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Triplex Formation by Oligodeoxyribonucleotides Involving the Formation of X·U·A Triads[†]

Paul S. Miller[*] and Cynthia D. Cushman

Department of Biochemistry, The School of Hygiene and Public Health, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, Maryland 21205

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ABSTRACT: The stabilities of oligodeoxyribonucleotide triplexes containing a single pyrimidine–purine base pair, which interrupts an otherwise purine–pyrimidine base pair motif, were studied by UV melting experiments. The oligomer systems consisted of an oligodeoxyribonucleotide target duplex d-GAAGAAAAAAYAAAA/d-TTTTZTTTTTTCTTC, I·II(Y·Z), or d-GAAGAAAAAGUGAAA/d-TTTCACTTTTTCTTC, IV·V(U·A), where Y·Z is C·G, T·A, or U·A and U is deoxyuridine. The third strand oligodeoxyribopyrimidine was d-$\underline{\mathrm{C}}$TT$\underline{\mathrm{C}}$TTTTTTXTTTT, III(X), or d-$\underline{\mathrm{C}}$TT$\underline{\mathrm{C}}$TTTTT$\underline{\mathrm{C}}$X$\underline{\mathrm{C}}$TTT, VI(X), where $\underline{\mathrm{C}}$ is 5-methyldeoxycytidine. Triplexes were observed in the system III·I·II(X·C·G) when X was T or U. This may involve formation of T· or U·C·G triads in which the 4-carbonyl of T or U serves as a hydrogen bond acceptor for the *N*^{4}-amino group of C. Triplex formation between III(X) and I·II(T·A) was only observed when X was G. In contrast to T·A or C·G, it appears a U·A base pair in the duplex target is a much more versatile participant in triad formation. Thus, stable triplexes were observed in III·I·II(X·U·A) and in VI·IV·V(X·U·A) when X was C, $\underline{\mathrm{C}}$, T, or U. The formation of a T·U·A or U·U·A triad can occur if the T or U of III translates approximately 1.4 A into the major groove, thereby allowing the 3-NH of T or U to donate a hydrogen bond to the 4-carbonyl oxygen of U in the duplex. Formation of C· or $\underline{\mathrm{C}}$·U·A base triads could involve formation of a single hydrogen bond between the third strand *N*^{4}-amino group of C or $\underline{\mathrm{C}}$ and the 4-carbonyl group of U of the target. Alternatively, a hydrogen bonding scheme similar to that proposed for the T· or U·U·A triads can be drawn in which protonation of C or $\underline{\mathrm{C}}$ at N-3 could provide a hydrogen bond to the 4-carbonyl of U of the duplex. The latter hydrogen bonding scheme is supported by the observed greater reduction in stability with increasing pH of the III·I·II(C· or $\underline{\mathrm{C}}$·U·A) triplexes compared to that of the III·I·II(T·U·A) triplex. The observations that III·I·II(G·U·A) does not form a stable triplex suggests that factors in addition to the availability of a 4-carbonyl hydrogen bond acceptor group influence the formation of G·T·A versus G·U·A triads.

Oligodeoxyribonucleotides can, under certain conditions, interact with double-stranded DNA to form triple-stranded complexes. Thus, for example, oligodeoxyribopyrimidines can bind to a purine tract in the major groove of double-stranded DNA (Moser & Dervan, 1987; Francois et al., 1988; Rajagopal & Feigon, 1989a,b; de los Santos et al., 1989; Plum et al., 1990; Roberts & Crothers, 1991). The polarity of the sugar–phosphate backbone of the oligodeoxyribopyrimidine third strand is the same as that of the homopurine tract in the DNA target. Formation of T·A·T and C$^+$·G·C base triads occurs as a result of Hoogsteen hydrogen bonding interactions between the pyrimidine bases of the third strand and the purine bases of the target. Oligodeoxyribopurines are also capable of forming triplexes, again through interaction with a homopurine tract of double–stranded DNA (Orson et al., 1991; Postel et al., 1991; Beal & Dervan, 1991; Radharkrishnan et al., 1991; Chen, 1991). The ability of oligodeoxyribonucleotides to form triplexes wtih double–stranded DNA is of considerable interest, because such formation allows design of reagents which can cleave or modify DNA in a sequence-specific manner (Moser & Dervan, 1987; Francois et al., 1989a,b; Povsic & Dervan, 1990; Dervan & Baker, 1990; Shaw et al., 1991; Strobel et al., 1991; Beal & Dervan, 1991, 1992; Takasugi et al., 1991; Kiessling et al., 1992; Singleton & Dervan, 1992). In addition, triplex formation can be used to prevent protein–DNA interactions (Maher et al., 1989; Francois et al., 1989a,b; Hanvey et al., 1990; Strobel & Dervan, 1991), to inhibit DNA transcription in vitro (Durland et al., 1991; Young et al., 1991; Maher et al., 1992; Duval-Valentin et al., 1992; Blume et al., 1992; Maher, 1992), and to regulate gene activity in cells in culture (Cooney et al., 1988; Postel et al., 1991; Orson et al., 1991; Grigoriev et al., 1992; McShan et al., 1992).

