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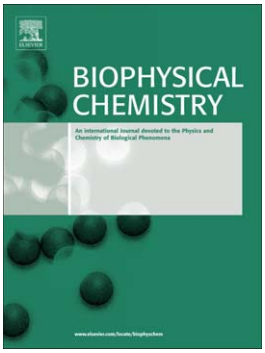

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The stability of triplex DNA is affected by the stability of the underlying duplex

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Abstract

We have studied the formation of DNA triple helices in different sequence contexts and show that, for the most stable triplexes, their apparent stability is affected by the stability of the underlying duplex. For a 14-mer parallel triplex-forming oligonucleotide (generating C^+.GC and T.AT triplets) at pH 5.0 the T_m is more than 10 °C lower with an intermolecular 14-mer duplex target, than it is with an intramolecular duplex, or one which is flanked by 6 GC base pairs at either end. A similar effect is seen with triplex-forming oligonucleotides that contain stabilising analogues, for which the T_m is higher for an intramolecular than an intermolecular duplex target. These results suggest that the use of simple intermolecular duplex targets may underestimate the triplex stabilisation that is produced by some nucleotide analogues.

1. Introduction

Triple helical nucleic acids are formed by binding a third oligonucleotide strand within the major groove of duplex DNA, in which bases in the third strand make specific contacts with the exposed edges of the duplex base pairs [1-5]. In the most commonly studied form the third strand is oriented parallel to the purine strand of the duplex generating T.AT and C^+.GC triplets [6-8]. The three stranded structure is usually less stable than the underlying duplex, as a result of the repulsion between the three negatively charge strands. The formation of these complexes is also limited by the requirement for low pH (required for protonation of cytosine in the C^+.GC triplet) and is usually restricted to oligopurine.oligopyrimidine tracts. A large number of nucleotide analogues have been devised in attempts to overcome these limitations [9-19].

One of the most commonly used methods for assessing the stability of these triple helical complexes is to measure their thermal stability by either UV or fluorescence melting experiments [20]. These are usually performed with short synthetic duplex targets, which are of similar length to the third strand. In some experiments the duplex has been made longer than the triplex in order to separate the duplex and triplex melting transitions [21-23], while others have employed intramolecular duplexes in order to increase the stability of the target [24-26]. In contrast in most footprinting experiments and for *in vivo* applications the duplexes in which the target sequences are located will be much longer than the third strand [19,27,28].

As a result of progress in the design and synthesis of modified nucleotides it is now possible to generate triplexes that are as stable as the underlying duplex and the entire complex melts in a single transition. We were therefore concerned that for some very stable triplexes the apparent affinity of the third strand will be limited by the stability of the underlying duplex, since melting of the duplex will probably cause the third strand to dissociate from its Hoogsteen partner. We have therefore examined how the stability of the duplex target affects triplex melting. We have done this by comparing the interaction of one oligonucleotide (TFO1, Fig. 1a) with its duplex target when this is placed in a variety of different duplex contexts. This TFO is suited to this analysis as at low pHs, and on incorporation of stabilising nucleotides, the melting of this triplex overlaps with that of the simple intermolecular duplex. For

these studies we have used duplexes that are stabilised by adding GC- or AT- tails to either end, or by joining the two strands to generate an intramolecular duplex.

2. Materials and Methods

2.1 Oligonucleotides

The sequences of the oligonucleotides used in these studies are shown in Fig. 1. These were prepared as previously described [23]. For the experiments with unmodified triplex-forming oligonucleotides all the duplex targets contained the oligopurine tract AGAGAGAAGGAGGA, which was embedded within a variety of different sequence contexts. The 14-mer third strand oligonucleotide (TFO1) contained natural bases only (T and C). The oligonucleotides containing nucleotide analogues at a single central location in place of T (TFOs 2-7) were 18-mers and were targeted against the duplexes inter2 and intra2. The structures of the various nucleotide analogues are shown in Fig. 1b. In each case the purine strand of the duplex was labelled with fluorescein, which was either placed at the 5'-end or attached to the 5-position of a U located close to the 5'-end of the triplex target site (FAM-dU). The TFOs were labelled with dabcyl at the 5'-end. Phosphoramidites for 5'-fluorescein, FAM-dU and 5'-dabcyl were purchased from Glen Research.

2.2 Fluorescence melting

Triplex stability was assessed by fluorescence melting experiments as previously described [20]. In these experiments, triplex formation brings the fluorophore (fluorescein) and quencher (dabcyl) close together and the fluorescence is quenched. When the triplex melts these reporter groups move apart and there is a large increase in the fluorescence signal. Unless otherwise stated the concentration of the fluorescein-labelled strand was 0.25 μM, while the dabcyl-labelled strand was 3 μM, as in previous work [19,29,30]. Melting curves were measured using a Roche LightCycler with complexes prepared in 50 mM sodium acetate containing 200 mM NaCl at pH 5.0 or 5.8 (total volume 20 μL). The LightCycler has one excitation source (488 nm) and changes in fluorescence were measured at 520 nm. The mid-points of the melting transitions were estimated from the maximum of the first derivative, using the LightCycler software. Each experiment was repeated at least twice and melting temperatures are accurate to within 0.5 °C. Rates of temperature change of slower than 6 °C.min^{-1} were achieved by increasing the temperature in 1 °C steps, leaving the samples for a fixed time interval between each increase in temperature. The LightCycler is designed for real-time PCR in which heat is rapidly

transferred to the glass capillary and it is compatible with very fast heating and cooling (up to 12 °C.min^{-1} in these experiments).

