Four base recognition by triplex-forming oligonucleotides at physiological pH

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ABSTRACT

We have achieved recognition of all 4 bp by triple helix formation at physiological pH, using triplex-forming oligonucleotides that contain four different synthetic nucleotides. BAU [2’-aminoethoxy-5-(3-aminoprop-1-ynyl)uridine] recognizes AT base pairs with high affinity, MeP (3-methyl-2 aminopyridine) binds to GC at higher pHs than cytosine, while APP (6-(3-aminopropyl)-7-methyl-3H-pyrrrolod[2,3-d]pyrimidin-2(7H)-one) and S [N-(4-(3-acetamidophenyl)thiazol-2-yl-acetamide)] bind to CG and TA base pairs, respectively. Fluorescence melting and DNase I footprinting demonstrate successful triplex formation at a 19mer oligopurine sequence that contains two CG and two TA interruptions. The complexes are pH dependent, but are still stable at pH 7.0. BAU, MeP and APP retain considerable selectivity, and single base pair changes opposite these residues cause a large reduction in affinity. In contrast, S is less selective and tolerates CG pairs as well as TA.

INTRODUCTION

Triplex-forming oligonucleotides (TFOs) are sequence-specific DNA-binding agents that can be exploited for the recognition of unique DNA sequences (1–4), and several recent reports have emphasized their therapeutic potential (5–8). These oligonucleotides bind in the major groove of double-stranded DNA, forming hydrogen bonds with exposed groups on the base pairs, generating a three-stranded structure. Pyrimidine-rich oligonucleotides bind parallel to the purine strand of the target duplex, forming T.AT and C.GC triplets (the notation X.ZY refers to a triplet, in which the third strand base X interacts with the duplex ZY base pair, forming hydrogen bonds to base Z). Recognition of pyrimidine residues is much harder to achieve as C and T have only one H-bond donor or acceptor site available for binding in the major groove. Recognition of the T of a TA base pair is also hampered by steric clash of the 5-methyl group. Therefore, there are currently several major limitations to this approach: (i) there are no stable means for recognizing TA or CG base pairs (pyrimidine inversions) using natural DNA bases; (ii) formation of the C.GC triplet requires conditions of low pH (<6.0), necessary for protonation of the third strand cytosine; (iii) the binding of the third strand may not be strong, due to electrostatic repulsion between the three polyanionic DNA strands. Therefore, there is a need for combinations of nucleoside analogues that can overcome all these limitations (2,9,10).

A wide variety of approaches have been employed to overcome each of these problems. The pH dependency of the C.GC triplet has been partially alleviated by using more basic analogues of cytosine or by using non-protonated cytosine mimics (11–21). Some success has also come from the attachment of charged moieties at the N4-position of cytosine (22,23). The recognition of pyrimidine inversions is much harder to achieve, as these bases offer only the formation of a single hydrogen bond within the major groove. The best combinations for recognizing TA and CG using natural bases are G.TA and T.CG (24,25), though these are much less stable than T.AT and C.GC and multiple inversions are strongly destabilizing (26). Analogues designed to form additional unconventional hydrogen bonds and/or target substituents on both partners of the base pair have been synthesized (27–30), often providing most benefit when incorporated alongside sugar and/or backbone modifications (31–35). An alternative less selective approach has been to use base analogues or linkers that skip or intercalate at such inversions (36–38). Attempts to increase the strength of binding of TFOs have included the addition of positively charged groups (39–42), increasing the base stacking (43,44) or changing the phosphodiester backbone (2).
Despite the substantial efforts in the synthesis and preparation of these analogues, there are very few examples in which they have been combined to achieve high binding affinity to mixed sequence duplex DNA targets at physiological pHs. We have, therefore, examined the ability of a TFO containing four different modified nucleosides (BAU, MeP, APP and S; see Figure 1A) to selectively target a mixed sequence at physiological pHs. BAU forms a very stable triplet with AT (41,42); MeP has a $pK_a$ that is higher than cytosine and targets GC base pairs at higher pHs (14–16); S has been proposed for recognizing TA inversions (30,45), while APP recognizes CG (46).

