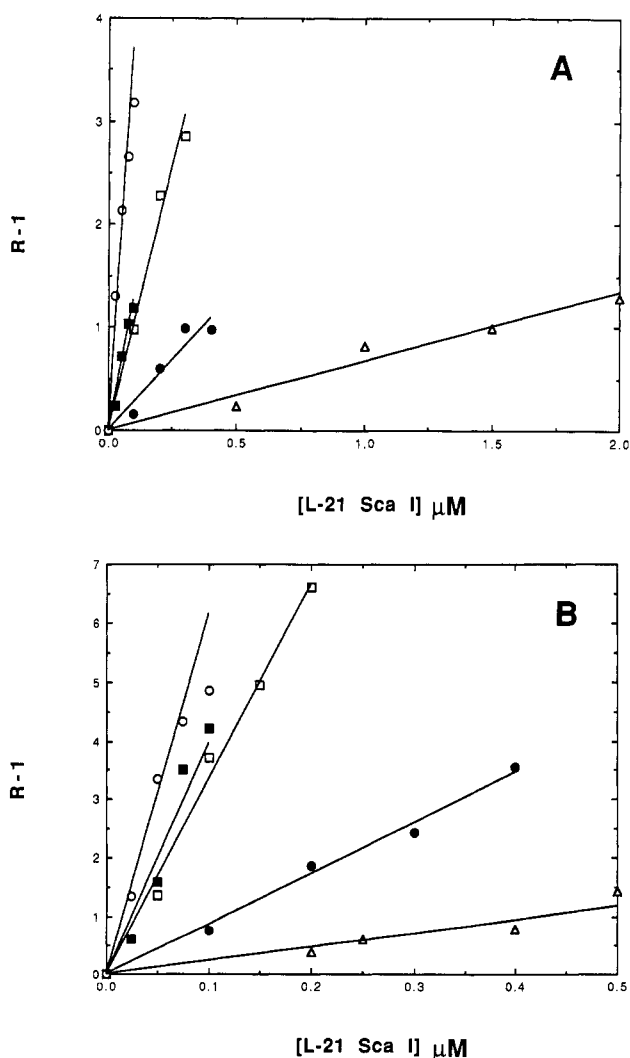


FIGURE 1: Plots of R-1 vs [L-21 *ScaI*] for rCrUrCrU (○), dCrUrCrU (■), rCdUrCrU (△), rCrUdCrU (●), and rCrUrCdU (□) in 50 mM HEPES/25 mM Na⁺, pH 7.4, 15 °C. Top (A): 5 mM MgCl₂/135 mM NaCl. Bottom (B): 50 mM MgCl₂. R is the ratio of counts per minute on the L-21 *ScaI* side of the membrane to the counts per minute on the side of the membrane without L-21 *ScaI*.

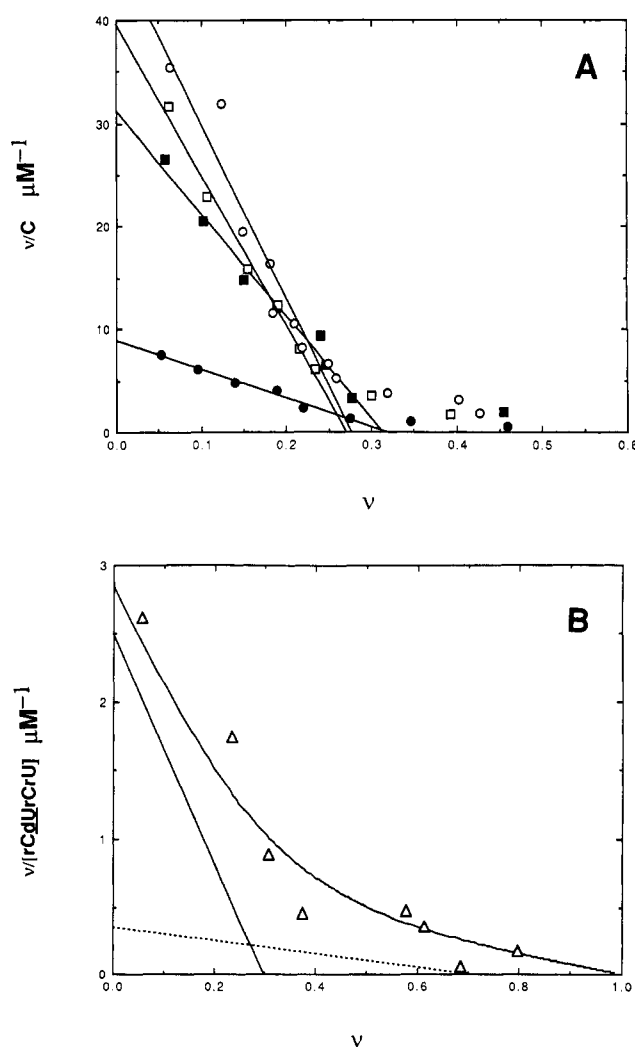


FIGURE 2: Top (A): Plot of ν/C vs ν , where C is the free concentration of rCrUrCrU (○), dCrUrCrU (■), rCrUdCrU (●), and rCrUrCdU (□). Bottom (B): plot of $\nu/[rCdUrCrU]$ (△) vs ν . Solid and dashed lines describe two independent binding modes (Rosenthal, 1967). Solutions are 50 mM MgCl₂/50 mM HEPES/25 mM Na⁺, pH 7.4, 15 °C. ν is the moles of tetramer bound per mole of L-21 *ScaI*.

rials). Dissociation constants derived from R-1 and Scatchard plots are listed in Table I, and are in good agreement.

