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Oligoribonucleotides containing 2′,5′-phosphodiester linkages exhibit binding selectivity for 3′,5′-RNA over 3′,5′-ssDNA

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ABSTRACT

Oligoribonucleotides containing 2′,5′-phosphodiester linkages have been synthesized on a solid support by the 'silyl-phosphoramidite' method. The stability of complexes formed between these oligonucleotides and complementary 3′,5′-RNA strands have been studied using oligoadenylates and a variety of oligonucleotides of mixed base sequences including phosphorothioate backbones. In many cases, particularly for 2′,5′-linked adenylates, the UV melting profiles are quite sharp and exhibit large hyperchromic changes. Substituting a few 3′,5′-linkages with the 2′,5′-linkage within an oligomer lowers the T_m of the complex and the degree of destabilization depends on the neighboring residues and neighboring linkages. The 2′,5′-linked oligoribonucleotides prepared in this study exhibited remarkable selectivity for complementary single stranded RNA over DNA. For example, in 0.01 M phosphate buffer — 0.10 M NaCl (pH 7.0), no association was observed between 2′,5′-r(CCC UCU CCC UUC U) and its Watson – Crick DNA complement 3′,5′-d(AGAAGGGAGAGGG). However, 2′,5′-r(CCC UCU CCC UUC U) with its RNA complement 3′,5′-r(AGAAGGGAGAGGG) forms a duplex which melts at 40°C. The decamer 2′,5′-r(Ap)$_9$A forms a complex with both poly dT and poly rU but the complex [2′,5′-r(Ap)9A]:[poly dT] is unstable (T_m, −1°C) and is seen only at high salt concentrations. In view of their unnatural character and remarkable selectivity for single stranded RNA, 2′,5′-oligo-RNAs and their derivatives may find use as selective inhibitors of viral mRNA translation, and as affinity ligands for the purification of cellular RNA.

INTRODUCTION

The synthesis of oligonucleotide analogs has been of intense interest not only from the structural point of view but also because of their utility as effective gene regulatory substances (the antisense oligonucleotides) (1). The most extensively studied oligonucleotide analogs are those containing modified phosphate linkages. These include the oligonucleoside methylphosphonates (2), phosphorothioates (3), and alkylphosphoramidates (4). Several other oligonucleotides in which the phosphate (1, 5–9), or sugar moiety (1, 10–12) has been replaced altogether have also been described. Interest in such analogs is primarily a consequence of their resistance to degradation by nucleases and favorable hybridization properties with complementary DNA and RNA. Relatively few examples of oligoribonucleotide analogs were reported in earlier oligonucleotide work due in part to the inherent difficulties of RNA synthesis. This is changing rapidly in view of the recent availability of an efficient method for automated construction of RNA sequences (13, 14). RNA analogs with a wide range of applications have recently been reported. These include t-RNA's (14–16), RNA phosphorothioates (17), 2′-O-alkyl RNA (18), 'mixed' DNA/RNA ribozymes (19, 20), branched oligo-RNA (21), and base-modified RNA analogs (22).

Very little is known about RNA analogs containing the unnatural 2′,5′-phosphodiester linkage (Scheme 1). However, from the very few examples found in the literature the potential benefits of such molecules as antisense therapeutics are clearly evident. In 1967, Michelson and Monny demonstrated that short 2′,5′-linked oligoadenylates form stable complexes with poly rU, although in comparison with 3′,5′-linked oligoadenylates, their T_m's were slightly lower (23). Ts'o and co-workers subsequently showed that 2′,5′-linked r(ApA) forms a complex with poly rU having the same stoichiometry (1A:2U) as the complex of 3′,5′-ApA with poly rU (24). The few data available on dimers and trimers also indicate that the 2′,5′-phosphodiester linkage is stable to a few hydrolytic nucleases, although it can be cleaved under basic conditions owing to nucleophilic attack of the neighboring 3′-alkoxide on the phosphodiester (25). More recently, three reports describing the preparation and hybridization properties of 2′,5′-linked oligo-DNA (26, 27) and RNA (28) have appeared. However, neither of these studies were directed to the problems of antisense oligonucleotides; for instance, binding to a target 3′,5′-linked RNA, or DNA, was not considered. In this work, we describe the synthesis of a variety of 2′,5′-linked oligoribonucleotides having homo and 'mixed' base sequences, and in some cases, a phosphorothioate backbone. The results demonstrate that these analogs not only

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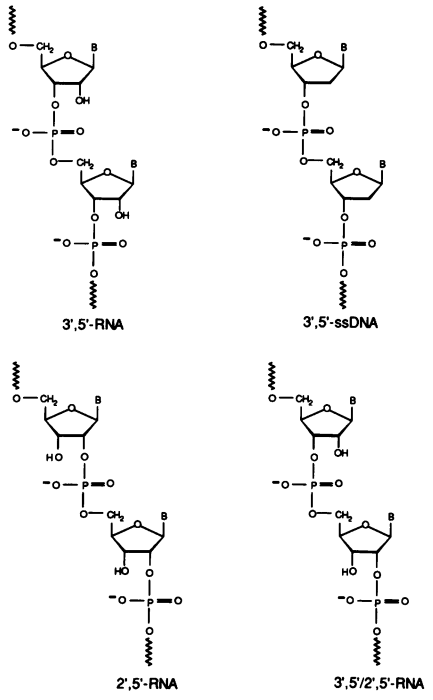

Scheme 1. Structure of 2',5'-, 3',5'- and 3',5'/2',5'-linked oligoribonucleotides.

form stable complexes with complementary RNA, but they also exhibit marked binding selectivity for RNA over single stranded DNA (ssDNA). Preliminary accounts on our findings have appeared (29).

