Energetics of Formation of Sixteen Triple Helical Complexes Which Vary at a Single Position within a Purine Motif

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Abstract: The association constants for the formation of sixteen purine • purine • pyrimidine triple helical complexes which vary at a single common position (Z•XY where Z = A, G, C, T and XY = AT, GC, CG, TA) were determined by quantitative DNAse I footprint titration. The sequence context in which the sixteen pairings Z•XY were studied is 5′-d(AGGGGAGGGGXGGGA)-3′ within a 648 base pair DNA fragment. The association constants for the purine-rich oligonucleotides 5′-d(TGGGZGGGGTGGGGT)-3′ ranged from 2.7×10^5 (Z•XY = G•TA) to 8.9×10^7 M^{-1} (Z•XY = T•AT) at 37 °C, pH 7.4, 50 mM tris acetate, 10 mM NaCl, and 3 mM $MgCl_2$. For the sequences studied, T•AT is more stable than T•CG, T•GC, and T•TA by ≥1.2 kcal mol^{-1}; A•AT is more stable than A•CG, A•GC, and A•TA by ≥1.2 kcal mol^{-1}; and G•GC is more stable than G•AT, G•CG, and G•TA by ≥2.1 kcal mol^{-1}. The triplet T•CG is ≥1.5 kcal mol^{-1} more stable than the other three bases opposite a CG base pair.

Introduction

Oligonucleotide-directed triple helix formation is a versatile method for the sequence-specific recognition of double helical DNA.[1] Triple helices can be classified into two structural motifs: those in which the third strand is primarily composed of pyrimidine bases, and those in which the third strand is composed primarily of purine bases.[1-3] In addition, triple helices composed of combinations of these two motifs can be formed within some sequence contexts.[4] Triple helix formation is sensitive to the length of the third strand,[1a,5] single base mismatches,[1a,5,6] pH,[7] cation concentration and valence,[8] temperature,[9] and backbone composition (DNA or RNA) of the three strands.[10] Oligonucleotide-directed triple helix formation has been shown to inhibit sequence specific DNA binding proteins[11] and has been used to mediate single site cleavage of human chromosomal DNA.[12] The ability to target a broad range of DNA sequences[1,13] and the high specificity and stability of the resulting local triple helical structures make this a powerful technique for the recognition of single sites within megabase segments of double helical DNA.

In an effort to understand the interactions which give rise to recognition in the major groove of DNA by oligonucleotides, the specificity afforded by the four natural bases in triple helix formation has been qualitatively characterized by affinity cleavage,[14] optical melting,[15] gel mobility shift,[16] and intramolecular triple helix formation.[17] Specific interactions in triple helix formation implicated by these studies have been characterized by NMR spectroscopy.[18-20] In the pyrimidine motif, Hoogsteen-type hydrogen bonds have been observed between

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Table 1. Association Constants (K_T) for the Formation of 16 Triple Helical Complexes Containing the Z·XY Triplets at 37 °C, 10 mM NaCl, 3 mM $MgCl_2$, 50 mM Tris Acetate, and pH 7.4[a,b]

	Z =			
XY	A	G	C	T
AT	5.3 (±1.4) × 10^7	1.9 (±0.2) × 10^6	4.0 (±1.2) × 10^6	8.9 (±0.5) × 10^7
GC	8.2 (±0.8) × 10^6	5.5 (±0.4) × 10^7	1.7 (±0.4) × 10^6	2.9 (±0.2) × 10^6
CG	1.1 (±0.3) × 10^6	5.8 (±0.8) × 10^5	8.5 (±0.9) × 10^5	1.3 (±0.1) × 10^7
TA	8.5 (±2.7) × 10^5	2.7 (±0.7) × 10^5	8.3 (±2.4) × 10^5	1.7 (±0.7) × 10^6

[a] K_T values are reported as the mean (± the standard error of the mean) of three measurements. The K_T values are reported in units of M^{-1}. [b] The identity of the base Z is indicated across the top of the columns; the identity of the Watson–Crick base pair XY is indicated on the left.

thymine (T) bases in the third strand and adenine–thymine (AT) base pairs in the duplex,[18] and between N3 protonated cytosine (C+) of 5-methylcytosine (^{me}C+) in the third strand and guanine–cytosine (GC) base pairs in the duplex.[19] In the purine motif, reversed-Hoogsteen-type hydrogen bonds have been observed between G bases in the third strand and GC base pairs in the duplex,[20] and between T[20a] or A[20b] in the third strand and AT base pairs in the duplex.

The introduction of quantitative methods has allowed the determination of equilibrium association constants for the binding of an oligonucleotide at a single site on a DNA plasmid fragment.[5,21] Previous reports on the purine motif suggest that in addition to the aforementioned three triplets there are several others that have intermediate stability,[2b,c] although little is known about the quantitative difference among all 16 triplets composed of natural bases. Here we report the equilibrium association constants and free energies of formation of 16 triple helical complexes which vary at a single position (Z·XY where Z = A, G, C, T and Watson–Crick XY = AT, GC, CG, and TA) (Figure 1). We are primarily interested in comparing the differences in free energy values ($\Delta\Delta G°$) which are relevant to the issue of specificity, as in an analogous study within the pyrimidine motif.[22]

