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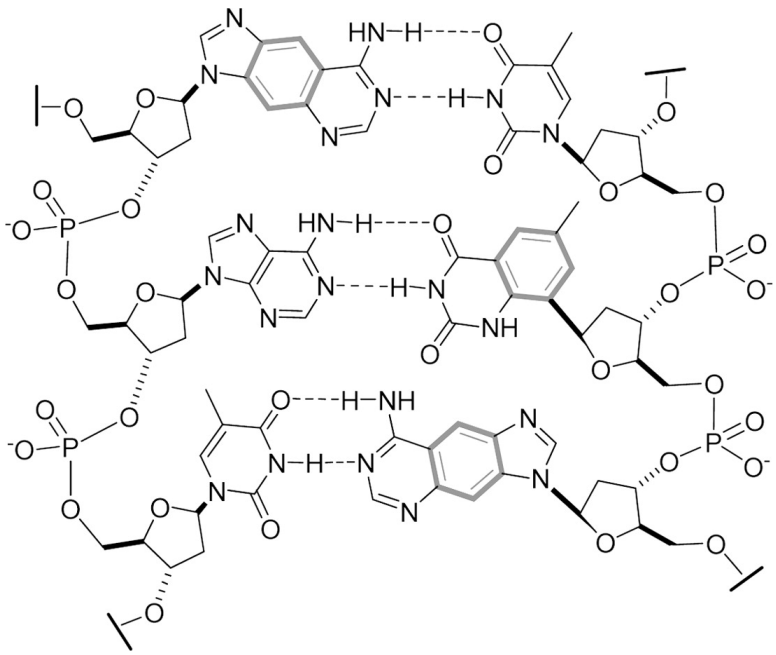

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Helix-Forming Properties of Size-Expanded DNA, an Alternative Four-Base Genetic Form

Haibo Liu, Jianmin Gao, and Eric T. Kool*

Contribution from the Department of Chemistry, Stanford University, Stanford, California 94305-5080

Received June 22, 2004; E-mail: kool@stanford.edu

Abstract: We describe the chemical and biophysical characterization of a new four-base genetic system, in which all base pairs are larger than the natural pairs. A recent preliminary study showed that three sequences containing size-expanded DNA (xDNA) bases could form stable cooperative complexes. However, many of the standard and essential properties that natural DNA possesses were unexplored for this new class of helical assembly. We therefore undertook a study of several properties of this new genetic complex: strand stoichiometry, preferred strand polarity (i.e., parallel vs antiparallel), mismatch selectivity, base size selectivity, ionic strength dependence, fluorescence behavior, CD spectra, and sequence generality. Results showed that several sequences formed double-stranded helical complexes, and interestingly, a pyrimidine-rich strand of xDNA bases was shown to form a triple helical complex as well. A test of strand polarity showed a preference for antiparallel orientation, as does natural DNA. Mismatch and size selectivity were generally moderate to strong, with one exception. Ionic strength dependence varied by relatively small degrees from that of natural DNA, although a triple helical complex of xDNA showed more marked dependence. Spectral characteristics (fluorescence, CD) were found to be quite different than those of natural DNA, apparently because of large differences in the electronic character of the expanded π-systems. Finally, several sequence contexts were found to form helices in a sequence-predictable manner. Two exceptions were noted and may be explained by competition from alternative folding structures and/or strong, single-stranded stacking. The viability of xDNA as an alternative genetic system and its possible biotechnological applications are discussed.

Introduction

A complete understanding of the chemical structure and function of our natural genetic material, DNA, will yield important implications in biology and medicine. For example, such basic knowledge is beginning to make it possible for chemists to alter its structure and properties to make useful tools for biological studies[1−5] and to apply modified DNAs to the diagnosis of disease.[6−8] Studies in this field are raising important questions in evolution as well. For example, one can ask why DNA evolved on Earth to have the structure that it does and whether other genetic forms might have evolved under different (or even the same) conditions.[9−12] One way to test this question is to make changes and ask how the chemical and biochemical functions are affected. For example, can the altered molecule self-assemble into a two-stranded complex that is stable, and is it selective for sequence? Can it encode genetic sequence information and allow it to be transferred from one strand to a new one?