3. Results

3.1 Comparison of different duplexes

We have examined how the stability of the duplex target affects the apparent triplex stability by studying the interaction of TFO1 (Fig. 1a) with its target site when this is placed in a variety of different duplex contexts. Each of these duplexes (shown in Fig. 1a) contains the same 14 base pair oligopurine.oligopyrimidine target sequence. Inter1 is a simple 14 base pair intermolecular duplex. This is flanked by 6 AT or 6 GC base pairs on either or both sides in sequences 3'ATATAT, 5'-ATATAT, 3'-GCGCGC, 5'-GCGCGC and 5'& 3'-GCGCGC. Intra1 is the same 14 base pair sequence in which the two strands are linked by hexaethylene glycol generating an intramolecular duplex. Representative melting profiles for the interaction of TFO1 with these different duplexes are shown in Fig. 2a at pH 5.0 for a rate of heating of 0.2 °C.min^{-1}. Under these conditions there is no hysteresis between the heating and cooling profiles, but the triplex melting profile is different for the various duplexes. The triplex formed with inter1 melts at the lowest temperature, followed in turn by 3'-ATATAT, 3'-GCGCGC and intra1. The T_m values for these and the other duplexes are summarised in Table 1. These data show that increasing the length of the duplex increases the triplex melting temperature and that GC tails produce more stable complexes than AT tails. There is no significant difference between 5'-ATATAT and 3'-ATATAT or between 3'-GCGCGC and 5'-GCGCGC. The most stable complexes are formed with the duplex that contains 6 GC pairs at both ends and the intramolecular duplex intra1.

When these experiments were repeated at pH 5.8, producing less stable triplexes as a result of the pH dependence of the C$^+$.GC triplets, there was little difference between the various triplex to duplex melting transitions (Fig. 2b, Table 1). The effect of duplex stability on triplex melting therefore appears to be greatest under conditions that generate triplexes that are more stable; this is exaggerated when using third strands that contain stabilising analogues (see below). In each of these profiles (as well as those shown in Fig.2a) a decrease in fluoresence is often evident at higher temperatures subsequent to the triplex melting. This has been noted in our previous studies [20] and corresponds to melting of the underlying duplex, caused by unstacking of the fluorescein. The apparent melting temperatures of the duplexes

alone are summarised in Supplementary material Table S1 and confirm that the addition of the tails stabilises the duplex and that GC is more effective than AT.

When these triplexes were heated and cooled at faster rates there was hysteresis between the melting and annealing profiles, as previously observed [23,30], indicating that one or more steps in the association or dissociation pathways are slow and that the system is not at thermodynamic equilibrium. Representative melting and annealing curves at a fast rate of temperature change (12 °C.min^{-1}) are shown in Fig. 3, for the duplex targets inter1 and intra1. It can be seen that at pH 5.0 (Fig. 3a) there is considerable hysteresis (12.7 °C) with inter1, but very little with intra1 (1.4 °C). The differences are much smaller at pH 5.8 (Fig. 3b). The T_m values for these and the other target duplexes, determined at heating rates of 12 °C.min^{-1}, are presented in Table 1 alongside those determined at 0.1 min^{-1}, while the values determined at intermediate rates of heating and cooling are presented in the Supplementary material (Tables S2 and S3). At pH 5.0 the difference between the triplex T_ms for heating and cooling is greatest for the less stable duplexes.

3.2. Comparison of different nucleoside analogues on intermolecular and intramolecular duplexes.

Triplex melting curves are commonly used to assess the stabilising effects of nucleotide substitutions [16,19,23,30-35]. Such modifications can produce complexes in which the triplex and duplex melts overlap, producing a single melting transition. Under these circumstances it is possible that the apparent triplex stability is limited by the stability of the underlying duplex. This is suggested by the experiments described above, which demonstrates that as the triplex becomes more stable (by lowering the pH), so the melting temperatures become more dependent on the nature of the underlying duplex. We have therefore compared the melting transitions of triplexes that contain modified oligonucleotides, formed on inter and intramolecular duplexes. In order to increase further the stability of these complexes the oligopurine.oligopyrimidine target site was extended to 18 base pairs, generating triplexes with the 18-mer TFOs 2-7 (Fig. 1b). Each of these TFOs contains a single nucleotide analogue in the centre that bears a positive charge on either the base (TFOs 3-5) or the base and the sugar (TFOs 6-7). The melting temperatures of these complexes, determined at the slowest rate of temperature change, at three different pHs, are presented in Table 2. Further values determined at faster rates of heating and

cooling are presented in the Supplementary material (Table S4). As previously noted, each of these modifications increases triplex stability and the most stable complexes are generated with BAU and BGU, which possess amino groups on the 2'- and 5-positions. At pH 6.0 there is no significant difference between the T_ms determined with inter2 and intra2. When the pH is decreased to 5.5, although the unmodified TFO2 binds equally well to both duplexes, the most stabilising analogues show higher T_ms with the intramolecular duplex. This effect is further exaggerated at pH 5.0; at this pH the unmodified TFO shows a higher T_m with intra1 than inter1 and this difference is even greater for the modified TFOs.

4. **Discussion**

The results presented in this paper demonstrate that the apparent stability of a triple helix can be affected by the length and sequence of the underlying duplex. Duplexes with enhanced stability, produced by adding extra base pairs at either end or by generating an intramolecular complex, generate higher triplex T_ms, even though the target site itself is unchanged. This effect is greatest at low pHs, at which the triplex is more stable and melts at a temperature that is closer to the duplex T_m. Under these conditions of enhanced triplex stability the triplex to duplex and duplex to single strand transitions can occur at similar temperatures. It is then possible that the Watson-Crick duplex may melt before the third strand dissociates, leaving a Hoogsteen paired duplex between the TFO and the duplex purine strand. This is likely to be unstable, when removed from the context of the entire triplex, and so will also dissociate. The melting transition of the third strand will therefore be limited by the stability of the underlying duplex.