EXPERIMENTAL

Reagents

Reagents for solid-phase syntheses including tetraethylthiuram disulfide (TETD, sulfurizing reagent) and DNA (cyanoethyl) phosphoramidite monomers were obtained from Applied Biosystems (Toronto). Silylated ribonucleoside 3'-(cyanoethyl)phosphoramidites used in the preparation of 3',5'-linked oligo-RNA were obtained from Dalton Chemical Laboratories, Inc. (Toronto). Long chain alkylamine controlled-pore glass (LCAA-CPG) bearing deoxyribonucleosides were obtained from Applied Biosystems. LCAA-CPG containing ribonucleosides were prepared as described previously (30). Tetra-*n*-butylammonium fluoride (1 M) in THF (desilylating reagent) and diethylpyrocarbonate were purchased from Aldrich. Reversed-phase C_{18} SEP-PAK® cartridges were obtained from Waters (Milford, Massachusetts). Polyacrylamide gel electrophoresis (PAGE) reagents were purchased from Bio-Rad (Toronto). HPLC grade acetonitrile was purchased from Caledon (Toronto).

3'-O-*t*-Butyldimethylsilyl ribonucleoside 2'-O-N,N'-diisopropyl(methyl) phosphoramidite monomers (U, C^{Bz} and A^{Bz}) were prepared according to literature procedures (31). The isomeric purity of these monomers was established by comparison with the known 2'-silylated ribonucleoside 3'-phosphoramidite regioisomers using TLC analysis, ^1H and ^{31}P-NMR (14, 31).

Solid-phase synthesis of oligonucleotides

Synthesis of DNA, 3',5'-RNA and 2',5'-RNA oligomers was carried out on an Applied Biosystems DNA 381A synthesizer. For the assembly of oligonucleotides incorporating phosphorothioate internucleotide linkages, the bottle on port # 15 (iodine/water) of the synthesizer was changed for one containing TETD prior to synthesis. After the coupling and capping steps, the CPG beads were allowed to react with TETD for 10 min and then washed extensively with acetonitrile. Average coupling yields (trityl assay method) for ribonucleoside 2'- (and 3'-) phosphoramidite couplings were in the range of 95–98%. The 'trityl on' DNA oligomers were deblocked by the standard protocol and purified by reversed-phased chromatography using reversed-phase OPC® cartridges (Applied Biosystems). 3',5'-and 2',5'-RNA oligomers were synthesized in the 'trityl off' mode as described elsewhere (14, 32). 2',5'-RNA oligomers were deprotected by treating the CPG-bound oligomer successively with thiophenol/triethylamine/dioxane (1:2:2:, vol/vol/vol; 30 min, r.t.), conc. NH_4OH/ ethanol (3:1, 55°C, 16 h), and TBAF (1.0 M in THF, r.t., 16 h) (14). 3',5'-RNA oligomers (prepared from cyanoethyl amidites) did not required the thiophenoxide step and were deprotected as described (32). Following deprotection, the 3',5'- and 2',5'-linked oligoribonucleotides were purified by PAGE and desalted by reversed-phase chromatography on C_{18} SEP-PAK® cartridges (Waters) (32). The sequences prepared are listed in Table 1.

Polyacrylamide native gel hybridizations

Equimolar quantities (3.0 μmols) of complementary oligomers were dissolved in 7.0 μL of 30% sucrose/10 mM $MgCl_2$ and initially incubated at 40°C for 30 min followed by 20 min at 20°C and a further 12 hours at 4°C. The samples were applied to a 24% polyacrylamide (without urea) and run at 4°C for 18–24 h at a current of 5 mA. Following electrophoresis, the gels were wrapped in plastic wrap, placed over a fluorescent TLC plate and illuminated with a UV lamp (254 nm). The gels were photographed using Polaroid PolaPlan® 4×5 cm Instant Sheet Film (#52, medium contrast, ISO 400/27°) through Kodak Wratten gelatin filter (#58 green).

Enzyme assays

Enzymes. These were obtained as from Boehringer Mannheim (Quebec) except as indicated. (i) RNase T_2 from *Aspergillus ozyrae* (Sigma) was obtained as a dry powder and dissolved in water to a concentration of 2 units/μL. (ii) Nuclease P1 from *Penicillium citrinum* was obtained as a lyophilized powder and prepared as a 1 mg enzyme/mL solution in 30 mM ammonium acetate, *ca.* pH 5.3. (iii) Calf-spleen phosphodiesterase (CSPDE) was obtained as a suspension (2 mg/mL) in 3.2 M ammonium sulfate, pH *ca.* 6.0. (iv) Snake venom phosphodiesterase (SVPDE) from *Crotalus durissus* was obtained as a solution of 2 mg/mL in 50% (v/v) glycerol, pH *ca.* 6. (v) Nuclease S_1 from *Aspergillus oryzae* was obtained as a solution in 50% glycerol (v/v), 20 mM Tris–HCl, 50 mM NaCl, 0.1 mM zinc chloride, pH *ca.* 7.5. (vi) Alkaline phosphatase (AP) from calf intestine was obtained as a suspension 2 mg/mL in $(NH_4)_2SO_4$ (3.2 M); $MgCl_2$ (1 mM); $ZnCl_2$ (0.1 mM), pH *ca.* 7.0. (vii) Adenosine deaminase (ADA) from calf-intestine was obtained as a 50% glycerol solution, pH *ca.* 6.

Incubation buffers. Incubation buffers for the enzyme digestions were prepared from autoclaved water and filtered through a sterile 0.2 μm membrane (Acrodisc® , Gelman Sciences). Buffers: (i) RNase T2, 0.05 M NH_4OAc, 2 mM EDTA, pH 4.5. (ii) Nucl. P1, 50 mM NH_4OAc, pH 5.3. (iii) CSPDE, 0.5 M $(NH_4)_2SO_4$,

pH *ca.* 6.0. (iv) SVPDE, 50 mM Tris−HCl, 10 mM $MgCl_2$ pH 8.0. (v) Nucl. S1, 50 mM NaOAc, 1 mM $ZnSO_4$, 250 mM NaCl, 50 μg/mL BSA, adjusted to pH 4.6 with AcOH.