Results and Discussion

Methods. A detailed description of the quantitative DNAse I footprint titration has been presented elsewhere for protein–DNA,[23] small molecule–DNA,[24] and oligonucleotide–DNA[21] interactions, hence, the protocol is outlined briefly in the Experimental Section. A 3′-^{32}P end labeled 648 base pair restriction fragment containing a single purine rich 15 base pair target site 5′-d(AGGGGAGGGGXGGGA)-3′ was allowed to equilibrate at 37 °C and pH 7.4 with a series of concentrations of third strand purine-rich oligonucleotides 5′-d(TGGG-ZGGGGTGGGGT)-3′ that ranged from 8 μM to 800 pM (Figure 1). Following a 48 h equilibration, DNAse I was added and digestion was allowed to proceed for 6 min. After quenching, the reaction mixtures were separated by denaturing polyacrylamide gel electrophoresis and the resulting gels were imaged by storage phosphor autoradiography. Integration of site and reference blocks allowed the determination of the apparent fractional occupancy of the site at each oligonucleotide concentration. A binding isotherm was fit to the resulting pairs of θ_{app}, $[O]_{tot}$ values (see Experimental Section) and the equilibrium association constant (K_T) was calculated. K_T values from three independent titrations were averaged to obtain each of the association constants reported in Table 1.

Table 2. Free Energy ($\Delta G°$) of Formation of 16 Triple Helical Complexes Containing the Z·XY Triplets at 37 °C, 10 mM NaCl, 3 mM $MgCl_2$, 50 mM Tris Acetate, and pH 7.4[a,b]

	Z =			
XY	A	G	C	T
AT	−11.0 ± 0.2	−8.9 ± 0.1	−9.4 ± 0.3	−11.3 ± 0.1
GC	−9.8 ± 0.1	−11.0 ± 0.2	−8.8 ± 0.1	−9.2 ± 0.1
CG	−8.6 ± 0.2	−8.2 ± 0.1	−8.4 ± 0.1	−10.1 ± 0.1
TA	−8.4 ± 0.2	−7.7 ± 0.2	−8.4 ± 0.2	−8.8 ± 0.3

[a] Free energy values are calculated from the measured association constants at 37 °C and are reported in kcal mol^{-1}. [b] The identity of the base in the third strand, Z, is indicated across the top row; the identity of the Watson–Crick base pair, XY, is indicated to the left.

Affinity. Experimental conditions such as temperature, salt concentration, and pH are chosen such that the lowest and highest equilibrium binding constants for the 16 triple helical complexes are within the range that can be measured with this technique. The footprint titration experiments were carried out at 37 °C, pH 7.4, 50 mM tris acetate, 10 mM NaCl, and 3 mM $MgCl_2$. The values of the 16 association constants (K_T) range from 2.7 × 10^5 to 8.9 × 10^7 M^{-1} (Table 1). An examination of the data confirms that three base triplets (G·GC, T·AT, and A·AT) are particularly stable. Triple helical complexes containing the G·GC, T·AT, and A·AT triplets at the Z·XY position are the most stable of the 16 complexes measured. The total standard free energies ($\Delta G°$) of these triple helix forming reactions are −11.0, −11.3, and −11.0 kcal mol^{-1}, respectively (Table 2). In addition, the triplets T·CG[2c,25] and A·GC[2c,26] have intermediate stability. The total standard free energies of triple helix forming reactions containing these triplets at the Z·XY position are −10.1 and −9.8 kcal mol^{-1}, respectively. Triple helices containing these triplets at the single variable position are approximately 1 kcal mol^{-1} less stable than those containing G·GC, T·AT, or A·AT at that position.

Specificity. If specific local triple helices are to be useful structures for accomplishing the recognition of single sites in megabase DNA, it is important that the complex be stabilized by specific interactions and be sensitive to single base mismatches. The results presented here demonstrate that either A or T in the third strand can accomplish the sequence specific recognition of AT base pairs in double helical DNA, G specifically recognizes GC base pairs, and T specifically recognizes CG base pairs (comparison across rows of Table 2). For example, T·AT and A·AT are more stable than C·AT and

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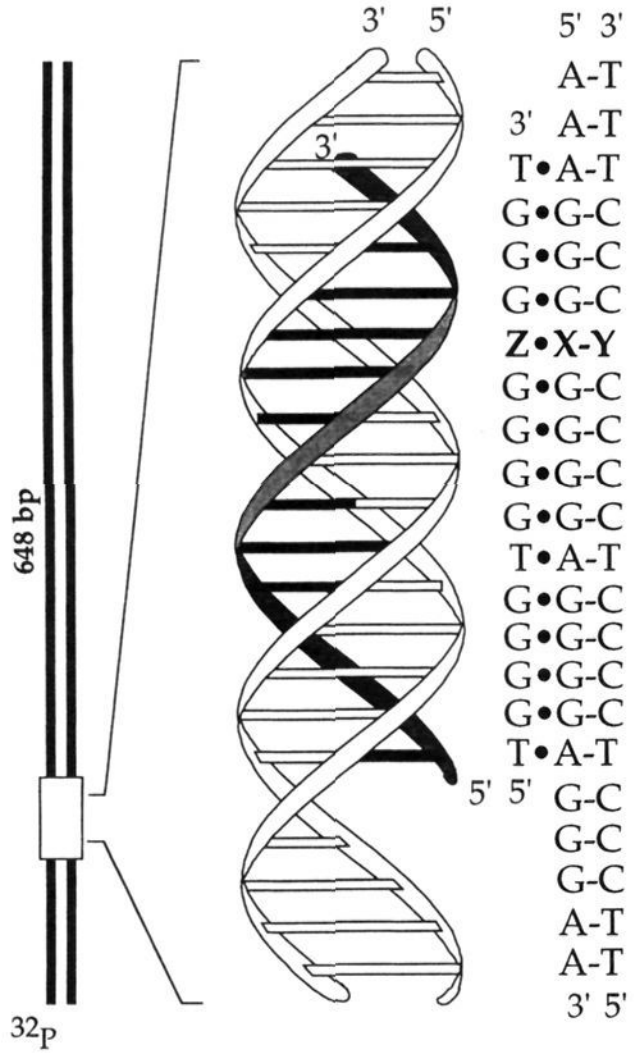

