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Oligodeoxyribonucleotide length and sequence effects on intermolecular purine–purine–pyrimidine triple-helix formation

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ABSTRACT

The binding of guanosine/thymidine-rich oligodeoxyribonucleotides containing various deletions, extensions, and point mutations to polypurine DNA targets was investigated by DNase I footprinting. Intermolecular purine–purine–pyrimidine triple-helical DNA formation was best achieved using oligonucleotides 12 nucleotides in length. Longer oligonucleotides were slightly weaker in binding affinity, whereas shorter oligonucleotides were considerably weaker. Oligonucleotide extensions had a slight effect on triplex formation, while single point mutations located near the oligonucleotide ends had a greater effect. In the cases of extensions and point mutations, changes to the 3′ end of the oligonucleotide had a consistently greater effect on triplex formation than changes to the 5′ end. Such differences in triplex-forming ability were not caused by an intrinsic property of these oligonucleotides, since the same point mutated oligonucleotides could bind with high affinity to duplex DNAs containing complementary sites. Taken together, our data suggest that there may be an asymmetry involved in the process of purine-motif triplex formation, with interactions between the 3′ end of the oligonucleotide and complementary sequences on the target duplex DNA being dominant.

INTRODUCTION

Oligonucleotide-mediated triple-helix formation has been described as a potentially powerful way to modulate gene expression at the level of transcription, presumably through its interference with specific transcription factor binding to promoter or enhancer elements (1,2). Two classes of DNA triple helices, or triplexes, have been described. Both contain oligonucleotides binding in the major groove of duplex DNA through hydrogen bonding interactions with runs of purine acceptors. In the less commonly described motif, purine oligonucleotides bind with an antiparallel orientation through reverse Hoogsteen base pairing (3,4). Base pairing in this motif includes guanosine binding to guanosine (G:G–C) and either adenosine or thymidine binding to adenosine (A:A–T or T:A–T). These purine–purine–pyrimidine (Pu–Pu–Py) triplexes have been shown to exist at physiological pH, Mg^{2+}, and polyvalent cation concentrations, though not at physiological monovalent cation concentrations (5,6).

Several laboratories have described the use of purine-motif triplexes to inhibit transcription from specific genes, both *in vitro* and *in vivo*. Examples include triplex-mediated inhibition of human c-*myc* (7,8), IL2Rα (9), HIV (10), progesterone receptor-dependent (11), 6-16 (12) and HER-2/neu (13) transcription. In order to achieve the required specificity of binding to a particular target sequence, these investigators have used oligonucleotides ranging from 21 to 39 nucleotides in length. Since most native DNA sequences lack a homopurine stretch of this length, thymidines are often incorporated in these long triplex-forming oligonucleotides at sites opposite the intervening pyrimidines, ostensibly to minimize interference between the oligonucleotide and duplex DNA. Taken together, these characteristics of long lengths and thymine substitutions define one paradigm for the design of purine-motif triplex-forming oligonucleotides.

While many of these longer oligonucleotides contained more than 30 nucleotides potentially capable of reverse Hoogsteen bonding, most of these oligonucleotides in fact possessed apparent binding affinities comparable to those found for far shorter oligonucleotides (3,14). To explain this discrepancy between length and binding affinity, we undertook a systematic investigation of the effects of oligonucleotide sequence and length in the formation of purine-motif triplexes. DNase I cleavage protection assays were used to investigate Pu–Pu–Py triplex formation for a variety of G/T-rich oligonucleotides, including those containing deletions, oligonucleotide extensions and point mutations on either the 5′ or 3′ ends. We found that the optimal triplex-forming oligonucleotide may actually be 12 nucleotides in length, with homology between the target duplex and the oligonucleotide 3′ end being more important than complementarity with the 5′ end. These data suggest a model

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in which G/T-rich oligonucleotides anneal with duplex DNA in an asymmetric fashion when forming Pu–Pu–Py triple helices.