Enzymatic hydrolysis and analyses. Typically, 0.3 A_{260} units of oligonucleotide was dissolved in *ca.* 20 μL incubation buffer to which the appropriate enzyme(s) was added 2 μL RNase T_2 (4 U), or 5 μL Nucl. P1 (1.5 U), or 5 μL CSPDE (0.02 U), or 2 μL SVPDE (0.006 U), or 0.2 μL Nucl. S1 (0.02 U), 2 μL AP (36 U), 5 μL ADA] and incubated at 37°C. HPLC analyses of the enzyme digestions were performed on a Waters instrument equipped with dual 501 pumps, UK6 injector, and a 480 UV detector governed by a gradient controller being output through a 740 Data Module. The column employed was a reversed-phase Whatman Partisil ODS-2 (10 μm, 4.6×250 mm) with a linear gradient 0−50% in B over 30 minutes (solvent A: 20 mM KH_2PO_4 pH 5.5, solvent B: methanol) and 2 mL/min flow rate at room temperature. Injections of 5 μL (0.07 A_{260} units) were found to be adequate for determinations. Products were easily separated and identified in most cases by co-injection with authentic samples of nucleosides and nucleotides. For PAGE analyses, the entire amount was loaded on a 1-cm well of a non-denaturing 16% polyacrylamide gel. The gel was then viewed by UV-shadowing as described above.

UV spectral and T_m measurements

Molar extinction coefficients for 2′,5′ and 3′,5′-linked oligomers were estimated using the nearest-neighbor approximation (33). The base concentrations of poly dT and poly rU were determined by first measuring the absorbance (260 nm) of a given amount of each polymer at 95°C where base stacking is minimal. Using the Beer Lambert law and the extinction coefficient of the component nucleotides (ε, dT = 8.70×10^{-3} M^{-1} cm^{-1}; ε, rU = 10.21×10^{-3} M^{-1} cm^{-1}), the number of base residues in the aliquoted solution was estimated. One could then prepare the appropriate mixtures of polymers with oligomers *on a per base basis*, 1Pu:1Py or 1Pu:2Py.

Thermal denaturations were performed on a Varian-Cary 1 UV/Vis spectrophotometer equipped with a Peltier type thermal unit and a six cell transport mechanism. UV cells containing oligomers were heated to 45°C for 10−20 min and cooled slowly to the start temperature over a 20−30 min period. The absorbance at 260 nm was measured at 0.5°C intervals in 1-cm (1.5 mL) cuvettes with a temperature ramp of 0.5°C/min. Normally, 30 absorbance readings were obtained at each temperature and averaged to improve the signal-to-noise ratio. The data was analyzed with the software provided by VARIAN/CARY and transferred to the spreadsheet package Quattro Pro Γ for presentation. For comparison purposes, the curves are presented in normalized absorbance (A_T/A_L, where A_T = absorbance at temperature T, and A_L = absorbance at initial temperature) versus temperature. T_m's were calculated from first derivative plots of absorbance versus temperature. Hyperchromicity (%H) values of the transitions are reported as the percent increase in absorbance at 260 nm with respect to the initial absorbance. UV mixing curves were obtained by the method of continuous variation (34) as described by Pilch *et al.* (35).

Hypochromicity measurements

An aliquot of the oligomer (0.30 A_{260} units) was dissolved in the indicated buffer (Table 2) and the solution was transferred to a 1-cm path length UV cell. For condition C the solution was slowly heated to 80°C over 2 hours, cooled to 37°C over a period of 1 h and incubated at 37°C for 10 min prior to enzyme addition. For conditions A and B the solutions were only incubated at 37°C for 10 min. Snake venom phosphodiesterase (5 μL) was then added to the cell and the absorbance measurements were taken at 260 nm every minute, inverting the cell after each absorbance reading. Typically, the absorbance reading increased sharply within the first five minutes and leveled-off during the next 30 minutes. Complete digestion, as indicated by no further increase in absorbance, was observed for all digestions within 40 min. Hypochromicity (H′) is reported as $(A_{(monomers)}-A_{(ologomer)})/A_{(monomers)}\times100$.

RESULTS AND DISCUSSION

Synthesis and characterization of 2′,5′-linked oligomers

The protocol of Ogilvie and co-workers (13, 14), originally developed for the solid-phase chemical synthesis of normal 3′,5-linked ribonucleotides, was adapted for the preparation of 2′,5′-linked oligomers. The only difference between the preparation of 2′,5′-linked RNA and that of 3′,5′-linked RNA was the use of nucleoside-2′-O-phosphoramidites (31) instead of the isomeric nucleoside-3′-O-phosphoramidites. All other aspects of chain assembly, deprotection procedures, and handling remained invariant (32). For the preparation of sequences containing phosphorothioate linkages (Table 1), the sulfurizing reagent tetramethylthiuram disulfide (36) was used in place of the iodine oxidizing reagent. To evaluate the effect of 3′,5′ to 2′,5′ substitution on complex stability, we also constructed a series of oligomers containing alternating 2′,5′ and 3′,5′-phosphodiester linkages. Twenty-one oligomers were prepared for this study and their base sequences are shown in Table 1.

As confirmatory evidence of the 2′,5′-connectivities, each deprotected oligomer was subjected to digestion with various hydrolytic enzymes. Treatment of 2′,5′-r$(Ap)_9$A, 2′,5′-r$(Up)_9$U and 2′,5′-r$(Cp)_9$C with RNase T_2 (30 min, 37°C) resulted in no observable degradation whereas under similar conditions the analogous 3′,5′-linked oligonucleotides were completely degraded to nucleoside monophosphate (HPLC, PAGE). Additionally, we observed no hydrolysis of 2′,5′-r$(Up)_9$A or 2′,5′-r$(Cp)_9$C by either Nuclease P1 (an endonuclease) or calf-spleen phosphodiesterase (a 5′-exonuclease), confirming the 2′,5′-connectivity of the phosphodiester linkages. Snake-venom phosphodiesterase, a 3′-exonuclease which is known to cleave both 3′,5′- and 2′,5′-phosphodiester bonds, completely degraded 2′,5′-r$(Ap)_9$A, 3′,5′/2′,5′-r$(Ap)_9$A, 2′,5′-r$(Up)_9$U and 2′,5′-r$(Cp)_9$C to nucleoside 5′-monophosphates (HPLC). Nuclease S1 hydrolyzed 2′,5′-r$(Ap)_9$A to the extent of 50% after 30 min at 37°C whereas 3′,5′-r$(Ap)_9$A was completely hydrolyzed within 5 min under similar conditions. Digestion with (nuclease P1/adenosine deaminase [ADA]/alkaline phosphatase [AP]) or (RNase T_2/ADA/AP) to 2′,5′-ApA and inosine confirmed the incorporation of alternating linkages in 3′,5′/2′,5′-r$(Ap)_9$A.