Figure 1. (Left) Ribbon model of the triple helix formed upon binding of the 15mer oligonucleotide probe to the 15 bp target site within a 648 bp restriction fragment. The Watson−Crick duplex strands are depicted as white ribbons while the oligonucleotide is depicted as a dark ribbon. (Right) The sequence modeled by the ribbons is shown. The position of the variable triplet is indicated as Z·XY, where Z = A, G, C, T and XY = AT, GC, CG, TA.

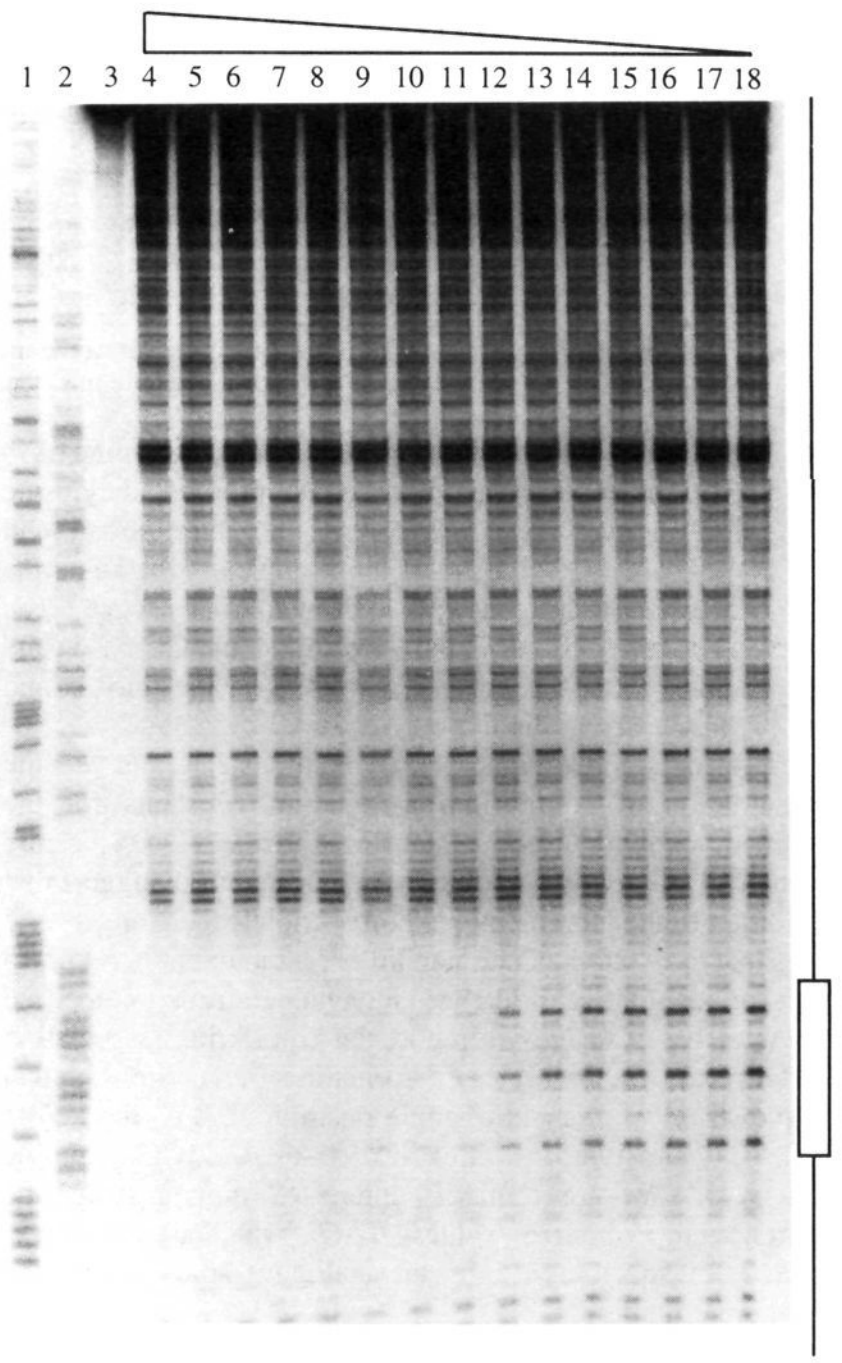

Figure 2. Autoradiogram of an 8% denaturing polyacrylamide gel used to separate fragments generated by DNAse I digestion in a quantitative footprint titration experiment. The bar to the right indicates the position of the 15 bp binding site within the 648 bp restriction fragment where XY = AT. (Lane 1) Products of an adenine-specific reaction. (Lane 2) Products of a guanine-specific reaction. (Lane 3) Intact 3′ labeled DNA after incubation in the absence of third strand oligonucleotide. (Lanes 4−18) DNAse I digestion products obtained in the presence of varying concentrations of oligonucleotide where Z = T: 8 μM (lane 4); 4 μM (lane 5); 2 μM (lane 6); 1 μM (lane 7); 500 nM (lane 8); 200 nM (lane 9); 100 nM (lane 10); 50 nM (lane 11); 20 nM (lane 12); 10 nM (lane 13); 5 nM (lane 14); 2 nM (lane 15); 1 nM (lane 16); 800 pM (lane 17); no oligonucleotide (lane 18).

