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Circular RNA oligonucleotides. Synthesis, nucleic acid binding properties, and a comparison with circular DNAs

Shaohui Wang and Eric T.Kool*
Department of Chemistry, University of Rochester, Rochester, NY 14627, USA

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ABSTRACT

We report the synthesis and nucleic acid binding properties of two cyclic RNA oligonucleotides designed to bind single-stranded nucleic acids by pyr·pur·pyr-type triple helix formation. The circular RNAs are 34 nucleotides in size and were cyclized using a template-directed nonenzymatic ligation. To ensure isomeric 3′-5′ purity in the ligation reaction, one nucleotide at the ligation site is a 2′-deoxyribose. One circle (1) is complementary to the sequence 5′-A_{12}, and the second (2) is complementary to 5′-AAGAAAGAAAAG. Results of thermal denaturation experiments and mixing studies show that both circles bind complementary single-stranded DNA or RNA substrates by triple helix formation, in which two domains in a pyrimidine-rich circle sandwich a central purine-rich substrate. The affinities of these circles with their purine complements are much higher than the affinities of either the linear precursors or simple Watson–Crick DNA complements. For example, circle 1 binds rA_{12} (pH 7.0, 10 mM $MgCl_2$, 100 mM NaCl) with a T_m of 48°C and a K_d (37°C) of 4.1×10^{-9} M, while the linear precursor of the circle binds with a T_m of 34°C and a K_d of 1.2×10^{-6} M. The complexes of circle 2 are pH-dependent, as expected for triple helical complexes involving C(+)G·C triads, and mixing plots for both circles reveal one-to-one stoichiometry of binding either to RNA or DNA substrates. Comparison of circular RNAs with previously synthesized circular DNA oligonucleotides of the same sequence reveals similar behavior in the binding of DNA, but strikingly different behavior in the binding of RNA. The cyclic DNAs show high DNA-binding selectivity, giving relatively weaker duplex-type binding with complementary RNAs. The relative order of thermodynamic stability for the four types of triplex studied here is found to be DDD >> RRR > RDR >> DRD. The results are discussed in the context of recent reports of strong triplex dependence on RNA versus DNA backbones. Triplex-forming circular RNAs represent a novel and potentially useful strategy for high-affinity binding of RNA.

INTRODUCTION

A new approach to nucleic acid recognition which has gained significant experimental attention recently is the binding of single strands by triple helix formation (1–12). This strategy involves the use of oligonucleotides which contain two domains complementary to a purine-rich target sequence: one domain is complementary in the Watson–Crick (parallel) sense, and the second domain is complementary in the Hoogsteen (antiparallel) sense. The two binding domains are linked by nucleotide or nonnucleotide linking groups, and this intramolecular arrangement allows the formation of bimolecular triplexes with single-stranded nucleic acid target strands.

This strategy for nucleic acid recognition has the advantages of higher binding affinity (2,5) and higher sequence selectivity (1) than can be achieved by standard Watson–Crick binding alone. The association involves a greater number of hydrogen bonding and base stacking interactions with the target sequence, and so results in a more favorable enthalpic term. In addition, since the domains are interconnected, the binding is entropically favored relative to the independent binding of separate Watson–Crick and Hoogsteen strands. To date, this approach has involved the use of several varied structures, including hairpin (3,4,11,12), stem-loop (7), and circular DNA oligonucleotides (1,2,5,8–10) in the binding of DNA sequences.

The binding of RNA by this approach, however, has been largely unexplored to date. Triple helical structures have recently been shown to be highly sensitive to DNA versus RNA backbone (13–15); for example, the DNA·DNA·DNA (DDD)-type and RNA·RNA·RNA (RRR)-type triple helices are stable, while the DRD and DDR-type triplexes are reported not to be formed (13). These results, although carried out in different types of triple helices, suggest that the binding of single-stranded RNAs or single-stranded DNAs by triplex formation may also be dependent on backbone composition (15).

In order to study the effects of backbone composition on the binding of RNAs and DNAs, we have constructed two circular RNA molecules which are designed to bind complementary squences by triple helix formation. Because the mode of binding is different, the relative dependence on backbone composition is found to be considerably different than reported in other triple helix studies. We find in general that the circular RNAs bind

*To whom correspondence should be addressed

both to RNA and DNA sequences with high affinity by this mode of recognition. By contrast, circular DNAs having the same sequence bind DNAs and RNAs with very different affinities.

EXPERIMENTAL PROCEDURES

Oligonucleotide synthesis

DNA oligonucleotides were synthesized on a Pharmacia LKB automated synthesizer or an Applied Biosystems 392 synthesizer using standard β-cyanoethylphosphoramidite chemistry (16). RNA oligonucleotides were prepared using t-butyl-dimethylsilyl-protected phosphoramidites (Applied Biosystems), and following the oligoribonucleotide synthesis procedure of Usman (17). For the synthesis of the 34mer RNAs to be cyclized, 2′-deoxynucleoside supports (dU-CPG and dC-CPG, Glen Research) were used in the synthesis, so that the 3′-end residue lacks a 2′-OH group. 5′-phosphorylation was carried out with a phosphoramidite reagent (18) purchased from Glen Research. Tetrabutylammonium fluoride in THF (Aldrich) was dried over molecular sieves prior to use in the desilylation step (19). Oligomers were purified by preparative 20% denaturing polyacrylamide gel electrophoresis and quantitated by absorbance at 260 nm. Molar extinction coefficients for the oligomers were calculated by the nearest neighbor method (20).