Base stacking in 2′,5′-linked RNA and 3′,5′-linked RNA

Studies reported in the literature addressing the differences and the extent of base stacking interactions between oligonucleotides containing 2′,5′ linkages and those containing 3′,5′-linkages have yielded mixed results. For example, spectroscopic studies on the conformation of dinucleoside monophosphates indicate that

Table 1. Oligonucleotides prepared for binding studies

Oligonucleotide Sequences	Designation
Normal 3',5'-linked oligomers	
5'-r(AAA AAA AAA A)	3',5'-r(Ap)$_9$A
5'-r(UUU UUU UUU U)	3',5'-r(Up)$_9$U
5'-d(AAA AAA AAA A)	3',5'-d(Ap)$_9$A
5'-d(TTT TTT TTT T)	3',5'-d(Tp)$_9$T
5'-r(CCC UCU CCC UUC U)	3',5'-r(U/C)-13-mer
5'-r(AGA AGG GAG AGG G)	3',5'-r(A/G)-13-mer
5'-d(AGA AGG GAG AGG G)	3',5'-d(A/G)-13-mer
5'-r(GCG UUU UUU GCU)	1
5'-d(GCG TTT TTT GCT)	2
5'-r(AGC AAA AAA CGC)	3
5'-d(AGC AAA AAA CGC)	4
Entirely 2',5'-linked oligomers*	
5'-r(A*A*A*A*A*A*A*A*A*A)	2',5'-r(Ap)$_9$A
5'-r(U*U*U*U*U*U*U*U*U*U)	2',5'-r(Up)$_9$U
5'-r(C*C*C*U*C*U*C*C*C*U*U*C*U)	2',5'-r(U/C)-13-mer
5'-r(As*As*As*As*As*As*As*As*As*A)	2',5'-r(As)$_9$A
Alternating 3',5'/2',5'-linked oligomers	
5'-r(AA*AA*AA*AA*AA)	3',5'/2',5'-r(Ap)$_9$A
5'-r(AsAs*AsAs*AsAs*AsAs*AsA)	3',5'/2',5'-r(As)$_9$A
5'-r(UU*UU*UU*UU*UU)	3',5'/2',5'-r(Up)$_9$U
5'-r(GCG U*UU* UU*U GCU)	5
5'-r(AGC A*AA* AA*A CGC)	6
5'-r(AGC AA*A A*AA* CGC)	7

All linkages are 3',5'-phosphodiesters except as indicated: '*' 2',5'-phosphodiester linkage, 's' 3',5-phosphorothioate linkage,'s*' 2',5-phosphorothioate linkage.

Table 2. Hypochromicity (%H') data of oligoriboadenylates

Oligomer	%H'			
	A	B	C	D
3',5'-r(Ap)9A	29	32	27	22
3',5'/2',5'r(Ap)9A	30	31	30	22
2',5'-r(Ap)9A	30	31	31	24

Oligomes were treated with 5 μL of SVPDE at 37°C and the digestion followed by UV at 260 nm to completion. Conditions; A, Snake venom buffer, 800 μL; B,10 μL SVPDE buffer in 790 μL of water; C, 10 μL SVPDE buffer in 790 μL of 100 mM NaCl pH 7.0; oligomers were incubation at 80°C prior to enzyme addition; D, 10 μL SVPDE buffer in 790 μL of 100 mM NaCl pH 7.0 without heating to 80°C prior to enzyme addition;
Hypochromicity = $[(A_{monomer} - A_{oligomers})/A_{monomer}] \times 100$.

2',5'-linked fragments base stack more readily than the corresponding 3',5'-linked isomers (24). In contrast, studies on homo-sequences indicated that the degree of stacking in 3',5'-linked oligomers exceeded that found in 2',5'-linked oligomers (37). Similarly, the mixed base hexamer 3',5'-(AA-CCUU) exhibited increased stacking relative to its 2',5'-analog (38). We have determined the extent of stacking using UV thermal denaturation and enzyme digestion analysis of riboadenylates differing only in the identity of the linkages. We obtained hypochromicities of 4% and 5%, respectively, when heat denaturing 3',5'-r(Ap)$_9$A or 2',5'-r(Ap)$_9$A in 100 mM NaCl and 8% for either sequence in 1 M NaCl. Similarly, in either 100 mM or 1 M NaCl, both 3',5'-r(Up)$_9$U and 2',5'-r(Up)$_9$U exhibited the same hypochromicity of about 1%. These results suggest that under the above conditions there is no significant difference in the base-stacking interactions between the isomerically linked molecules. We also used snake venom phosphodiesterase to digest 3',5'-r(Ap)$_9$A and 2',5'-r(Ap)$_9$A to monomers as the resulting hyperchromic effect is a better measure of the full extent of base stacking interactions. The results listed in Table 2 indicate that there is no significant difference in the

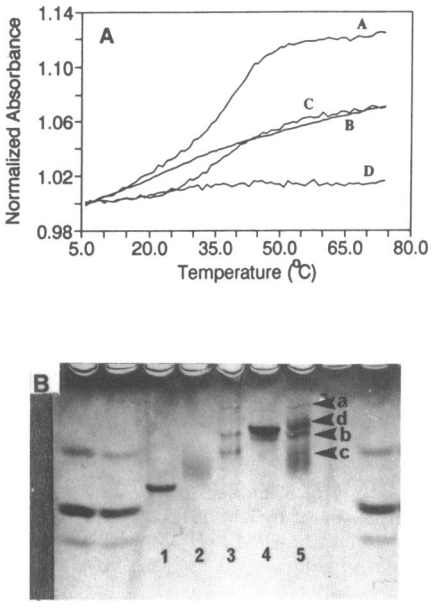

Figure 1. A) Thermal melting curves in 100 mM NaCl, 10 mM Na_2HPO_4, pH 7.0, for the complex [2',5'-r(U/C)-13-mer]:[3',5'-r(A/G)-13-mer] (T_m = 40°C) (A) and [2',5'-r(U/C)-13-mer] : [3',5'-d(A/G)-13-mer] (**B**). The single strands 3',5'-r(A/G)-13-mer (**C**); and 2',5'-r(U/C)-13-mer (**D**) are also shown. The complex [3',5'-r(U/C)-13-mer] : [3',5'-d(A/G)-13-mer] was also observed, (800 mM NaCl, T_m = 53°C with 15%H, not shown) B). Non-denaturing PAGE (24%) hybridizations. Lanes: 1, 3',5'-r(UC)-13-mer; 2, 2',5'-r(U/C)-13-mer; 3, 3',5'-r(A/G)-13-mer; 4, [3',5'-r(U/C)-13-mer] : [3',5'-r(A/G)-13-mer]; 5, [2',5'-r(U/C)-13-mer] : [3',5'-r(A/G)-13-mer].