G·AT by $\geq$1.6 kcal mol^{-1}. G·GC is more stable than A·GC, C·GC, and T·GC by $\geq$1.2 kcal mol^{-1}. T·CG is more stable than A·CG, C·CG, and G·CG by $\geq$1.5 kcal mol^{-1} (Figure 3). In the reciprocal sense (Figure 3), A·AT is 1.2 kcal mol^{-1} more stable than A·GC, the next most stable A·XY triplet, G·GC is 2.1 kcal mol^{-1} more stable than G·AT, and T·AT is 1.2 kcal mol^{-1} more stable than T·CG.

The intermediate stability of the T·CG and A·GC triplets can be rationalized by single specific hydrogen bonding contacts (Figure 4), whereas A·AT, T·AT, and G·GC each have two. While T offers a means of recognizing CG base pairs in this sequence context, it is not specific in that T recognizes AT base pairs more strongly than CG. This degenerate recognition by T suggests that a novel base that *specifically* recognizes CG base pairs with high affinity is needed,[2d] particularly if sequences containing more than one CG base pair are to be targeted. In such cases the use of T to recognize CG would compromise the overall specificity of the oligonucleotide.

The data in Tables 1 and 2 and Figure 3 reveal that, under these conditions and in this sequence context, there is no favorable Z·TA triplet. Thus the specific recognition of the TA base pair remains a challenge for design and synthesis.

Conclusion. These results demonstrate that the G·GC, T·AT, and A·AT base triplets are significantly more stable than the 13 other triplets studied. The T·CG and A·GC possess stabilities between that of G·GC, T·AT, and A·AT, and the worst mismatches. Importantly, the G·GC, T·AT, and A·AT interactions are specific. Oligonucleotides containing G and A or T are capable of recognizing target sites composed of Watson−Crick AT and GC base pairs in a specific manner; the energetic penalty for such an oligonucleotide binding to a site with a single mismatch is in the range of 1.2−2.1 kcal mol^{-1}. In addition, the relatively stable T·CG triplet offers a means of recognizing CG base pairs; however, because of the degenerate recognition properties of a third strand T, the overall specificity of the oligonucleotide would be compromised. We emphasize that our results are for one sequence composition and set of conditions and that the dependence of the energetics of triple helix formation on sequence composition and salt conditions remains to be elucidated.

This work represents an important first step in quantitatively characterizing the purine · purine · pyrimidine motif, which is less well studied than the pyrimidine · purine · pyrimidine motif. The values reported here will serve as a basis with which to compare and evaluate novel base designs within this structure.

