

The influence of single base triplet changes on the stability of a Pur·Pur·Pyr triple helix determined by affinity cleaving

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ABSTRACT

The influence of sixteen base triplet changes at a single position within a pur·pur·pyr triple helix was examined by affinity cleaving. For the 15 base pair target site studied here, G·GC, A·AT and T·AT triplets stabilize a triple helix to a greater extent than the other 13 natural triplets (pH=7.4, 25°C). Weaker interactions were detected for the C·AT, A·GC and T·CG triplets. The absence of specific, highly stabilizing interactions between third strand bases and the CG or TA base pairs demonstrates a current sequence limitation to formation of this structure. Models for the two dimensional base triplet interactions for all possible 16 natural triplets are presented.

INTRODUCTION

Oligonucleotide-directed triple helix formation is one of the most powerful methods for the sequence specific recognition of double helical DNA(1,2). At least two families of DNA triple helices exist, which differ in the sequence composition of the third strand, relative orientations of the three strands, and positioning of the third strand in the major groove. Pyrimidine oligonucleotides bind to purine tracts of double helical DNA in the major groove parallel to the Watson–Crick (WC) purine strand(1). Sequence specificity is derived from thymine (T) recognition of adenine–thymine base pairs (T·AT base triplets) and protonated cytosine (C+) recognition of guanine–cytosine base pairs (C+GC base triplets)(1–4). The use of other natural base triplets(5), nonnatural base triplets(6,7), and alternate strand triple helix formation with pyrimidine oligonucleotides linked 3' to 3' or 5' to 5'(8,9), has expanded the number of double helical sequences recognized beyond purine tracts. Purine-rich oligonucleotides bind purine tracts of double helical DNA in the major groove antiparallel to the WC purine strand(10–13). Sequence specificity is derived from guanine (G) recognition of GC base pairs (G·GC base triplets) and adenine (A) recognition of AT base pairs (A·AT base triplets). Within this structure, T has been shown to recognize AT base pairs (reverse Hoogsteen T·AT base

triplets)(11,13). Recently, the triplet specificities and required strand orientations for the two families of triple helical structures (pyr·pur·pyr and pur·pur·pyr) have been combined to form intramolecular alternate strand triple helices(14), and to recognize double helical sequences containing all four base pairs by alternate strand triple helix formation with oligonucleotides containing only natural 3'-5' phosphodiester linkages(15).

We report here the results of affinity cleaving experiments designed to determine the effect of single base triplet changes on the stability of a triple helix containing a purine-rich third strand with the goal of increasing our understanding of the sequence specificity of binding for this structure. By determining the relative strengths of the natural base triplets, we can define the limitations in the use of this structure as a general method for recognizing a broad range of specific sequences of duplex DNA containing all four base pairs.

MATERIALS AND METHODS

General

Distilled, deionized water was used for all aqueous reactions and dilutions. Enzymes were purchased from Stratagene, Boehringer–Mannheim or New England Biolabs. Enzyme reactions were performed using the manufacturer's recommended protocol in the activity buffer provided. 5'-(γ -³²P)ATP (>5000 Ci/mmol) was obtained from Amersham. Calf thymus DNA was purchased from Pharmacia. Polyacrylamide gel electrophoresis was performed in 1×TBE buffer(16). 5' end labeling was accomplished using standard procedures(16). Cerenkov radioactivity was measured with a Beckman LS 2801 scintillation counter. Autoradiography was carried out using Kodak X-Omat film.

Synthesis and Purification of Oligonucleotides

Oligonucleotides were prepared on an Applied Biosystems Model 380B DNA synthesizer with β -cyanoethyl phosphoramidites. Thymidine-EDTA was prepared as described and incorporated at the 3' end of oligonucleotides via the 5'-O-DMT-thymidine-EDTA-triethylester 3'-succinyl controlled pore glass(17).