At 50 mM Mg²⁺/25 mM Na⁺, Scatchard plots for all but rCdUrCrU are relatively linear, but the x-intercepts are close to 0.3. This suggests that at 50 mM Mg²⁺/25 mM Na⁺, n

Table II: Thermodynamic Parameters for Binding to GGAGAA^a

oligomer	1/T _M vs log (C _T /4) parameters				curve fit parameters			
	-ΔG ₁₅ ^b (kcal/mol)	-ΔH ^o (kcal/mol)	-ΔS ^o (eu)	T _M ^b (°C)	-ΔG ₁₅ ^b (kcal/mol)	-ΔH ^o (kcal/mol)	-ΔS ^o (eu)	T _M ^b (°C)
rCrUrCrU	6.15 ± 0.1	35.74 ± 2	102.70 ± 8	26.7	6.20 ± 0.2	35.60 ± 5	102.04 ± 16	27.2
dCrUrCrU	5.44 ± 0.08	30.14 ± 4	85.71 ± 13	21.8	5.49 ± 0.1	31.67 ± 2	90.87 ± 6	21.9
rCdUrCrU	5.26 ± 0.03	30.31 ± 2	86.95 ± 5	19.9	5.31 ± 0.08	32.33 ± 3	93.78 ± 9	20.1
rCrUdCrU	5.64 ± 0.04	32.99 ± 1	94.90 ± 5	23.0	5.70 ± 0.1	34.28 ± 3	99.20 ± 9	23.2
rCrUrCdU	5.73 ± 0.1	35.77 ± 4	104.26 ± 13	23.1	5.69 ± 0.2	33.60 ± 4	96.85 ± 14	23.3

^aSolutions are 50 mM MgCl₂, 50 mM HEPES/25 mM Na⁺, pH 7.4. Estimated errors are derived as described in SantaLucia et al. (1991a,b). Additional significant figures are given to allow accurate calculation of T_M and other parameters. ^bCalculated for 10⁻³ M oligomer concentration.

= 0.3 and 70% of L-21 *ScaI* is trapped in a conformation unable to bind oligomer strongly. Thus only 30% of the total concentration of L-21 *ScaI* is used in eq 2 to derive binding constants from R-1 plots. Dissociation constants derived in this way and from Scatchard plots are listed in Table I. There is reasonable agreement between the two methods.

As shown in Figure 2B, the Scatchard plot for rCdUrCrU at 50 mM Mg²⁺/25 mM Na⁺ is clearly biphasic. Biphasic Scatchard plots may occur for several reasons: there may be two or more independent sites or there may be two or more negatively interacting sites. Because there is no evidence for interacting sites, it is simplest to assume there are two independent sites. Using the graphical method of Rosenthal (1967; Zierler, 1989), the data may be resolved into two components: one with 0.3 binding sites and a dissociation constant of 120 nM, and one with 0.7 binding sites and a dissociation constant of 2.3 μM. The curve corresponding to this deconvolution is shown in Figure 2B. The smaller dissociation constant agrees with that from R-1 plots, and its degree of saturation is 0.3, consistent with the Scatchard plots at 50 mM Mg²⁺ for rCrUrCrU, dCrUrCrU, rCrUdCrU, and rCrUrCdU. Presumably, the weaker binding mode is well separated from the tighter mode for other CUCU analogues because the tighter binding mode is 3–20 times stronger than for rCdUrCrU. Thus all other Scatchard plots reflect primarily the strongest binding site. Treatment of the data in terms of two independent classes of sites thus gives a physical description of rCdUrCrU binding that is consistent with that of the other four substrates.

Equilibrium Dialysis: rUCU. To avoid using high concentrations of L-21 *ScaI*, which lead to covalent coupling, the dissociation constant for UCU was measured by competition with CUCU. In these experiments, [UCU] ≫ [L-21 *ScaI*] ≫ [p**CUCU*]. Thus binding of UCU reduces the concentration of unoccupied L-21 *ScaI* binding sites available for p**CUCU* by a factor of (1 + [UCU]/K₋₁^{UCU}). This modifies eq 2 to (Uhlenbeck, 1972)

$$R-1 = [L-21 \text{ ScaI}] / \{(1 + [UCU]/K_{-1}^{\text{UCU}})K_{-1}^{\text{CUCU}}\} \quad (4)$$

K₋₁^{CUCU} is known from the experiments described above. [UCU] is the same on both sides of the dialysis membrane and is unaffected by binding, since UCU is in large excess. Thus, for experiments at constant [UCU], a plot of R-1 versus [L-21 *ScaI*] should be linear, and K₋₁^{UCU} can be obtained from the slope. The supplementary material shows such plots for UCU concentrations of 1.5 and 3.0 μM at 50 mM Mg²⁺/25 mM Na⁺. The values of K₋₁^{UCU} obtained are 0.80 and 1.1 μM, respectively.