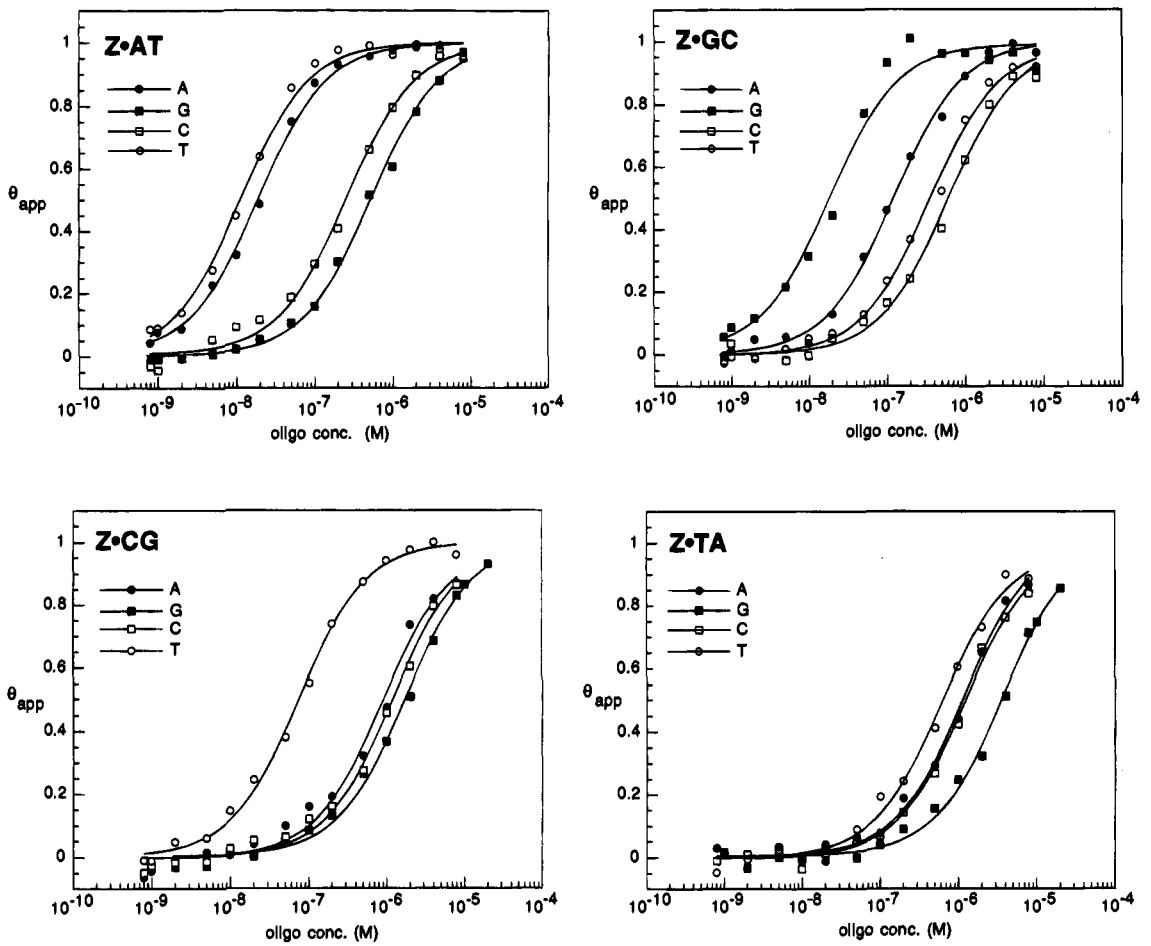

Figure 3. Binding isotherms for the 16 Z·XY triplets studied, depicted in the form Z·AT, Z·GC, Z·CG, and Z·TA. Each isotherm represents the average of three experiments conducted at 37 °C, 50 mM tris acetate, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$.

Experimental Section

General. Sonicated, phenol extracted calf thymus DNA (Pharmacia) was dissolved in H_2O to a final concentration of 1.0 mM in base pairs and was stored at 0 °C. Glycogen was obtained from Boehringer Mannheim as a 20 mg/mL aqueous solution. Deoxynucleotide triphosphates were Pharmacia Ultra-Pure grade and were used as supplied. α-^{32}P nucleotide triphosphates (3000 Ci/mmol) were purchased from Amersham. Cerenkov radioactivity was measured with a Beckman LS 2801 scintillation counter. UV−visible spectroscopy was performed on a Hewlett-Packard 8452A diode array spectrophotometer. Restriction endonucleases were purchased from Boehringer Mannheim or New England Biolabs and were used according to the supplier's recommended protocol in the buffer provided. Sequenase version 2.0 was purchased from United States Biochemical. DNAse I was purchased from Pharmacia. Phosphoramidites were purchased from Glen Research. Tris (ultrapure) was purchased from Boehringer Mannheim. All other chemicals were of reagent grade or better and were used as supplied. General manipulations of duplex DNA[27] and oligonucleotides were performed according to established procedures.

Oligodeoxyribonucleotide Synthesis. Oligodeoxyribonucleotides were synthesized by standard automated solid-support chemistry using an Applied Biosystems Model 380B DNA synthesizer and *O*-cyanoethyl-*N*,*N*-diisopropyl phosphoramidites. The final 5′-dimethoxytrityl group was removed, and deprotection was carried out in concentrated aqueous ammonia at 55 °C for 24 h. Crude oligodeoxyribonucleotide products were purified by ion exchange FPLC using a Mono Q 10/10 column (Pharmacia) and a gradient of 0−1 M LiCl in 10 mM LiOH and pH 12.0. Purified oligodeoxyribonucleotides were desalted by extensive dialysis against H_2O with a Spectra/Por MWCO 1000 membrane. The concentration of single-stranded oligodeoxyribonucleotides were determined by UV absorbance at 260 nm using extinction coefficients calculated by the nearest-neighbor method from the monomer and dimer values.[28] Oligodeoxyribonucleotides were divided into 5 nmol aliquots, lyophilized to dryness, and stored at −20 °C.

Plasmid Preparation and 3′ End Labeling. Preparation of plasmid pPBAG19, where XY = AT, is described in ref 2b. Plasmids pPB19GC, pPB19CG, pPB19TA were prepared in analogous fashion. The procedure for preparing 3′-end labeled DNA was as follows: to 10 μg plasmid was added 60 units of *Hind*III, 20 μL of 10X *Hind*III reaction buffer, and sufficient water for a total volume of 200 μL. After 2 h of incubation at 37 °C, the linearized plasmid was extracted with phenol twice and with 24:1 chloroform: isoamyl alcohol once, precipitated with 20 μL of 3 M NaOAc at pH 5.2 and 500 μL ethanol, and washed with 70% ethanol. To the linearized plasmid was added 10 μL of 5X sequenase buffer, 7 μL (70 μCi) each of α-^{32}P dATP, dCTP, dGTP, and TTP, and 2 μL of sequenase. After 30 min of reaction at room temperature, 2 μL of each cold dNTP (10 mM solution) was added. After an additional 5 min, the reaction was quenched with 50 μL of 100 mM EDTA. Unincorporated radioactivity was removed using a NICK column (Pharmacia) and the labeled, linearized plasmid was ethanol precipitated and washed with 70% ethanol. After brief drying, the pellet was digested with 30 units of *Ssp*I in a total volume of 200 μL for 2 h at 37 °C. After phenol extraction twice, chloroform extraction once, ethanol precipitation, and 70% ethanol wash, the labeled fragments were suspended in 20 μL of water and 5 μL of 15% ficoll nondenaturing loading buffer. The desired 648 bp fragment was isolated by preparative polyacrylamide gel electrophoresis (5% nondenaturing gel, 1:29 crosslinking, 160 V, 2 h) and visualized by autoradiography. The desired band was excised, crushed, and eluted overnight at 37 °C into 1 mL of 10 mM tris HCl, pH 8.0, 250 mM