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Deprotection was carried out in 0.1 N NaOH at 55°C for 24 hours. Oligonucleotides were purified by reverse phase chromatography on a Pharmacia FPLC system using a ProRPC 10/10 (C2–C8) column with a 0–40% CH₃CN gradient in 100 mM triethylammonium acetate, pH 7. The concentration of single-stranded oligonucleotides was determined by UV absorption at 260 nm, using the following molar extinction coefficients for each base: 15400 (A), 11700 (G), 7300 (C), 8800 cm⁻¹M⁻¹(T and T*).

5' End Labelling and Formation of Oligonucleotide Duplexes

Each oligonucleotide of sequence composition 5'-AATTCTCTCTAAAA GGGXGGGGAGGGGAGGGAAAACTCTCT-3' where X = A, G, C, or T was 5' end labelled using T4 polynucleotide kinase with γ -³²P ATP and annealed to its unlabelled complementary strand. The resulting duplexes were purified by non-denaturing 15% PAGE. Gel bands were visualized by autoradiography, cut out and eluted with 200 mM NaCl. The suspension was filtered to remove polyacrylamide and the DNA was purified by ethanol precipitation.

Affinity Cleaving Reactions

The cleavage reactions were carried out by combining a mixture of oligonucleotide-EDTA (100 nM) and Fe(NH₄)₂(SO₄)₂·6H₂O (250 nM) with the ³²P-labelled oligonucleotide duplex [\sim 20,000 cpm] in a solution of Tris-acetate (50 mM, pH 7.4), NaCl (10 mM), spermine·4HCl (100 μ M) and calf thymus DNA (100 μ M in base pairs) and then incubating at room temperature for one hour. Cleavage reactions were initiated by the addition of dithiothreitol (DTT) (4 mM) and allowed to proceed for 12 hours at room temperature. The reactions were stopped by freezing and lyophilization. The cleavage products were separated by gel electrophoresis, visualized by autoradiography and analyzed by storage phosphor autoradiography.

Quantitation of Cleavage Efficiencies by Storage Phosphor Autoradiography

Storage phosphor imaging plates (Kodak Storage Phosphor Screen S0230 obtained from Molecular Dynamics) were exposed to gel samples in the dark for 12–17 hours(18). A Molecular Dynamics 400S PhosphorImager was used to obtain all data from the storage screens. The data were analyzed by performing volume integrations of the regions corresponding to intact DNA, the target site and reference sites using the ImageQuant v. 3.0 software running on an AST Premium 386/33 computer. Absolute cleavage efficiencies were determined by calculating the ratio of site specific cleavage products generated for each triplet combination to the total amount of radiolabelled DNA in that lane on each gel from three independent experiments. To account for background cleavage generated by unbound oligonucleotide-EDTA·Fe, the value for site specific cleavage was calculated by subtracting the integral value corresponding to a reference site in each lane from the integral value at the main cleavage site. Relative efficiencies were calculated by normalizing the absolute cleavage efficiency for each triplet combination to that of the combination yielding the most efficient cleavage in each experiment.

RESULTS AND DISCUSSION

Previously, it was shown that three A·AT or three T·AT base triplets stabilized a 19 mer pur·pur·pyr triple helix to a greater extent than three C·AT triplets(11). However, the effect of each of the 16 possible natural base triplets on triple helix stability has not been determined. For this purpose, the four oligonucleotides 1-4 shown in Figure 1A were synthesized. These 15 mer oligonucleotides differ in sequence at a single position (Z), where Z = A, G, C, or T, and are equipped with thymidine-