degree of stacking within the adenylates. When the digestions were carried out in buffers containing 100 mM sodium chloride (C and D) we did observe a lower%H' value for 3',5'-r(Ap)$_9$A relative to 2',5'-r(Ap)$_9$A suggesting a somewhat lower degree of stacking in the former oligomer. This is in agreement with the work of Kondo *et al.* (24c) in which alkaline hydrolysis was used to show that base stacking in 2',5'-ApA was greater than that in 3',5'-ApA (15.9 vs 11.9%H').

Association of 2',5'-r(CCC UCU CCC UUC U) with Complementary RNA and ssDNA

Equimolar amounts of 2',5'-linked r('-CCC UCU CCC UUC U-3') [abbreviated 2',5'-r(U/C)-13 mer] and complementary 3',5'-linked r(3'-GGG AGA GGG AAG A-') [abbreviated 3',5'-r(A/G)-13 mer] were annealed in phosphate buffered sodium chloride solution (pH 7.0). At 100 mM NaCl, 2',5'-r(U/C)-13-mer hybridized to 3',5'-r(A/G)-13 mer, as the resulting complex exhibited a hyperchromic effect and a T_m of 40°C (curve A, Figure 1A). At 800 mM NaCl, the T_m of the complex [2',5'-r(U/C)-13-mer] : [3',5'-r(A/G)-13-mer] was 47.7°C compared with 77.0°C for the regular complex [3',5'-r(U/C)-13-mer] : [3',5'-r(A/G)-13-mer], suggesting that annealing of 2',5'-RNA to 3',5'-RNA is thermodynamically less favorable than annealing of 3',5'-RNA to 3',5'-RNA. Such a conclusion should take into account the fact that the 3',5'-r(A/G)-13 mer is able to self-associate (curve C, Figure 1A), thus complicating the interpretation.

The association between 2',5'-r(U/C)-13-mer and 3',5'-r(A/G)-13-mer was reinforced by a mobility shift polyacrylamide gel electrophoretic assay. As shown in Figure

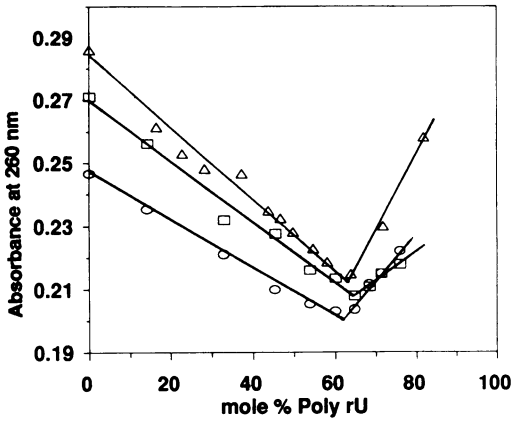

Figure 2. Stoichiometry of interaction for oligoriboadenylates and poly rU. Stoichiometry was determined by Job's method of continuous variation (34, 35) at 100 mM NaCl, 10 mM Na_2HPO_4, pH 7.0, with 40 min equilibration time. Legend: (O) [3',5'-r(Ap)$_9$A]:[poly rU], (Δ) [2',5'-r(Ap)$_9$A]:[poly rU], and (□) [3',5'/2',5'-r(Ap)$_9$A]:[poly rU].

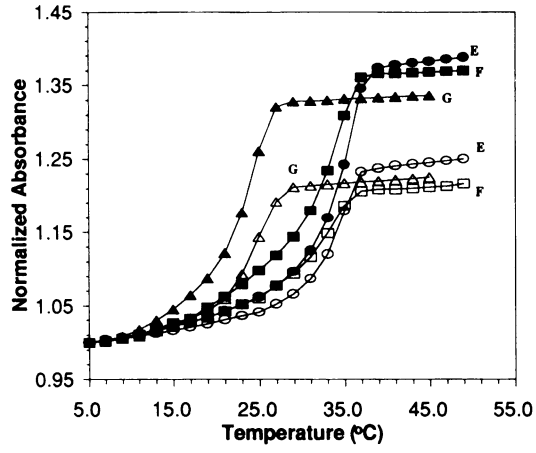

Figure 3. Interaction of 3',5'-r(Ap)$_9$A (E), 3',5'/2',5'-r(Ap)$_9$A (F), and 2',5'-r(Ap)$_9$A (G) with poly rU in 100 mM NaCl, 10 mM Na_2HPO_4, pH 7.0. See Table 3 for T_m and %H values of complexes. Thermal melt curves with filled symbols were run with 1A:2U stoichiometry and curves with open symbols were run with 1A:1U stoichiometry.

1B, 3',5'-r(A/G)-13-mer alone appeared as three bands (**a**, **b** and **c**) confirming its self-association (only one band was observed under denaturing [urea] PAGE conditions, ruling out the possibility of contamination). Mixing equimolar amounts of 2',5'-r(U/C)-13-mer and 3',5'-r(A/G)-13-mer gave an equilibrium mixture of the 2',5'/3',5' complex (**b** and **d**) and its component strands (lane 5). On the other hand, association of 3',5'-r(U/C)-13-mer and 3',5'-r(A/G)-13-mer was quantitative as evidenced by the complete disappearance of the component purine and pyrimidine strands (lane 4, Figure 1B). Interestingly, no cooperative association between 2',5'-r(U/C)-13-mer and its complementary DNA oligomer could be observed (curve B, Figure 1A). The marked selectivity of this oligomer for RNA over ssDNA is also a characteristic of other 2',5'-linked oligomers studied here (*vida infra*).