Equilibrium Dialysis: rCCCUCU and rCUCUCU. Dissociation constants for the hexamers rCCCUCU and rCUCUCU binding to L-21 *ScaI* were determined from R-1 plots at 10 mM MgCl₂ in 5 mM pdG and at 5 mM MgCl₂ both with and without 5 mM pdG. Plots of R-1 vs [L-21 *ScaI*] were

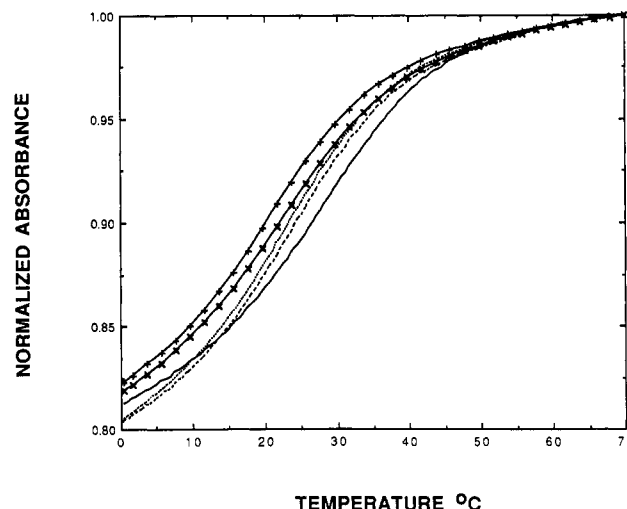
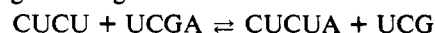


FIGURE 3: Plots of normalized absorbance vs temperature for rGGAGAA with rCrUrCrU at C_T = 1.18 mM (—), dCrUrCrU at C_T = 0.89 mM (···), rCdUrCrU at C_T = 0.78 mM (+), rCrUdCrU at C_T = 0.90 mM (---), and rCrUrCdU at C_T = 0.76 mM (×). Curves are normalized at 70 °C. Solutions are 50 mM MgCl₂/50 mM HEPES/25 mM Na⁺, pH 7.4.

linear as expected (see supplementary material). Denaturing gels revealed no ligation of oligomer and L-21 *ScaI* in either the presence or absence of pdG. Measured dissociation constants are listed in Table I.

Optical Melting of Model Oligomers. To determine if the effects observed with L-21 *ScaI* are due to the ribozyme, optical melting experiments were used to determine thermodynamic parameters at 50 mM Mg²⁺/25 mM Na⁺ for each tetramer bound to rGGAGAA. GGAGAA mimics the internal guide sequence of L-21 *ScaI*, GGAGGG, but only allows one binding site. It is assumed that tertiary interactions force CUCU to bind to the GGAG part of the internal guide sequence in L-21 *ScaI*. GGAGAA also avoids potential aggregation problems often seen with oligomers containing three consecutive G's (Breslauer et al., 1986; SantaLucia et al., 1991). Figures 3 and 4 show typical melting curves and T_M⁻¹ versus log C_T/4 plots, respectively. Thermodynamic parameters derived from fits to the melting curves and from T_M⁻¹ plots are listed in Table II. The ΔH^o's obtained from the two methods agree within 7%, consistent with the two-state model used to analyze the data (Petersheim & Turner, 1983; Turner et al., 1988). Thermodynamic parameters for duplex formation in 5 mM Mg²⁺/160 mM Na⁺ are expected to be similar (Williams et al., 1989).

Reactivity of Oligomers under Single-Turnover Conditions. To determine if the mixed deoxy-ribo oligomers were active as substrates for L-21 *ScaI*, they were reacted with 200 μM UCGA at 50 mM Mg²⁺/25 mM Na⁺ under conditions designed to give a single turnover for the reaction



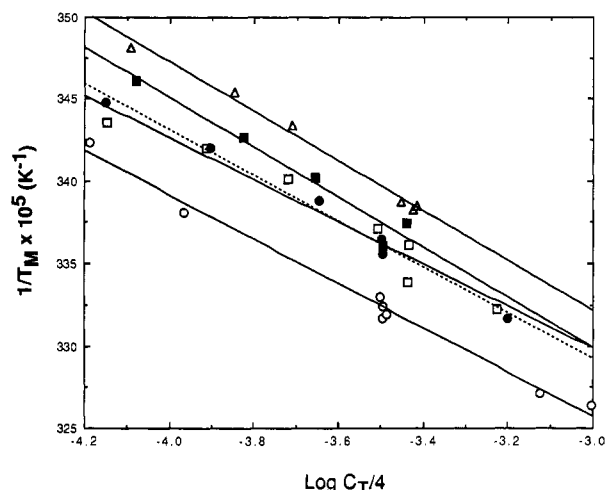


FIGURE 4: Reciprocal melting temperature vs $\log (C_T/4)$ plots for rGGAGAA with rCrUrCrU (○), dCrUrCrU (■), rCdUrCrU (△), rCrUdCrU (●, ---), and rCrUrcdU (□). Solutions are 50 mM $MgCl_2$ /50 mM HEPES/25 mM Na^+ , pH 7.4.