MATERIALS AND METHODS

Oligodeoxyribonucleotides and DNA probes

Guanosine/thymidine-rich oligodeoxyribonucleotides (ODN) used in this study are listed in Table 1. All were synthesized by phosphoramidite chemistry on an Applied Biosystems DNA synthesizer and purified by n-butanol precipitation (15). Concentrations were determined spectrophotometrically, using an average nucleotide molar extinction coefficient at 260 nm of 3.3×10^5 $M^{-1}cm^{-1}$. Duplex DNA probes used in DNase I cleavage protection assays consisted of the 201-bp *Eco*RI–*Pvu*II fragment from pBluescript II SK, containing a 19-bp polypurine–polypyrimidine cassette cloned into the *Sac*I site. These were singly 3′ end-labeled at the *Eco*RI site by Klenow end-filling and purified by NA-45 membrane (Schleicher & Schuell) following manufacturer's instructions. The structure of these probes, together with the sequence of their triplex-forming cassettes, is shown schematically in Figure 1.

DNase I cleavage protection (footprinting) assays

To effect triplex formation, we incubated 50 nM duplex DNA probe and oligonucleotide (concentration indicated in each figure) for 60 min at 30°C in a 10-μl volume containing 40 mM HEPES·Na^+ (pH 8.2), 12 mM $MgCl_2$ and 5 mM dithiothreitol. DNase I (2 ng) was then added to each sample and cleavage was allowed to proceed for 30 s at room temperature before termination by the addition of 3 μl stop buffer (3.0 M ammonium acetate, 0.5 M EDTA, 1.0 mg/ml tRNA). Adenine-specific chemical cleavage reactions were used as markers (16). Samples were purified by phenol/chloroform extraction and ethanol precipitation, dried briefly, resuspended in 95% formamide, and heated at 95°C for 4 min before being loaded onto a denaturing 8% polyacrylamide gel. Electrophoretically separated DNA cleavage products were visualized by autoradiography and quantitated by densitometric scanning. Determination of the extent of triplex formation followed normalization of DNase I cleavage efficiency using sites on either side of the footprints. Reactions containing no oligonucleotide (0) served as controls.

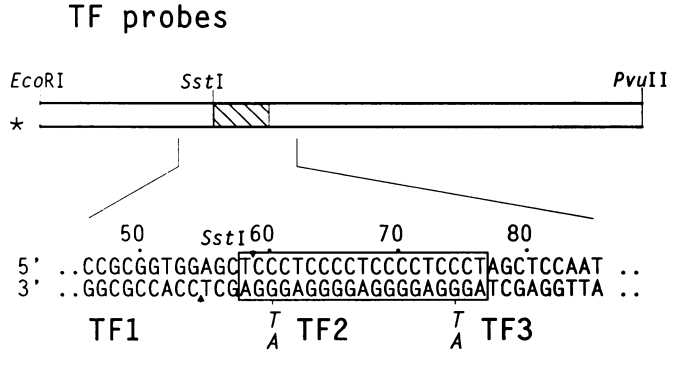

Figure 1. (**Top**) Schematic representation of duplex DNA probes used for analyzing Pu–Pu–Py triplex formation. Triplex-forming region is boxed. Site of unique, 3′-^{32}P end label is indicated by an asterisk. (**Bottom**) Nucleotide sequence of homopurine-homopyrimidine triplex-forming region for probe TF1. Numbers above indicate distance from the labeled 3′ end. Probes TF2 and TF3 are identical to probe TF1 except for single GC→TA point mutations at position 60 and 74, respectively.

RESULTS

The effects of oligonucleotide length on Pu–Pu–Py triplex formation

As is found with Watson–Crick base-paired duplex DNA, it was anticipated that oligonucleotides containing more complementary nucleotides would form triplexes with a higher binding affinity than shorter oligonucleotides (17). For these studies we chose the purine-rich oligodeoxyribonucleotide $TG_3TG_4TG_4TG_3T$ (**ODN 1**) as our model compound, given its strong, sequence-specific binding in an antiparallel fashion to a G-rich homopurine, duplex DNA target (3,14). To test the effects of oligonucleotide length, oligonucleotides derived from ODN 1 containing deletions at either the 5′ or 3′ end were synthesized. Their nucleotide sequences are shown in Table 1. Oligonucleotides and a radiolabeled DNA probe, TF1, were incubated under conditions favoring purine-motif triplex formation (12 mM Mg^{2+} buffered to pH 8.2; no additional monovalent cations) (3,5). Triplex formation was investigated by DNase I cleavage protection under conditions that should allow a determination of relative binding affinity (18).