Circularization of linear 5′-phosphorylated precircle oligomers to give compounds **1** and **2** was carried out as previously described for cyclization of DNAs (5), using short DNA templates to align the reactive ends, and BrCN/imidazole/Ni^{2+} chemistry to achieve the ligation (21). The reactions contained 50 μM precircle, 55 μM template strand, 200 mM imidazole·HCl (from a pH 7.0 stock), 100 mM $NiCl_2$. BrCN was added last as a solid to the mixture to give a final calculated concentration of 125 mM. The template for **1** was 5′-dA_{12}, and the template for **2** was 5′-dAAGAAAGAAAAG. Conversion from linear to circular product in both cases was ≥ 75−85% in 12 hr, as judged by UV-shadowing. Reactions were then dialyzed against water and lyophilized. Purification of the circular products was carried out using preparative denaturing PAGE (isolation by the crush-and-soak method). The circular products migrated on the 20% gel at 0.85 times the rate of their linear precursors. When desired for analysis, RNA and DNA bands were visualized by staining with Stains-all dye (Sigma). Oligonucleotides were obtained as the sodium salt, and the yields of the purified circular products were 22% for **1** and 24% for **2**. Intact synthesis of RNA strands was confirmed by complete ribonuclease P1 digestion (enzyme obtained from Gibco BRL) followed by HPLC analysis of the monophosphates. Circularity of RNA oligomers **1** and **2** was confirmed by partial digestion with RNase Phy M (USB) followed by gel electrophoresis; the initial product of cleavage was in both cases a single band with the same mobility as the linear precursor to the circle.

Thermal denaturation studies

Solutions for the thermal denaturation studies contained a one-to-one ratio of 34-nucleotide circular pyrimidine oligomer and 12-nucleotide complementary purine oligomer (1.5 μM each). Also present were 100 mM NaCl and 10 mM $MgCl_2$. Solutions were buffered with 10 mM Na·PIPES (1,4-piperazine-bis(ethanesulfonate), Sigma) at pH 7.0 or 5.5. This buffer was chosen because its pK_a has the lowest temperature dependence of the Good buffers (22). The buffer pH is that of a 1.4× stock solution at 25°C containing the buffer and salts. After the solutions were prepared they were heated to 90°C and allowed to cool slowly to room temperature prior to the melting experiments.

The melting studies were carried out in teflon-stoppered 1 cm pathlength quartz cells under nitrogen atmosphere on a Varian Cary 1 UV-vis spectophotometer equipped with thermoprogrammer. Absorbance (260 nm) was monitored while temperature was raised from 5 to 80°C at a rate of 0.5°C/min; a slower heating rate did not affect the results. In all cases the complexes displayed sharp, apparently two-state transitions, with all-or-none melting from bound complex to free oligomers. Melting temperatures (T_m) were determined by computer fit of the first derivative of absorbance with respect to 1/T, and are reported at 0.971 of the maximum of the first derivative. Uncertainty in T_m is estimated at ± 0.5°C based on repetitions of experiments.

Free energy values were derived by computer-fitting the denaturation data, using the two-state approximation for melting (23). Fits were excellent, with χ^2 values of 10^{-6} or better. In two cases, van't Hoff thermodynamic parameters were also derived by measuring T_m as a function of concentration ($1/T_m$ vs. $\ln(C_T/4)$); close agreement was seen (within 4%) with the results from curve-fitting, indicating that the two-state approximation is a reasonable one, at least for these specific sequences. Uncertainty in individual free energy measurements is estimated at ± 10%. Uncertainty in enthalpy and entropy values is estimated at ± 20%.

Mixing experiments

Solutions of circle and substrate oligomer were prepared at 1.5 μM total oligomer concentration, with 10 mM $MgCl_2$ and 100 mM NaCl at pH 7.0 (10 mM Na·PIPES buffer). These solutions

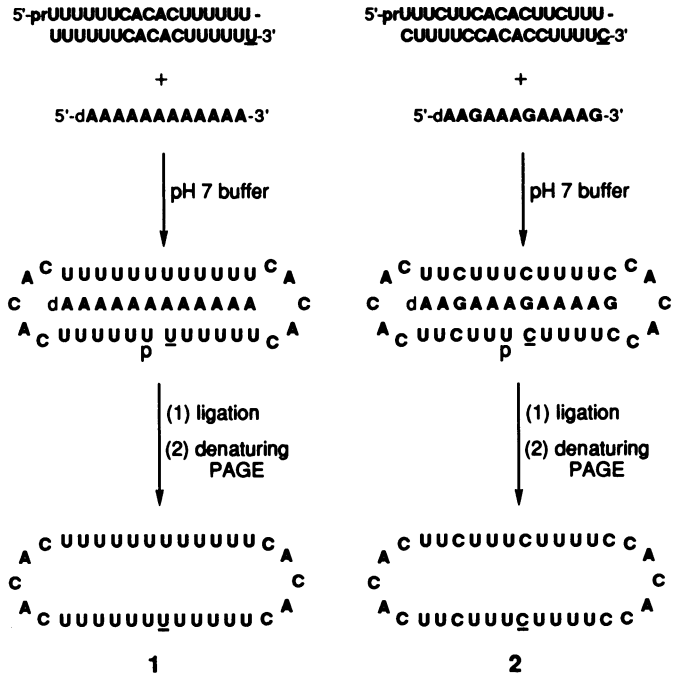

Figure 1. The strategy used for nonenzymatic template-directed cyclization of 5′-phosphorylated 34-base RNA precursors to give the circles **1** and **2**. One nucleotide in each (underlined) contains a 2′-deoxyribose sugar to ensure isomeric purity in the cyclization. Two 12-nucleotide DNA complements were used as templates. See text for details of the ligation reaction.