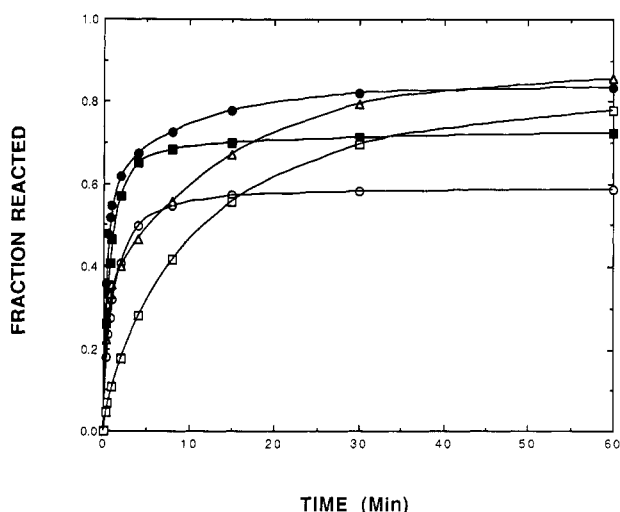


FIGURE 5: Fraction reaction vs time plots for 200 μM UCGA reacting with nanomolar concentrations of 5'- ^{32}P -labeled rCrUrCrU (○), dCrUrCrU (■), rCdUrCrU (△), rCrUdCrU (●), or rCrUrcdU (□). Solutions are 2.5 μM L-21 *ScaI*/50 mM $MgCl_2$ /50 mM HEPES/25 mM Na^+ , pH 7.4, 15 °C.

Plots of fraction reaction versus time are shown in Figure 5. All five oligomers reacted. Surprisingly, none reacted to completion. This may reflect the presence of significant concentrations of UCG in the reaction mixture. UCG is generated by hydrolysis of UCGA catalyzed by L-21 *ScaI* (unpublished experiments). Analysis of the time dependence of reaction is also complex. Only the reaction curve for rCrUrcdU is fit by a single exponential. Nevertheless, the time required for the oligomers to reach maximum percentage reaction differs by less than a factor of 10, suggesting the deoxy substitutions do not grossly affect reactivity at 50 mM Mg^{2+} .

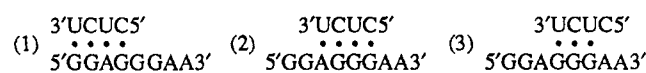
DISCUSSION

The possibility that oligomers mimicking the 5' exon bind unusually tightly to ribozyme was first suggested by Sullivan and Cech (1985) from kinetic data for reaction of dimers and trimers with a covalently closed circular form of the ribozyme, C-IVS. Further studies of this system suggested interactions with the ribozyme provide 4–6 kcal/mol of binding free energy above that expected for base pairing (Sugimoto et al., 1988, 1989a), and that a 2' OH group is responsible for almost 2 kcal/mol of this free energy (Sugimoto et al., 1989b). Kinetic

(Herschlag & Cech, 1990a–c) and gel shift binding (Pyle et al., 1990; Pyle & Cech, 1991) studies of substrate binding by L-21 *ScaI* indicate similar effects with this form of the ribozyme. In this paper, we report the first equilibrium measurements that confirm these findings.

At 5 mM Mg^{2+} /160 mM Na^+ , All L-21 *ScaI* Binds CUCU Tightly, but at 50 mM Mg^{2+} /25 mM Na^+ , Only 30% Binds CUCU Tightly. Except for rCdUrCrU at 50 mM Mg^{2+} /25 mM Na^+ , all the Scatchard plots are relatively linear, so that the number of tight binding sites per L-21 *ScaI*, n , can be obtained from the x -intercept. At 5 mM Mg^{2+} /160 mM Na^+ , n is 1 for both rCrUrCrU and rCrUdCrU. At 50 mM Mg^{2+} /25 mM Na^+ , however, n is 0.3 for rCrUrCrU, dCrUrCrU, rCrUdCrU, and rCrUrcdU. While n is constant, the dissociation constants for these oligomers differ by a factor of 6. This suggests L-21 *ScaI* folds into two different conformations at 50 mM Mg^{2+} /25 mM Na^+ . One conformation binds CUCU tightly and the other does not. Simulations show that if these two conformations were in equilibrium, then linear Scatchard plots with an x -intercept of 1 would be observed. Thus the two conformations are kinetically trapped and do not interconvert during the 3 days required for equilibrium dialysis. All the oligomer substrates react with UCGA so that at least one of the conformations is catalytically active. Walstrum and Uhlenbeck (1990) have shown that a circular form of this ribozyme can be trapped in a slowly reacting conformation when it is purified by denaturing gel electrophoresis. Tanner and Cech (1985) suggested that a linear form of the ribozyme may be present as two slowly interconverting species in 50 mM Mg^{2+} at 42 °C. Downs and Cech (1990) have suggested that photo-cross-linking of A57A95 provides one assay for correct local folding of L-21 *ScaI*. The equilibrium dialysis results suggest Scatchard plots to determine n for various substrates can provide another assay for local folding.