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides were synthesized on an Applied Biosystems ABI 394 automated DNA/RNA synthesizer on the 0.2 or 1 $\mu$m scale using the standard cycles of acid-catalysed detritylation, coupling, capping and iodine oxidation procedures. Phosphoramidite monomers and other reagents were purchased from Applied Biosystems or Link Technologies. Phosphoramidites for BAU (41,42), MeP (16), S (30) and APP (46) were prepared as described previously. The deprotected oligonucleotides were purified by reversed-phase high-performance liquid chromatography on a Brownlee Aquapore column (C8) using a gradient of acetonitrile in 0.1 M ammonium acetate.

The sequences of the oligonucleotides used in this work are shown in Figure 1B. For the fluorescence melting experiments, the purine-containing strand of the duplex was labelled at the 5' end with 6-amido-2-hexylfluorescein (6-FAM phosphoramidine, Link Technologies), and the third strand was labelled at the 5' end with methyl red serinol. The same third strand nucleotide was used for the footprinting experiments.

Fluorescence melting studies

The thermal melting temperature of the triplexes was determined using the fluorescence melting technique that we have developed previously (47) and have used for assessing the stability of triplexes that contain modified nucleotides (41,42,45,46). The third strand oligonucleotide is labelled at the 5' end with a quencher (methyl red), while the 5' end of the purine-rich strand of the duplex is labelled with a fluorescent group (fluorescein). These are in close proximity when the triplex is formed and the fluorescence is quenched. When the triplex melts these groups become separated and there is a large increase in fluorescence. In this manner, the dissociation of the third strand is observed directly, without interference from dissociation of the duplex. By placing the quencher on the third strand, the TFO can be added in excess, thereby facilitating triplex formation, without increasing the

Figure 1. (A) Chemical structure of the four base triplets employed in this work. (B) Sequences of the oligonucleotides used in the fluorescence melting experiments. The duplexes are boxed and are labelled with fluorescein (F) at the 5' end of the purine strand, whereas the TFOs are labelled with methyl red serinol (Q) at the 5' end. Oligo 1 was used to estimate the melting temperature of duplex, whereas oligos 2 and 3 were used to verify the orientation of TFO binding. Duplexes 2–5 are identical to duplex 1, except that single base pair changes are introduced at different positions, opposite one of the modified third strand bases; positions 1.2, 3.4, 5.6 and 7.8 correspond to each base pair (A,T, T,A, G,C and C,G) in turn.
were first denatured by rapidly heating to 95°C in 20 mM NaCl containing 2 mM MgCl₂ and 2 mM MnCl₂. Each value was recorded in triplicate and usually differed by of the melting profiles using the Roche LightCycler software. The Roche LightCycler, which permits the determination of up to 32 melting profiles in parallel. The melting points were equilibrate for 10 min. The complexes were then heated again to 95°C at 0.2°C/min in the same manner. Recordings were taken during both the heating and cooling steps to check for hysteresis and no significant hysteresis was observed. The LightCycler has one excitation source (488 nm) and the changes in fluorescence emission were measured at 520 nm. Tm values were determined from the first derivatives of the melting profiles using the Roche LightCycler software. Each value was recorded in triplicate and usually differed by <0.5°C.

**DNase I footprinting**

DNA fragments for the footprinting experiments were prepared by cloning synthetic oligonucleotides into the BamHI site of pUC19. These contained the same target sites as used for the fluorescence melting studies. Radiolabelled fragments were produced by digesting each plasmid with EcoRI and HindIII and labelling at the 3' end of the EcoRI site using reverse transcriptase and [α-32P]dATP. Each fragment was separated from the remainder of the plasmid DNA on an 8% (w/v) non-denaturing polyacrylamide gel. After elution, the fragment was dissolved in 10 mM Tris–HCl (pH 7.5) containing 0.1 mM EDTA to give ~10 c.p.s./μl as determined on a hand held Geiger counter (<10 nM).