were prepared in various mole ratios of the two oligomers, so that final oligomer concentrations remained constant at a total of 1.5 μM. The solutions were heated to 90°C and allowed to cool slowly to room temperature, and their absorbances at 260 nm were then recorded at 20°C.

RESULTS

Design and synthesis of cyclic RNAs

The two cyclic RNAs, **1** and **2** (Figure 1), were designed to form parallel-motif pyr·pur·pyr triplexes (24) with purine-rich nucleic acids. A complex with **1** would involve U·A·U triads only, while a complex with **2** would involve both U·A·U and C(+)G·G triads. The linear RNA precursors for cyclization were synthesized with 5′-phosphate groups (18), and using a 2′-deoxynucleoside CPG support, with a dU-CPG for **1** and a dC-CPG for **2**. This was done to ensure chemical purity at the ligation site, since the nonenzymatic ligation may otherwise result in mixtures of 2′-5′ and 3′-5′ diesters when ribonucleotides are joined (21). In the present case, the final 34-nucleotide cyclic products thus differ from completely RNA strands only by a single 2′-hydroxyl group.

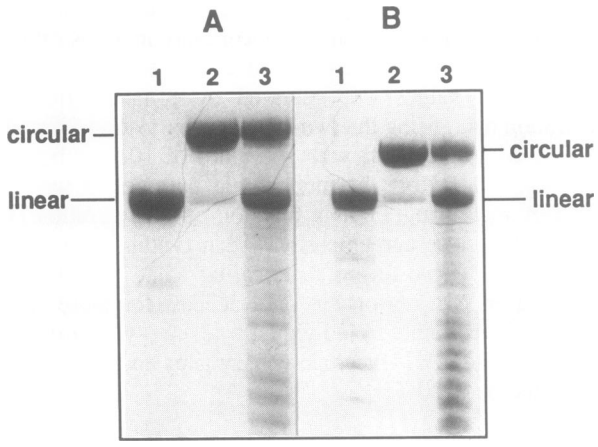

Figure 2. Photograph of stained 20% denaturing PAGE gels showing relative mobilities of linear RNA precursors and cyclic products of the ligation. **A.** (sequence **1**) Lane 1, 34-nt precursor; lane 2, purified circle; lane 3, circle partially digested by RNase Phy M. **B.** (sequence **2**) Lane 1, 34-nt precursor; lane 2, purified circle; lane 3, circle partially digested by RNase Phy M.

Cyclization of the RNAs was carried out by the BrCN/imidazole/Ni^{2+} template-directed ligation method (21) which was previously used in the cyclization of triplex-forming DNAs (5). Conversions in the cyclization reactions were ~75% for **1** and ~85% for **2** as judged by UV shadowing after gel electrophoresis. Recoveries after preparative denaturing PAGE were considerably lower, and the final yields of purified products were 22% for **1** and 24% for **2**. As is the case for cyclic DNAs of the same size and sequence (2,5), the cyclic products migrate at 0.8–0.9 times the rate of the linear precursor on a 20% gel (Figure 2). In general, the circular RNAs migrated slightly more slowly (~0.9 times the rate) on the gel than do circular DNAs of the same sequence. Confirmation of the cyclic structure of the RNAs was carried out by partial hydrolysis by RNase Phy M; as expected for circular oligonucleotides (25), the initial products of hydrolysis were bands having the same mobility as the linear starting material (shown in Figure 2).

Thermal denaturation experiments

The circular RNAs and their linear precursors (nicked circles) were tested for their binding affinity with complementary strands of RNA and DNA (Figure 3 and Table I). Thermal denaturation experiments revealed that the RNA circles bind both types of