Association of 2',5' and 3',5' linked oligoadenylates with poly rU and poly dT

In order to evaluate the effect of the 2',5'-phosphodiester linkage on complexation with natural nucleic acids and the selectivity for RNA, we studied the association of 3',5'-r(Ap)$_9$A and 2',5'-r(Ap)$_9$A with poly rU and poly dT. The stoichiometry of interaction between 3',5'-linked adenylic and 3',5'-linked uridylic acid has been shown to be 1A:1U or, 1A:2U at neutral pH in low to moderate monovalent cation concentrations (39) and in one report 2A:1U (40). The stoichiometry of interaction between 2',5'-oligoribonucleotides with RNA an ssDNA is not known; however, the dinucleotide 2',5'-r(ApA) forms a complex with poly rU having the same stoichiometry (1A:2U) as the complex of 3',5'-r(ApA) with poly rU (24). In this study, the stoichiometry of association between each of the oligoadenylates 3',5'-r(Ap)$_9$A, 3',5'/2',5'-r(Ap)$_9$A and 2',5'-r(Ap)$_9$A with complementary poly rU was determined to be 1A to 2U in 100 mM NaCl (Figure 2). The 1A:2U stoichiometry for these complexes was further supported by the hyperchromic rise observed in the thermal melts. For example, in each case, a significant increase in the absorbance (14%) was observed when the adenylate:poly rU ratio was increased from 1A:1U to 1A:2U (Figure 3, Table 3). This increase in absorbance contributed only to the cooperative portion of the melting curves without changing the T_m, the slope of the upper base-line and the overall monophasic appearance of the curves. Consequently, the increase in hypechromicity observed is likely due to the additional stacking interactions of the second uridylic acid strand in the triplex.

There are some similarities between the complex formed by poly rU with 2',5'-r(Ap)$_9$A and that formed with 3',5'-r(Ap)$_9$A. For example, T_m values for both complexes increased linearly with an increasing value of log [Na^+] (data not shown). Also, the slopes of T_m versus log [Na^+] were found to be essentially identical (*ca.* 23°C), which indicates that the single-strand to complex equilibrium in each case is similarly influenced by the Na^+ ion concentration. The kinetics of association of 2',5'-r(Ap)$_9$A with poly rU was also found to be similar to that of 3',5'-r(Ap)$_9$A with poly rU. When sufficient time was allowed for equilibration (40 min) the stoichiometry of both complexes was clearly 1A:2U. However, when the equilibration time was significantly reduced from 40 to 2 min, the end-point of the titration curves ('stoichiometry') were found to be identically dependent on the order of addition of the component strands. Under these non-equilibrium conditions, the stoichiometries were 1A to 2U when the adenylates was added to a fixed concentration of poly rU, and 1A to 1U when poly rU was added to the adenylates (data not shown). Slow kinetics is a well known phenomenon for triplex formation in 3',5'-linked nucleic acids (41) and has also been observed for 2',5'-r(ApA) and 3',5'-r(ApA) in forming 1A:2U complexes with poly rU (24b). We can now extend the known slow kinetics of triplex formation to include the complexes [3',5'-r(Ap)$_9$A] : 2[poly rU] and [2',5'-r(Ap)$_9$A] : 2[poly rU].

By comparing the thermal denaturation of the complexes [2',5'-r(Ap)$_9$A] : [poly rU] (Figure 3) and [2',5'-r(Ap)$_9$A] : [poly dT] at 1 M NaCl (Figure 4), it is clear that the former is significantly more stable (T_m 54 vs −1°C, Table 3) (42). At lower salt concentration (100 mM), only the [2',5'-RNA] : [poly rU] complex was detected. This is in contrast to the normal complexes [3',5'-r(Ap)$_9$A] : [poly rU] and [3',5'-r(Ap)$_9$A] : [poly dT] which were detected at both 100 mM and 1.0 M NaCl (Table 3).

Table 3. T_m[a] and %H[b] values for oligonucleotide complexes with poly rU and poly dT.

Oligomer	poly rU			Poly dT		
	Curve (Fig. 3)	*Tm* (%H) 0.1 M (2U:1A)	*Tm* (%H) 0.1 M (1U:1A)	*Tm* (%H) 1.0 M (1U:1A)	Curve (Fig. 4)	*Tm* (%H) 1.0 M (2T:1A)	*Tm* (%H) 1.0 M (1T:1A)
3',5'-r(Ap)$_9$A	E	36.1 (38)	35.0 (25)	65.2 (38)	H	37.1 (30)	38.0 (25)
3',5'/2',5'-r(Ap)$_9$A	F	33.9 (34)	33.2 (20)	62.3 (36)	I	22.4 (28)	21.7 (24)
2',5'-r(Ap)$_9$A	G	24.3 (34)	24.7 (20)	54.1 (18)	J		-1 (19)
2',5'-r(As)$_9$A		21.6 (12)	21.9 (7)	50.0 (21)			< -1 (3)
3',5'/2',5'-r(As)$_9$A		9.8 (7)	--- (3)	--- (18)			< -1 (3)
3',5'-d(Ap)$_9$A		31.6 (28)	31.3 (15)	65.3 (40)			55.4 (42)

[a]T_m values were obtained by the first derivative method and the values are given in °C (±0.5). [b]The hyperchromicity values (% H shown in parentheses) are given relative to the initial absorbance value for each curve, respectively. '---' T_m not observed. Purine:pyrimidine strand ratios (Pu:Py) and Molar (M) concentration of Na^+ are given. Solutions contained 10 mM Na_2HPO_4 (pH 7.0).

Due to their low T_m's, we were unable to determine the stoichiometry of the complexes formed by the adenylates and poly dT. However, it is likely that the stoichiometry of these complexes is 1A:1T since there was no significant increase in the hyperchromicity of the T_m curves when the strand ratios were changed from 1A:1T to 1A:2T (Table 3). Pilch *et al.* have shown that, under conditions similar to ours (i.e., 100 mM NaCl), the oligonucleotides $(dA)_{10}$ and $(dT)_{10}$ also form a 1A:1T complex (35).