The structure of DNA includes the phosphodiester backbone, which remains constant over all natural genetic material and thus acts as a *context* for storage of information, and the bases, which vary and thus encode the actual biological instructions. Studies over the past two decades have clearly shown that the backbone context of DNA can be altered in surprisingly diverse ways and still allow formation of double-stranded helices, sometimes with high thermodynamic stability.[1−12] However, only more recently have chemists begun to study whether the bases and base pairs of DNA—the information-encoding part—can be altered and still function.[3,13−16]

In this regard, we recently began a study to test whether DNA base pairs that are larger than the natural ones can function chemically and biochemically in ways similar to the function of DNA itself.[17] This was inspired by the important early work of Leonard, who made a size-expanded analogue of the base

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adenine and studied it as the ribonucleoside triphosphate derivative;[18] we adopted his design of the adenine base (abbreviated here as xA), developed a new design for expanded thymine (xT), and successfully incorporated them into DNA strands. We previously showed that size-expanded pairs including these bases can be destabilizing to the helix when forced into the smaller natural DNA context, where the backbone must adapt unfavorably to the added size.[19,20] However, when all four base pairs were replaced, preliminary studies showed that cooperative complexes of high stability could be formed.[17]

In chemical and biophysical terms, however, there are many more features to the double helix than simply the formation of a stable noncovalent complex. Among them are sequence selectivity, sequence generality, strand stoichiometry, ionic strength dependence, strand orientation, and spectral characteristics. Here we examine those features for several sequences of size-expanded DNA (xDNA) composed of four base pairs: T−xA, xA−T, xT−A, and A−xT, and we compare them to natural DNAs of analogous sequence. The results show that some features of four-base expanded DNA are not unlike those of natural DNA, for example, strand orientation, stoichiometry, sequence selectivity, and ionic strength dependence. Some are markedly different, however, and include thermal stability, which is affected by the strong stacking propensities of xDNA bases, and unusual spectral characteristics, which result from their extended π-systems. Implications of these similarities and differences are discussed.

Experimental Section

Nucleoside Phosphoramidite Derivatives of dxA and dxT. Syntheses of these two compounds were carried out as previously reported.[21]

Oligonucleotide Synthesis. Oligodeoxynucleotides were synthesized on 1.0 μM scale on an Applied Biosystems 394 DNA/RNA synthesizer using standard β-cyanoethyl phosphoramidite chemistry, but with extended (180 s) coupling time for nonnatural nucleotides. Stepwise coupling yields for nonnatural compounds were all greater than 95% as determined by trityl cation monitoring. All oligomers were deprotected in concentrated ammonium hydroxide (55 °C, 16 h), purified by preparative 20% denaturing polyacrylamide gel electrophoresis or preparative reverse-phase HPLC, and isolated by excision and extraction from the gel, followed by dialysis against water. The recovered material was subsequently quantified by absorbance at 260 nm with molar extinction coefficients determined by the nearest-neighbor method. Values for oligomers containing modified nucleotides were estimated by measuring the molar extinction coefficients of modified nucleosides at 260 nm and adding these values to the calculated values of natural DNA fragments. Intact incorporation of modified nucleotides was confirmed by characterization of short unpurified trimers (sequence TxAT and TxTT) by ^{1}H NMR. These short oligomers were also characterized by ESI-MS: calcd for TxAT + H 910.1, found 910.1; calcd for TxTT 901.2, found 901.0. Other modified oligonucleotides in the study were characterized by MALDI-TOF mass spectrometry: calcd for 5′-dxATxAxATxATTxAT 3277, found 3277; calcd for 5′-dxTxATxATxAxTTxAA+H 3327, found 3330; calcd for 5′-dxTTxAATxATxATA+H 3227, found 3228; calcd for d(xT)$_{10}$ 3482, found 3485.

