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Relative stabilities of triple helices composed of combinations of DNA, RNA and 2′-*O*-methyl-RNA backbones: chimeric circular oligonucleotides as probes

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ABSTRACT

Described is a systematic study of the effects of varied backbone structure on the stabilities of pyr•pur•pyr triple helices. The effects were measured using six circular 34 base oligonucleotides containing DNA (D), RNA (R) and/or 2′-*O*-methyl-RNA (M) residues designed to bind a complementary single-stranded purine target strand by triple helix formation. Eighteen different backbone combinations were studied at pH 5.5 and 7.0 by optical melting experiments and the results compared with the stabilities of the corresponding Watson–Crick duplexes. When the target purine strand is DNA, all circles form pH-dependent triple helical complexes which are considerably stronger than the duplexes alone. When RNA is the target, five of the nine complexes studied are of the pH-dependent triplex type and the other four complexes are not significantly stronger than the corresponding duplexes. The results are useful in the design of the highest affinity ligands for single- and double-stranded DNAs and RNAs and also point out novel ways to engender DNA- or RNA-selective binding.

INTRODUCTION

Recent studies have established that pyr•pur•pyr triple helices are quite sensitive in their stability to whether each of the three strands is composed of DNA or RNA nucleotides (1–6). There are eight possible combinations of strands with these two backbones, designated the DDD, DDR, DRD, RDD, RRD, RDR, DRR and RRR types (where the first letter represents the pyrimidine Hoogsteen strand, the second the purine central strand and the third is the pyrimidine Watson–Crick complementary strand). Studies have shown that the relative stabilities of these vary quite widely (as much as 11 kcal/mol) (1,5) and the DRD and DDR triplexes are sufficiently unstable that they have not yet been observed (1–3). The specific reasons for these differences are as yet unclear, although H bonding, conformational, steric and stacking differences as a result of the influence of 2′-OH and C-5 methyl groups are likely involved (3,6).

Another nucleic acid backbone variation which has interesting hybridization properties is the 2′-*O*-Me-RNA modification (7,8). This synthetic analog has several unusual properties, for example it can hybridize to RNA single strands with an affinity higher than a DNA strand (7). It has recently been shown also to bind with high affinity to duplex DNA by triplex formation (9,10). Furthermore, the addition of the methyl group to the 2′-hydroxyl significantly inhibits the action of nucleases in degrading the strand (8). Although this modification has been tested for effects on third strand binding of duplex DNA, the general effects on triple helix formation have not yet been surveyed.

We have previously examined four different RNA–DNA triplex combinations (DDD, RRR, DRD and RDR) using bimolecular complexes involving pyrimidine-rich circular RNA or DNA molecules binding to purine-rich DNA or RNA targets (5). In order to examine the remaining four RNA and DNA cases (DDR, RDD, RRD and DRR) using such pyrimidine-rich two-domain ligands, it is necessary to synthesize chimeric oligomers containing both DNA and RNA backbones. In this paper we describe the synthesis of such compounds in circular form. Other studies examining structural effects on triplexes have used different model systems, for example Roberts and Crothers measured third strand binding to hairpin-type duplexes (1). In the case of our bimolecular system there is the advantage that the thermal denaturation from triplex to complete random coil occurs cooperatively in a single transition, making thermodynamic analysis simpler (5).

In addition to studying the RNA–DNA triplex combinations, we also carried out a general survey of the 2′-*O*-methyl modification (designated M here) on triple helix formation. This was again carried out by synthesizing chimeric circular oligomers containing combinations of DNA, RNA and/or 2′-*O*-Me-RNA. Altogether, 18 different combinations of these three backbone types which can potentially form triplexes were examined. We find widely varying stabilities as a result of the structural differences.

MATERIALS AND METHODS

Oligonucleotide synthesis

DNA oligonucleotides were synthesized on a Pharmacia LKB automated synthesizer or an Applied Biosystems (ABI) 392 synthesizer using standard β-cyanoethylphosphoramidite chemistry

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(11). RNA oligonucleotides were prepared using *t*-butyl-dimethylsilyl-protected phosphoramidites (Applied Biosystems) and following published oligoribonucleotide synthesis procedures (12,13). Oligomers terminating with RNA structure were synthesized with a single dC residue at the 3′-terminus, so that the 3′-end residue lacks a 2′-OH group. 2′-*O*-Me-RNA oligomers were synthesized using the ABI standard RNA coupling cycle; the monomer phosphoramidites and solid supports were purchased from Glen Research. 5′-Phosphorylation was carried out with a commercially available phosphoramidite reagent (14) (Glen Research). Tetrabutylammonium fluoride in THF (Aldrich) was dried over molecular sieves prior to use in the desilylation step for compounds containing RNA residues (15). 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 (16).

Circularization of linear 5′-phosphorylated oligomers to give compounds **1**–**6** (see Fig. 1) was carried out as previously described (5,17), using short DNA templates to align the reactive ends and BrCN/imidazole/Ni^{2+} chemistry to achieve the ligation. The precursors for circles **1**–**6** were as follows: (**1**) 5′-pdTTTCTTCACACTTCTTTCTTTTCCACACCTTTTC; (**2**) 5′-prUUUCUUdCACACrUUCUUUCUUUUCdCACACrCUUUUdC; (**3**) 5′-pdTTTCTTCACACrUUCUUUCUUUUCdCACACCTTTTC; (**4**) 5′-prUUUCUUdCACACrUUCUUUCUUUUCdCACACrCUUUUC; (**5**) 5′-prUUUCUUdCACACrUUCUUUCUUUUCdCACACrCUUUUdC; (**6**) 5′-pdTTTCTTCACACrUUCUUUCUUUUCdCACACCTTTTC (2′-*O*-Me-RNA segments are underlined). The cyclization reactions contained 50 μM pre-circle, 55 μM template strand (5′-dAAGAAAGAAAAG), 200 mM imidazole–HCl (from a pH 7.0 stock) and 100 mM $NiCl_2$. BrCN was added last as a solid to the mixture to give a final calculated concentration of 125 mM. After 12 h at room temperature, the mixtures were 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 0.8–0.9 times the rate of their linear precursors, as previously seen for cyclic oligomers of this size (5). Circularity was confirmed by nicking with S1 nuclease; circles give a single initial degradation product which migrates with the linear precursor. When desired for analysis, RNA and DNA bands were visualized by staining with Stains-all dye (Sigma). Oligonucleotides were obtained after dialysis as the sodium salt.