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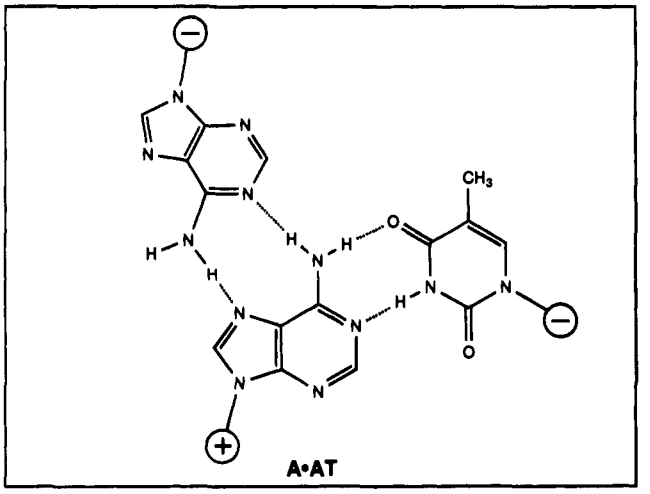

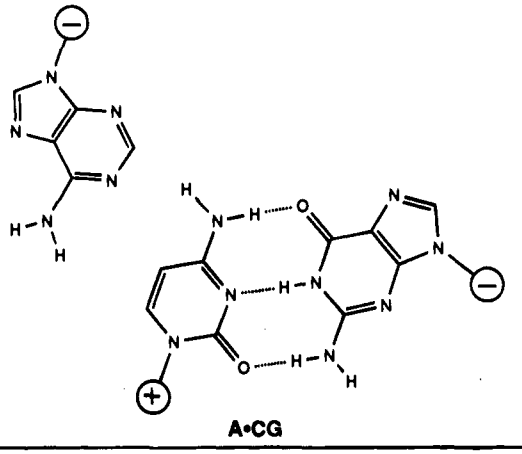

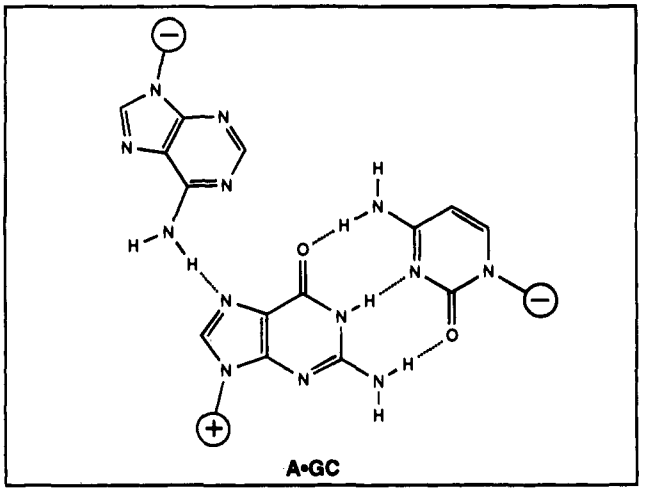

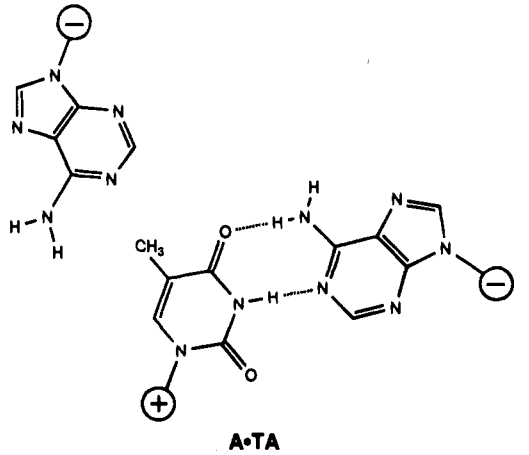

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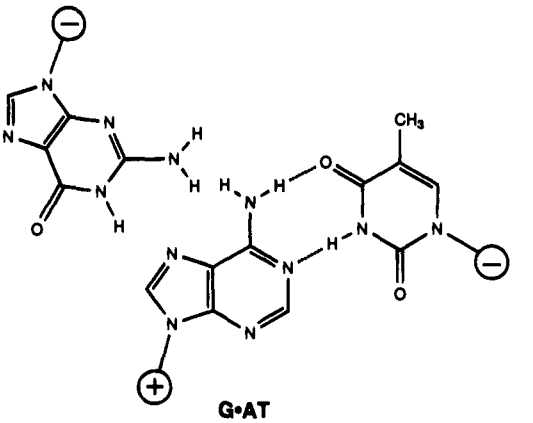