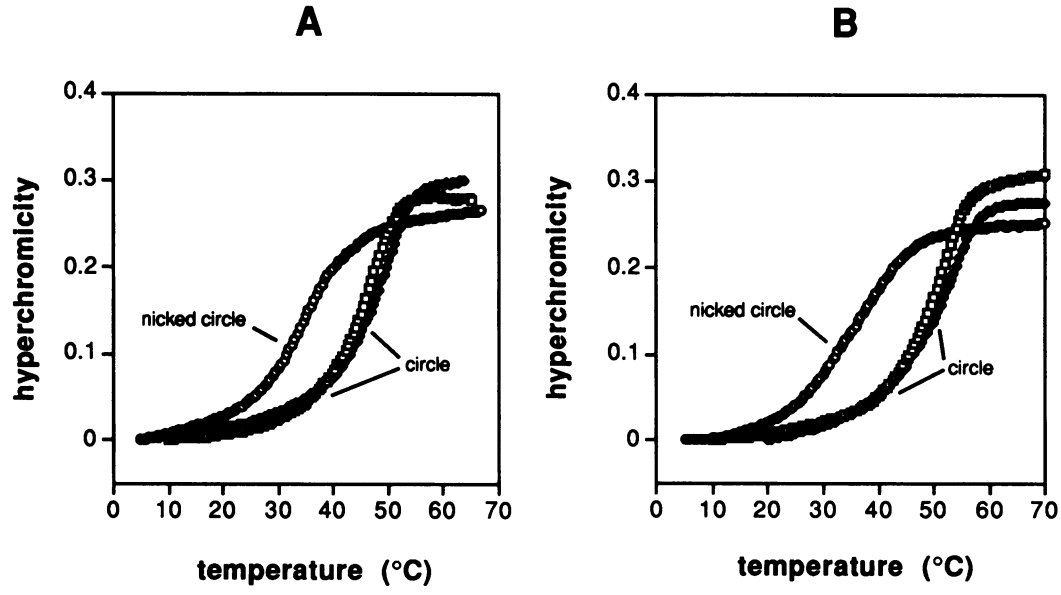

Figure 3. Thermal melting profiles at pH 7.0 and 5.5 (monitored at 260 nm) for one-to-one mixtures of 34mer RNAs with RNA and DNA complements. (○) circle precursors with RNA 12mer complements; (◆) circles with RNA complements; (□) circles with DNA complements. **A.** Circular sequence **1** with substrate 5′-rA_{12} and 5′-dA_{12}. **B.** Circular sequence **2** with substrate 5′-rAAGAAAGAAAAG and 5′-dAAGAAAGAAAAG. Solutions contain 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Na·PIPES, with a 3.0 μM total strand concentration.

Table I. Melting transition temperatures (T_m (°C)) and free energies ($-\Delta G°_{37}$ (kcal/mol) for complexes of nicked circular and circular RNAs with complementary purine RNA and DNA single strands at two pH values. Phosphate groups are designated with a 'p' and are located at the 5' end of the sequences; underlined residues lack a 2'-OH. See experimental section for details

complex	pH = 7.0		pH = 5.5	
	T_m (°C)	$-\Delta G°_{37}$ (kcal)	T_m (°C)	$-\Delta G°_{37}$ (kcal)
A C UUUUUUUUUUUU C A C rAAAAAAAAAAAA C A C UUUUUU UUUUUU C A p	34.0	8.1	35.4	8.3
A C UUUUUUUUUUUU C A C rAAAAAAAAAAAA C A C UUUUUUUUUUUU C A	48.2	11.7	48.7	11.8
A C UUUUUUUUUUUU C A C dAAAAAAAAAAAA C A C UUUUUUUUUUUU C A	45.5	11.1	46.4	11.2
A C UUCUUUCUUUUC C A C rAAGAAAGAAAAG C A C UUCUUU CUUUUC C A p	34.4	8.3	45.5	10.3
A C UUCUUUCUUUUC C A C rAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	51.2	12.6	62.9	17.7
A C UUCUUUCUUUUC C A C dAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	48.5	11.8	62.3	16.4

Table II. Thermodynamic parameters for one-to-one complexes of nicked circular and intact circular RNAs with a single-stranded RNA complement, obtained from plots of $1/T_m$ vs. ln ($C_t/4$). Conditions are pH=7.0, with 10 mM $MgCl_2$ and 100 mM NaCl

complex	$-\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)	$-\Delta G°_{37}$ (kcal/mol)	K_d (M)
A C UUUUUUUUUUUU C A C rAAAAAAAAAAAA C A C UUUUUU UUUUUU C A p	63	176	8.4	1.2×10^{-6}
A C UUUUUUUUUUUU C A C rAAAAAAAAAAAA C A C UUUUUUUUUUUU C A	108	311	11.9	4.1×10^{-9}

complements with high affinity, and that the benefit of cyclization is substantial. Figure 3 shows the melting profiles for both sequences in this study; behavior for the two sequences is nearly identical, with the complexes involving linear precursors melting at a substantially lower temperature (by 14–17°C) than with the intact circles.

Hyperchromicity values for melting of the complexes of the circles with RNA complements at pH 7.0 are 28% for **1** and 27% for **2**. With the DNA complements, hyperchromicity values are very similar, at 28% for **1** and 29% for **2**. Both circles bind an RNA complement with greater thermal stability than do the linear precursors, with advantages in T_m of 14–17°C. For both sequences, the binding of an RNA complement is favored over a DNA complement by 3°C in T_m. Comparison of the behavior for the two different sequences at pH 7.0 shows that the mixed A,G sequence (i.e., in complexes with circle 2) is bound with slightly higher thermal stability (3°C in T_m). The free energies of association at pH 7.0 (Table I) show the same trends: the circular compounds bind an RNA strand with free energies 3.6 to 4.3 kcal more favorable (at 37°C) than do the linear precursors. RNA substrates are bound slightly more strongly than DNA substrates, with a preference of 0.6–0.8 kcal/mol. Overall, the mixed sequence complexes of circle **2** have a 0.7–0.9 kcal higher affinity (37°C, pH 7.0) than the complexes of compound **1**.