DNase I footprinting was performed by mixing radiolabelled DNA (1.5 μl) with the TFO (3 μl) dissolved in the appropriate buffer. Experiments at pH 5.0 were performed in 50 mM sodium acetate buffer (pH 5.0, 5.5 or 6.0) or 50 mM sodium phosphate (pH 6.5, 7.0 and 7.5) containing 200 mM NaCl. Melting experiments were performed in a total volume of 20 μl and contained 0.25 μM duplex and 3 μM third strand. These complexes were first denatured by rapidly heating to 95°C and left to equilibrate for 10 min. The complexes were then heated to 30°C at a rate of 0.2°C/min by decreasing the temperature in 1°C steps, leaving the samples to equilibrate for 5 min before each fluorescence reading. After 10 min, the complexes were then heated again to 95°C at 0.2°C/min in the same manner. Recordings were taken during both the heating and cooling steps to check for hysteresis and no significant hysteresis was observed. On increasing the temperature, the strands separate and there is a large increase in fluorescence. The melting profiles clearly demonstrate successful triplex formation with Tm values >60°C at low pHs. As expected, the Tm is pH dependent, due to the presence of the MeP.GC tripllet; there is no difference in Tm between pH 5.0 and 6.0, though Tm decreases at higher pHs.

**RESULTS**

Duplex 1 (Figure 1) contains an oligopurine. oligopyrimidine tract that is interrupted by two CG and two TA base pairs. TFO-1 was designed to form a specific triplex with this target generating BAU.AT, MeP.GC, S.TA and AP.CG triplets as well as the conventional T.AT triplet. In contrast, TFO-2 is designed to recognize this target generating the best triplets using only natural DNA bases (T.AT, C.GC.G.TA and T.CG). The interaction of these oligonucleotides with this target site, and several others that differ by single base pair substitutions, was analysed by fluorescence melting and DNase I footprinting experiments.

**Fluorescence melting studies**

Representative melting profiles showing the interaction of TFO-1 with duplex 1 are shown in Figure 2. In these experiments, the fluorophore and quencher are in close proximity when the triplex is formed and the fluorescence is quenched. On increasing the temperature, the strands separate and there is a large increase in fluorescence. The melting profiles clearly demonstrate successful triplex formation with Tm values >60°C at low pHs. As expected, the Tm is pH dependent, due to the presence of the MeP.GC triplet; there is no difference in Tm between pH 5.0 and 6.0, though Tm decreases at higher pHs.

**Figure 2.** Fluorescence melting curves for the interaction of TFO-1 with duplex 1 at different pHs. Open squares, pH 7.5; open circles, pH 7.0; filled triangles, pH 6.5; filled squares, pH 6.0; filled circles, pH 5.5; open triangles, pH 5.0. In each case, the duplex concentration was 0.25 μM and the third strand was 3 μM. All oligonucleotides were prepared in an appropriate buffer containing 200 mM NaCl. The y-axis shows the normalized fluorescence (arbitrary units), whereas the x-axis shows the temperature (°C). The samples were heated at a rate of 0.2°C/min. The apparent relative fluorescence for the triplex is higher at pH 7.0 and 7.5 as the affinity of the third strand is weaker and there is a significant amount of unbound fluorescent duplex in the equilibrium.
pHs, presumably because the pKₐ of MeP is between 6 and 7 in this system. In contrast, TFO-2, which only contains natural nucleotides, failed to generate a stable triplex, even at pH 5.0. The Tₘ values, calculated from the first derivatives of these profiles, are highlighted in Table 1. There was no hysteresis between the heating and melting curves at this rate of heating and cooling (0.2°C/min), though significant hysteresis was observed at faster rates of temperature change (0.1°C/s). This is consistent with the known slow rates of triplex formation. Stable triplex formation normally requires the addition of divalent metal ions to screen the charge interaction between the three polyanionic backbones. This was not required for the formation of these triplets, presumably due to the presence of multiple positive charges within the TFO on the BAU residues.