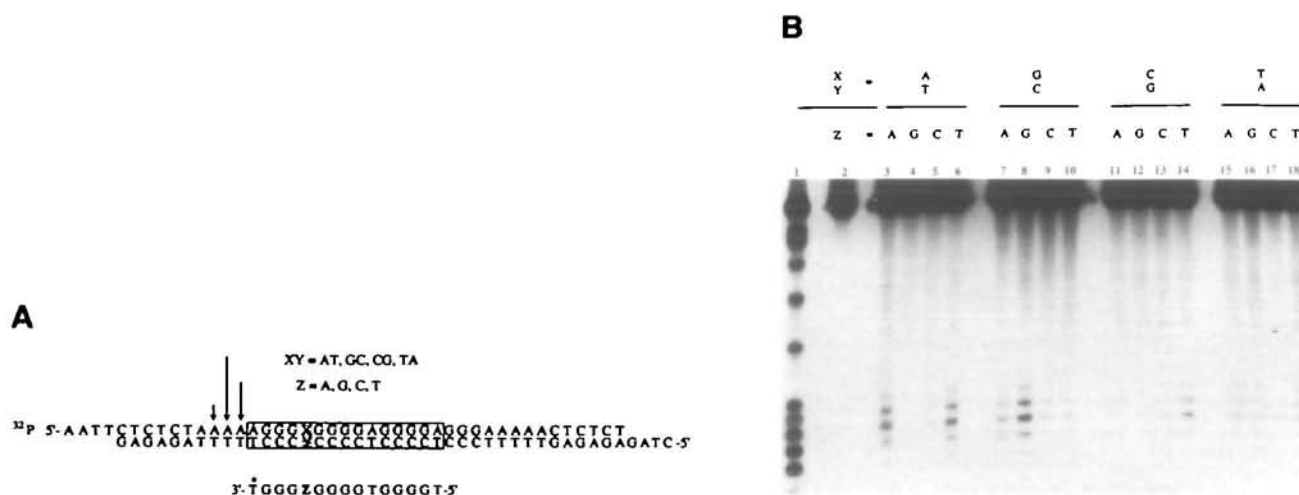


Figure 1. (A) The sequences of oligonucleotide-EDTA's 1-4 are shown, where T* indicates the position of thymidine-EDTA. The oligonucleotides differ at one base position, where Z = A, G, C, and T for 1, 2, 3, and 4, respectively. The sequences of the target duplexes are indicated with a common variable base pair position. The box indicates the double stranded sequence bound by oligonucleotide-EDTA's 1-4. The Watson-Crick base pair (AT, GC, CG, or TA) opposite the variant base in the oligonucleotide is shaded. The arrows show the sites of cleavage and heights indicate the relative cleavage efficiencies at the indicated bases as determined by quantitative analysis using storage phosphor autoradiography. (B) Autoradiogram of a 20% denaturing polyacrylamide gel used to separate affinity cleavage products. (Lanes 1–18) Duplexes containing 5' end labelled 5'-AATTCTCTCTAAAA GGGXGGGGAGGGGAGGGAAAACTCTCT-3'. (Lane 1) Products of an adenine specific sequencing reaction for duplex with X=A(20). (Lane 2) Intact 5' labelled duplex obtained after incubation under the conditions of the cleavage reactions in the absence of oligonucleotide-EDTA3Fe(II). (Lanes 3–18) DNA cleavage products produced by oligonucleotide-EDTA3Fe(II) 1-4; 1 (Z=A) (lanes 3, 7, 11, and 15); 2 (Z=G) (lanes 4, 8, 12, and 16), 3 (Z=C) (lanes 5, 9, 13, and 17); 4 (Z=T) (lanes 6, 10, 14, and 18). XY=AT (Lanes 3–6). XY=GC (Lanes 7–10). XY=CG (Lanes 11–14). XY=TA (Lanes 15–18).