Studies on triplex formation have been mainly directed to the interaction of oligodeoxyribonucleotides with homopurine tracts in the target DNA, and a number of investigations have examined the effects of various "mismatches" on triplex formation (Griffin & Dervan, 1989; Radhakrishnan et al., 1991; Macaya et al., 1991; Mergny et al., 1991; Wang et al., 1992; Yoon et al., 1992; Radakrishnan & Patel, 1992). Our laboratory has been interested in studying triplex formation between oligodeoxyribopyrimidines and oligo–DNA duplexes which contain a single pyrimidine–purine base pair which interrupts an otherwise purine–pyrimidine motif. In the studies described in this paper, we focus attention on oligodeoxyribonucleotide triplexes which involve formation of X·U·A triads, where U is deoxyuridine. Triplex formation in this system is compared with that involving X·C·G and X·T·A base triads.

MATERIALS AND METHODS

Protected nucleoside 3′-(β-cyanoethyl-*N*,*N*-diisopropylphosphoramidites) and nucleoside-derivatized controlled pore glass, CPG, supports were purchased from Glen Research. All chemicals used were reagent grade or better. Reversed-phase high-performance liquid chromatography, HPLC, was

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carried out on Rannin C-18 Microsorb columns using a linear gradient of 2% to 20% acetonitrile in 50 mM sodium phosphate buffer, pH 5.8. Gel electrophoresis was carried out on 20 × 20 × 0.75 cm gels containing 20% acrylamide and 7 M urea. The running buffer was 0.045 Tris, 0.045 boric acid, and 0.05 mM ethylenediaminetetraacetate buffered at pH 8.0.

Syntheses of Oligodeoxyribonucleotides. Oligodeoxyribonucleotides were synthesized on CPG supports in a Biosearch Model 8700 DNA synthesizer using standard phosphoramidite chemistry (Brown & Brown, 1991). Oligodeoxyribonucleotides containing 8-oxo-2′-deoxyadenosine were prepared using a protected 8-methoxy-2′-deoxyadenosine 3′-(β-cyanoethyl *N,N*-diisopropylphosphoramidite) as previously described (Miller et al., 1992). The DNA synthesizer was programmed to remove the 5′-terminal dimethoxytrityl group from the protected oligomers at the end of the synthesis. The oligomers were deprotected and removed from the support by treatment with a solution containing concentrated ammonium hydroxide/pyridine (1:1 v/v) at 55 °C for 6 h. Oligomers which contained 8-oxo-2′-deoxyadenosine were further incubated for 96 h at 37 °C with a solution containing thiophenol/triethylamine/dioxane (1:2:2 v/v). This latter treatment removed the 8-methoxy protecting group from 8-oxo-2′-deoxyadenosine. The oligomers were then purified by C-18 reversed-phase high-performance liquid chromatography and desalted using a Sep-Pak C-18 cartridges. The oligomers were checked for purity by polyacrylamide gel electrophoresis after they were phosphorylated using polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]$ATP. The extinction coefficients of the oligomers were determined by recording their UV absorption spectra before and after hydrolysis by snake venom phosphodiesterase as previously described (Miller et al., 1992).