Results of binding studies at pH 5.5 show behavior consistent with the expected triple helical complexes. Table I lists T_m and free energy values for the complexes at this lower pH. The complexes of circle **1** with A_{12} sequences show almost identical behavior to that seen at pH 7.0; free energies of the three complexes at pH 5.5 are within 0.2 kcal of the values measured at the higher pH, indicating no pH dependence for this complex. By contrast, the mixed-sequence complexes of circle 2 and its precursor are all strongly pH-dependent. The linear precursor binds its complement with an increase in T_m of 11.1°C and in free energy, −2.0 kcal relative to binding at pH 7.0. The intact circular complexes involving 2, both with RNA and DNA, show a strong increase in affinity at pH 5.5. T_m values are increased by 12–14°C, and free energies are 4.6–5.1 kcal more favorable, relative to values at neutral pH. This strong pH dependence is consistent with pH dependence previously reported for pyr·pur·pyr·triplexes involving RNA strands (26,13).

Thermodynamic parameters were also measured from van't Hoff plots of $1/T_m$ vs. ln ($C_T/4$) for the complexes of circle **1** and its linear precursor with the sequence rA_{12} (Table II). Results show that free energies derived by this method are in quite good agreement (within 0.3 kcal) with those measured by the curve fitting method; thus, the two-state approximation for melting may be a reasonable model for these complexes. Examination of the enthalpic and entropic contributions shows that, as seen for circular DNA complexes (27), a large and favorable enthalpic term is opposed by a slightly smaller unfavorable entropic term. Comparison of enthalpy terms for the linear and circular RNAs shows a more favorable enthalpy of binding (by −45 kcal) for the circular compound. The entropy terms nearly compensate for this difference, with the circle giving a 135 eu less favorable entropy of binding. The free energy benefit of cyclization (the difference for linear and circular compounds) is −3.5 kcal/mol under these conditions. Comparison of dissociation constants (K_d, 37°C) for the linear and circular compounds with the RNA complement at pH 7.0 reveals that the circular compound binds with approximately 300-fold higher affinity (Table II).

Mixing studies

Mixing studies were undertaken to examine the stoichiometry of binding of circular RNAs **1** and **2** with RNA and DNA complementary strands. Figure 4 shows plots of mole fraction versus absorbance at 260 nm (pH 7.0, 20°C) for these RNA circles and also for circular DNAs of the same sequence, for comparison of binding behavior. In general, the plots show very clear behavior (with one exception, see below), with points fitting straight lines with very little scatter. The data were fit to 2:1, 1:1, and 1:2 stoichiometry models, and the best fit is as shown in the Figure. The mole fraction corresponding to the intersection of the two lines is shown in each plot.

Results indicate that both RNA circles bind their complements with one-to-one stoichiometry, as expected for this type of triple helical complex (see the top four plots in Figure 4). If simple Watson–Crick binding alone were involved, a given circle would be expected to bind more than one linear complement, especially