In comparison with 2',5'-r(Ap)$_9$A, the phosphorothioate 2',5'-r(As)$_9$A exhibited only a slightly lower T_m with poly rU, suggesting similar hybridization affinities (Table 3). For the 1A:2T mixtures of 2',5'-r(As)$_9$A and 3',5'/2',5'-r(As)$_9$A with poly dT, there was no measurable T_m and a hyperchromicity of only 3%H was observed. This was expected since the all-phosphate complex [2',5'-r(Ap)$_9$A] : [poly dT] exhibited a T_m of only −1°C under the same conditions (Table 3). It would appear that incorporation of P=S for P=O linkages in 2',5'-linked RNA destabilizes the complexes formed as they do in 3',5'-linked RNA (43).

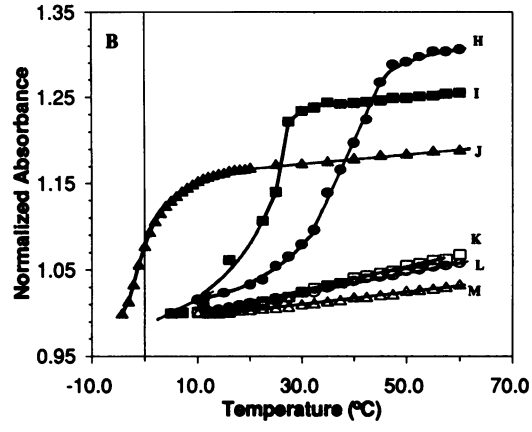

Figure 4. Thermal melt curves of [oligoriboadenylates]:poly dT complexes in 1M NaCl, 10 mM Na_2HPO_4, pH 7.0. Interaction of 3',5'-r(Ap)$_9$A (H), 3',5'/2',5'-r(Ap)$_9$A (I), and 2',5'-r(Ap)9A (J) with poly dT. See Table 3 for T_m and %H values of complexes. Single strands 2',5'-r(Ap)$_9$A (K), 3',5'-r(Ap)$_9$A (L), and 3',5'/2',5'-r(Ap)$_9$A (M) are also shown.

Effect of 3',5' to 2',5' substitutions on helix stability

It was of interest to determine whether an oligoribonucleotide of mixed base sequences containing both types of linkages (2',5' and 3',5') forms stable structures with RNA and ssDNA, and to what extent 3',5' to 2',5' linkage substitution affects complex stability. In either 100 mM or 1 M NaCl, a complex is formed between a decaadenylate with alternating 2',5'- and 3',5'-linkages [i.e., 3',5'/2',5'-r(Ap)$_9$A] and poly rU (Table 3) (44). This complex has a T_m of intermediate value relative to the corresponding ['all'-3',5'] : [3',5'] and the ['all'-2',5']: [3',5'] complexes. Plots of T_m vs the number of 2',5'-linkage substitutions (Figure 5) are linear indicating that each substitution has a constant effect on T_m (−1.3°C/2',5'-linkage). For the interactions between the oligoadenylates and poly dT in 1 M NaCl, there was a larger progressive decrease in T_m as the internucleotide connectivities within the adenylates were changed from exclusively 3',5', to alternating 3',5'/2',5', to exclusively 2',5' (Figure 5, Table 3). The significantly steeper slope for the poly dT line (−4.2°C/2',5'-linkage) suggests that substitution with 2',5'-linkages destabilizes binding to RNA less than to ssDNA. One must be cautious in making this conclusion, however, because ΔΔG° can manifest itself as different ΔT_m's depending on the enthalpy of the transition (ΔH°) (45).

A series of 3',5'/2',5'-decaribonucleotides of mixed base composition were also studied. The results shown in Table 4 indicate that destabilization from X-3',5'-X to X-2',5'-X substitutions is dependent on the residue X, as well as on the 'arrangement' of the 2',5'-linkages within the duplex. In this series, substitution between adenosine residues is destabilizing (11°C per 2',5'-linkage) relative to substitution between uridines in the opposite strand (4°C per 2',5'-linkage) (e.g., compare **3/1** to **3/5**, **6/1** and **7/1**). This substitution effect appears to be additive. For example, the T_m of duplex **7/5** which contains six 2',5'-phosphodiester linkages (3 per strand) is decreased by 16−17°C relative to **6/1** and **7/1** (with three linkages), and by 50°C relative to **3/1** (with none). Interestingly, duplexes **7/5** and **6/5** which differ only in the arrangement of their 2',5'-linkages (i.e., staggered versus juxtaposed) exhibited significantly different T_m's (Table 4). 'Juxtaposed' 2',5'-linkages as in **6/5**, appear to stabilize the helix. In the series of decamers presented in Table 5, it is clear that substitution of the natural 3',5'-linkage with the 2',5'-linkage is destabilizing. Furthermore, substitution of 2',5' linkages between uridines are more destabilizing (large decrease in T_m of N>>R≡P) relative to substitutions between adenosines (small decrease in

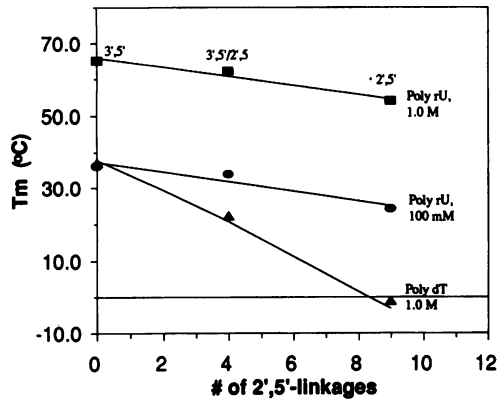

Figure 5. Selectivity of 2',5'-r(Ap)$_9$A for binding with poly rU over poly dT. The T_m values of 1Pu:2Py complexes of the riboadenylates containing either all 3',5'-linkages (3',5'), alternating 3',5' and 2',5'-linkages (3',5'/2',5') or all 2',5'-linkages (2',5') with poly rU (in 1 M and 100 mM NaCl) and poly dT (in 1 M NaCl) are shown as a function of number of 2',5'-phosphodiester linkages.