Melting Experiments. Melting experiments were carried out in one of the following buffers. pH 6.4 buffer: 0.1 M sodium chloride, 20 mM magnesium chloride, 20 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid), PIPES. pH 7.0 buffer: 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM tris(hydroxymethyl)aminomethane, Tris, or 50 mM 3-(*N*-morpholino)propanesulfonic acid, MOPS. pH 7.5 buffer: 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM Tris or 50 mM MOPS. pH 8.0 buffer: 0.1 M sodium chloride, 20 mM magnesium chloride, 50 mM Tris, or 50 mM MOPS. The pH of the buffers was measured at 22 °C. Triplexes were formed by adding 0.5 mL of a 1 μM or 2 μM solution of the third strand oligomer to 0.5 mL of a 1 μM or 2 μM solution of the preformed DNA duplex at room temperature. The final strand concentration of each oligomer was thus 0.5 μM or 1 μM. The solution was then stored at 4 °C overnight. The A_{260} versus temperature profile of the solution was measured using a Varian 219 UV/vis spectrophotometer filled with a thermostated cell holder which was connected to a Neslab RTE-100 programmable circulating temperature bath. Spectra and melting profiles were also recorded on a Cary 3E UV/vis spectrophotometer fitted with a thermostatable cell block and temperature controller. The latter instrument allowed four melting curves to be recorded simultaneously. This instrument was used in experiments in which the effects of pH on melting temperature were measured. The oligonucleotide solutions were placed in the sample cuvettes at 0 °C. The cuvettes were bathed with dry nitrogen gas to prevent condensation at low temperature and the temperature was increased at a rate of 0.5 °C/min over the range 0–60 °C. The melting temperatures, T_m, of the third strands were determined from the midpoint of the transition curves. Identical melting curves and melting transitions were observed for duplicate samples, and measurements using either spectrophotometer gave the same results.

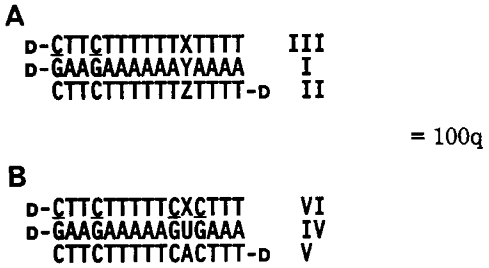

FIGURE 1: Oligodeoxyribonucleotides used to study triplex formation. C̲ is 5-methyldeoxycytidine.

RESULTS AND DISCUSSION

Triplex formation was examined using the oligodeoxyribonucleotides shown in Figure 1A. The target duplex consists of a purine strand I(Y) and pyrimidine strand II(Z). The Y·Z base pair of duplex I·II(Y·Z) is either C·G, T·A, or U·A and interrupts the otherwise purine–pyrimidine motif of this duplex. The third strand oligomer III(X), contains thymidine, 5-methyldeoxycytidine, C̲, and a test base X. 5-Methyldeoxycytidine is included in order to enhance the stability of the triplexes at pH 7 and above (Povsic & Dervan, 1989). The sugar–phosphate backbone polarity of III is the same as that of the purine strand of the duplex.

The ability of the these oligomers to form triple-stranded complexes was determined from ultraviolet spectroscopic melting experiments in a solution containing 0.1 M sodium chloride, 20 mM magnesium chloride, and 50 mM Tris, buffered at pH 7.0. The results of these melting experiments are shown in Figure 2A,B and in Table I. For purposes of comparison, a triplex which does not contain a pyrimidine–purine interruption, III·I·II(T·A·T), was also examined. Triplex formation was observed for III·I·II(T·A·T) and between III(X) and duplex I·II(C·G) when X is T or U. This was indicated by the presence of two sigmoidal transitions in the A_{260} vs temperature profile when solutions containing equal concentrations of each strand were heated from 0 to 60°C at a rate of 0.5 °C/min. The melting profile for III·I·II(T·C·G) is shown in Figure 2A. The first transition corresponds to the melting of the third strand, III(T), and the second transition corresponds to melting of the duplex, I·II(C·G). A profile identical to that of the second transition was obtained when a solution containing I·II(C·G) alone was heated under identical conditions. The third strands of III·I·II(U·C·G) and III·I·II(T·C·G) had T_m's 8 °C and 10 °C lower than that of the triplex III·I·II(T·A·T). Triplex formation was not observed when X was C, C̲, A, or G.