The data clearly show that TFO-1 forms a stable triplex at pH 7 with this mixed sequence duplex target containing four pyrimidine inversions and four GC base pairs. This compares with the unmodified TFO-2, where triplex formation is not observed even at pH 5. Although formation of the triplex with TFO-1 is still pH dependent, the complex is stable under physiological conditions. The stability of the underlying duplex was assessed by similar melting experiments replacing the unlabelled pyridine-rich strand of duplex 1 with oligo 1 (bearing a 3′-methyl red) and showed Tₘ values between 58.5 and 62.5°C, depending on the pH, and between pH 5 and 6 the triplex formed with TFO-1 is more stable than the duplex alone.

Several other oligonucleotides were also prepared, with appropriately positioned fluorophores and quenchers, to ensure that parallel triplex formation was being observed. Addition of TFO-1 to the purine strand of the duplex, which could theoretically generate a parallel Hoogsteen duplex, failed to show the formation of a complex. The formation of an antiparallel duplex was assessed by combining TFO-1 with oligo-2 and this also showed no complex formation. Finally, oligo-3 corresponds to the opposite orientation of the purine strand of the duplex, and this showed no interaction with TFO-1.

The sequence specificity of the triplex formation was examined by determining the melting profiles of TFO-1 with a further 12 duplexes, each of which differed from duplex 1 by a single base pair opposite one of the modified nucleotides. Duplexes 2, 3, 4 and 5 were used to assess the selectivity of S, BAU, MeP and P, respectively, in the context of this sequence. Each duplex, therefore, generated a single triplet X.Y.Z, where X is BAU, MeP, S or P, and YZ is each base pair in turn. The fluorescent melting profiles of these complexes at pH 7.0 are shown in Figure 3, and Tₘ values, together with those determined at other pHs, are shown in Table 1. It can be seen that the sequence specificity of BAU is maintained over the entire pH range, and each single base pair mismatch decreased the Tₘ by at least 10°C, an effect that was greater at higher pHs. A similar effect is seen with MeP, which shows at least a 15°C decrease in Tₘ for each of the triplet mismatches. P always forms the most stable triplets with CG, but its selectivity for the other 3 bp depends on the pH. At low pH, the next highest Tₘ (to GC) is 13°C lower showing a high level of discrimination. At higher pH, the selectivity is retained but the next best base pair is TA. The monomers BAU, MeP and P therefore retain exquisite sequence selectivity and the stability of these 19mer triplets decreases by ~15°C for single mismatches.

In contrast, nucleotide S exhibits a much lower level of selectivity; at low pH, it recognizes CG with a greater affinity than TA, and there is only a few degrees difference between the best and the worst complexes. The discrimination increases at higher pH and at pH 7.0 it produces higher Tₘs with TA and CG than with GA and AT. We have previously shown that the selectivity of S is pH dependent and have suggested alternative hydrogen bonding arrangements for the protonated form (45).

### DNase I footprinting

The affinity and selectivity of these modified oligonucleotides was further assessed by DNase I footprinting, using DNA fragments that contain similar target sites. For these experiments the same oligonucleotides were used as in the fluorescence melting studies, because we find that addition of the terminal methyl red does not significantly affect their binding (D.A. Rusling and V.E.C. Powers, unpublished data). The interaction of TFO-1 and TFO-2 with the perfect match target site is shown in Figure 4. It can be seen that TFO-2, which contains only natural nucleotides, does not affect the cleavage pattern, even at the highest concentration (30 μM) at pH 5.0. In contrast, clear footprints are evident with TFO-1 at the intended target site. At pH 5.0, the footprint persists to concentrations <10 nM and, although higher oligonucleotide concentrations are required at elevated pHs, a footprint is still evident at micromolar concentrations even at pH 7.0. C₅₀ values derived from these data, indicating the oligonucleotide concentration that reduces the band intensity at the target site