Thermal denaturation studies

Solutions for the thermal denaturation studies contained a 1:1 ratio of 34 nt circular pyrimidine oligomer and 12 nt 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 (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 (18). 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 path length quartz cells under a nitrogen atmosphere on a Varian Cary 1 UV-vis spectophotometer equipped with a thermoprogrammer. Absorbance (260 nm) was monitored while the temperature was raised at a rate of 0.5°C/min; a slower heating rate with this apparatus does 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 $1/T$. The uncertainty in T_m is estimated at ±0.5°C, based on repetitions of experiments.

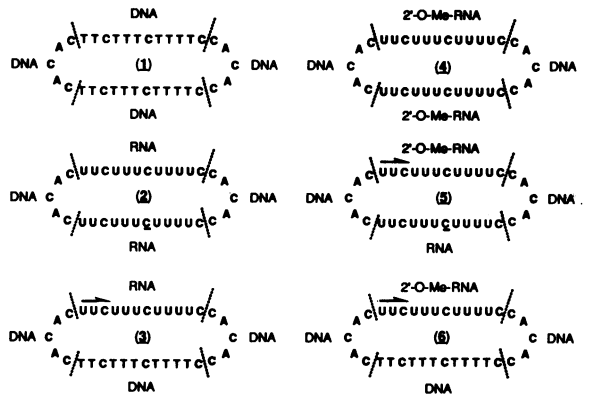

Figure 1. The circular chimeric sequences constructed for this study, with backbone structures as shown. Underlined C residues in RNA domains lack a 2′-OH group. Arrows denote 5′→3′ strand orientation in ambiguous cases.

Free energy values were derived by computer fitting the denaturation data, using the two-state approximation for melting (19). Fits were excellent, with C^2 values typically 10^{-6} or better. Van't Hoff thermodynamic parameters derived from the concentration dependence of T_m were previously measured for DNA oligonucleotides having this sequence; close agreement was seen (within 4%) with the results from curve fitting. Uncertainty in individual free energy measurements is estimated at ±10%.

RESULTS

Design of the chimeric ligands

Six different 34 nt circular oligonucleotides, all having the same sequence, were synthesized for this study (Fig. 1). One is composed only of DNA residues (2′-deoxy) and was previously described (5); the other five are chimeric circular compounds containing DNA, RNA and/or 2′-*O*-Me-RNA residues. We divide each conceptually into four domains: two 5 nt loop domains, which serve to bridge the other two domains, which are 12 nt pyrimidine-rich binding domains. The opposing pyrimidine domains are designed to sandwich a purine complement between them in a high affinity cooperative triple helix (17). In this study we constructed the loop domains in all six compounds from DNA residues. Thus only the 12 nt binding domains were varied in these experiments. Since the sequence of all domains was also held constant, the only variable from experiment to experiment is the substituent at the ribose 2′ position and the presence or absence of a C-5 methyl group on the uracil base.

Considered by themselves, such circular sequences can have a pseudo-mirror plane of symmetry (20). This symmetry is broken on binding a purine complementary strand, since one domain binds the substrate with anti-parallel Watson–Crick bonds and the other by parallel Hoogsteen hybridization. Interestingly, the original sequence symmetry allows such a circle to bind a 5′→3′

sequence or its 3′→5′ reverse equally well (20,21). This switch requires reversing the roles of the circle's Watson–Crick and Hoogsteen domains. With the present sequences, such a reversal brings about the formation of a complex with slightly different nearest neighbors (a difference of one GA versus AG), however, we have shown that this makes no measurable difference to the T_m or calculated ΔG^0. We made use of this bifunctional binding property to test a greater number of structural combinations with the given set of circular probes.

Synthesis of chimeric circular ligands

The circular oligomers in this study were constructed in a single non-enzymatic ligation from 5′-phosphorylated linear precursors, essentially as described earlier (5,17). Standard phosphoramidite coupling protocols were used and for 2′-*O*-Me-RNA phosphoramidites we used the RNA coupling protocol. For the cyclizations, complementary DNA 12mers (5′-dAAGAAAGAAAAG) were used as templates to bring the reactive ends into close proximity and BrCN/imidazole/Ni^{2+} was used (22) to form the final phosphodiester bond. In the case of the two circles which were closed in an RNA domain, we used a deoxycytidine residue at the 3′-terminus, to ensure the proper 5′→3′ ligation geometry (5).

Also synthesized were linear 12 nt pyrimidine oligomers of DNA, RNA or 2′-*O*-Me-RNA composition for comparison to the pyrimidine circles (see Table 1). Triple helical complexes are known to be sensitive to the chemical make-up of the three individual strands (1–6) and some are unstable enough that only the duplex forms, without the third strand Hoogsteen interaction (1–3). For complexes between circles and target strands one can distinguish duplex from triplex structure by comparing the strength of the complexes with the strength of simple duplexes having the same backbones. Any significant increase in binding by the circle would arise as a result of the third strand interaction, allowing a measure of the strength of that interaction, as well as providing additional evidence for three-stranded structure.