EDTA (T*) at each 3' end such that binding can be monitored by the affinity cleaving method(17). Because the EDTA·Fe moiety generates a diffusible oxidant yielding a Gaussian, sequence-independent cleavage pattern, the amount of cleavage observed is assumed to be proportional to the fractional

W-C duplex (XY)	Third strand base (Z)			
	A	G	C	T
AT	+++	-	+	+++
GC	+	+++	-	-
CG	-	-	-	+
TA	-	-	-	-

Figure 2. Relative strengths of base triplet interactions in the pur·pur·pyr triple helix as determined by quantitative analysis using storage phosphor autoradiography of the relative cleavage efficiencies of oligonucleotide-EDTA's 1-4 on duplexes containing each of the four base pairs at the single variable position. (+++) >80%; (++) 60-80%; (+) 40-60%; (-) <40% relative to the most efficient combination.

occupation of the target site by the oligonucleotide-EDTA·Fe(19). Therefore, the relative stabilities of the triple helices formed by oligonucleotides 1-4 with duplexes differing in sequence at a single variable position (XY = AT, GC, CG, and TA) opposite the variant third strand base were assessed by comparing the relative cleavage efficiencies.

The oligonucleotides 1-4 cleave the target duplexes at the 5' end of the purine tract indicating that they bind in an orientation antiparallel to the WC purine strand. Cleavage efficiencies are dependent on solution conditions, particularly the concentration of the polycation spermine. Stringent conditions could be found under which differences in cleavage efficiencies were detected based on single triplet changes (100 nM oligonucleotide-EDTA, 100 μ M spermine, 20 mM NaCl, pH 7.4). Under these conditions, the three combinations ZXY = AAT, TAT and GGC show the most efficient cleavage (Figure 1B, Lanes 3, 6 and 8 respectively). No other strong interactions could be detected. Some weak interactions were observed for ZXY = CAT, AGC and TCG (Figure 1B, Lanes 5, 7, and 14 respectively). The relative cleavage efficiencies allow a qualitative ranking of the relative stabilities of the 16 triplet combinations at a single variable position within the sequence context studied here (Figure 2).