The limiting effect of duplex stability on triplex formation is especially important when comparing triplexes that have been generated with oligonucleotides that contain stabilising nucleotide analogues. T_m determination is one of the simplest and most commonly used means for assessing improvements in triplex stability and these experiments are usually performed with intermolecular duplexes that are often no longer that the triplex target. Although this technique has successfully established the stabilising effects of many such analogues, the results presented in this paper suggest that these experiments may have underestimated the improvements in stability of the best nucleotide analogues.

The observation that there is little hysteresis for third strand melting from the most stable duplexes at low pH is at first sight puzzling. At pH 5.0, with a temperature change of 12 °C.min^{-1} there is a 12.6 °C difference between melting and annealing with duplex inter1, but only 1.4 °C for intra1. In contrast there is little difference in the hysteresis at these targets at pH 5.8. Hysteresis is caused by the slow association or dissociation of the third strand, such that at fast rates of temperature change, the reaction is not at thermodynamic equilibrium [25,36]. It is well known that triplex formation is very slow (typically three orders of magnitude slower than duplex formation) [36,37] and we would expect the fundamental rate constants for the reaction to be independent of the stability of the target. Our observations suggest that the apparent association and/or dissociation reactions are faster for the intra- than the

inter-molecular duplex at pH 5.0. The differences largely arise from changes in the annealing (rather than the melting) temperature, which becomes much lower for the less stable duplexes, suggesting that the differences are caused by changes in the association reaction. A possible origin of this is the rate of end-fraying of double stranded DNA caused by ingress of water. In all DNA duplexes the base pairs at or near the termini open and close much more rapidly than do the internal base pairs. This is especially true for AT base pairs. In contrast, the base pairs at the ends of hairpin loops (particularly at the "closed" end) are less prone to fraying. It is likely that the end fraying propagates well into the duplex, possibly as a wave of instability as the temperature is raised in a UV melting experiment. This will make the duplex an unstable, or structurally incorrect scaffold for attachment of a third strand, thus slowing the rate of triplex association. It is also possible that the Hoogsteen duplexes that are formed with the oligonucleotides containing stabilising analogues may be as stable as the normal Watson-Crick duplex target. However these parallel structures should be less stable than the entire triplex as they lack the stacking interactions with the duplex pyrimidine strand. Hairpin loop or cyclic duplexes will therefore be better model systems for studying triplex formation in genomic DNA.

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References

[1] K.R. Fox, Targeting DNA with triplexes, Curr. Med. Chem. 7 (2000) 17-37.

[2] J.M. Kalish, P.M. Glazer, Targeted genome modification via triple helix formation, Ann. N. Y. Acad. Sci. 1058 (2005) 151-161.

[3] V.N. Potaman, Applications of triple-stranded nucleic acid structures to DNA purification, detection and analysis, Expert. Rev. Mol. Diagn. 3 (2003) 481-496.

[4] D.A. Rusling, V.J. Broughton-Head, T. Brown, K.R. Fox, Towards the targeted modulation of gene expression by modified triplex-forming oligonucleotides, Curr. Chem. Biol. 2 (2008) 1-10.

[5] N.T. Thuong, C. Hélène, Sequence-specific recognition and modification of double-helical DNA by oligonucleotides, Angew. Chem. Intl. Ed. Engl. 32 (1993) 666-690.

[6] J.L. Asensio, T. Brown, A.N. Lane, Comparison of the solution structures of intramolecular DNA triple helices containing adjacent and non-adjacent CG.C^+ triplets, Nucleic Acids Res. 26 (1998) 3677-3686.

[7] H.E. Moser, P.B. Dervan, Sequence-specific cleavage of double helical DNA by triple helix formation, Science 238 (1987) 645-650.

[8] I. Radhakrishnan, D.J. Patel, Solution structure of a pyrimidine.purine.pyrimidine DNA triplex containing T.AT, C^+.GC and G.TA Triplets, Structure 2 (1994) 17-32.

[9] O.A. Amosova, J.R. Fresco, A search for base analogs to enhance third-strand binding to 'inverted' target base pairs of triplexes in the pyrimidine/parallel motif, Nucleic Acids Res. 27 (1999) 4632-4635.

[10] S. Buchini, C.J. Leumann, Recent improvements in antigene technology, Curr. Opin. Chem. Biol. 7 (2003) 717-726.

[11] S.A. Cassidy, P. Slickers, J.O. Trent, D.C. Capaldi, P.D. Roselt, C.B. Reese, S. Neidle, K.R. Fox, Recognition of GC base pairs by triplex forming oligonucleotides containing nucleosides derived from 2-aminopyridine, Nucleic Acids Res. 25 (1997) 4891-4898.

[12] B. Cuenoud, F. Casset, D. Hüsken, F. Natt, R.M. Wolf, K.H. Altmann, P. Martin, H.E. Moser, Dual recognition of double-stranded DNA by 2'-aminoethoxy- modified oligonucleotides, Angew. Chem. Intl. Ed. Eng. 37 (1998) 1288-1291.

[13] R. Eritja, E. Ferrer, R.G. García, M. Orozco, Modified oligonucleotides with triple-helix stabilization properties, Nucleosides Nucleotides 18 (1999) 1619-1621.

[14] K.R. Fox, T. Brown, An extra dimension in nucleic acid sequence recognition, Q. Rev. Biophys. 38 (2005) 311-320.

[15] D.M. Gowers, K.R. Fox, Towards mixed sequence recognition by triple helix formation, Nucleic Acids Res. 27 (1999) 1569-1577.

[16] T. Hojland, S. Kumar, B.R. Babu, T. Umemoto, N. Albaek, P.K. Sharma, P. Nielsena, J. Wengel, LNA (locked nucleic acid) and analogs as triplex-forming oligonucleotides, Org. Biomol. Chem. 5 (2007) 2375-2379.

[17] L. Lacroix, J.L. Mergny, Chemical modification of pyrimidine TFOs: Effect on i-motif and triple helix formation, Arch. Biochem. Biophys. 381 (2000) 153-163.