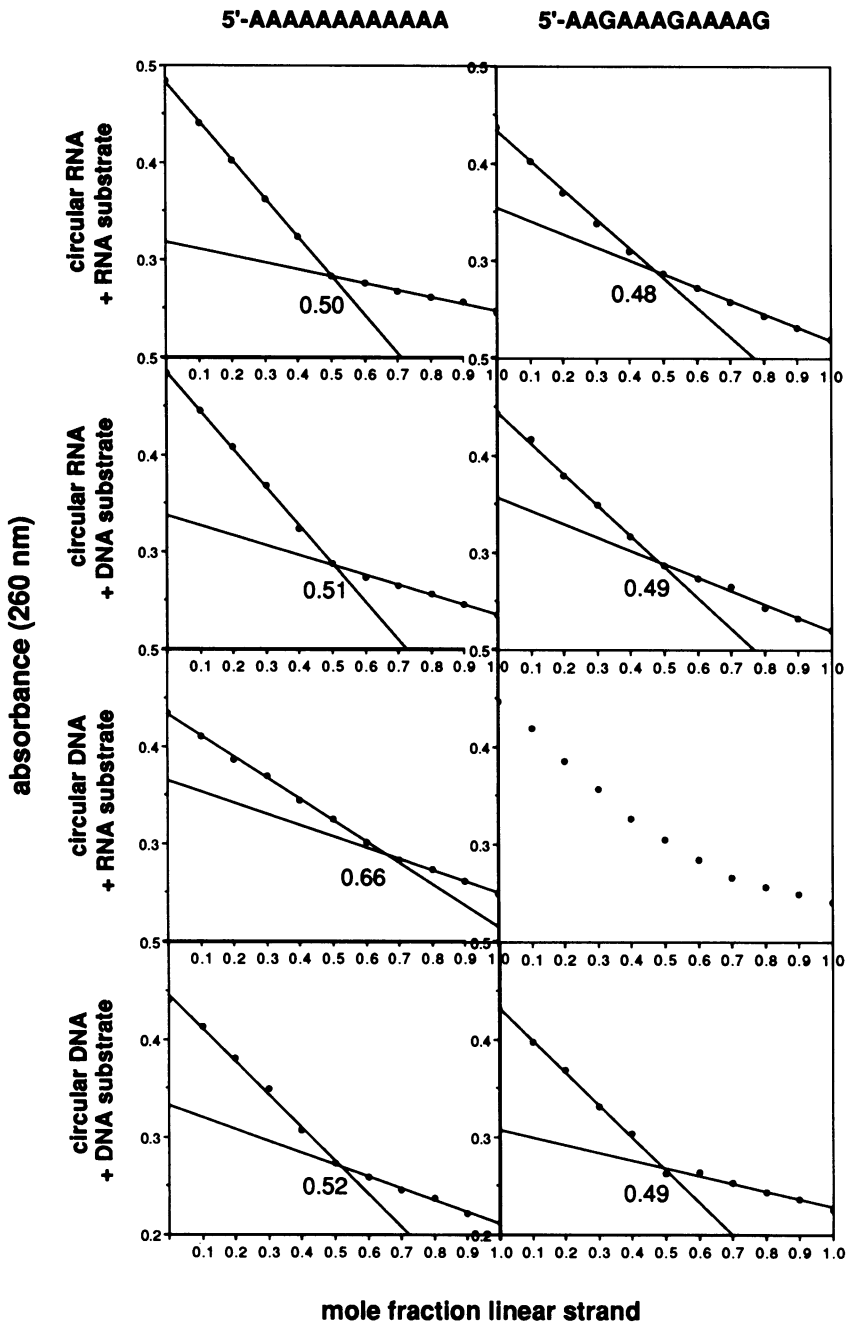

Figure 4. Mixing plots (absorbance vs. mole fraction linear strand) for the complexes of circular RNAs and DNAs with 12mer purine RNA or DNA substrates, as shown. The four plots on the left involve circles having the sequence of circle **1**, and those on the right, circle **2**. Experiments were carried out at pH 7.0 and 20°C, with 10 mM $MgCl_2$, 100 mM NaCl, at 1.5 μM total strand concentration. The mole fraction corresponding to the intersection of the best-fit lines is indicated on each plot.

in the case of circle **1** and A_{12}. It is clear from the plots that both antiparallel and parallel domains of the RNA circles are involved in binding the complementary strands, even though at pH 7.0 the C(+)G·C triads in the complex of **2** are not fully protonated (27).

Comparison of circular RNAs and circular DNAs

The DNA versions of the RNA circles **1** and **2** were synthesized by the same method, resulting in somewhat higher yield. These two compounds have been described previously (2), although their binding properties have not previously been measured under the conditions of the present study. Structurally, there are, of course, two major differences between the circular DNAs and RNAs: while the RNAs have no C-5 methyl groups (uracil in place of thymine bases), the DNAs have twenty-four and eighteen, respectively, for sequences **1** and **2**. The second structural difference is the thirty-three 2'-hydroxyl groups in the circular RNAs which are absent in the DNAs.

Table III. Melting transition temperatures (T_m (°C)) and free energies ($-\Delta G°_{37}$ (kcal/mol) for complexes of two circular RNAs and two circular DNAs with complementary purine RNA and DNA single strands at two pH values. Underlined residues in circular RNAs lack a 2′-OH

complex	type	pH = 7.0		pH = 5.5	
		T_m (°C)	$-\Delta G°_{37}$ (kcal)	T_m (°C)	$-\Delta G°_{37}$ (kcal)
A C UUUUUUUUUUUU C A C rAAAAAAAAAAAA C A C UUUUUUUUUUUU C A	RNA RNA RNA	48.2	11.7	48.7	11.8
A C UUUUUUUUUUUU C A C dAAAAAAAAAAAA C A C UUUUUUUUUUUU C A	RNA DNA RNA	45.5	11.2	46.4	11.2
A C TTTTTTTTTTTT C A C dAAAAAAAAAAAA C A C TTTTTTTTTTTT C A	DNA DNA DNA	53.6	15.0	54.1	15.7
A C TTTTTTTTTTTT C A C rAAAAAAAAAAAA C A C TTTTTTTTTTTT C A	DNA RNA DNA	23.6	6.1	20.4	6.1
A C UUCUUUCUUUUC C A C rAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	RNA RNA RNA	51.2	12.6	62.9	17.7
A C UUCUUUCUUUUC C A C dAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	RNA DNA RNA	48.5	11.8	62.3	16.4
A C TTCTTTCTTTTC C A C dAAGAAAGAAAAG C A C TTCTTTCTTTTC C A	DNA DNA DNA	55.5	14.5	69.7	21.2
A C TTCTTTCTTTTC C A C rAAGAAAGAAAAG C A C TTCTTTCTTTTC C A	DNA RNA DNA	44.0	10.4	42.0	10.1

Thermal denaturation studies were carried out for both sequences and at both pH 7.0 and 5.5 on all possible combinations of RNA and DNA circles with RNA and DNA complements. Table III lists the data from these sixteen experiments. Overall, results from sequence **1** are very similar to those with sequence **2** when the comparison is made at pH 7.0. At pH 5.5, as expected (27), most (not all) of the complexes involving sequence **2** are increased in affinity, while those involving sequence **1** are unchanged from the affinities at neutral pH. The four types of potentially triple helical complexes are RNA·RNA·RNA (RRR), RNA·DNA·RNA (RDR), DNA·RNA·DNA (DRD), and DNA·DNA·DNA (DDD), as indicated in Table III.