The formation of triplexes by III(T) or III(U) most likely involves formation of a T·C·G or U·C·G triad. A possible hydrogen bonding scheme for these triads is shown in Figure 3A. In both cases, a single hydrogen bond can form beween the 4-carbonyl acceptor group of T or U of the third strand and the N^4-amino donor group of C. Although C and C̲ are isosteric with T or U, a similar hydrogen bonding scheme is not possible when these bases are present in III(X) because both of these pyrimidines contain hydrogen bond donors at their 4-position. The inability of C or C̲ to form hydrogen-bonded triads with C·G most likely accounts for the failure to observe triplex formation in the III·I·II(C· or C̲·C·G) system.

It appears that triplex formation between III(X) and duplex I·II(C·G) is very sequence specific under the conditions of our

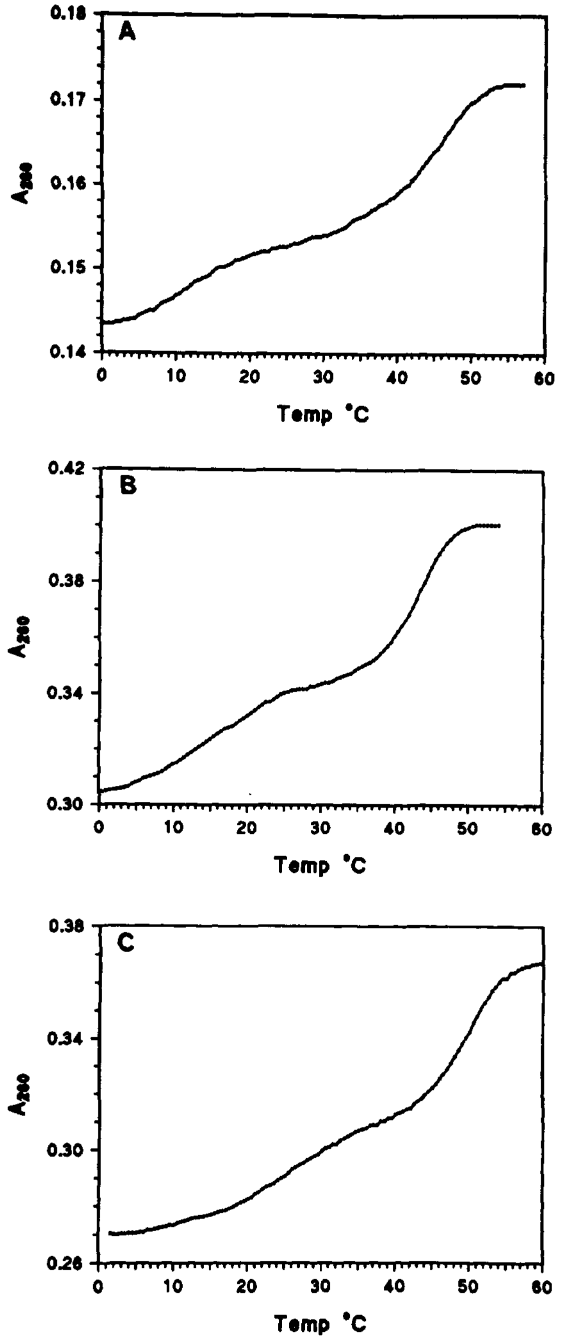

FIGURE 2: Absorbance vs temperature profiles of (A) 0.5 μM III·I·II(T·C·G) at pH 7.0; (B) 1.0 μM III·I·II(T·U·A) at pH 7.0; and (C) 1.0 μM VI·IV·V(U·U·A) at pH 6.4.

Table I: Triplex Formation at pH 7.0

complex			T_m[a] (°C) third strand
d-C̲TTC̲TTTTTTXTTTT d-GAAGAAAAAAYAAAA CTTCTTTTTTZTTTT-d			
X	Y ·	Z	
T	A	T	22[b]
T	C	G	12[b]
U	C	G	14[b]
C	C	G	--[c]
C̲	C	G	--
A	C	G	--
G	C	G	--
T	T	A	--
C	T	A	--
A	T	A	--
G	T	A	15[b]
T	U	A	15
U	U	A	13
C	U	A	5
C̲	U	A	14
A	U	A	--
G	U	A	--