Binding studies at pH 5.5 and 7.0

Perhaps the most distinguishing feature of pyr•pur•pyr triple helices relative to Watson–Crick duplexes is the pH sensitivity of the former (23–25). We therefore investigated the affinities of all complexes in this study at neutral and acidic pH values. Those complexes which show increased affinity at the lower pH value are likely to be triple helical in structure and those which show no pH sensitivity are almost certainly not triple helical. These findings can also be independently checked by a comparison of the three strand binding affinities to simple duplexes alone, as described above.

Duplexes. First we examined the effects of these three backbones on the strengths of duplexes, all having the same sequence (Table 1). These were carried out for comparison with the later triple helix experiments. Results show that at pH 7.0 (with 100 mM Na^+ and 10 mM Mg^{2+}) the duplexes vary widely in stability, with T_m values ranging from 20 to 55°C and free energies (37°C) from –4.9 to –12.8 kcal/mol. At pH 7.0 the weakest duplex is that between a RNA pyrimidine strand and a DNA purine complement. The strongest is that between a 2′-*O*-Me-RNA pyrimidine strand and a RNA complement. When a DNA purine strand is the target, the order of pyrimidine strand affinities is DNA > 2′-*O*-Me-RNA > RNA. When a RNA purine strand is the target, the order is 2′-*O*-Me-RNA > RNA > DNA. The DNA pyrimidine strand hybridizes almost equally well to DNA or RNA complements; in contrast, RNA and 2′-*O*-Me-RNA pyrimidine strands strongly prefer hybridization to RNA over DNA strands.

Table 1. Melting temperatures (T_m, °C) and free energies ($-\Delta G°_{37}$, kcal) for duplexes composed of DNA, RNA and 2′-*O*-Me-RNA backbones at two pH values, the latter backbone type is indicated by a line under the sequence

duplex	type	pH = 7.0		pH = 5.5	
		T_m (°C)[a,b]	$-\Delta G°_{37}$ (kcal)	T_m (°C)	$-\Delta G°_{37}$ (kcal)
3′-dGAAAAGAAAGAA 5′-dCTTTTCTTTCTT	DD	38.8	9.2	40.0	9.4
3′-rGAAAAGAAAGAA 5′-dCTTTTCTTTCTT	RD	42.2	10.2	41.5	9.8
3′-dGAAAAGAAAGAA 5′-rCUUUUCUUUCUU	DR	19.9	4.9	30.3	6.7
3′-rGAAAAGAAAGAA 5′-rCUUUUCUUUCUU	RR	46.5	11.4	47.3	11.9
3′-dGAAAAGAAAGAA 5′-rCUUUUCUUUCUU	DM	21.9	6.0	24.8	6.1
3′-rGAAAAGAAAGAA 5′-rCUUUUCUUUCUU	RM	54.9	12.8	55.6	12.9

[a]Conditions: 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Na•PIPES buffer, 3 μM total DNA concentration.

[b]Uncertainties in T_m values and in free energies are estimated at ±1.0°C and ±15%, respectively.

The results at pH 5.5 are very similar. Five of the duplexes show the same affinities, within experimental error, as those measured at neutral pH. One minor exception is the complex of a RNA pyrimidine strand with a DNA complement, which shows some increase in affinity at the lower pH value. This behavior was observed previously for this sequence (6) and is possibly due to partial disproportionation of the duplex into a triplex structure driven by the lower pH.

Triplexes containing RNA/DNA backbones. Table 2 lists results for the binding of circular oligomers containing DNA and/or RNA backbones with RNA and DNA purine complements. There are potentially eight different types of triplex (DDD, RRR, DRD, RDR, DDR, DRR, RDD and RRD) which can be formed from these combinations. We previously described properties of the DDD, DRD, RRR and RDR complexes using all-DNA or all-RNA circles (5). In that previous study, not only the binding domains but also the loops were changed on comparison of a DNA to an RNA circle. Since it is unclear what effect the backbone difference will have on loop stability, in the present case we have synthesized a new circle with RNA domains but with DNA loops. This holds the loop structure constant throughout the entire series of eight complexes.

Table 2 shows the results of hybridization experiments carried out with the DNA/RNA-containing circles and the DNA and RNA purine complements and Figure 2 graphically compares the T_m values. A broad comparison of the left four pairs of data in Figure 2A and B shows that six of the eight cases increase in affinity on lowering the pH from 7.0 to 5.5. The two that do not are the DRD and DRR cases. Interestingly, these are the two cases which have been reported not to form triplexes in other studies (1–3,5). At neutral pH we find that the strongest complexes globally are the DDD, RDD and RRR cases; on lowering the pH the DDD and RDD cases increase their affinity by 6.7 and 9.0 kcal, while the RRR case increases by only 2.4 kcal. At pH 5.5, where protonation of any triplexes is expected to be nearly