Optical Melting Experiments and Preliminary Thermodynamic Measurements. Solutions for thermal denaturation studies contained either a 1:1 ratio of two complementary oligomers or only a self-complementary oligomer. Total oligomer concentrations ranged from 2.0 to 30 μM. The buffer contained NaCl (100 mM), $MgCl_2$ (10 mM), and Na·PIPES buffer (10 mM), at pH 7.0, except where noted otherwise. Melting studies were carried out in Teflon-stopped 1.0-cm path length quartz cells (under nitrogen atmosphere when temperature was below 20 °C) on a Varian Cary 1 UV−vis spectrophotometer equipped with a thermoprogrammer. Absorbance was monitored while temperature was changed at a rate of 0.5 °C/min. Melting data were essentially the same for heating or cooling at this rate. Experiments were monitored at 260 or 325 nm. In most cases the complexes displayed apparent two-state transitions, with all-or-none melting curves from bound duplex to free single strands. Computer fitting of the melting data using Meltwin 3.0b provided both melting temperatures T_m and free-energy values for the complexes. Free energies were calculated from van't Hoff plots by plotting $1/T_m$ vs ln(C/4) and from curve fitting; in most cases (except where noted) close agreement was observed, indicating that the two-state approximation is reasonable for these specific sequences.

Ionic Strength Dependence Measurements. Samples for ionic strength dependence measurement contained a 1:1 ratio of complementary oligomers at a total concentration of 10.0 μM. The buffer contained Tris·HCl (3.0 mM), EDTA (1.0 mM), and varied [NaCl] from 100 mM to 1.00 M at pH 7.0. Each sample was then measured for melting transition temperature following the same procedure as described in the previous paragraph.

Fluorescence Measurements. Samples of oligomers were measured in the same buffer as for melting experiments. UV−vis absorption spectra were recorded on a Varian Cary 1 UV−vis spectrometer. Fluorescence emission spectra were measured on a Spex Fluorolog 3 spectrometer. To prevent aggregation and reabsorption of light, samples were diluted to absorption at λ_{max} of less than 0.05. Unless otherwise noted, all samples were excited at 320 nm.

Circular Dichroism Measurements. CD spectra were measured on AVIV 62A DS circular dichroism spectrometer at 20 °C at 5.0 μM total oligomer concentration in the same buffer as used for optical melting measurements.

Job Plots. Data for Job plots were measured by reading signal output of samples of various oligomer strand ratios by either UV absorption change or native gel shift. Multiple mixed samples were prepared at varied molar ratios but keeping total oligomer concentration constant. For UV absorption measurement, each sample contained 10.0 μM total oligomer concentration in the same buffers as used for optical melting measurements. For gel shift experiments using 20% native PAGE, the total oligomer concentration was 200 μM. Line fits were tested at 1:2, 2:1, and 1:1 stoichiometries, and the best fits were plotted for the figures.

Results

Helix-Forming Tendencies. To test the generality of helix formation by strands composed of xDNA base pairs, we prepared several new sequences (Figure 1) and observed their thermal denaturation behavior in a pH = 7.0 buffer containing 10 mM Mg^{2+} and 100 mM Na^+. Melting data and preliminary thermodynamic data are listed in Table 1; the estimated free energies were derived from curve fits and from studies of concentration dependence (van't Hoff plots). Natural DNAs of the analogous sequences (**a**−**f**) were studied for comparison under the same conditions.

The four complementary cases listed in Table 1 were observed to form cooperative complexes with sigmoidal melting transitions. The shapes of the curves and the agreement between curve fits and the van't Hoff data suggest that these are reasonably described as two-state, all-or-none transitions. Interestingly, for all four sequences the xDNA duplexes are more thermally stable

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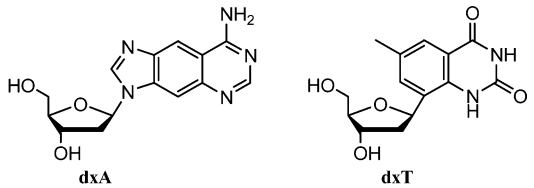
A



B

1	5'-xATxAxATxATTxAT	**a**	5'-ATAATATTAT
2	5'-xTTxAATxATxATA	**b**	5'-TTAATATATA
3	5'-xTxATxATxAxTTxAA	**c**	5'-TATATATTAA
4	5'-xAxATxATxAAxTxTA	**d**	5'-AATATAATTA
5	5'-xTAAxTTxATxATT	**e**	5'-TAATTATATT
6	5'-xTxTxTxTxTxTxTxTxT	**f**	5'-TTTTTTTTTT
7	5'-AAAAAAAAAA		
8	5'-ATxATxATxATxT		

Figure 1. (A) Structures of free nucleosides dxA and dxT. (B) Sequences of oligomers used in this study.