As shown in Figure 2, a DNase I footprint encompassing the GA strand of the polypurine–polypyrimidine cassette was observed with increasing oligonucleotide concentration for each of the oligonucleotides tested. Qualitatively, the footprints and surrounding DNase I cleavages were similar in appearance for the oligonucleotides ODN 1, Δ1–4 and Δ1–7. In the case of Δ1–9, however, protection corresponding to the 3′ end of the GA strand (i.e., towards the bottom of autoradiogram) was somewhat diminished. This is understandable, given the antiparallel binding orientation of purine-motif triplexes. Quantitatively, differences were quite apparent regarding the concentration of each oligonucleotide necessary to demonstrate

Table 1. Triplex-forming abilities of G/T-rich oligodeoxyribonucleotides binding to probe TF1

Name	Length (nt)	Sequence (5′ -> 3′)	Binding Affinity
	Probe TF1	*5′----TCCCTCCCCTCCCCTCCCT----3′*	
		3′----AGGGAGGGGAGGGGAGGGA----5′	
ODN 1	19	TGGGTGGGGTGGGGTGGGT	++
Δ1-4	15	TGGGGTGGGGTGGGT	++
Δ1-5	14	GGGGTGGGGTGGGT	++
Δ1-7	12	GGTGGGGTGGGT	+++
Δ1-9	10	TGGGGTGGGT	+
Δ16-19	15	TGGGTGGGGTGGGGT	++
Δ15-19	14	TGGGTGGGGTGGGG	++
Δ13-19	12	TGGGTGGGGTGG	+++
Δ11-19	10	TGGGTGGGGT	-
ODN 1+5′	27	TTCTTCTTTGGGTGGGGTGGGGTGGGT	++
ODN 1+3′	27	TGGGTGGGGTGGGGTGGGTTTCTTCTT	+
(Δ1-7)+5′	20	TTCTTCTTGGTGGGGTGGGT	+++
(Δ1-7)+3′	20	GGTGGGGTGGGTTTCTTCTT	++
(Δ13-19)+5′	20	TTCTTCTTTGGGTGGGGTGG	+
(Δ13-19)+3′	20	TGGGTGGGGTGGTTCTTCTT	-
T3	19	TGTGTGGGGTGGGGTGGGT	++
T17	19	TGGGTGGGGTGGGGTGTGT	-

Relative binding affinities determined by the concentration of oligonucleotide necessary for 70% protection from DNase I cleavage. (+++), less than 0.2 μM; (++), 0.2 to 0.6 μM; (+), 0.6 to 1.0 μM; (−) greater than 1.0 μM.

DNase I cleavage protection. With ODN 1 and Δ1−4, substantial cleavage protection (greater than 70%) was only observed once 0.4 μM oligonucleotide was present. However, comparable levels of protection required less than 0.2 μM of the oligonucleotide Δ1−7. In the case of the longest 5′ deletion, Δ1−9, concentrations in excess of 1 μM were necessary to achieve substantial footprinting (data not shown). The relative binding affinities for these oligonucleotides are presented in Table 1. A similar relationship between oligonucleotide length and binding affinity was also found for a series of 3′ deleted oligonucleotides (see Table 1, oligonucleotides Δ16−19, Δ15−19, Δ13−19 and Δ11−19). Taken together, these data would suggest that G/T-rich oligonucleotides 12 nucleotides in length are most capable of triplex formation under these reaction conditions. Shorter oligonucleotides were significantly impaired in their ability to form triplexes, while longer oligonucleotides exhibited no direct correlation between increasing length and binding affinity.

The effects of oligonucleotide extensions on Pu−Pu−Py triplex formation

One possible explanation for our observed lack of correlation between binding affinity and increasing oligonucleotide length would be that only a 12-nucleotide stretch within the longer oligonucleotide is ever capable, at any moment, of engaging in triplex formation. In this model the remaining nucleotides would not be involved in hydrogen bonding; rather their phosphate backbone might actually contribute to a decrease in triplex binding affinity through like-charge repulsion with the duplex DNA.

To test this hypothesis, we constructed oligonucleotides derived from ODN 1 containing short oligonucleotide stretches appended to either the 5′ or 3′ end. Their sequences are shown in Table 1. The sequence TTCTTCTT was chosen for the additional nucleotides in order to minimize the possibility of hydrogen bonding, either Watson−Crick or Hoogsteen, between individual oligonucleotides or with the duplex DNA probe. Binding of these oligonucleotides to probe TF1 was analyzed by quantitative DNase I cleavage protection.