[18] I. Prévot, C.J. Leumann, Evaluation of novel third-strand bases for the recognition of a CG base pair in the parallel DNA triple-helical binding motif, Helv. Chim. Acta 85 (2002) 502-515.

[19] D.A. Rusling, V.E.C. Powers, R.T. Ranasinghe, Y. Wang, S.D. Osborne, T. Brown, K.R. Fox, Four base recognition by triplex-forming oligonucleotides at physiological pH, Nucleic Acids Res. 33 (2005) 3025-3032.

[20] R.A.J. Darby, M. Sollogoub, C. McKeen, L. Brown, A. Risitano, N. Brown, C. Barton, T. Brown, K.R. Fox, High throughput measurement of duplex, triplex and quadruplex melting curves using molecular beacons and a LightCycler, Nucleic Acids Res. 30 (2002).

[21] S.R. Gerrard, N. Srinivasan, K.R. Fox, T. Brown, CG base pair recognition within DNA triple helices using N-methyl-3H-pyrrolo[2,3-d]pyrimidin-2(7H)-one nucleoside analogues, Nucl. Nucl. Nucleic Acids 26 (2007) 1363-1367.

[22] H. Li, V.J. Broughton-Head, G.M. Peng, V.E.C. Powers, M.J. Ovens, K.R. Fox, T. Brown, Triplex staples: DNA double-strand cross-linking at internal and terminal sites using psoralen-containing triplex-forming oligonucleotides, Bioconjug. Chem. 17 (2006) 1561-1567.

[23] D.A. Rusling, G. Peng, N. Srinivasan, K.R. Fox, T. Brown, DNA triplex formation with 5-dimethylaminopropargyl deoxyuridine, Nucleic Acids Res. 37 (2009) 1288-1296.

[24] D. Renneberg, C.J. Leumann, Exploring Hoogsteen and reversed-Hoogsteen duplex and triplex formation with tricyclo-DNA purine sequences, Chembiochem 5 (2004) 1114-1118.

[25] E. Bernal-Mendez, C.J. Leumann, Stability and kinetics of nucleic acid triplexes with chimaeric DNA/RNA third strands, Biochemistry 41 (2002) 12343-12349.

[26] J. Bijapur, M.D. Keppler, S. Bergqvist, T. Brown, K.R. Fox, 5-(1-propargylamino)-2 '-deoxyuridine (U-P): a novel thymidine analogue for generating DNA triplexes with increased stability, Nucleic Acids Res. 27 (1999) 1802-1809.

[27] M. Sollogoub, R.A.J. Darby, B. Cuenoud, T. Brown, K.R. Fox, Stable DNA triple helix formation using oligonucleotides containing 2'-aminoethoxy,5-propargylamino-U, Biochemistry 41 (2002) 7224-7231.

[28] D.A. Rusling, L. Le Strat, V.E.C. Powers, V.J. Broughton-Head, J. Booth, O. Lack, T. Brown, K.R. Fox, Combining nucleoside analogues to achieve recognition of oligopurine tracts by triplex-forming oligonucleotides at physiological pH, FEBS Lett. 579 (2005) 6616-6620.

[29] D.A. Rusling, T. Brown, K.R. Fox, DNA triple-helix formation at target sites containing duplex mismatches, Biophys. Chem. 123 (2006) 134-140.

[30] D.A. Rusling, V.J. Broughton-Head, A. Tuck, H. Khairallah, S.D. Osborne, T. Brown, K.R. Fox, Kinetic studies on the formation of DNA triplexes containing the nucleoside analogue 2'-O-(2-aminoethyl)-5-(3-amino-1-propynyl)uridine, Org. Biomol. Chem. 6 (2008) 122-129.

[31] S. Buchini, C.J. Leumann, Stable and selective recognition of three base pairs in the parallel triple-helical DNA binding motif, Angew. Chem. Int. Ed. Engl. 43 (2004) 3925-3928.

[32] S. Buchini, C.J. Leumann, 2 '-O-aminoethyl oligoribonucleotides containing novel base analogues: Synthesis and triple-helix formation at pyrimidine/purine inversion sites, Eur. J. Org. Chem. (2006) 3152-3168.

[33] T. Hojland, B.R. Babu, T. Bryld, J. Wengel, Triplex-forming ability of modified oligonucleotides, Nucl. Nucl. Nucleic Acids 26 (2007) 1411-1414.

[34] A. Mayer, C.J. Leumann, Pyrrolidino DNA with bases corresponding to the 2-oxo deletion mutants of thymine and cytosine: Synthesis and triplex-forming properties, Eur. J. Org. Chem. (2007) 4038-4049.

[35] S.D. Osborne, V.E.C. Powers, D.A. Rusling, O. Lack, K.R. Fox, T. Brown, Selectivity and affinity of triplex-forming oligonucleotides containing 2'-aminoethoxy-5-(3-aminoprop-1-ynyl)uridine for recognizing AT base pairs in duplex DNA, Nucleic Acids Res. 32 (2004) 4439-4447.

[36] M. Rougée, B. Faucon, J.L. Mergny, F. Barcelo, C. Giovannangeli, T. Garestier, C. Hélène, Kinetics and thermodynamics of triple-helix formation - effects of ionic-strength and mismatches, Biochemistry 31 (1992) 9269-9278.

[37] L.J. Maher, P.B. Dervan, B.J. Wold, Kinetic-analysis of oligodeoxyribonucleotide-directed triple-helix formation on DNA, Biochemistry 29 (1990) 8820-8826.

Legends to Figures

Fig. 1. A) Sequences of the triplex forming oligonucleotides TFO1-7 and the various duplexes used in this work. H = hexaethyelene glycol; U = FAM-dU. B) Structures of the nucleotides analogues used for TFOs 2-7.