[a] Melts were carried out in a buffer containing 50 mM Tris, pH 7.0, 20 mM $MgCl_2$, 0.1 M NaCl, at a strand concentration of 1 μM. [b] The strand concentration was 0.5 μM. [c] Indicates triplex formation was not observed.

experiments. A similar sequence specificity was observed by Yoon et al. (1992), who used electrophoretic mobility shifts on nondenaturing polyacrylamide gels to study oligodeoxyribonucleotide triplex formation. In these studies, the oligo-DNA duplexes were 21 or 25 nucleotides long and the C·G base pair was flanked on each side by A·T base pairs. Consistent with our studies, stable triplex formation was only observed for the T·C·G triad. Mergny et al. (1991), on the other hand, found much less sequence specificity in their studies on oligodeoxyribonucleotide triplex formation. In their studies, the target duplex was 31 nucleotide long. A single C·G base pair was placed in the middle of a purine–pyrimidine tract 12 nucleotides in length. This tract was located 2 base pairs in from one end of the duplex. The C·G was flanked on the 5′-side by an A·T base pair and on the 3′-side by a G·C base pair. Triplex formation by the third strand oligomer d-CTTTTTXCTTCTC was observed at pH 5.6 when X was A, C, G, or T as determined by melting experiments. The T_m's of the third strands of these triplexes ranged between 18 and 23 °C, the latter being observed when X was C. The reduced base discrimination in these experiments may be due to increased stability of the C^+·G·C triads at pH 5.6 compared to the stability of $\underline{C}^+$·G·C triads at pH 7.0 in our experiments. In addition, the sequences flanking the C·G interruption may affect the stability of triplex formation as has been observed in other systems (Kiessling et al., 1992).

Triplex formation between I·II(T·A) and III(X) was only observed when X was G. Formation of a triplex involving a G·T·A triad has been previously observed in intermolecular triplexes (Griffin & Dervan, 1989; Mergny et al, 1991; Yoon et al., 1992). Proton NMR studies on G·T·A triads in an intramolecular triplex showed that a single hydrogen bond is formed between the N^2-amino group of G and the O-4 carbonyl of T (Radhakrishnan et al., 1991; Wang et al., 1992; Radhakrishnan & Patel, 1992). An additional, weak hydrogen bond was also observed between the other N^2-amino proton of G and the O-4 carbonyl of T of the neighboring A·T base pair.

The lack of stable triplex formation by III·I·II(X·T·A) when X is T, C, or A again points out the sequence specificity of triplex formation in this system. Deoxycytidine in the X position of III could potentially donate a single hydrogen bond from its N^4-amino group to the 4-carbonyl group of T. However, this interaction may be prevented by the presence of the 5-methyl group of T which projects into the major groove and could sterically interact with the cytosine base of the third strand. Similar steric interference has been suggested by Mergny et al. (1991) to explain the lack of triplex formation by T·A base pairs in their oligodeoxyribonucleotide system.

Considerably different behavior was observed when triplex formation between I·II(U·A) and III(X) was studied. In this case, as is shown in Figure 2B and Table I, stable triplexes were observed when X was T, U, C, or C̲. With the exception