The results show that the overall order of affinity for these four types is DDD >> RRR > RDR >> DRD. This holds true for both sequences studied. For the sequence **1**, none of the complexes shows any significant pH dependence, giving the same results within experimental error both at pH = 7.0 and 5.5. Examination of data for sequence **2** shows that three of the complexes, the RRR, RDR, and DDD types, show a strong increase in binding affinity at pH 5.5 relative to pH 7.0, with a stabilization of −4.6 to −6.7 kcal at the lower pH. The fourth complex, involving a DNA circle with an RNA complement (DRD type), already the weakest complex of the four, shows no significant increase in affinity at the lower pH value.

Mixing experiments were also carried out for the four types of complex and for both sequences at pH 7.0 (Figure 4). Results of these studies overall show that three of the four types of complex — RRR, RDR, and DDD — give clearly one-to-one stoichiometry of binding; again, this holds true for both sequences studied. The fourth type, DRD, shows clearly different binding behavior (see the third row of plots in Fig. 4). The data for the DNA circle of sequence **1** hybridized to the complement rA_{12} shows clearly that the stoichiometry of binding is one circle to two linear complements. The corresponding data for the DRD complex of sequence **2** is less clear, and no lines were fit to the points. The overall shape of the points shows a two-line best fit with intersection at a mole fraction of 0.62; this would seem to indicate a stoichiometry of one circle to two linear complements. However, a three-line fit also appeared reasonable, with breaks indicating stoichiometries of either 1:1 or 1:2.

Comparison of circular DNAs with linear 12mers

Since one explanation for the behavior of the DRD-type complexes is Watson−Crick binding without significant Hoogsteen-type interaction (28), a comparison of binding affinity for the RNA strands was made between the two DNA circles and simple 12-nucleotide oligomers having the sequence dT_{12} and dCTTTTCTTTCTT. The two circles contain these sequences in their Watson−Crick domains. Results of melting studies of the two short linear DNA strands at pH 7.0 with the RNA complements (data not shown) indicate that they have similar binding affinity as the circular DNAs. The oligomer dT_{12} binds rA_{12} with a T_m of 22.8°C and a free energy (by curve fitting) at 37°C of −6.0 kcal/mol, which is close to the values for the DNA circle of sequence **1** binding the same RNA strand, 23.6°C and −6.1 kcal/mol (Table III). The oligomer 5′-dCTTTTCTT-TCTT binds the RNA complement 5′-rAAGAAAGAAAAG with a T_m of 42.2°C and a free energy of −10.2 kcal/mol, similarly close to the values in Table III for the DNA circle of sequence **2** (44.0°C and −10.4 kcal/mol).

DISCUSSION

Synthesis

The synthesis of circular DNA oligonucleotides has received increasing experimental attention recently (29−33,25,6); however, methods for constructing cyclic RNAs have received relatively less attention (30,34,35). In the present work, the synthesis of the circular RNAs by the nonenzymatic ligation method presented no particular difficulties, and proceeded with high conversion. The strategy may be general for the construction of circular RNAs for triplex-type binding; it is possible, however, that sequence-dependent variations in cyclization efficiency (35) will become evident as additional studies are carried out. Since one can envision loops of almost any length and sequence for such circles, this method may be seen as a general strategy for the construction of circular RNAs of many sizes and sequences, as long as triplex-type binding domains are included in the sequence. Further studies will be needed to test the generalizability of the approach.

It also remains to be seen whether the 3′-terminal 2′-OH group need be deleted from the precircle structure to give a homogeneous 5′-3′ phosphodiester linkage. Results of other chemical ligation studies with RNAs have shown good isomeric purity at the linkage (35), and thus indicate that this isomer question may not be a problem in general.

Binding properties of circular RNAs

The two RNA circles in this study bind both their RNA and DNA complements with a one-to-one stoichiometry at pH 7.0. In addition, the pH dependence observed for the complexes

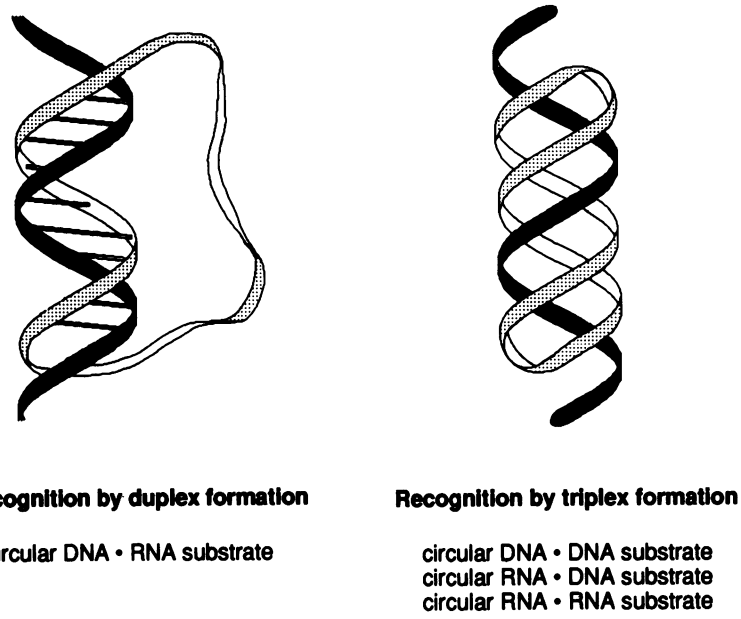

Figure 5. The two modes of circle·single strand recognition observed in this study, depending on ribose or 2'-deoxyribose backbone composition. The duplex recognition mode likely involves binding of more than one substrate strand to the circle (see text).