As shown in Figure 3, both extended oligonucleotides were capable of triplex formation at micromolar concentrations. However, while the oligonucleotide with the 5′ extension (ODN 1+5′) demonstrated a binding affinity comparable to the parent oligonucleotide, ODN 1 (see Table 1), the triplex-forming ability of ODN 1+3′ was slightly reduced. This would suggest that an oligonucleotide extension on the 3′ end has a greater effect on purine-motif triplex formation than extension on the 5′ end. To confirm this, we analyzed the effect of oligonucleotide extensions on triplex formation by the shorter oligonucleotides Δ1−7 and Δ13−19. In these cases, one could have expected that the destabilizing effects of additional nucleotides would be more substantial, given their closer proximity to the core 12-nucleotide stretch presumed essential for triplex formation. As was also found for the longer oligonucleotides, eight-nucleotide extensions on the 3′ ends of either 12-mer oligonucleotide had a consistently greater effect on triplex formation than extensions on the 5′ ends (Table 1).

Concerning the expected increased destabilization of the shorter oligonucleotides, such was most apparent with the oligonucleotides derived from Δ13−19, (Δ13−19)+5′ and (Δ13−19)+3′. However, oligonucleotide extensions on the 5′ end of oligonucleotide Δ1−7 was found to have no significant effect on triplex formation. Thus the effects of oligonucleotide extension was very dependent on the location of the additional nucleotides, both with respect to the oligonucleotide ends (5′ or 3′) and to their proximity to the core triplex-forming region.

The effects of single base mismatches on Pu−Pu−Py triplex formation

Given that oligonucleotides extended at either their 5′ or 3′ ends differed in their abilities to form triplexes, we next investigated whether oligonucleotides containing single-base mismatches with the duplex DNA target exhibited similar differences. Oligonucleotides T3 and T17, which were synthesized for this study, contained sequences identical to ODN 1, with the exception

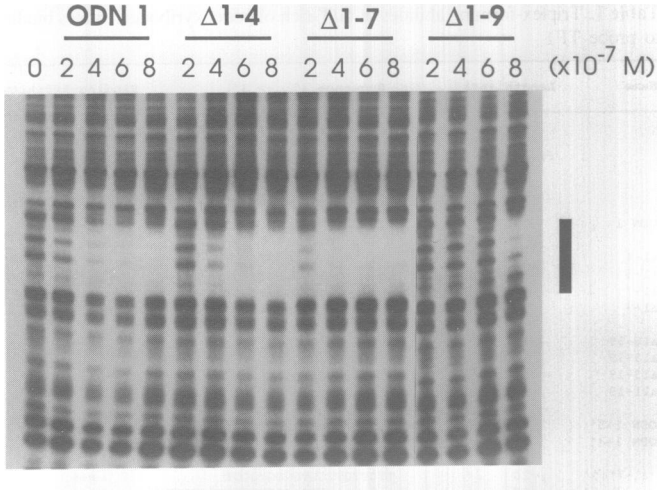

Figure 2. DNase I cleavage analysis of Pu−Pu−Py triplex formation on probe TF1 by G/T-rich oligonucleotides containing 5′ deletions. Sequences of oligonucleotides ODN 1, Δ1−4, Δ1−7 and Δ1−9 are shown in Table 1. Triplex formation was performed in a buffer containing 40 mM HEPES·Na^+ (pH 8.2), 12 mM $MgCl_2$ and 1 nM probe TF1. Final oligonucleotide concentrations were as indicated above each lane. The solid bar at right indicates the region protected against DNase I cleavage by oligonucleotide binding.