Positioning of CUCU on the Internal Guide Sequence. There are three reasonable possibilities for positioning CUCU on the internal guide sequence, GGAGGGAA:



Site 1 is used in the first step of splicing (Davies et al., 1982; Waring et al., 1983; Been & Cech, 1987), site 2 has not been reported to be reactive, and site 3 is used for cyclization (Been & Cech, 1987; Sugimoto et al., 1988). If CUCU bound primarily to site 3, then the dissociation constants for UCU and CUCU are expected to be similar on the basis of thermodynamic parameters for terminal mismatches and unpaired terminal nucleotides (Freier et al., 1986a; Turner et al., 1988). This is observed for UCU and CUCU binding to C-IVS (Sugimoto et al., 1988). For L-21 *ScaI*, however, the measured dissociation constants for CUCU and UCU differ by a factor of 200, corresponding to a free energy difference of 3 kcal/mol at 15 °C. This suggests site 3 is not the strong binding site. Either site 1 or 2 is consistent with the data, however. They are predicted to give free energy differences of 2.5 and 2.1 kcal/mol, respectively. The reactivity of the substrates indicates they are all able to bind at an active site, though it does not have to be the same as the equilibrium site.

CUCU and UCU Bind to L-21 *ScaI* More Than 1000 Times Tighter Than Expected. The dissociation constant of about 5 nM measured for CUCU binding to L-21 *ScaI* in 50 mM Mg^{2+} /25 mM Na^+ can be compared with the dissociation constant of about 20 μM for CUCU binding to the hexamer, GGAGAA (see Tables I and II). Evidently, at 15 °C, L-21 *ScaI* has additional interactions with CUCU that contribute

Table III: Comparison of $\Delta\Delta G_{15}^\circ$ for Binding to L-21 ScaI and rGGAGAA^a

oligomer	$\Delta\Delta G_{15}^\circ$ relative to rCrUrCrU ^b		$\Delta\Delta\Delta G_{15}^\circ$ ^d (L-21 ScaI-rGGAGAA)
	L-21 ScaI	rGGAGAA ^c	
rCrUrCrU			
dCrUrCrU	-0.26 ± 0.09	-0.71 ± 0.1	0.5 ± 0.15
rCdUrCrU	-1.88 ± 0.05	-0.89 ± 0.1	-1.0 ± 0.1
rCrUdCrU	-1.13 ± 0.05	-0.51 ± 0.1	-0.6 ± 0.1
rCrUrCdU	-0.35 ± 0.05	-0.42 ± 0.15	0.07 ± 0.2

^aSolutions are 50 mM MgCl₂/50 mM HEPES/25 mM Na⁺, pH 7.4, 15 °C. $\Delta\Delta G_{15}^\circ$ values are given in kilocalories per mole. ^b $\Delta\Delta G_{15}^\circ = \Delta G_{15}^\circ$ (all ribo) - ΔG_{15}° (deoxyribo). ^cCalculated from $1/T_M$ vs log ($C_T/4$) parameters. ^d $\Delta\Delta\Delta G_{15}^\circ = \Delta\Delta G_{15}^\circ \text{L-21ScaI} - \Delta\Delta G_{15}^\circ \text{rGGAGAA}$.

about 4.8 kcal/mol in favorable binding free energy above that available from base pairing. While the binding of UCU to GGAGAA is too weak to measure reliably, it can be predicted from the measured value for CUCU and nearest-neighbor parameters for the deleted base pair and new unpaired terminal nucleotide (Freier et al., 1986a). The predicted dissociation constant is 1.7 mM. This is about 1800 times weaker than the measured dissociation constant of 0.95 μ M for UCU binding to L-21 ScaI. This corresponds to an additional 4.3 kcal/mol in favorable binding free energy at 15 °C.

The Middle Two 2' OH Groups Enhance Binding to L-21 ScaI. The results in Table I show that the substitution of deoxyribose for ribose in the oligomers rCrUdCrU and rCdUrCrU increases the dissociation constant relative to rCrUrCrU for the L-21 ScaI oligomer complex by factors ranging from about 7 to 50, depending on position and salt concentration. The largest effects are seen with rCdUrCrU and at 5 mM Mg²⁺/160 mM Na⁺. The free energy increments corresponding to these substitutions range from 1 to 2 kcal/mol and are listed in Table III. Smaller effects are seen with dCrUrCrU and rCrUrCdU, where dissociation constants increase by less than a factor of 4 relative to rCrUrCrU. Evidently, the middle two 2' OH groups of rCrUrCrU enhance binding to L-21 ScaI.

CUCU Binds to GGAGAA as Expected from Base Pairing and Stacking Interactions. The stabilities of short RNA duplexes in solution are predicted well with a nearest-neighbor model (Borer et al., 1974; Freier et al., 1986a). The ΔG_{15}° predicted for the duplex

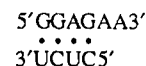


is -6.4 kcal/mol, close to the measured value of -6.2 kcal/mol. The prediction neglects stabilization from the 3' terminal A of GGAGAA. The favorable free energy contribution expected from this second "dangling end" has not been measured but is expected to be less than 0.8 kcal/mol (SantaLucia et al., 1991a; S. M. Freier and D. H. Turner, unpublished experiments). Thus the difference between the predicted and measured ΔG_{15}° 's is within the range expected for the nearest-neighbor model (Kierzek et al., 1986; Freier et al., 1986a). This indicates there is nothing inherently special about this particular base-pairing sequence.