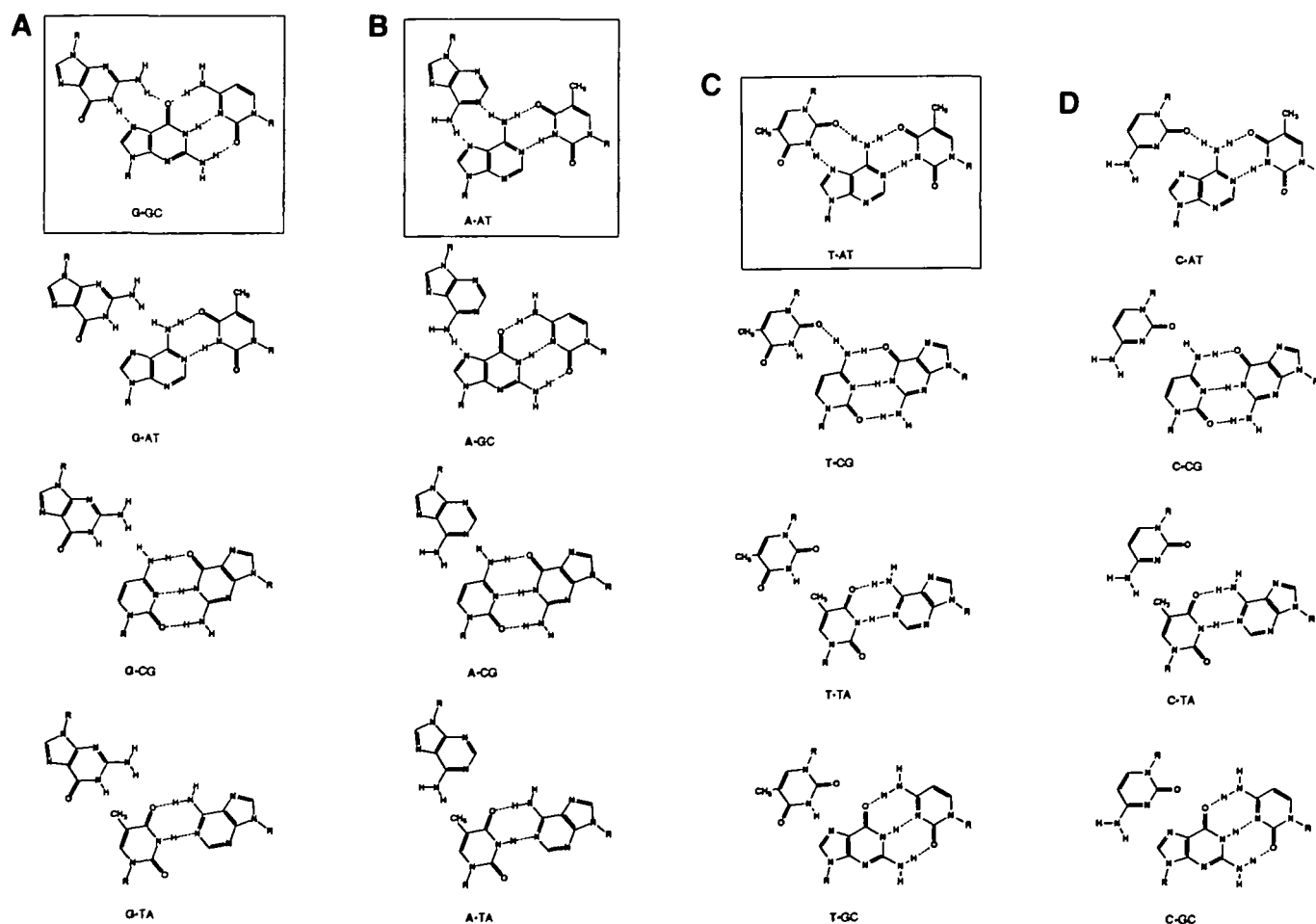


Figure 3. (A-D) Models depicting the 16 possible natural base triplets for the pur·pur·pyr triple helix. The third strand base has been positioned to maximize complementarity of hydrogen bonding surfaces while maintaining a backbone position similar to the G·C·C, A·T·T or T·A·A triplet shown boxed. Hydrogen bonds are shown in triplets for which stabilizing interactions were detected; two for strong triplets, one for weak triplets. All bases are depicted with *anti* conformations about the glycosidic bonds.

Models

Recent NMR spectroscopic studies indicated that the glycosidic bond conformations in a triple helix of the type pur·pur·pyr were *anti* (13). Models for the 16 triplet interactions compared in this study are shown in Figure 3. For each of the strong triplets A·AT, G·GC and T·AT, the third strand base is presumed to form two hydrogen bonds to the purine of the WC base pair. The three weaker base triplet interactions C·AT, A·GC and T·CG might be rationalized in part by the formation of a single hydrogen bond in each case between the third strand base and WC base pair, while maintaining a backbone position similar to that of the G·GC, A·AT or T·AT triplets.

Because the most stable base triplets form between third strand bases and purines of the Watson Crick GC or AT base pairs, the best potential double helical target sites are homopurine tracts. A limited number of CG base pairs may possibly be tolerated, if thymidine in the third strand is used (T·CG triplets). However, since thymidine binds tightly to the AT base pair (T·AT triplets), it is not a solution to specific recognition of CG. Also, no stabilizing interactions were detected between the third strand bases and the TA base pair. This lack of specific, highly stabilizing interactions at the CG or TA base pair shows a current sequence limitation to the formation of this structure. It should be emphasized that these triplet stabilities were determined only for the sequences shown in Figure 1A, where the variable triplet is flanked by two G·GC triplets. The relative strengths of base triplets may vary with flanking sequence, as is observed in the pyr·pur·pyr triple helix(7).

CONCLUSION

The basis for sequence specificity in the pur·pur·pyr triple helix has been confirmed by demonstrating that G·GC, A·AT and T·AT triplets stabilize a 15 mer triple helix to a greater extent than the other 13 natural triplet possibilities. Due to the lack of specific, stabilizing base triplet interactions between third strand bases and the CG or TA base pairs, this structure has sequence limitations to formation when only natural bases are in the third strand.

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