Table 2. Melting transition temperatures (T_m, °C) and free energies ($-\Delta G°_{37}$, kcal/mol) for complexes of chimeric DNA/RNA circles with complementary purine RNA and DNA single strands at two pH values

complex	type	pH = 7.0 T_m (°C) [a,b]	pH = 7.0 $-\Delta G°_{37}$ (kcal)	pH = 5.5 T_m (°C)	pH = 5.5 $-\Delta G°_{37}$ (kcal)
$^{A}{}_{C}$TTCTTTCTTTTC$^{C}{}_{A}$ C dAAGAAAGAAAAG C $^{A}{}_{C}$TTCTTTCTTTTC$^{C}{}_{A}$	D D D	54.5	14.5	68.8	21.2
$^{A}{}_{C}$TTCTTTCTTTTC$^{C}{}_{A}$ C rAAGAAAGAAAAG C $^{A}{}_{C}$TTCTTTCTTTTC$^{C}{}_{A}$	D R D	42.8	10.4	40.5	10.1
$^{A}{}_{C}$UUCUUUCUUUUC$^{C}{}_{A}$ C rAAGAAAGAAAAG C $^{A}{}_{C}$UUCUUUCUUUUC$^{C}{}_{A}$	R D R	51.1	12.8	63.2	15.9
$^{A}{}_{C}$UUCUUUCUUUUC$^{C}{}_{A}$ C dAAGAAAGAAAAG C $^{A}{}_{C}$UUCUUUCUUUUC$^{C}{}_{A}$	R R R	54.0	14.3	63.9	16.7
$^{A}{}_{C}$UUCUUUCUUUUC$^{C}{}_{A}$ C dAAGAAAGAAAAG C $^{A}{}_{C}$TTCTTTCTTTTC$^{C}{}_{A}$	R D D	54.2	14.6	66.2	23.6
$^{A}{}_{C}$UUCUUUCUUUUC$^{C}{}_{A}$ C rAAGAAAGAAAAG C $^{A}{}_{C}$TTCTTTCTTTTC$^{C}{}_{A}$	R R D	48.3	13.0	59.8	16.3
$^{A}{}_{C}$TTCTTTCTTTTC$^{C}{}_{A}$ C dAAGAAAGAAAAG-5' C $^{A}{}_{C}$UUCUUUCUUUUC$^{C}{}_{A}$	D D R	41.7	10.4	54.1	14.9
$^{A}{}_{C}$TTCTTTCTTTTC$^{C}{}_{A}$ C rAAGAAAGAAAAG-5' C $^{A}{}_{C}$UUCUUUCUUUUC$^{C}{}_{A}$	D R R	47.4	11.7	46.1	11.0

Underlined residues and all loop residues lack 2′-OH groups. Linear strands are shown left to right in 5′→3′ orientation unless marked otherwise. Arrows denote 5′→3′ strand directionality in circles.

[a]Conditions: 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Na•PIPES buffer, 3 μM total DNA concentration.

[b]Error in T_m values and in free energies are estimated at ±1.0°C and ±15%, respectively.

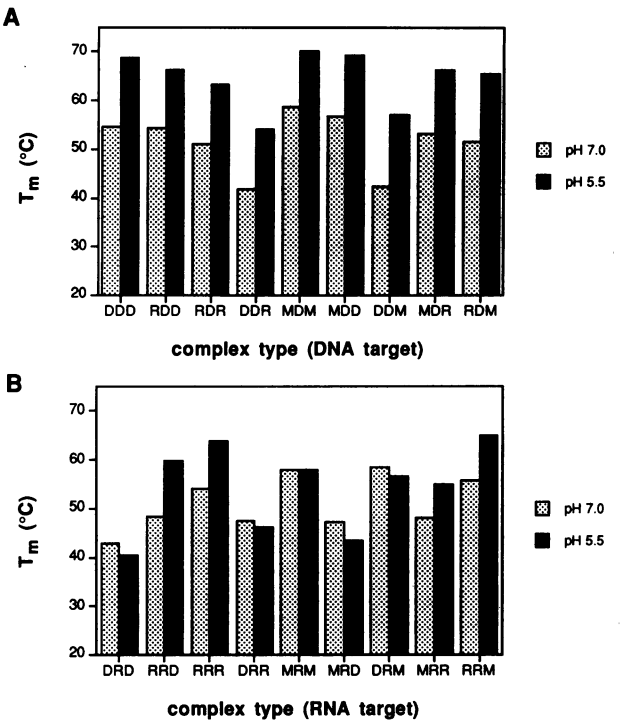

Figure 2. Thermal stabilities of the 18 potential triplexes in this study, as indicated by T_m values measured at pH 7.0 and 5.5. (**A**) Cases where the central purine strand has a DNA backbone. (**B**) Cases where the central purine strand has a RNA backbone. The complex types are denoted by abbreviations using the letters R (RNA), D (DNA) and M (2′-*O*-Me-RNA), in the order Hoogsteen pyrimidine strand, purine Watson–Crick strand, pyrimidine Watson–Crick strand. See Table 2 for conditions.

complete (26), the range of binding affinities is quite broad, varying over 28°C in T_m and 13.5 kcal/mol in free energy. This range can be seen graphically in Figure 2, where the DNA/RNA cases fall in the left halves of the two graphs. Overall, at pH 5.5 we find that the complexes have stabilities which fall in the order RDD > DDD > RRR ≥ RRD ~ RDR > DDR > (DRR, DRD) (see Fig. 5). The last two are likely not triple helical, since they are not pH dependent.

Comparison can also be made between the circle complexes and duplexes of the corresponding backbone compositions. Any increased binding by a circle relative to the analogous Watson–Crick complementary strand might indicate the presence of positive contributions from the Hoogsteen domain of the circle, thus indicating triplex structure. The relative magnitudes of the differences in T_m and ΔG^0 can be considered measures of the relative contributions of the third strand to such a complex. In Figure 3 are shown such comparisons from the pH 7.0 and 5.5 data, with the DNA/RNA data in the left halves of the two graphs.

Results of this comparison are consistent with the pH sensitivities of the complexes, described above. The DRD and DRR cases are not pH sensitive, indicating a lack of third strand interaction. Comparison of the affinities with the corresponding RD and RR duplexes shows (Fig. 3) that they are the same within experimental error. This is again consistent with a lack of third strand binding. The six other complexes were found to be pH sensitive and all six show increased binding relative to their analogous duplexes.