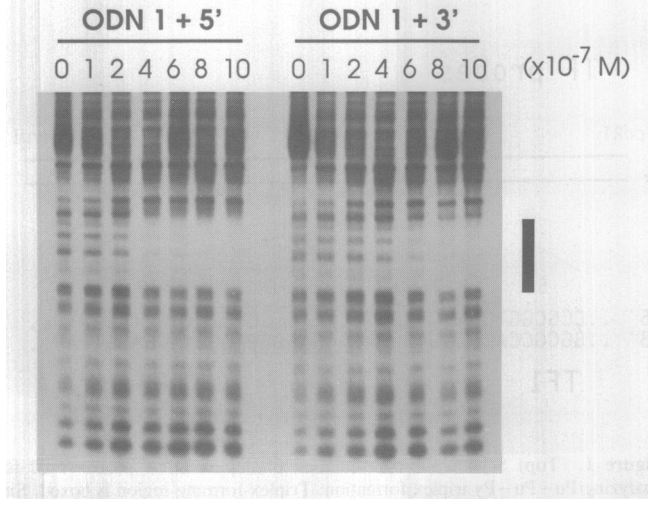

Figure 3. DNase I cleavage analysis of Pu−Pu−Py triplex formation on probe TF1 by G/T-rich oligonucleotides containing nucleotide extensions on either their 5′ or 3′ ends. Sequences of oligonucleotides ODN 1+5′ and ODN 1+3′ are shown in Table 1.

of a single G→T point mutation at positions 3 or 17, respectively (see Table 1). Note that the T:G−C base triplet was chosen in order to maintain the G/T-rich nature of these oligonucleotides and also because this triplet has previously been shown to destabilize purine-motif triplexes [14].

The binding of these oligonucleotides to the probe TF1 was analyzed by quantitative DNase I footprinting. As shown in Figure 4, significant DNase I protection was afforded by 0.4 μM T3, whereas 2.0 μM T17 was required to exhibit similar levels of protection. In fact, the relative binding affinity of T3 to probe TF1 was similar to that of ODN 1 (Fig. 2 and Table 1). These data demonstrated that a single T:G−C triplex mismatch located three nucleotides from the 3′ end of a G/T-rich oligonucleotide had a far greater effect on triplex formation than a corresponding mismatch located near its 5′ end. Thus both types of potentially destabilizing phenomena, oligonucleotide extensions and base mismatches, had identical end-specific effects on purine-motif triplex formation.

Our findings could be interpreted in two ways. First, interactions between the 3′ end of a G/T-rich oligonucleotide and the duplex DNA may be essential for purine-motif triplex formation. Alternatively, changes near the 3′ termini of these oligonucleotides may affect some other process that indirectly affects triplex formation. For example, G/T-rich oligonucleotides are well known to self-associate under certain conditions into four-stranded species termed tetraplexes (19,20). Given their stability, oligonucleotides bound in tetraplexes would not be expected to be readily available for subsequent triplex formation. In order to differentiate between these two possibilities, the binding of oligonucleotides ODN 1, T3 and T17 to different duplex DNA probes was investigated. Probes TF2 and TF3 were homologous to probe TF1, with the exception of single GC→AT mutations at positions 60 and 74 base pairs from the labeled 3′ end, respectively (Fig. 1). Note that with these mutations, oligonucleotide T3 could form a triplex-stabilizing T:A−T triplet with probe TF2 while ODN 1 would contain a destabilizing G:A−T triplet near its 5′ end (Table 2). Similar effects would also be expected for the binding of oligonucleotides T17 and ODN 1 to probe TF3, except that the mismatch would now be located near the 3′ end of ODN 1. The results of a representative DNase I footprinting assay are shown in Figure 5. Substantial binding between T3 and probe TF2 was found to occur at 0.4 μM T3, whereas greater than 0.6 μM ODN 1 was required to demonstrate similar levels of protection. Similarly, the binding of T17 to its complimentary sequence on probe TF3 was greater than the binding of ODN 1 to TF3 (Table 2). Note also that ODN 1 bound to probe TF3 with less affinity than to probe TF1. This is consistent with our observation that a mismatch near the oligonucleotide 3′ end has a greater effect on triplex formation than a mismatch near the 5′ end. Taken together, these data would suggest that the difference in triplex-forming abilities between 5′- and 3′-modified, G/T-rich oligonucleotides is not the result of an intrinsic property of these molecules but rather reflects the importance of interactions between the oligonucleotide 3′ end and duplex DNA during purine-motif triplex formation.