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**Table 1.** Tₘ values of different triplet combinations determined by fluorescence melting

| pH | X = | BAU | MeP | P | S |
|----|-----|-----|-----|---|---|
| pH 5.0 | X.AT | 63.5 | 43.9 | 48.3 | 61.5 |
|       | X.TA | 53.6 | 49.8 | 48.4 | 63.5 |
|       | X.GC | 53.3 | 63.5 | 50.8 | 60.4 |
|       | X.CG | 53.6 | 47.2 | 63.5 | 66.5 |
| pH 5.5 | X.AT | 63.9 | 43.7 | 48.1 | 62.0 |
|       | X.TA | 52.8 | 49.5 | 48.3 | 63.9 |
|       | X.GC | 53.0 | 63.9 | 48.1 | 60.6 |
|       | X.CG | 51.5 | 46.3 | 63.9 | 66.4 |
| pH 6.0 | X.AT | 62.4 | 40.4 | 45.7 | 60.5 |
|       | X.TA | 50.7 | 47.0 | 46.4 | 62.4 |
|       | X.GC | 49.0 | 62.4 | 45.3 | 57.7 |
|       | X.CG | 48.0 | 44.5 | 62.4 | 64.5 |
| pH 6.5 | X.AT | 58.2 | n.d. | 38.8 | 54.1 |
|       | X.TA | 42.9 | 39.1 | 40.4 | 58.2 |
|       | X.GC | 42.6 | 58.2 | 37.3 | 50.3 |
|       | X.CG | 40.2 | 37.7 | 58.2 | 59.6 |
| pH 7.0 | X.AT | 48.8 | n.d. | n.d. | 43.2 |
|       | X.TA | n.d. | n.d. | n.d. | 48.8 |
|       | X.GC | n.d. | 48.8 | n.d. | 39.7 |
|       | X.CG | n.d. | n.d. | 48.8 | 49.8 |
| pH 7.5 | X.AT | 37.4 | n.d. | n.d. | n.d. |
|       | X.TA | n.d. | n.d. | n.d. | 37.4 |
|       | X.GC | n.d. | 37.4 | n.d. | n.d. |
|       | X.CG | n.d. | n.d. | 37.4 | 37.8 |

The values in bold correspond to those for TFO-1 at its intended target (employing BAU, AT, MeP, GC, P, CG and S.TA triplets). For all the other cases, 1 bp in the target was changed opposite one of the modified bases, as shown in Figure 1 using duplexes 2–5 (1.2 opposite S, duplex 2; 3.4 opposite BAU, duplex 3; 5.6 opposite MeP, duplex 4; 7.8 opposite P, duplex 5). n.d. indicates that no melting transition was detected (Tₘ < 30°C). Each value is the average of three determinations, which usually differed by <0.5°C.
by 50%, are summarized in Table 2. As with fluorescence melting studies, divalent metal ions were not required for binding, though these are present for a short while during the DNase I digestion.

In order to study the selectivity of triplex formation, we prepared four further footprinting substrates, which contained single base pair substitutions opposite one of the novel nucleotides, generating S, BAU, MeP, TA and APP, TA triplets in turn. The results of these experiments at pH 5.0 and 7.0 are shown in Figure 5, and the C50 values, together with those obtained at pH 6.0, are summarized in Table 2. In each case, with the exception of the complex containing the S,AT triplet at pH 5.0, it can be seen that the mismatch reduces the binding affinity, requiring higher oligonucleotide concentrations to generate a footprint. The selectivity is less pronounced at the lower pH (pH 5–6), though at pH 7.0 the single base pair changes opposite BAU, MeP and APP abolished the footprint. An unusual effect is seen with the combination generating an APP, TA triplet at low pH, for which a partial footprint is evident, covering only the upper part of the target. These results, together with fluorescence melting studies, demonstrate that triplex formation can be achieved at this mixed sequence target site at pH 7.0, and that BAU, MeP and APP are highly selective. S permits stable triplex formation at TA inversions but shows much less discrimination between the 4 bp.