Using this comparison as a measure of the effectiveness of third strand binding, we find that the relative order of third strand binding affinity is R+DR > D+DR > D+DD ~ R+DD > R+RD ~ R+RR > D+RD, D+RR. A similar comparison can be made using free energies (Fig. 4) and at pH 7.0 the resulting order is essentially the same.

Triplexes containing 2′-O-Me-RNA backbones. There are 27 possible types of pyr•pur•pyr triplexes composed of combinations of DNA, RNA and 2′-*O*-Me-RNA strands. We did not examine the nine cases in which the central purine strand is composed of 2′-*O*-Me-RNA, since such cases may have limited practical application. We did, however, examine all 18 of the remaining possibilities. Eight of these are the DNA/RNA combinations discussed above. The last 10 are potential triple helices formed from at least one strand of 2′-*O*-Me-RNA. We examined these with circular oligomers containing (i) two domains of 2′-*O*-Me-RNA, (ii) one domain of 2′-*O*-Me-RNA and one of DNA and (iii) one domain of 2′-*O*-Me-RNA and one of RNA (see Fig. 1). As before, all intervening loops are composed of DNA nucleotides.

Table 3 lists the data for the 10 cases and Figure 2 graphically displays the results (see the right half of each graph). An overview

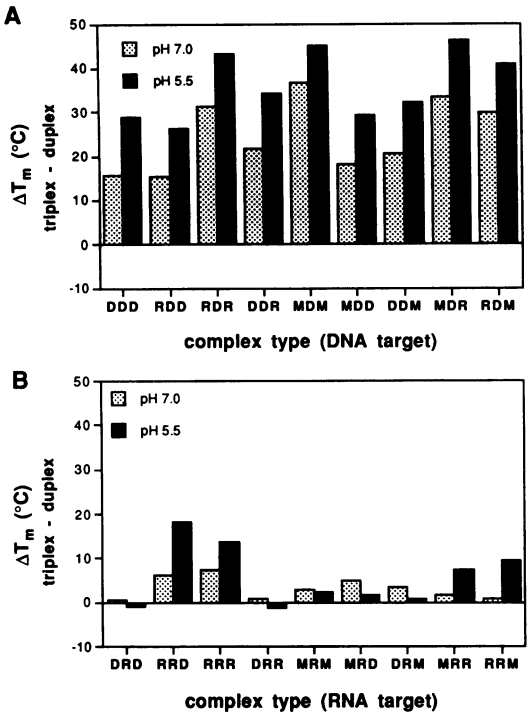

Figure 3. Contributions of Hoogsteen third strand binding to the stabilities of the complexes in this study, as indicated by ΔT_m values measured at pH 7.0 and 5.5. Values were derived by subtracting T_m values for the corresponding duplex from the value for a given circle complex. Note that where the duplex is the DR case we used only the pH 7.0 T_m value, since the low pH value is anomalous (see text). (**A**) Cases where the central purine strand has a DNA backbone. (**B**) Cases where the central purine strand has a RNA backbone. See Table 2 for conditions.

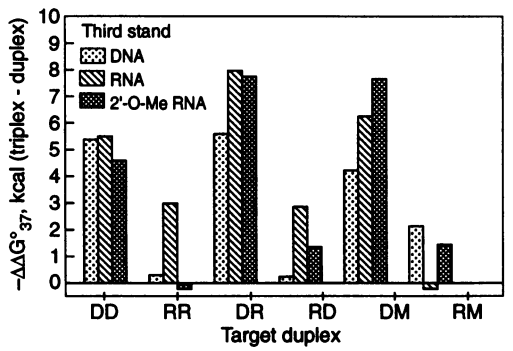

Figure 4. Third strand interactions at pH 7.0 with target duplexes of the types shown. $\Delta\Delta G^0_{37}$ values were obtained by subtracting free energies of analogous duplexes from values for circle complexes. Data are taken from Tables 1–3; see Tables for conditions.

of the data shows that at neutral pH the strongest complexes are the MRM and DRM types, followed closely by the MDD and MDM complexes; the weakest complex at neutral pH is the DDM case. At acidic pH the overall stability order is MDD > MDR ~ MDM ~ RDM ≥ RRM ≥ DRM ~ MRM ~ DDM ~ MRR > MRD (see also Fig. 5). Examining pH effects (Table 3 and Fig. 2), one finds that seven of the 10 are pH dependent, with higher affinity at acidic pH; the other three are insensitive to lowered pH. These

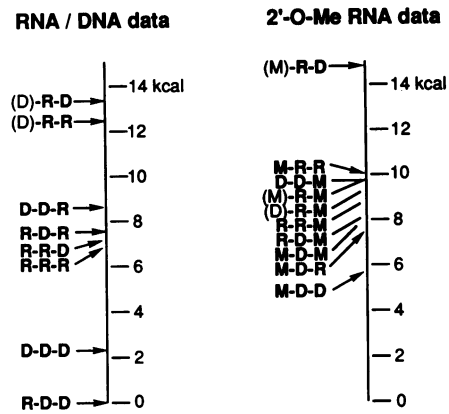

Figure 5. Relative free energies at pH 5.5 for all 18 potential triplexes in this study, displayed in the style of Roberts and Crothers (1). On the left are the RNA/DNA combinations and on the right the cases containing 2′-*O*-Me-RNA strands (shown to the same relative scale). The most stable complex is set to zero kcal and the others are shown relative to that reference point. Cases which have the Hoogsteen strand in parentheses are complexes which have no detectable third strand binding.

three cases are the MRM, MRD and DRM cases and the lack of pH sensitivity is consistent with none of the three being a triplex.