Fig. 2. Representative melting curves for the interaction of TFO1 with the intermolecular duplexes inter1, 3'-ATATAT, 3'-GCGCGC and intra1. In each case the concentration of the third strand was 3 μM and the duplex concentration was 0.25 μM. The rate of hearing was 0.2 °C. min^{-1}.The experiments were performed in 50 mM sodium acetate containing 200 mM NaCl pH 5.0 or 5.8. Dotted line, inter1; long dashes, 3'-ATATAT; short dashes 3'-GCGCGC; solid line, intra1. The inset shows the first derivatives of the melting profiles, for which the peak corresponds to the Tm.

Fig. 3. Representative melting curves for TFO1 with duplexes inter1 and intra1, performed at a fast rate of temperature change (12 °C.min^{-1}). In each case the concentration of the third strand was 3 μM and the duplex concentration was 0.25 μM. The experiments were performed in 50 mM sodium acetate containing 200 mM NaCl pH 5.0 or 5.8. inter 1: anneal (open triangles), melt (filled triangles); intra1: anneal (open circles), melt (filled circles). In A) for inter1 the arrows indicate the direction of heating or cooling.

Triplex-forming oligonucleotides

TFO1 5′-Q-TCTCTCTTCCTCCT

5′-Q-TCTCTCTT**N**TCCTCCTCC
TFO2 N = T
TFO3 N = DMAPdU
TFO4 N = APdU
TFO5 N = GPdU
TFO6 N = BAU
TFO7 N = BGU

Target duplexes

Inter1
5′-F-AGAGAGAAGGAGGA
3′-TCTCTCTTCCTCCT

Intra1
5′-F-AGAGAGAAGGAGGA$_H$
3′-TCTCTCTTCCTCCT

3′-ATATAT
5′-F-AGAGAGAAGGAGGAATATAT
3′-TCTCTCTTCCTCCTTATATA

5′-ATATAT
5′-TATATA**U**AGAGAGAAGGAGGA
3′-ATATATATCTCTCTTCCTCCT

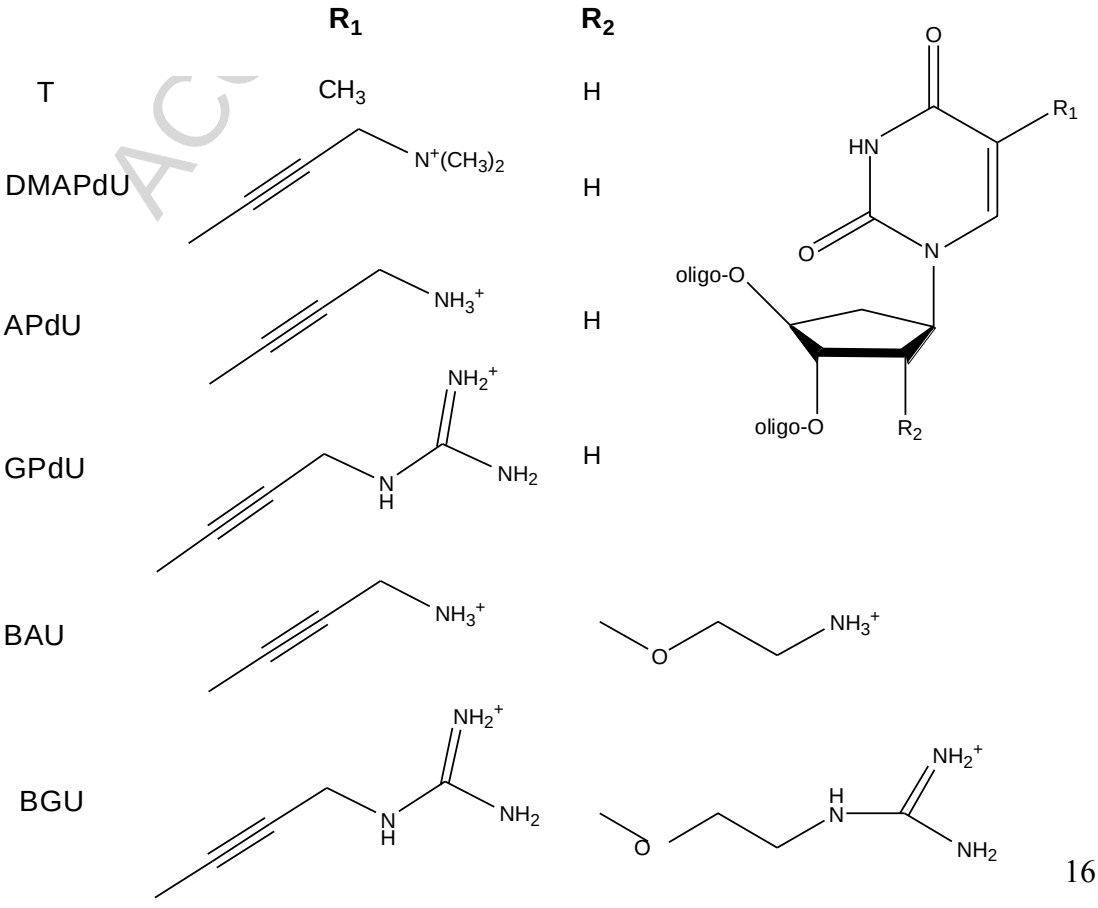
5′& 3′-GCGCGC
5′-GCGCGC**U**AGAGAGAAGGAGGAGCGCGC
3′-CGCGCG**A**TCTCTCTTCCTCCTCGCGCG