**DISCUSSION**

The formation of stable triple helices at mixed sequence target sites, at physiological pH is a major challenge for the general use of the antigen triplex strategy. There have been many studies investigating the effects of single nucleotides on triplex stability, each addressing one or other aspects of the problem (pH dependency, affinity and recognition of pyrimidine inversions). We and others have previously demonstrated that BAU (39,40), MeP (14–16), S (30,45) and APP (46) are able to form stable triplets at AT, GC, TA and CG, respectively. The results presented in this paper are one of the few examples in which several different synthetic nucleotides have been incorporated within a single TFO. These studies suggest that, by incorporating these modified nucleotides in a single a TFO, it is possible to form stable triplexes at a mixed sequence duplex targets that contain four pyrimidine inversions at
physiological pH. This does not require any other stabilizing factors, such as high concentrations of divalent metal ions or the addition triplex binding ligands. At pH 7.0 BAU, MeP and APP display clear selectivity, whereas S monomer, which is designed to recognize TA inversions, also recognizes CG base pairs with a similar affinity. Therefore, there is the need for further development of monomers designed to recognize TA base pairs. Although these triplexes are stable at pH 7.0, the affinity is ~100-fold lower than at pH 5.0 as a result of the pH dependency of the MeP.GC triplet. Although the affinity of complexes containing this triplet at pH 7.0 is enhanced by the presence of the very strong BAU.AT triplet, there is clearly still need for further derivatives with higher pK values. These results confirm the selectivity of APP for CG base pairs; the APP triplet contains two hydrogen bonds, one of which is a C–H–O bond, yet this is more stable than both T.CG and C.CG and has greater selectivity as a third strand base than T or A, which also bind well to AT and GC base pairs, respectively (46).

The target sites differed by a single base pair within the 19mer target site, generating a triplex mismatch opposite one of the novel nucleotides. The identity of the mismatch (S.AT, BAU.TA, MeP.TA or APP.TA) is indicated. The experiments were performed at pH 5.0, 6.0 and 7.0 in an appropriate buffer containing 50 mM NaCl. n.d. indicates that no footprint was detected.

For this target, TFO-1 only produced a partial footprint that did not cover the entire site.

Table 2. C50 values (µM) determined from quantitative analysis of the DNase I footprinting experiments with TFO-1

|            | C50 (µM) | pH 5.0 | pH 6.0 | pH 7.0 |
|------------|----------|--------|--------|--------|
| Perfect match | <0.01    | 0.011 ± 0.002 | 1.1 ± 0.1 |
| S.AT       | <0.01    | 0.020 ± 0.005 | 4.5 ± 0.7 |
| BAU.TA     | 0.013 ± 0.002 | 0.04 ± 0.02 | n.d.   |
| MeP.TA     | 0.019 ± 0.005 | n.d.      | n.d.   |
| APP.TA     | a        | a       | n.d.   |

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2'-aminoethoxy modifications further increase triplex stability (40,49,50). This has been attributed to its effect on the oligonucleotide conformation, as well as the presence of the positive charge. The oligonucleotides used in the present study did not contain any contiguous BAU residues, and these were all separated by at least one other base. It is, therefore, possible that further improvements in triplex affinity might be achieved by either increasing the number of BAU modifications or by changing their distribution within the oligonucleotide. Further improvement might also be possible by using 2'-aminoethoxy S (45).

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