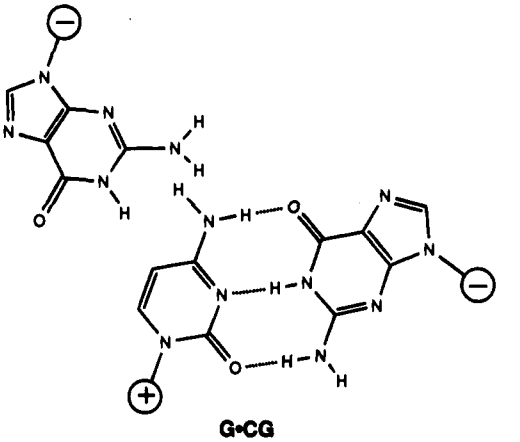

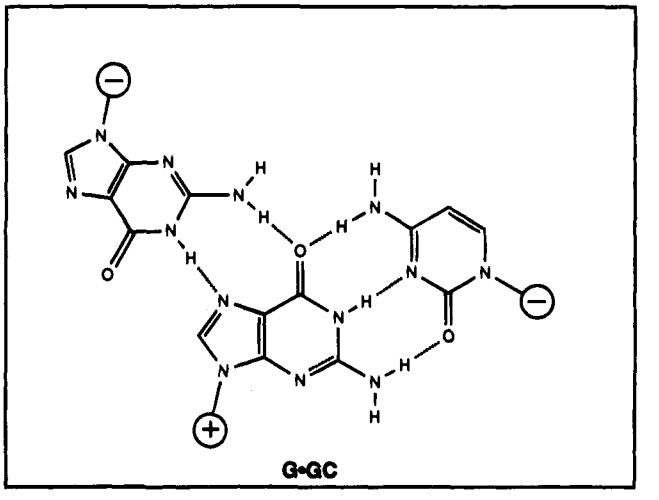

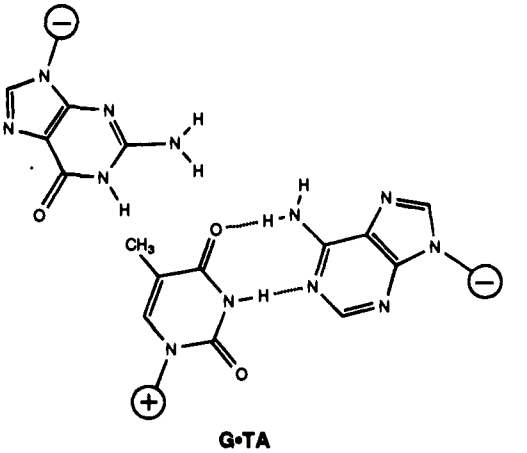

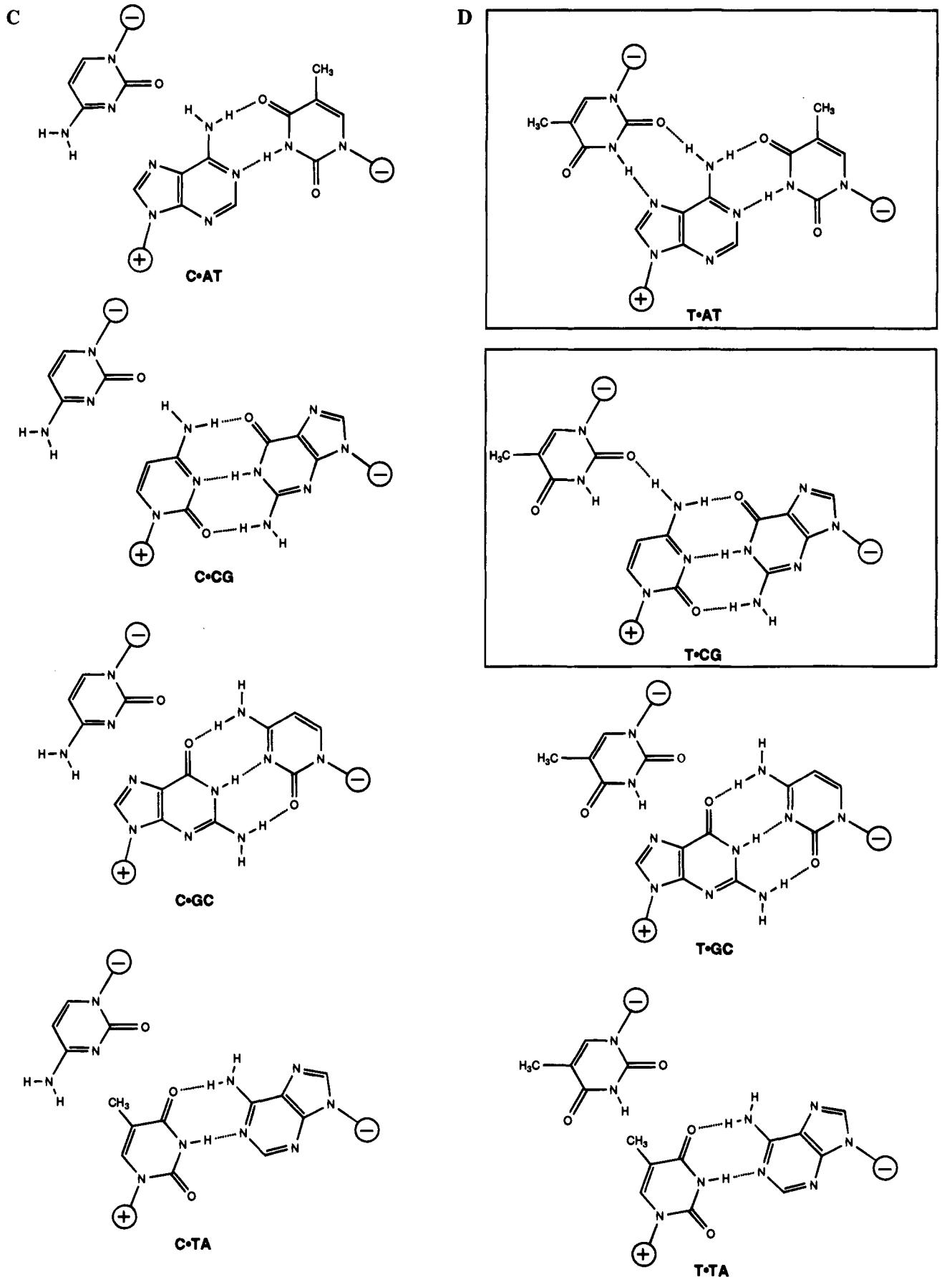

Figure 4. Putative structures of the A•XY (A), G•XY (B), C•XY (C), and T•XY (D) base triplets studied. The plus and minus signs indicate the relative strand polarity. The three strong and two intermediate interactions are shown in boxes.