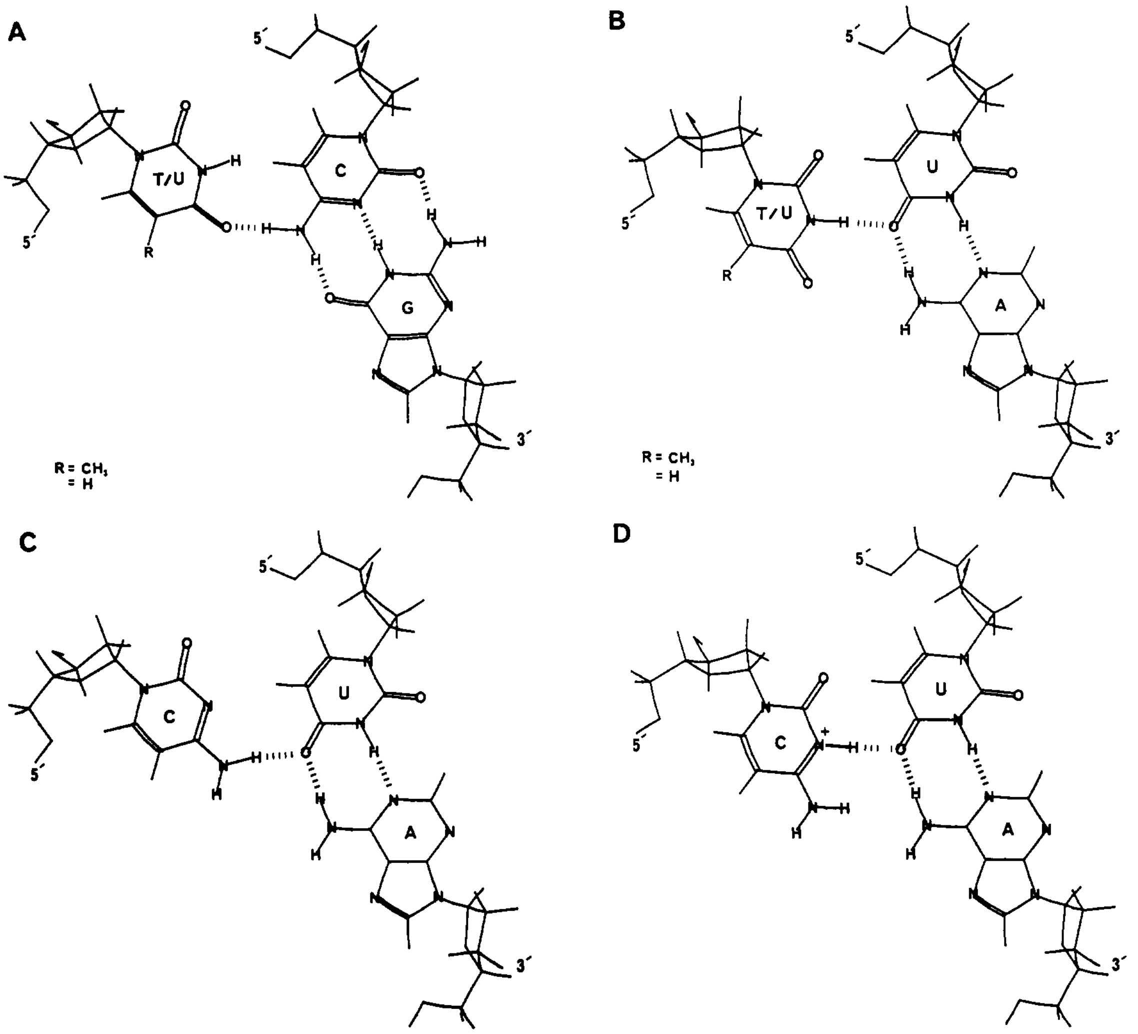

FIGURE 3: Possible hydrogen bonding schemes for (A) T· or U·C·G triad; (B) T· or U·U·A triad; (C) C·U·A triad; and (D) C^+·U·A triad.

of the triplex formed when X is C, the melting temperatures of the third strands of these triplexes are similar to those observed for III·I·II(T or U·C·G) and III·I·II(G·T·A). This suggests formation of base triads which employ a single hydrogen bond between pyrimidine X of III and the U of I. If the structure of the T·U·A or U·U·A triad is isomorphous with that of the U·C·G triad shown in Figure 3A, then the 4-carbonyl group of T or U of strand III, which is a hydrogen bond acceptor, cannot interact with the 4-carbonyl group of U of I, which is also a hydrogen bond acceptor. However, if the T or U of III translates approximately 1.4 A into the major groove, the 3-NH is then positioned to donate a hydrogen bond to the 4-carbonyl oxygen of U in the duplex. This hydrogen bonding scheme is shown in Figure 3B. Examination of molecular models suggests that the required translation can occur with minimal perturbation of the sugar–phosphate backbone of III. Similar hydrogen bonding interactions are less likely to occur in the formation of T·T·A or U·T·A triads in III·I·II(T· or U·T·A) due to unfavorable steric interactions between the 5-methyl group of T of I and the T or U of III.