Table 2. Triplex formation with G/T-rich oligodeoxyribonucleotides to point mutated sites

Name	Length (nt)	Sequence (5′ -> 3′)	Binding Affinity
A.	*Probe TF2*	*5′----TCTCTCCCCTCCCCTCCCT----3′* *3′----AGAGAGGGGAGGGGAGGGA----5′*	
ODN 1	19	TGGGTGGGGTGGGGTGGGT	+
T3	19	TGTGTGGGGTGGGGTGGGT	++
B.	*Probe TF3*	*5′----TCCCTCCCCTCCCCTCTCT----3′* *3′----AGGGAGGGGAGGGGAGAGA----5′*	
ODN 1	19	TGGGTGGGGTGGGGTGGGT	-
T17	19	TGGGTGGGGTGGGGTGTGT	++

Relative binding affinities determined by the concentration of oligonucleotide necessary for 70% protection from DNase I cleavage on (**A**) probe TF2 or (**B**) probe TF3. (++), 0.2 to 0.6 μM, (+), 0.6 to 1.0 μM; (−) greater than 1.0 μM.

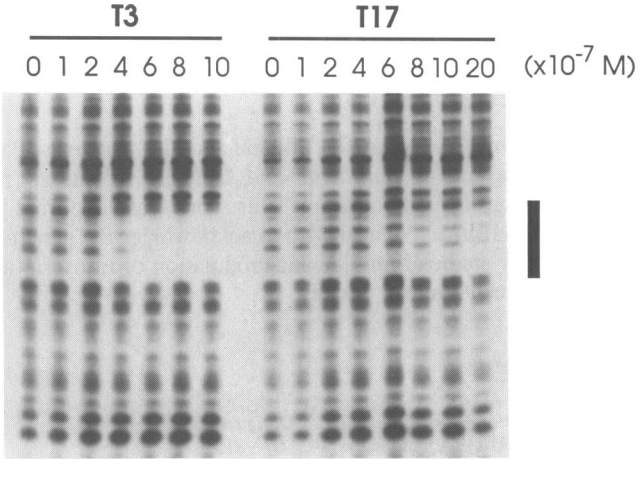

Figure 4. DNase I cleavage analysis of Pu−Pu−Py triplex formation on probe TF1 by G/T-rich oligonucleotides containing single base mutations near either their 5′ or 3′ ends. Sequences of oligonucleotides T3 and T17 are shown in Table 1.

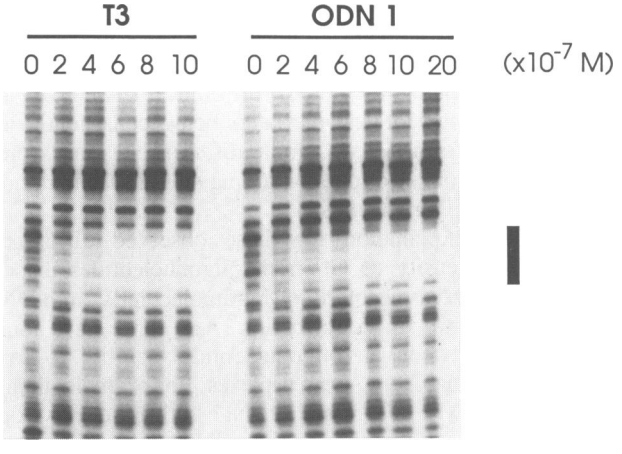

Figure 5. DNase I cleavage analysis of Pu−Pu−Py triplex formation on probe TF2 by the G/T-rich oligonucleotides ODN 1 and T3. Sequences of oligonucleotides ODN 1 and T3 and duplex DNA probe TF2 are shown in Table 2.

DISCUSSION

As part of our studies on the modulation of transcription by triplexes, we investigated what oligonucleotide parameters are important for purine-motif triplex formation. Oligonucleotide length was an obvious first candidate. Several laboratories have used relatively long (greater than 30-nucleotide) G/T-rich oligonucleotides to target cellular genes (7–13), yet their binding affinities were often no greater than those of shorter oligonucleotides (3,14). Using the well-characterized 19-mer $TG_3TG_4TG_4TG_3T$ as a starting point, we found that certain short deletions from either the 5′ or 3′ ends actually facilitated triplex formation several fold. Only when the oligonucleotides were reduced to 10-mers was triplex formation substantially decreased. This apparent 12-nucleotide optimum is unique to purine-motif triplex formation; both the annealing of Watson–Crick base-paired duplex DNA (17) and the formation of pyrimidine-motif triplexes (21) demonstrate increased binding affinity with increasing length. One possible explanation for this difference may lie with the runs of guanines present in our oligonucleotides. Purines such as guanine would be expected to have strong stacking interactions with adjacent purines. These interactions could place the oligonucleotide in a conformation that is not fully conducive to the formation of reverse Hoogsteen base pairs, which are required for purine-motif triplex formation. If the loss of base stacking stabilization is not fully compensated for by hydrogen bonding, then the formation of long stretches of G:G–C base triplets would become energetically unfavorable. This may also have been observed in early studies with homopolymers, where the use of shorter poly-rG homopolymers was required for rG–rG–rC triplex formation (22). Similarly, this could help explain the benefit of periodic thymines located in G/T-rich oligonucleotides. Their placement would separate the stretches of stacked purines, thereby allowing a greater degree of flexibility for these oligonucleotides.