Comparison of these 10 potential triplexes with the analogous duplexes (as previously done for the RNA/DNA series) adds further evidence as to their structures. Figure 3 (right half) displays the ΔT_m comparisons; the results show that five of the cases show a clear benefit from the Hoogsteen interaction, with advantages of 29–46°C in T_m (at pH 5.5) over the simple duplexes. These cases are MDM, MDD, DDM, MDR and RDM. All of these are also pH dependent. The combination of these two findings strongly indicates triple helical structures for these five complexes.

Two cases give borderline behavior; both the MRR and RRM cases show weaker, but still significant, pH dependence. At neutral pH they have little or no advantage over simple duplexes, but at acidic pH they do show small (8–9°C) advantages. It therefore seems likely that these cases form weak triplexes which exist at acidic pH; at neutral pH the third strand probably does not interact with the Watson–Crick duplex portion of the complex.

Finally, the MRM, MRD and DRM cases are not stabilized by lowering the pH and show no advantage in binding relative to the duplexes at either pH. This strongly indicates that all three complexes are simple duplexes, with the third strand dissociated and not binding in the major groove (5).

Once again, we can use the relative ΔT_m values shown in Figure 3 as a measure of the relative affinities of the third strand interactions for these 10 cases. The relative order of these interactions by this analysis is M+DM ~ M+DR > R+DM > D+DM > M+DD > M+RR ~ R+RM > M+RM, M+RD, D+RM. A similar order is found by comparing the free energies of the triplexes and duplexes at pH 7.0 (Fig. 4).

Comparison of RNA/DNA and 2′-O-Me-RNA results. The total results from all 18 complexes can be compared most easily for general trends from Figures 2, 3 and 5. Figure 2 shows the overall T_m values for the complexes and from this comparison there is little overall difference seen for cases that contain 2′-*O*-Me-RNA and those that do not. Comparison of the free energies shows that the highest affinity complexes form for complexes which contain

Table 3. Melting transition temperatures (T_m, °C) and free energies ($-\Delta G°_{37}$, kcal/mol) for complexes of circular ligands containing domains of 2′-*O*-Me-RNA with complementary purine RNA and DNA single strands at two pH values

complex	type	pH = 7.0 T_m (°C) [a,b]	pH = 7.0 $-\Delta G°_{37}$ (kcal)	pH = 5.5 T_m (°C)	pH = 5.5 $-\Delta G°_{37}$ (kcal)
A C UUCUUUCUUUUC C A C dAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	M D M	58.6	13.6	70.0	15.6
A C UUCUUUCUUUUC C A C rAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	M R M	57.8	14.2	57.8	13.9
A C UUCUUUCUUUUC C A C dAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	M D R	53.2	12.6	66.2	15.8
A C UUCUUUCUUUUC C A C rAAGAAAGAAAAG C A C UUCUUUCUUUUC C A	M R R	48.1	11.2	54.8	13.4
A C UUCUUUCUUUUC C A C dAAGAAAGAAAAG-5′ C A C UUCUUUCUUUUC C A	R D M	51.6	12.2	65.6	15.3
A C UUCUUUCUUUUC C A C rAAGAAAGAAAAG-5′ C A C UUCUUUCUUUUC C A	R R M	55.6	12.6	65.0	14.6
A C UUCUUUCUUUUC C A C dAAGAAAGAAAAG C A C TTCTTTCTTTTC C A	M D D	56.9	13.7	69.2	17.8
A C UUCUUUCUUUUC C A C rAAGAAAGAAAAG C A C TTCTTTCTTTTC C A	M R D	47.1	11.5	43.3	9.9
A C TTCTTTCTTTTC C A C dAAGAAAGAAAAG-5′ C A C UUCUUUCUUUUC C A	D D M	42.3	10.2	57.0	13.8
A C TTCTTTCTTTTC C A C rAAGAAAGAAAAG-5′ C A C UUCUUUCUUUUC C A	D R M	58.3	14.9	56.4	14.1

2′-*O*-Me-RNA residues are designated by a line over the sequence. Underlined residues and all loop residues lack a 2′-OH group. Arrows denote 5′→3′ directionality. Linear strands are shown left to right in 5′→3′ orientation unless marked otherwise.

[a]Conditions: 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Na•PIPES buffer, 3 μM total DNA concentration.

[b]Uncertainties in T_m values and in free energies are estimated at ±1.0°C and ±15%, respectively.

only DNA or RNA backbones however. This is illustrated most clearly in Figure 5, which shows relative free energies for all the complexes at acidic pH. The globally strongest complex when a DNA purine strand is the target is the RDD case; when a RNA strand is the target, it is the RRR case. Cases involving 2′-*O*-Me-RNA strands are all at least 5 kcal lower in affinity than the best RNA/DNA case.

The data do show one important general trend: all complexes with DNA as the purine strand (Fig. 3, top) form stable triplexes, but if the central strand is composed of RNA, few form triplexes at all (Fig. 3, bottom) and those that do are relatively weak compared with the DNA cases. Thus, in agreement with previous observations (1,3,5), we find that pyr•pur•pyr triplexes are generally destabilized when the purine strand is RNA and the present data show that this holds true whether the pyrimidine strands are composed of DNA, RNA or 2′-*O*-Me-RNA.

Comparison of free energies of the circle complexes with those for the underlying duplexes gives a measure of the relative strengths of the third strand interactions at neutral pH. If the underlying duplex is a standard DNA–DNA duplex, the strongest third strand binding occurs (compare DDD, RDD and MDD in Fig. 4) with RNA or DNA Hoogsteen complements, although all three are relatively close in affinity. When an RNA–RNA duplex is the target, an RNA third strand binds the strongest; a 2′-*O*-Me-RNA strand shows no binding at pH 7.0 and only weak binding at pH 5.5, while a DNA strand shows no binding at all. When a DNA–RNA duplex (with the purine strand being DNA) is the target the order of binding is RNA ~ 2′-*O*-Me-RNA > DNA. When the reversed RNA–DNA duplex is the target, the order is RNA > 2′-*O*-Me-RNA and a DNA Hoogsteen strand does not bind. For the non-natural DM duplex, all three backbones bind well, with the 2′-*O*-Me-RNA strand being the best; for the RM duplex type, binding is weak, with a DNA strand displaying the highest affinity.