Deoxyribose Substitution for Ribose in CUCU Also Decreases Binding to the Oligomer rGGAGAA. Previous studies of hybrid duplexes formed by deoxyribose and ribose strands indicate a hybrid duplex can be more or less stable than the RNA-RNA duplex, depending on sequence (Chamberlin & Patterson, 1986; Riley et al., 1966; Chamberlin, 1966; Martin & Tinoco, 1980; Hall & McLaughlin, 1991). For example, Hall and McLaughlin (1991) found that the hybrid duplexes

dCACAG-rCUGUG and rCACAG-dCTGTG are both less stable than rCACAG-rCUGUG by about 2 kcal/mol of duplex or 0.3–0.4 kcal/mol of base pair. Inspection of Tables II and III indicates even larger effects are observed for individual substitutions of deoxyribose for ribose in the rCrUrCrU strand of the rGGAGAA-rCrUrCrU duplex. For dCrUrCrU and rCdUrCrU, the free energies of duplex formation are 0.7–0.9 kcal/mol less favorable than for rCrUrCrU. Effects of 0.4–0.5 kcal/mol are observed for rCrUdCrU and rCrUrCdU. The large energy effects observed for deoxy substitutions at the 5' end are surprising and suggest the lower affinity of chimeric deoxy-ribo oligomers to L-21 ScaI may not be due entirely to tertiary interactions with the ribozyme.

There is no obvious reason for the large duplex destabilization observed with dCrUrCrU and rCdUrCrU. The difference in stability of RNA and DNA duplexes is known to be very sequence dependent (Freier et al., 1986a; Breslauer et al., 1986). For example, at 15 °C, the ΔG° for the ribo $\begin{smallmatrix} 5' \text{GA} 3' \\ 3' \text{CU} 5' \end{smallmatrix}$ nearest neighbor is 1.4 kcal/mol more favorable than for the deoxy nearest-neighbor $\begin{smallmatrix} 5' \text{GA} 3' \\ 3' \text{CT} 5' \end{smallmatrix}$. The role of the 2' OH in determining duplex stability is not understood (Saenger, 1984). It has been suggested that the 2' OH group can hydrogen-bond to the O4' of the 3' adjacent sugar (Rabcszenko & Shugar, 1972; Abraham, 1971; Young & Kallenbach, 1978; Jack et al., 1976) or to the 3' phosphate through a water bridge (Bolton & Kearns, 1978, 1979). Either bond would be absent for the 3' terminal 2' OH, consistent with the small effect observed for rCrUrCdU. Furthermore, the terminal GU pair may alter helix geometry enough to eliminate such a bond for rCrUdCrU. Alternatively, stability may be more sensitive to deoxy substitution at the 5' end of CUCU because this is close to the 3' terminal unpaired A's in the duplex



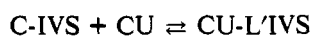
The 3' terminal A's are expected to add 1–2 kcal/mol to duplex stability (Turner et al., 1988; SantaLucia et al., 1991a). This stabilization, however, is sensitive to local geometry. It is much less at the 3' end for DNA helices (Mellema et al., 1984; Senior et al., 1988) and at the 3' ends of RNA helices containing a bulge (Longfellow et al., 1990). Substitution of deoxyribose for ribose may alter this local geometry. A third possibility is that certain 2' OH groups may be particularly important for hydration patterns in the helix groove (Rosenberg et al., 1976; Kim & Jhon, 1979; Drew & Dickerson, 1981; Conner et al., 1982). Clearly it will be necessary to study more model systems to sort out these effects.

Tertiary Interactions between L-21 ScaI and the Middle Two 2' OH Groups of rCrUrCrU Can Enhance Binding by About 1 kcal/mol Each. The results for tetramer binding to hexamer GGAGAA suggest that not all the free energy differences measured between rCrUrCrU and deoxy substituted analogues can be attributed to tertiary interactions with the ribozyme. The difference between effects observed for hexamer and L-21 ScaI binding, however, presumably does provide a measure of ribozyme interactions dependent on the 2' OH groups. These differences are listed in Table III. For rCdUrCrU and rCrUdCrU, these free energy differences are 1 and 0.6 kcal/mol, respectively. These values are similar to ΔG° 's derived for hydrogen bonds in base pairs and internal GA mismatches (Freier et al., 1986b; Turner et al., 1987; SantaLucia et al., 1991a). Thus the results are consistent with hydrogen bonding between the middle two 2' OH groups and L-21 ScaI enhancing binding. A recent three-dimensional model of the self-splicing intron has hydrogen bonds from the 2' OH of the middle C of CUCU to the phosphate of A304

and from the 4' O of the middle U to N2 of G303 (Michel & Westhof, 1990). Our results could be consistent with a hydrogen bond to the 4' O if substitution of deoxyribose for ribose changes the sugar pucker, thus moving the 4' O.