NaCl, 10 mM EDTA, of 0.1% SDS. The suspension was filtered and the eluted DNA precipitated by addition of 700 μL of 2-propanol. The pellet was resuspended in 100 μL of 0.5X TE, extracted once each with phenol and chloroform, and desalted on a NICK column. The

DNA was counted and stored at −20 °C at a final concentration in water of 10 000 cpm per μL.

Quantitative DNAse I Footprint Titrations. DNAse I footprint experiments were performed essentially as described.[5,21,23] A stock solution of radiolabeled DNA in buffer was prepared from 312.5 μL of 5X association buffer (250 mM tris acetate, 50 mM NaCl, 15 mM $MgCl_2$, pH 7.40 at 37 °C), 156 μL of calf thymus DNA (1.0 mM in base pairs), approximately 250 000 cpm 3′-end labeled target DNA, and enough water to bring the total volume to 1.25 mL. The stock solution was distributed among 15 microcentrifuge tubes in 80 μL aliquots. A dried 5 nmol aliquot of oligonucleotide was dissolved in water to a concentration of 80 μM, heat denatured at 90 °C for 4 min, and serially diluted. To each reaction tube was added 10 μL of oligonucleotide at the appropriate concentration. Oligonucleotide and target DNA were allowed to equilibrate at 37 °C for 48 h. Following equilibration, DNAse I, $CaCl_2$, and a nonspecific 34-mer oligonucleotide which was used to maintain uniform DNAse I reactivity were added and the digestion allowed to proceed for 6 min. Final reaction conditions in 100 μL solution were 50 mM tris acetate at pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM $CaCl_2$, 100 μM bps calf thymus DNA, 500 nM nonspecific oligonucleotide, approximately 15 000 cpm labeled duplex, and 0.1 milliunits/μL DNAse I. Reactions were quenched by the addition of EDTA, glycogen, and NaCl to final concentrations of 25 mM, 80 μg/ mL, and 200 mM. The DNA was precipitated with 2.5 volumes of ethanol and isolated by ultracentrifugation. The precipitate was dissolved in 30 μL of water and the solution frozen and lyophilized. The DNA in each tube was resuspended in 5 μL of 80% formamide−1X TBE loading buffer and assayed for Cerenkov radioactivity by scintillation counting. The DNA was denatured at 90 °C for 4 min, loaded on an 8% denaturing polyacrylamide gel, and electrophoresed in TBE buffer at 2000 V. After loading, residual radioactivity in the microcentrifuge tubes was counted. The gel was dried on a slab dryer and exposed to a storage phosphor screen.

Quantitation and Data Analysis. After 12−24 h exposure in the dark, storage phosphor screens were scanned on a Molecular Dynamics 400S PhosphorImager. The data were analyzed by performing volume integrations of target and reference sites using ImageQuant software running on an AST Premium 386/33 computer. The analysis of DNAse I footprint titrations was performed according to the previously described method.[5,21,23] Briefly, the apparent fractional occupancy (θ_{app}) at each oligonucleotide concentration ($[O]_{tot}$) was determined using the following equation:

$$\theta_{app} = 1 - \frac{I_{site}/I_{ref}}{I_{site}^{0}/I_{ref}^{0}}$$

where I_{site} and I_{ref} are the digestion intensities in the target and reference sites, respectively, and I_{site}^{0} and I_{ref}^{0} are the digestion intensities at target and reference sites in a DNAse I control to which no oligonucleotide probe was added. The resulting pairs of (θ_{app}, $[O]_{tot}$) values were plotted on a semilog scale. The following binding isotherm was fit to the experimental data using a nonlinear least-squares algorithm in Kaleidagraph 3.0.1 running on a Macintosh IIfx or IIci:

$$\theta_{app} = \theta_{min} + (\theta_{max} - \theta_{min}) \cdot \frac{K_T[O]_{tot}}{1 + K_T[O]_{tot}}$$

where θ_{min} is the apparent fractional occupancy at the lowest oligonucleotide concentrations, θ_{max} is the apparent fractional occupancy at saturation, and K_T is the equilibrium association constant. All data points from a gel were included in the fitting procedure unless visual inspection revealed a flaw in the gel at either target or reference sites, or the θ_{app} value for a single data point was more than two standard errors away from the data points on either side. Data from experiments for which less than 80% of the lanes were usable were discarded. The goodness of fit of the binding curve to the data points was judged by the χ^2 criterion, and fits were judged acceptable for $\chi^2 \leq 1.5$. Correlation coefficients reported for acceptable fits were ≥0.95.

Repeat experiments for a particular triplet used different serial dilutions of oligonucleotide probe prepared from a different aliquot of the probe and different preparations of 3′-end labeled DNA. All K_T values reported in the text are the means of three experimental observations plus or minus the standard error of the mean.

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