DISCUSSION

Relative duplex stabilities for the three backbones

Our results for the duplex studies confirm that RNA and 2′-*O*-Me-RNA pyrimidine oligonucleotides prefer to bind to RNA strands over DNA. DNA pyrimidine oligomers bind both strand types almost equally well, with a small advantage for RNA; DNA purine oligomers, however, strongly prefer binding to DNA over RNA complements. Comparison of the hybridization of an RNA strand relative to a 2′-*O*-Me analog shows very similar behavior, with a small binding advantage for the *O*-methyl substitution. A previous study also compared the relative hybridization abilities of DNA, RNA and 2′-*O*-Me-RNA strands in duplex formation, with similar findings (7). The differences in hybridization properties between RNA and 2′-*O*-methyl-RNA must arise from steric and/or conformational differences resulting from the presence or absence of 2′-*O*-methyl groups. Differences between DNA and RNA properties have been shown to be due both to 2′-substituents and to the presence or absence of C-5 methyl groups (6).

Comparison with previous DNA/RNA triplex studies

Previous studies of DNA/RNA triplex stabilities have used different types of complexes to probe the effects. Roberts and Crothers (1) used hairpin-type duplexes and hybridized various pyrimidine complements to them. Such a system entropically favors the Watson–Crick duplex, giving it a considerably higher affinity than the third strand interaction and resulting for the most part in two separate helix dissociations with rising temperature. The studies of Dervan (2), Hélène (3) and Maher (4) also used systems with strong underlying duplexes. That type of system may be considered a reasonable model for third strand binding to long duplex targets.

Several laboratories have recently begun studying other potentially useful modes of triplex formation however. There has been increasing interest in the formation of triplexes on single-stranded targets (5,17,20,26–41), which can result in complexes with high affinity (5,17,29,30) and sequence selectivity (28). The complexes in the present study are especially useful as models for that second triplex binding mode and with careful comparisons one can also derive information on third strand binding (see Figs 3 and 4 and Discussion below). The present system (circle + single strand) is easily analyzed, because all the dissociations appear to be cooperative all-or-none melting events, even at neutral pH.

Comparisons of the present data with three previous studies overall show reasonably good agreement, despite the difference in binding modes. For example, in agreement with all three studies (1–3), we find that the DRR and DRD triple helices are highly unstable and like those groups we observe only duplexes, with no third strand interaction, even at low pH.

Some differences between previous data and the present cases are evident on closer inspection however. For example, Roberts and Crothers previously reported an overall order of triple helix stability of RRR > RDR > RRD > RDD > DDD ≥ DDR for the six observed triplexes (1); our study finds a significantly different order (Fig. 5): RDD > DDD >RRR ~ RRD ≥ RDR > DDR. The largest differences are found for the RDD and DDD cases, which are among our most stable complexes, but which they find to be of intermediate and poor stability respectively. Some differences in the two studies may arise from variations in sequences and buffer conditions used, however, another important reason for this difference arises from the fact that the structures of the triplexes are considerably different. In the previous study the duplex portion is relatively emphasized, because it is intramolecular, while the affinity of the third strand interaction plays a relatively smaller role. In the present study both interactions are given strong emphasis, because the whole complex is cooperative. Since there are apparently large differences in backbone effects depending on which model structure is used, single strand + duplex hairpin systems should be considered as suitable models for third strand binding to duplexes, while structures such as those in the current study may be better models for triplex formation on a single strand target.

The 2′-*O*-Me-RNA modification in triplex formation

Studies have compared the ability of DNA, RNA and 2′-*O*-Me-RNA strands to bind to sites in duplex DNAs (7). A general survey of triplex formation with 2′-*O*-Me-RNA strands in all possible positions has not previously been carried out however. The present study thus represents the first general comparison of all three structural modifications in triple helix formation.

Third strand binding of duplex DNA. Studies reported by Ohtsuka (10) and by Hélène (9) compare the relative abilities of DNA, RNA and 2′-*O*-Me-RNA strands to bind to sites in duplex DNAs. At acidic pH both studies found that a 2′-*O*-Me-RNA strand binds to a duplex target sequence more strongly than does an RNA strand, which in turn binds more strongly than a DNA strand. At less acidic pH (pH 6.1) Ohtsuka (10) found that both the 2′-*O*-Me-RNA and RNA probes bound duplex equally well, but still with higher affinity than the DNA strand.

In the present study we find that at neutral pH all three structural analogs form roughly equally strong third strand interactions, thus the major difference is that in our study the DNA strand is found to have a higher relative binding affinity. A likely explanation for this difference lies in the different sequences used in the three experiments. Our study uses a probe sequence with 25% C residues, while the other two studies used probes with 45–53% C residues. We have shown in other work that the thymine C-5 methyl group is stabilizing by 0.2–0.3 kcal/methyl in third strand binding (6). Thus our DNA probe is favored because it is T-rich, benefiting from larger numbers of C-5 methyl groups. The other two analogs are less affected by this sequence difference, because the RNA pyrimidines are unmethylated. If all C-5 methylated nucleotides (both T and 5mC) were used, presumably the 2′-*O*-Me-RNA third strand would likely be favored no matter what the sequence.