involving circle **2** indicates a stabilization of the complexes by protonation. These two results strongly suggest that the cyclic RNAs bind their complements, whether DNA or RNA, by triple helix formation. The effect of covalent closure of the circles is to greatly increase binding affinity relative to the circle precursors, an effect which was also seen for the cyclic DNAs in the binding of DNA complements (5). It would seem likely that much of this benefit is due to the entropic benefit of preorganization (36); for the cyclic structures there is less freedom of internal bond rotations than for the linear precursors, and so complexation results in a lower entropic cost. Interestingly, the thermodynamic parameters measured for the circle **1** and its precursor (Table III) are not in accordance with this idea: in fact, much of the benefit of circularity appears in the enthalpy term. It should be noted, however, that interpretation of the large and opposing enthalpic and entropic contributions measured for such complexes is difficult (37), since they often compensate for one another; in addition, the roles of solvating water and metal ions are complicating factors (38).

Differences in properties of circular RNA and DNAs

The data indicate that the circular RNAs bind both RNA and DNA complements with similarly high affinity, with a small preference for RNA strands. Evidence indicates that the complexes are triple helical in structure. Circular DNAs, however, show strikingly different behavior: while DNA circles can bind single-stranded DNA complements strongly in triplex fashion, they cannot bind RNA complements with as high affinity, at least for the sequences examined here.

The present findings are consistent with recent reports that DNA-DNA-DNA (DDD) type triplexes are stable, while DNA-RNA-DNA (DRD) triplexes are much lower in stability, and have, in fact, never been observed (13–15). The observation that the circular DNA of sequence **1** binds rA_{12} with about the same affinity as a simple Watson–Crick complement suggests that the circle complex may only involve Watson–Crick-type interactions with the RNA strand (Figure 5). The mixing plot for this complex strongly supports this idea, since the circle binds two rA_{12} strands simultaneously. Also lending credence to this type of complex is the fact that the DNA circle of sequence **2** binds its RNA complement in pH independent fashion, which strongly suggests a lack of Hoogsteen interactions involving C+G-C triads. This circle also binds about as strongly as a simple Watson–Crick complement, and shows some evidence suggestive of either one or two complements binding the circle simultaneously in the mixing plot. The binding of a second linear strand by the circle in this case might be expected to give ambiguous results, since the Hoogsteen domain is not fully complementary to the linear 12mer in antiparallel mode.

The present experiments indicate that for these RNA circles, RRR- and RDR-type complexes are stable in circle-single strand complexes. For circular DNAs, DDD-type triplexes are stable, while DRD triplexes are not observed. Taken together, the relative stability at pH 5.5 for the four types of triplex studied here is DDD >> RRR > RDR >> DRD. Using a (single strand + hairpin duplex) form of triple helix, Roberts and Crothers reported a relative order of RRR > RDR >> DDD >> DRD at the same pH (13). Differences in the relative ordering of stability between the two studies is not unexpected, however, since the two systems are different. For example, in a (single strand + hairpin duplex) system, the duplex part is biased toward stronger complexes because it is an intramolecular complex, and thus the total free energies of the triplex are weighted toward the free energy of the duplex. In the present system, neither the Watson–Crick nor Hoogsteen interaction is particularly biased since dissociation is always cooperative. Additional differences between the two studies may also arise from differences in the sequences used. All studies to date do

agree, regardless of type of triplex involved, in the finding that the DRD-type triplexes are quite unstable relative to most of the other variants.

It is interesting to speculate on the reasons for the large variance in stability seen for the different types of triple helix. Previous studies have invoked the differences in the ribose versus deoxyribose backbone (13–15), and it does seem likely that conformational and steric differences in the strands as a result of the 2′-OH group may play a significant role. Studies underway in our own laboratory suggest that the presence or absence of C-5 methyl groups also may contribute significantly to these effects; clearly, more study of these two structural differences between DNA and RNA structures, as well as sequence-dependent variations in the effects, will be needed before useful conclusions can be drawn.

In practical terms, the results indicate that circular RNAs may be most proficient at forming complexes with single-stranded RNA targets, while circular DNAs show a strong preference for DNA target strands. The magnitude of the selectivity of circular DNAs for DNA over RNA strands is quite impressive and is relatively unprecedented. If it holds for more than the two sequences studied here, it may represent a useful strategy for discriminating between the two natural forms of nucleic acid.

CONCLUSIONS

The present studies establish that circular RNA oligonucleotides can be efficiently constructed by a nonenzymatic template-directed cyclization strategy. Binding studies indicate that the cyclization increases binding affinity very substantially, and that circular RNAs can bind both single-stranded RNA and DNA substrates with high affinity by triple helix formation. Comparison of circular RNA and DNA oligonucleotides of the same sequence shows that both bind DNA strands by triple helix formation and with similar affinity. Circular RNAs bind RNA single strands with considerably higher affinity than do circular DNAs, which apparently do not form a triple helical complex.

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