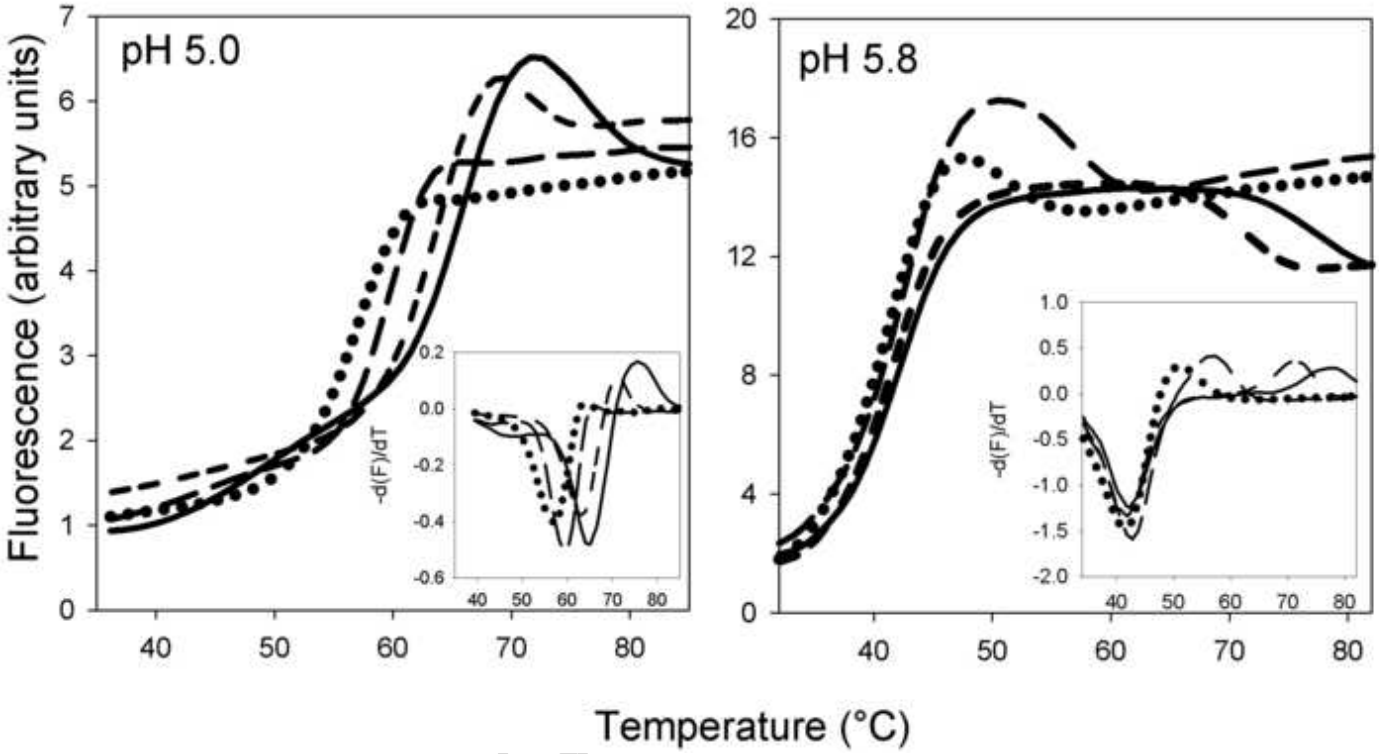
3′-GCGCGC
5′-F-AGAGAGAAGGAGGAGCGCGC
3′-TCTCTCTTCCTCCTCGCGCG

5′-GCGCGC
5′-GCGCGC**U**AGAGAGAAGGAGGA
3′-CGCGCG**A**TCTCTCTTCCTCCT

Inter2
5′-F-AGAGAGAAAAGGAGGAGG
3′-TCTCTCTTTTCCTCCTCC

Intra2
5′-F-AGAGAGAAAAGGAGGAGG$_H$
3′-TCTCTCTTTTCCTCCTCC





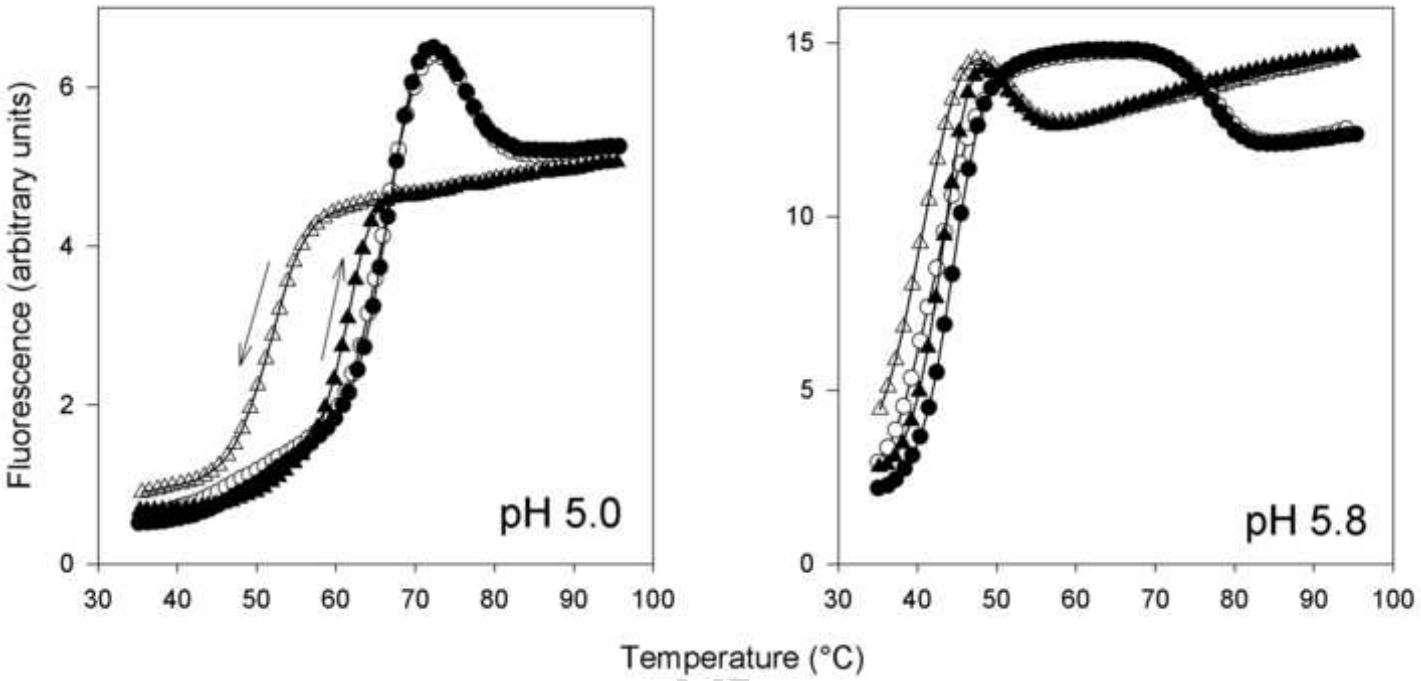


Table 1

T_m values (°C) for the interaction of TFO1 with the different duplexes, determined at a rate of heating of 0.2 °C.min^{-1} and 12 °C.min^{-1}. Values for annealing are shown in parenthesis. The reactions were performed in 50 mM sodium acetate containing 200 mM NaCl. The concentration of each fluorescently-labelled strand was 0.25 μM (duplex or TFO), while the concentration of quencher-labelled strand was 3 μM.

	T_m °C			
	0.2 °C.min^{-1}		12 °C.min^{-1}	
Target duplex	pH 5.0	pH 5.8	pH 5.0	pH 5.8
Inter1	55.7 (55.6)	41.6 (41.5)	62.8 (50.1)	43.9 (40.5)
Intra1	65.9 (66.2)	42.5 (42.6)	66.9 (65.5)	45.1 (41.3)
3′-ATATAT	59.3 (59.6)	42.5 (42.6)	64.7 (55.5)	44.8 (41.3)
5′-ATATAT	58.6 (58.4)	43.3 (43.4)	65.3 (52.6)	46.0 (42.5)
3′-GCGCGC	64.0 (64.2)	41.9 (41.6)	65.1 (63.8)	43.9 (41.1)
5′-GCGCGC	63.5 (64.0)	43.3 (43.3)	66.0 (63.7)	46.2 (42.2)
3′&5′-GCGCGC	67.4 (67.6)	42.2 (42.4)	68.5 (67.3)	45.6 (42.3)

Table 2

T_m values (°C) for the interaction of TFOs 2-7 with the duplexes inter2 and intra2, determined at a rate of heating and cooling of 0.2 °C.min^{-1}. The values for annealing are shown in parentheses. The reactions were performed in 50 mM sodium acetate containing 200 mM NaCl, at pH 5.0, 5.5 or 6.0. The concentration of the fluorescently-labelled duplexes was 0.25 μM, while the TFO concentration was 3 μM.