Ribose to Deoxyribose Substitution at the 5' Terminal C of CUCU Affects Binding to L-21 ScaI and GGAGAA Differently. rCrUrCrU and dCrUrCrU bind similarly to L-21 ScaI. The free energy for rCrUrCrU binding the oligomer GGAGAA, however, is 0.7 kcal/mol more favorable than for dCrUrCrU. This difference between ribozyme and oligomer binding cannot be clearly interpreted since the reason for the large effect with GGAGAA is not understood. It is possible, however, that tertiary interactions in the ribozyme are also responsible for the difference. The three-dimensional model of Michel and Westhof (1990) has tertiary hydrogen bonds to the riboses shown as underlined in the internal guide sequence: GGAGGG. Thus hydration and local rigidity in this region may be very different for ribozyme and oligomer.

Comparisons with Sugimoto et al. (1988, 1989a,b). Sugimoto et al. (1988, 1989b) studied the kinetics of the following and related reactions:



Here C-IVS is a circular form of the ribozyme and CU-L'IVS is the linear form obtained by attack of CU at the G414–A16 phosphodiester linkage in C-IVS (Sullivan & Cech, 1985). From the dependence of rate on oligomer concentration, they concluded that a noncovalent intermediate is formed, and that the binding of CU in this intermediate involves 5–6 kcal/mol more favorable free energy than expected for simple helix formation. A fluorescence titration of the binding of ϵ ACUCU to C-IVS indicated the binding was about 4 kcal/mol more favorable than expected (Sugimoto et al., 1989a). Kinetic studies with rCrUrCrU, rUrCrU, rCrU, dCrU, rCdU, and dCdT suggested this additional binding free energy was localized to CU and that almost 2 kcal/mol was due to an interaction involving the 2' OH of C (Sugimoto et al., 1988, 1989b). The equilibrium dialysis and oligomer melting results presented above indicate the binding of rUrCrU and rCrUrCrU to L-21 ScaI is 4–5 kcal/mol more favorable than simple helix formation but that the additional interactions are localized in the trimer UCU. Furthermore, comparison with the oligomer melting results suggests each of the 2' OH interactions for the middle two nucleotides of CUCU may only contribute about 1 kcal/mol. Thus results for the two systems are qualitatively similar but may differ in some details. The differences may reflect the fact that the two forms of the ribozyme use slightly different binding sites for these oligomers. (Been & Cech, 1987; Sugimoto et al., 1988).

Comparisons with Pyle and Cech (1990, 1991). While this manuscript was in preparation, Pyle and Cech (1991) reported a study of mixed ribo–deoxy oligomers binding to L-21 ScaI using a gel shift method (Pyle et al., 1990). Our results are consistent with their main conclusion that, for the oligomer rGGCCCUCU, the 2' OH groups of the underlined U and C are involved in tertiary interactions with L-21 ScaI. In addition, both studies suggest that the free energy change measured when a ribose is replaced by a deoxyribose may not reflect only these tertiary interactions, since changes are also observed for simple duplexes in solution. Thus good agreement is found at 42 °C between the free energy contributions of 2' OH groups to simple helix formation as measured by our melting studies and the preliminary gel retardation studies of Pyle and Cech (1991). Our oligomer melting studies, however, reveal that free energy contributions of 2' OH groups to duplex formation are larger at 15 °C than at 42 °C. After correcting

for these effects (see Table III), we conclude that 2' OH groups may not provide all the extra free energy measured for binding of rCrUrCrU to L-21 ScaI as compared to rGGAGAA.

Thermodynamic parameters for nearest-neighbor interactions of base pairs and terminal mismatches (Freier et al., 1986a,b; Turner et al., 1988; He et al., 1991) can be used to extrapolate our results for rCrUrCrU at 5 mM Mg^{2+} /160 mM Na^+ and 15 °C to expectations at 25 °C for the rGGCCCUCU and rCUCUCU substrates studied by Pyle et al. (1990). Such extrapolations neglect any ΔH° associated with the tertiary interactions of substrate with ribozyme. Such corrections, however, are expected to be small if the thermodynamics of these interactions are similar to those for hydrogen bonds in base pairs (Turner et al., 1987). The binding site is assumed to be GGAG. Thus the ΔH° of -35.74 kcal/mol measured with oligomers was used to extrapolate the dissociation constant of 27 nM for rCrUrCrU at 15 °C to 25 °C. Additional free energies for the pairing and mispairing expected for the longer substrates were then added. For rGGCCCUCU and rCUCUCU, the predicted dissociation constants are 0.002 and 0.3 nM, respectively. The value of 0.002 nM is about 1000 times smaller than the reported value of 2.5 nM for rGGCCCUCU at 5 mM Mg^{2+} . The predicted value of 0.3 nM is somewhat smaller than the reported dissociation constant of 1.6 nM for CUCUCU at 10 mM Mg^{2+} . To see if this disagreement resulted from the extrapolation, equilibrium dialysis experiments were conducted with rCCCUCU and rCUCUCU at 25 °C. [Pyle et al. (1990) report that rGGCCCUCU and rCCCUCU bind similarly.] The measured dissociation constants for rCCCUCU at 5 and 10 mM Mg^{2+} are 0.004 nM. For rCUCUCU at 5 and 10 mM Mg^{2+} , the dissociation constants are 0.1 and 0.04 nM, respectively. The results suggest that the rate assumptions implicit in the experimental protocol for the gel shift measurement are not reasonable at 25 °C. The equilibrium dialysis results for rCCCUCU and rCUCUCU at 25 °C can be extrapolated to 42 °C with nearest-neighbor parameters (Freier et al., 1986a; He et al., 1991). The extrapolated dissociation constants are 0.9 and 5 nM, respectively. These are in reasonable agreement with the values of 1.2 and 22 nM reported by Pyle et al. (1990) at 10 mM Mg^{2+} . The results suggest the gel shift measurement reasonably reflects the equilibrium at 42 °C.