Other duplex targets. 2′-*O*-Me-RNA third strands show differing preferences for the other three natural duplex targets (the RR, DR and RD types). Pyrimidine strands composed of this modification bind as well as unmodified RNA to a DR-type duplex. However, such strands hybridize poorly to RD-type targets and not at all to RNA–RNA duplexes, while RNA third strands bind both reasonably well. Thus while 2′-*O*-Me-RNA shows some hybridization similarities to RNA, there are also significant differences.

Single-stranded RNA or DNA targets. Circular oligonucleotides containing one or more pyrimidine domains of 2′-*O*-Me-RNA can in all cases bind to a DNA single strand by triple helix formation, resulting in higher binding affinity than can be achieved by simple Watson–Crick hybridization (see Fig. 3). The strongest ligand containing 2′-*O*-Me-RNA residues is the circle containing one 2′-*O*-Me-RNA domain and one DNA domain. However, considerably higher affinity (up to 6 kcal) can be achieved using circles with one RNA and one DNA or with two DNA domains and no 2′-*O*-Me-RNA residues. Thus for this binding mode we conclude that the 2′-*O*-Me modification does not offer binding advantages over the natural backbones.

As seen for RNA/DNA chimeras, the triplex binding of RNA purine strands with chimeric oligonucleotides containing 2′-*O*-Me-RNA is considerably rarer and lower in affinity. When RNA is the target, the strongest ligand containing 2′-*O*-Me-RNA residues is the circle containing one RNA and one 2′-*O*-Me-RNA domain; again, however, stronger binding (by ~1 kcal) can be achieved with a circle containing two RNA domains. In general, with single-stranded RNA as a target, triplex-forming ligands offer less binding advantage than is seen when the target is DNA, however, modifications such as pyrimidine C-5 methylation can enhance binding affinity for RNA very significantly (6).

In general then, we find that 2′-*O*-Me-RNA strands can in many cases form triple helical complexes, but in the binding of single-stranded targets by triplex formation, molecules containing this modification offer no binding advantage over ones containing only DNA and/or RNA strands. It should be noted, however, that 2′-*O*-Me-RNA does offer the significant advantage of resistance to degradation by endonuclease enzymes (8), making this analog attractive relative to unmodified RNA if some binding affinity can be sacrificed.

General recommendations for contructing ligands for RNA or DNA

Several recommendations for the design of pyrimidine ligands for nucleic acids can be offered after considering all the data. The following approaches will give the highest affinity binding of purine-rich sequences using the three backbones in this study:

Binding of duplex DNA. At neutral pH, pyrimidine-rich third strands composed of 2′-*O*-Me-RNA or RNA will give the highest affinity; if the target is A-rich rather than G-rich, DNA third strands will bind almost equally well. In addition, analogs of 2′-*O*-Me-RNA pyrimidines which are also C-5 methylated are likely to give yet higher affinity (6).

Binding of other duplexes. For highest affinity, RNA–RNA duplexes are best targeted with RNA third strands. In addition, our previous study of C-5 methylation effects established that methylation of the

pyrimidine bases adds considerable affinity to such a complex (6); this effect holds true no matter what type of duplex is being targeted. For binding DR-type duplexes, either RNA or 2′-*O*-Me-RNA strands bind equally well. Finally, for RD-type duplexes, RNA third strands bind the most proficiently of the three analogs.

Binding of single-stranded DNA. The best ligands in this case will be circular or hairpin compounds containing an RNA Hoogsteen domain and a DNA Watson–Crick domain. Again, methylation of all pyrimidine bases will add to the affinity (6).

Binding of single-stranded RNA. Highest affinity binding can in this case be achieved with triplex-forming compounds containing two RNA domains. Methylation of the pyrimidines has been shown to add several kcal/mol additional affinity (6).

It should be noted also that if the target purine sequence is rich in G rather than A residues, one can use the pur•pur•pyr motif in the binding of either duplex or single-stranded sequences. Recent studies of the effects of backbone in that motif have shown that RNA structure can be destabilizing in the third strand binding of the duplex (42). In the binding of single strands we recently found that although RNA targets are not bound as strongly as DNA targets, higher affinity can still be gained by use of triplex structure (40).

Selectivity for DNA versus RNA strands

Some of the new circular ligands have the unusual property of binding selectively to DNA over RNA (5). For example, at pH 7.0 the circle with two DNA binding domains hybridizes to the DNA complement with a $>10^6$-fold higher association constant. At acidic pH the selectivity for DNA increases further, to a remarkable 11.1 kcal (5). To our knowledge, this magnitude of DNA selectivity has not been observed previously.

Although the effect has been shown to vary with sequence (6,43–45), single strands composed of RNA or 2′-*O*-Me-RNA backbones often show the opposite selectivity, with a significant preference for RNA over DNA (7). For example, the 2′-*O*-Me-RNA pyrimidine strand in this study (Table 1) binds to an RNA strand with 6.8 kcal higher affinity than to a DNA strand and the RNA pyrimidine strand shows a similar magnitude of preference. Other synthetically modified nucleic acid analogs have also been reported to display such a RNA binding preference (46,47).

Interestingly, our results show that a triplex-forming oligonucleotide which contains two domains of 2′-*O*-Me-RNA residues actually reverses this binding preference. At pH 5.5 such a compound actually prefers a DNA purine complement over a RNA one by 12.2°C in T_m or 1.7 kcal/mol in free energy. Thus not only backbone differences, but also structural and conformational properties can influence such selectivity in binding.

By correct choice of ligand structure (as outlined above) it is now possible to choose to bind either to RNA or to DNA single strands with high selectivity under physiological conditions. This fact may prove useful in designing hybridization probes for biological research and diagnostic applications, where both types of strands may be present in a mixture. Studies exploring this possibility are currently under way.

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