Table 4. T_m and % H values for mixed oligoribonucleotide complexes with complementary RNA and ssDNA(1 M NaCl)

	Duplexes	T_m (%H)
3	5'-r(AGC A A A A A A A CGC)-3'	65 (16%)
1	3'-r(UCG U U U U U U GCG)-5'	
3	5'-r(AGC A A A A A A A CGC)-3'	52 (13%)
5	3'-r(UCG U*U U* U*U U GCG)-5'	
6	5'-r(AGC A*A A*A A*A CGC)-3'	29 (16%)
1	3'-r(UCG U U U U U U GCG)-5'	
7	5'-r(AGC A A*A A*AA* CGC)-3'	30 (16%)
1	3'-r(UCG U U U U UU GCG)-5'	
7	5'-r(AGC A A*A A*AA* CGC)-3'	13 (10%)
5	3'-r(UCG U*U U*U U*U GCG)-5'	
6	5'-r(AGC A*A A*A A*A CGC)-3'	29 (14%)
5	3'-r(UCG U*U U*U U*U GCG)-5'	
3	5'-r(AGC A A A A A A A CGC)-3'	54(16%)
2	3'-d(TCG T T T T T T GCG)-5'	
6	5',-r(AGC A* A A* A A *A CGC)-3'	28 (10%)
2	3'-d(TCG T TT T T T GCG)-5'	
7	5',-r(AGC A A* A A* AA* CGC)-3'	28 (10%)
2	3'-d(TCG T T T T TT GCG)-5'	
4	5'-d(AGC A A A A A A A CGC)-3'	--- (8%)
5	3'-r(UCG U*U U*U U*U GCG)-5'	

T_m N>O>Q). This is in contrast to what was observed for the oligoribonucleotides of mixed base composition (Table 4) where insertion of 2',5'-linkages between adenosines was more destabilizing. From these generalizations it can be concluded that 2',5' substitutions always destabilize the complex although the degree of destabilization depends on both the neighboring residues and neighboring linkages.

The partially substituted oligomers **6** and **7** also form duplexes with complementary ssDNA **2** but their T_m's are, as expected, lower than that of the corresponding unmodified duplex **3**/**2**. No evidence for interaction of 'mixed' 3',5'/2',5'-RNA **5** with DNA **4** was found, although an interaction with the RNA target **3** was observed (Table 4). This result further reinforces our observation that introduction of 2',5'-linkages into otherwise natural RNA imparts selectivity for complementary RNA over ssDNA. It would be interesting to determine the minimum

Table 5. T_m and % H values for decameric homooligoribonucleotide complexes (1 M NaCl)

Oligonucleotide Complex	Complex Designation	T_m (°C)	% H
3',5'-r(Ap)$_9$A / 3',5'-r(Up)$_9$U	N	28	30
3',5'/2',5'-r(Ap)$_9$A / 3',5'-r(Up)$_9$U	O	20	26
3',5'-r(Ap)$_9$A / 2',5'-r(Up)$_9$U	P	---	13
2',5'-r(Ap)$_9$A / 3',5'-r(Up)$_9$U	Q	17	55
3',5'-r(Ap)$_9$A / 3',5'/2'5'-r(Up)$_9$U	R	---	6
2',5'-r(Ap)$_9$A / 2',5'-r(Up)$_9$U	S	8	15

number of 2',5'-inserts necessary for this selectivity as a potential means of detecting only RNA strands in a mixture containing both RNA and ssDNA of the same base sequence.

In the course of this work we also observed the formation of the complex [2',5'-r(Ap)$_9$A] : [2',5'-r(Up)$_9$U] in 1 M NaCl by thermal denaturation analysis (T_m = 8°C with 15%H). Although the T_m value of this complex is significantly lower than that of the analogous natural complex [3',5'-r(Ap)$_9$A] : [3',5'-r(Up)$_9$U] (T_m = 28°C), its formation is significant in that it raises the possibility of the existence of a primitive self-replicating system based on 2',5'-linkages at some earlier stage in evolution. During the preparation of this manuscript we became aware of one other publication describing the association of 'exclusively' 2',5'-linked RNA (28). Their conclusion that '2',5'-linked RNA strands associate to give complexes of lower stability than those formed by 3',5'-linked oligoribonucleotides of the same sequence' is confirmed and extended by our investigations (29d).

CONCLUSIONS

2',5'-Linked oligoribonucleotides and a 2',5'-oligoadenylate phosphorothioate analog are capable of forming stable complexes with complementary strands of (3',5'-linked) RNA. Furthermore, several of the 2',5'-oligo-RNA's studied here possess the uncommon ability to discriminate between single stranded DNA and RNA, binding strongly only to RNA (46). This is in marked contrast to 3',5'-oligo-RNA which can form usually stable structures with both complementary RNA and DNA. Because of its RNA specificity and unnatural character, 2',5'-oligo-RNA's may find application as ligands for the affinity purification of cellular RNA, or as selective inhibitors of viral mRNA translation *in vivo*. With respect to the latter, the sugar–phosphate backbone of 2',5'-linked ribonucleotides may be further modified (e.g., 3'-modifications) to improve binding, RNA specificity, and nuclease stability. Nonspecific carriers such as cholesterol (47) may also be covalently attached to improve cellular uptake.

Very recently, a 2',5'-linked tetraadenylate was covalently linked to the end of an antisense oligonucleotide to yield a chimeric molecule, 2',5'-(pA)$_4$-3',5'-antisense DNA (48). The antisense 3',5'-linked oligodeoxynucleotide component of this molecule hybridized to a specific RNA target sequence, while the (unbound) 2',5'-linked segment activated 2–5A-dependent RNase, thereby causing selective cleavage of the RNA sequence. In view of these findings, and the ability of 2',5'-oligo-RNA to associate with 3',5'-RNA targets, it would now be instructive to determine whether a fully bound 2',5'-RNA:3',5'-RNA duplex can activate 2,5A-dependent RNase (49), or even RNase H (50), another enzyme implicated in the antisense mechanism of oligodeoxynucleotides.

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44. Stoichiometry of this complex was determined to be 1A:2U using Job's method of continuous variation (references 34 and 35).
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