Implications for Design of Antisense Therapeutics. A promising approach to drug design is the use of oligonucleotide analogues to target undesirable RNAs (Zamecnik & Stephenson, 1978; Zamecnik et al., 1986; Uhlmann & Peyman, 1990). One potential problem with this approach is the necessity of using 15–20-nucleotide oligomers to assure specificity. The results of this work show that RNA can also use tertiary interactions to bind substrates. Thus it may be possible to take advantage of tertiary contacts to achieve the desired specificity and binding with relatively short therapeutics. For example, the tertiary interactions reported in this paper must depend on formation of a three-dimensional pocket in the ribozyme. Recognition of rCrUrCrU depends on the nucleotides required to form this pocket, in addition to the GGAG internal guide sequence. This suggests tertiary interactions can be used to design a short substrate that recognizes a three-dimensional shape dependent on many nucleotides in the target sequence but that binds tightly enough to serve as a therapeutic.

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SUPPLEMENTARY MATERIAL AVAILABLE

Three figures showing Scatchard plots at 5 mM Mg²⁺, R-1 plots for UCU, and R-1 plots for CCCUCU and CUCUCU (3 pages). Ordering information is given on any current masthead page.

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Human Substance P Receptor (NK-1): Organization of the Gene, Chromosome Localization, and Functional Expression of cDNA Clones^{†,‡}

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ABSTRACT: The gene for the human substance P receptor (NK-1) was cloned using cDNA probes made by the polymerase chain reaction from primers based on the rat sequence. The gene spans 45-60 kb and is contained in five exons, with introns interrupting at sites homologous to those in the NK-2 receptor gene. Analysis of restriction digests of genomic DNA from mouse/human cell hybrids indicates the NK-1 receptor is a single-copy gene located on human chromosome 2. Polymerase chain reaction using primers based on the 5' and 3' ends of the coding sequence was used to generate full-length cDNAs from human lung and from IM9 lymphoblast cells. When transfected into COS-7 cells, the NK-1 receptor binds ¹²⁵I-BHSP with a K_d of 0.35 ± 0.07 nM and mediates substance P induced phosphatidylinositol metabolism. The receptor is selective for substance P; the relative affinity for neurokinin A and neurokinin B is 100- and 500-fold lower, respectively. Human IM9 lymphoblast cells express relatively high levels of the NK-1 receptor, and Northern blot analysis indicates modulation of mRNA levels by glucocorticoids and growth factors, suggesting that this cell line may be useful as a model for studying the control of NK-1 receptor gene expression.

Over the past several years, it has become increasingly evident that the class of neuropeptides known as tachykinins possess a remarkable array of biological roles in the central and peripheral nervous system as well as in the immune system (Lundberg et al., 1983; Payan et al., 1983; Saria et al., 1983; Coleridge & Coleridge, 1984; Lee et al., 1986; Maggio, 1988). The three mammalian tachykinins, substance P, neurokinin

A (substance K), and neurokinin B (neuromedin K), appear to mediate such diverse processes as transmission of sensory information, smooth muscle contraction, nociception, inflammation, sexual behavior, and possibly wound healing and nerve regeneration (Pernow, 1983; Mantyh et al., 1988, 1989; Skerrett, 1990). The tachykinin receptors have been classified with respect to their preferred ligands in the following order: NK-1, substance P; NK-2, neurokinin A (substance K); NK-3, neurokinin B (neuromedin K). However, because the tachykinins share a common C-terminal amino acid sequence, -Phe-X-Gly-Leu-Met-NH₂, which is essential for biological activity, there is a certain amount of cross reactivity among the receptors and their ligands (Buck et al., 1984; Burcher et al., 1986; Martling et al., 1987). This cross reactivity often confounds studies of the role of the ligand-receptor pairs involved in particular physiological or pathophysiological responses, and at present only pharmacologic methods are available to distinguish between involvement of one or more of the tachykinins.

The molecular characterization of the tachykinin receptors provides a new avenue to study the functions of these molecules. In initial work by Masu et al. (1987), the bovine NK-2 receptor was cloned by expression in *Xenopus* oocytes. The information provided from that cDNA was used to clone

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[‡] The nucleic acid sequence for the human NK-1 receptor gene reported in this paper has been submitted to GenBank under Accession Number M76675.

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