	T_m (°C)					
	pH 5.0		**pH 5.5**		**pH 6.0**	
	Intra	*Inter*	*Intra*	*Inter*	*Intra*	*Inter*
TFO2 (T)	65.1 (65.3)	61.0 (61.2)	51.2 (51.3)	51.4 (51.5)	39.4 (39.2)	39.8 (39.6)
TFO3 (DMAPdU)	67.0 (67.3)	62.4 (62.2)	53.8 (53.7)	53.2 (53.4)	41.3 (41.5)	41.8 (41.9)
TFO4 (APdU)	69.3 (69.2)	63.5 (62.9)	56.1 (56.3)	55.3 (55.6)	43.7 (43.9)	44.0 (44.1)
TFO5 (GPdU)	70.7 (70.9)	64.4 (63.7)	57.4 (57.5)	56.5 (56.8)	45.2 (45.4)	45.1 (45.3)
TFO6 (BAU)	73.5 (73.5)	66.6 (63.3)	61.6 (61.7)	59.3 (59.2)	48.1 (48.3)	48.4 (48.6)
TFO7 (BGU)	73.3 (73.4)	67.6 (63.6)	60.6 (60.7)	58.8 (58.7)	48.1 (48.0)	48.4 (48.3)

SUPPLEMENTARY

Table S1

T_m values (°C) for each of the target duplexes, estimated from the temperature-dependent change in fluorescence. The reactions were performed in 50 mM sodium acetate containing 200 mM NaCl at pH 5.0 or 5.8. The concentration of each fluorescently-labelled duplex was 0.25 μM.

Duplexes	pH 5.0	pH 5.8
inter1	46.7	50.0
intra	75.1	77.3
3′-ATATAT	54.9	57.2
5′-ATATAT	51.1	53.0
3′-GCGCGC	69.4	71.7
5′-GCGCGC	66.2	68.8
3′&5′-GCGCGC	76.2	78.7

Table S2

T_m values (°C) for the interaction of TFO1 with the different duplexes, determined at various rates of heating and cooling. The values for annealing are shown in parentheses. The reactions were performed in 50 mM sodium acetate pH 5.0 containing 200 mM NaCl. The concentration of each fluorescently-labelled strand was 0.25 μM (duplex or TFO), while the concentration of quencher-labelled strand was 3 μM.