If a 12-nucleotide G/T-rich oligonucleotide is optimal for purine-motif triplex formation, then those additional nucleotides present in a typical 30-mer could be interpreted as merely appended additional mass and negative charge. In order to test this hypothesis, we constructed G/T-rich oligonucleotides possessing eight additional nucleotides attached to either their 5′ or 3′ ends. The sequence of these nucleotide extensions was chosen to minimize the possibility of interactions, either with the duplex probe or with other oligonucleotides. Most of these extended oligonucleotides displayed a decreased triplex-forming ability. Most surprising, we noted a difference between 5′- and 3′-modified oligonucleotides, the latter being consistently less capable of triplex formation. A similar difference was also found with G/T-rich oligonucleotides containing single base mutations near either their 5′ or 3′ ends.

One possible explanation for these observed differences involves the propensity of G/T-rich oligonucleotides to self-associate, either intermolecularly or intramolecularly, into DNA species refractory to triplex formation. As yet we have found no correlation between an oligonucleotide's ability to form tetraplexes and its inability to form triplexes. For example, though oligonucleotides (Δ1–7)+5′ and ODN 1 both efficiently form triplexes (Table 1), under the proper conditions (Δ1–7)+5′ can form tetraplexes while ODN 1 cannot (A.-J.Cheng, unpublished observations; ref. 6, Fig. 6). Further evidence that an intrinsic oligonucleotide property was not responsible for these differences was obtained with the point mutated oligonucleotides T3 and T17. While these oligonucleotides bound to the wild-type duplex probe TF1 with very different affinities, they both bound duplex probes containing correspondingly mutated target sequences with equal affinity. Taken together, our data suggest that interactions between the 3′ end of the oligonucleotide and purines in the DNA duplex are instrumental in subsequent purine-motif triplex formation. Under our reaction conditions, the rate of oligonucleotide association with duplex DNA appears to be the determining factor in the extent of triplex formation observed (6). Given this, possible explanations for the apparent difference between oligonucleotides altered at 5′ or 3′ ends include purine-motif triplex nucleation occurring preferentially with the 3′ end of the third strand and triplex annealing proceeding with a preferred 3′→5′ orientation. Either explanation would be in contrast to the cases with duplex DNA (23,24) and pyrimidine-motif triplexes (25), where nucleation of hybridization is thought to occur anywhere along the length of the nucleic acid and annealing proceeds in either direction with equal efficiency.

The findings of our studies should have practical benefit for the design of G/T-rich triplex-forming oligonucleotides. If 12-mer oligonucleotides are optimal for triplex formation, then the recognition of cellular targets might be best achieved using several shorter oligonucleotides tethered together by non-anionic linkers. Also through the use of different linker lengths, the sequences of these tethered oligonucleotides could be chosen to avoid the necessity of base triplet formation with pyrimidines in the target homopurine strand of the duplex DNA. Evidence for the feasibility of this approach has recently been described (26), in which hybrid G/T-rich oligonucleotides containing a polymeric linker exhibited at least a 10-fold increase in apparent binding affinities when compared to conventional oligonucleotides. Regarding the significance of the oligonucleotide 3′ end, not only do our findings stress the importance of proper base triplet formation in this region, but they also suggest the possibility of improving triplex formation through the use of 3′ covalently-attached chemical moieties that interact strongly with DNA. Examples include both duplex and triplex DNA intercalating agents (27–29) and various polycations (30). Such improvements in binding affinity and specificity will be essential if the modulation of gene expression through the triplex-dependent inhibition of transcription is to have eventual therapeutic applications.

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