Triplex formation by III·I·II(C· or C̲·U·A) could involve formation of a single hydrogen bond between the N^4-amino group of C or C̲ and the 4-carbonyl group of U as is shown in Figure 3C. In this case, the structure of the triad is isomorphous with that of T·C·G or U·C·G. An alternative hydrogen bonding scheme which is isomorphous with the T·U·A or U·U·A triad shown in Figure 3B can also be drawn. In this scheme, which is shown in Figure 3D, protonation of C or C̲ at N-3 provides a hydrogen bond to the 4-carbonyl of U of I.

Formation of C^+·G·C triads requires protonation of C of the third strand and the stability of triplexes containing this triad decreases as the pH approaches 7. Formation of C̲$^+$·G·C triads also requires protonation of C̲. However the stability of triplexes containing this triad is less affected by increases in pH (Lee et al., 1984; Povsic & Dervan, 1989). The observation that III·I·II(C̲·U·A) is more stable than III·I·II(C·U·A) at pH 7.0 suggests that protonation of C or C̲ may be involved in formation of these triads.

The effect of pH on triplex formation by III·I·II(X·U·A) where X is T, U, C, or C̲ was examined and the results are shown in Table II. When X was T or U, the T_m of the third strand decreased approximately 7 °C for each 0.5 unit increase in pH over the pH range 6.4–8.0 in Tris buffer. A similar decrease in T_m with increasing pH was also observed in MOPS buffer. This buffer has a lower temperature coefficient, –0.013 pK/°C, than does Tris, –0.031 pK/°C (Fasman, 1976). Neither T nor U is expected to be protonated or ionized in this pH range. The observed decrease in T_m is therefore most likely due to the reduction in stability of the C̲$^+$·G·C triads near the 5′-end of the triplex as the pH is raised. Consistent with this argument is the observation that substitution of the two C̲ residues of III(T) with 8-oxo-2′-deoxyadenosine, A̲, resulted in formation of triplexes whose T_m, 12 °C was unchanged over the pH range 6.4–8.0. Previous studies have

Table II: Effect of pH on Triplex Formation

complex	T_m[a] (°C) third strand			
d-$\underline{C}$TT·$\underline{C}$TT·TTT·TXT·TTT d-GAA·GAA·AAA·AUA·AAA CTT·CTT·TTT·TAT·TTT-d	pH 6.4	7.0	7.5	8.0
X = T	22	15	7	~0
	22[b]	16[b]	12[b]	5[b]
X = U	20	13	nd[c]	nd
X = 5-MeC	20	14	-0	--[d]
X = C	18	5	--	--
	18[b]	14[b]	4[b]	--

[a] Melts were carried out in a buffer containing 20 mM PIPES (pH 6.4) or 50 mM Tris (pH 7.0, 7.5, or 8.0) and 20 mM $MgCl_2$, 0.1 M NaCl, at a strand concentration of 1 μM. [b] Melts were carried out in a buffer containing 20 mM PIPES (pH 6.4) or 50 mM MOPS (pH 7.0, 7.5 or 8.0) and 20 mM $MgCl_2$, 0.1 M NaCl, at a strand concentration of 1 μM. [c] Not determined. [d] Indicates triplex formation was not observed.

Table III: Triplex Formation at pH 6.4

complex	T_m[a] (°C) third strand
d-$\underline{C}$TT$\underline{C}$TTTTT$\underline{C}$X$\underline{C}$TTT d-GAAGAAAAAGYGAAA CTTCTTTTTCZCTTT-d	
X Y · Z	
T U A	10
U U A	27
C U A	21

[a] Melts were carried out in a buffer containing 20 mM PIPES, pH 6.4, 20 mM $MgCl_2$, 0.1 M NaCl, at a strand concentration of 1 μM.

shown that 8-oxoadenine forms $\underline{A}$·G·C triads, whose stability is not affected by changes in pH over this range (Miller et al., 1992).