	T_m °C				
Target duplex	12 °C.min^{-1}	6 °C.min^{-1}	3 °C.min^{-1}	1 °C.min^{-1}	0.2 °C.min^{-1}
inter	62.8 (50.1)	61.8 (52.2)	59.1 (54.2)	57.1 (56.3)	55.7 (55.6)
intra	66.9 (65.5)	66.6 (66.1)	66.3 (66.8)	66.9 (66.8)	65.9 (66.2)
3′-ATATAT	64.7 (55.5)	63.1 (57.3)	60.6 (59.3)	59.7 (59.7)	59.3 (59.6)
5′-ATATAT	65.3 (52.6)	64.0 (55.2)	61.4 (56.9)	59.8 (58.9)	58.6 (58.4)
3′-GCGCGC	65.1 (63.8)	65.3 (64.4)	64.5 (64.9)	64.3 (64.6)	64.0 (64.2)
5′-GCGCGC	66.0 (63.7)	65.9 (64.8)	64.6 (65.1)	64.3 (64.4)	63.5 (64.0)
3′&5′-GCGCGC	68.5 (67.3)	68.1 (66.7)	67.7 (68.3)	67.5 (67.1)	67.4 (67.6)

Table S3

T_m values (°C) for the interaction of TFO1 with the different duplexes, determined at various rates of heating and cooling. The values for annealing are shown in parentheses. The reactions were performed in 50 mM sodium acetate pH 5.8 containing 200 mM NaCl. The concentration of each fluorescently-labelled duplex was 0.25 μM, while the TFO concentration was 3 μM.

	T_m (°C)				
	12 °C.min^{-1}	6 °C.min^{-1}	3 °C.min^{-1}	1 °C.min^{-1}	0.2 °C.min^{-1}
inter1	43.9 (40.5)	43.5 (40.6)	42.4 (42.0)	41.6 (41.8)	41.6 (41.5)
intra	45.1 (41.3)	44.3 (42.0)	43.1 (43.2)	42.5 (43.1)	42.5 (42.6)
3′-ATATAT	44.8 (41.3)	44.1 (41.8)	43.0 (43.1)	42.6 (42.9)	42.5 (42.6)
5′-ATATAT	46.0 (42.5)	45.2 (43.8)	44.2 (44.0)	43.5 (43.9)	43.3 (43.4)
3′-GCGCGC	43.9 (41.1)	43.2 (41.0)	43.1 (42.0)	42.0 (42.1)	41.9 (41.6)
5′-GCGCGC	46.2 (42.2)	45.1 (43.0)	43.5 (42.9)	43.5 (43.8)	43.3 (43.3)
3′&5′-GCGCGC	45.6 (42.3)	44.6 (42.3)	43.2 (42.8)	42.5 (42.8)	42.2 (42.4)

Table S4

T_m values (°C) for the interaction of TFOs 2-7 with the duplexes inter2 and intra2, determined at various rates of heating and cooling. The values for annealing are shown in parentheses. The reactions were performed in 50 mM sodium acetate containing 200 mM NaCl, at pH 5.0, 5.5 or 6.0. The concentration the fluorescently-labelled duplexes was 0.25 μM, while the TFO concentration was 3 μM.

	T_m (°C)					
	0.2 °C.min^{-1}		1 °C.min^{-1}		6 °C.min^{-1}	
pH 5.0	*Intra*	*Inter*	*Intra*	*Inter*	*Intra*	*Inter*
T	65.1 (65.3)	61.0 (61.2)	64.8 (65.2)	61.9 (60.7)	66.5 (63.8)	65.6 (56.9)
DMAPdU	67.0 (67.3)	62.4 (62.2)	67.3 (67.4)	63.5 (61.5)	68.7 (65.9)	67.6 (57.4)
APdU	69.3 (69.2)	63.5 (62.9)	69.3 (69.6)	65.0 (62.4)	70.4 (68.4)	69.0 (58.2)
GPdU	70.7 (70.9)	64.4 (63.7)	70.8 (71.1)	66.1 (62.7)	72.0 (70.0)	70.1 (58.8)
BAU	73.5 (73.5)	66.6 (63.3)	73.0 (73.6)	68.9 (62.1)	74.5 (72.0)	73.4 (57.8)
BGU	73.3 (73.4)	67.6 (63.6)	73.4 (73.6)	70.4 (61.9)	75.7 (71.4)	74.5 (57.1)
pH 5.5						
T	51.2 (51.3)	51.4 (51.5)	52.3 (51.4)	52.4 (51.4)	55.3 (48.6)	55.1 (48.7)
DMAPdU	53.8 (53.7)	53.2 (53.4)	54.4 (54.5)	54.1 (53.8)	57.4 (52.7)	56.7 (51.2)
APdU	56.1 (56.3)	55.3 (55.6)	56.6 (56.9)	55.8 (55.9)	59.2 (55.3)	58.7 (53.7)
GPdU	57.4 (57.5)	56.5 (56.8)	58.1 (58.3)	57.1 (56.8)	60.4 (56.4)	59.7 (54.4)
BAU	61.6 (61.7)	59.3 (59.2)	62.2 (62.5)	60.2 (59.2)	63.8 (61.2)	63.5 (55.6)
BGU	60.6 (60.7)	58.8 (58.7)	61.5 (61.1)	60.2 (58.1)	64.3 (57.8)	63.7 (54.1)
pH 6.0						
T	39.4 (39.2)	39.8 (39.6)	40.8 (39.1)	41.0 (39.5)	44.6 (<35)	44.7 (<35)
DMAPdU	41.3 (41.5)	41.8 (41.9)	42.5 (41.3)	42.8 (41.9)	46.1 (37.3)	46.3 (38.7)
APdU	43.7 (43.9)	44.0 (44.1)	44.7 (43.8)	45.0 (44.1)	47.8 (40.5)	48.2 (41.1)
GPdU	45.2 (45.4)	45.1 (45.3)	45.8 (45.5)	46.0 (45.5)	49.3 (42.6)	49.1 (42.6)
BAU	48.1 (48.3)	48.4 (48.6)	49.2 (47.8)	49.5 (48.1)	52.6 (44.3)	53.0 (45.3)
BGU	48.1 (48.0)	48.4 (48.3)	49.3 (47.6)	49.6 (47.9)	53.5 (42.1)	53.5 (42.9)