A much greater sensitivity of the T_m of the third strand to pH was observed when X was C or $\underline{C}$. Thus, the T_m of the III(X) was 0 °C or less when X was $\underline{C}$ and no triplex formation was observed when X was C at pH 7.5 in Tris buffer or at pH 8.0 in MOPS buffer. In Tris at pH 7.0, III(C) formed a triplex whose T_m was only 5 °C, which is a decrease of 13 °C compared to its T_m at pH 6.4. A similar decrease was also seen in MOPS buffer when X is C. Thus, in MOPS buffer at pH 7.5, the T_m of III(C) is 4 °C, a decrease of 10 °C relative to that observed at pH 7.0 Besides the inherent destabilization caused by the presence of the $\underline{C}^+$·G·C triads, there appears to be additional destabilization of triplexes involving C·U·A or $\underline{C}$·U·A triads with increasing pH. These results suggest that formation of C·U·A or $\underline{C}$·U·A triads involves base protonation and are consistent with the hydrogen bonding scheme shown in Figure 3D. We also attempted to examine the effect of triplex formation by III(C or $\underline{C}$) in which the two 5′-end 5-methyldeoxycytidines were replaced by 8-oxo-2′-deoxyadenosine. However, the shape of the A_{260} vs temperature profiles of solutions containing these oligomers suggested that the third strand was melting before 0 °C and thus meaningful comparisons of the T_m could not be made over this pH range.

It is interesting to note that in contrast to III·I·II(G·T·A), triplex formation was not observed for III·I·II(G·U·A). Recent studies suggest that neighboring bases can influence the stability of G·T·A triads and that additional weak hydrogen bonding interactions between G and neighboring base pairs may stabilize the triad (Mergny et al., 1991; Radhakrishnan et al., 1991; Wang et al., 1992; Radhakrishnan & Patel, 1992). Although the same neighboring A·T base pairs occur in both the III·I·II(G·T·A) and III·I·II(G·U·A) systems, triplex formation is only observed in the former system. Examination of molecular models suggests that hydrogen bond formation between the N^2-amino proton of G and the 4-carbonyl of U is sterically feasible and could occur in much the same way as is observed for the G·T·A triad. Thus, it appears that by itself, the availability of a hydrogen bond acceptor group is not sufficient to allow formation of the G·U·A triad. Possibly local conformational changes caused by the absence of a 5-methyl group on U of II(U) disfavor formation of necessary, additional hydrogen bonding between G and the neighboring T·A base pair, and this could account for the lack of stability of the G·U·A triad.

Triplex formation involving T·U·A and U·U·A triads appears to be sensitive to neighboring bases. This was demonstrated using the oligonucleotide system, VI·IV·V(X·U·A), shown in Figure 1B in which the X·U·A triad is flanked on either side by $\underline{C}^+$·G·C triads. Melting experiments were carried out at pH 6.4 in order to stabilize the triplex, which contains four $\underline{C}^+$·G·C triads. As shown in Figure 2C and Table III, VI·IV·V(U·U·A) and VI·IV·V(C·U·A) each form stable triplexes whose third strands melt respectively at 27 °C and 21 °C. In contrast to this behavior, the third strand of VI·IV·V-(T·U·A) melts 11 °C to 17 °C lower than the third strand of VI·IV·V(C· or U·U·A). A similar reduction in T_m was not seen for III·I·II(X·U·A) at pH 6.4, a system in which the X·U·A triad is flanked by T·A·T triads. Taken together, these results suggest that the presence of the 5-methyl group of T in VI(T), which projects into the major groove, perturbs triplex formation. There appear to be no obvious steric interactions between the methyl group and the neighboring bases which would account for such a perturbation. An alternative explanation is the possibility that the presence of the hydrophobic methyl group limits the ability of the flanking $\underline{C}$ residues to be protonated during the formation of the $\underline{C}^+$·G·C triads, thus leading to overall reduction in stability of the triplex.

The results of our experiments are in general agreement with previous studies of others and show that triplex formation by oligodeoxyribopyrimidines is possible when a purine–pyrimidine motif of the double-stranded target is interrupted by a single pyrimidine–purine base pair. The formation of such triplexes most likely involves triads having a single hydrogen bond between a pyrimidine of the third strand and the pyrimidine of the interrupting base pair. It appears that a U·A base pair in the duplex target is a more versatile participant in triad formation than is either T·A or C·G and that hydrogen bonding to this base pair most likely occurs through donation of a hydrogen bond from the 3-position of the pyrimidine base of the third strand. Although U·A base pairs are not expected to occur in natural DNAs, information on triad formation involving this base pair furthers our general understanding of triplex formation. Such understanding can ultimately be applied to the design of sequence selective oligonucleotides which can interact with double-stranded nucleic acids.

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