Table 1. Melting Temperatures of xDNA Monitored by UV and Free Energies Estimated from van't Hoff Plots

sequences	T_m (°C, 5.0 mM)	$-\Delta G_{310K}$ (kcal/mol)
1:1	54.9 ± 0.5	9.5 ± 0.2
a:a	20.1 ± 0.5	3.7 ± 0.3
2:3	35.7 ± 0.5	8.2 ± 0.2
b:c	16.3 ± 0.5	3.8 ± 0.5
4:5	36.1 ± 0.5	8.3 ± 0.1
d:e	14.9 ± 0.5	5.0 ± 0.1
6:7[a]	35.8 ± 0.5	8.1 ± 0.1
f:7	24.3 ± 0.5	5.9 ± 0.1
6:7:6	45.4 ± 0.5	9.5 ± 0.1

[a] Measured in 125 mM NaCl, 3mM Tris-HCl, 1mM EDTA, pH = 7.0.

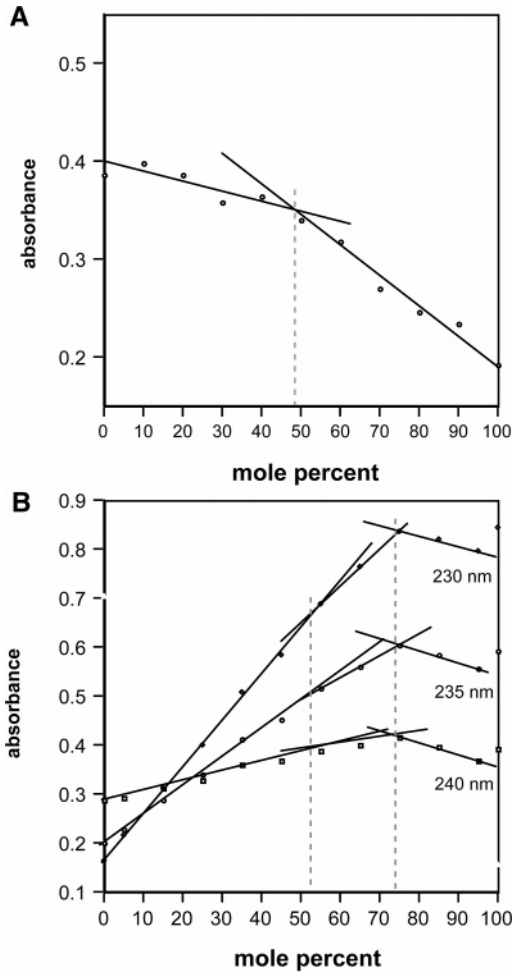


Figure 2. Job plot with mixing of complementary xDNA sequences. (A) Job plot with mixing of **4** and **5** monitored by UV absorption at 246 nm. (B) **6** and **7** showing fits that suggest two complexes (duplex and triplex) are possible. Data were analyzed at three wavelengths to increase confidence in the fits.

than their DNA counterparts by a wide margin of 12−25 °C (Table 1) and display estimated free energies that are considerably more favorable. Denaturation curves for each sequence are shown in the Supporting Information (Figure S1). In separate experiments, we confirmed that the melting transitions for these cases depended on the presence of both strands; that is, each single strand alone showed either no clear transition or gave a T_m value that was significantly lower than with both strands present.

Under varied ionic strength conditions, we observed that the all-xDNA strand $d(xT)_{10}$ and its all-DNA complement, $d(A)_{10}$, can form either 1:1 (denoted 6:7, Table 1) or 2:1 complexes (6:7:6). This latter complex, putatively a triple helix composed of xT:A:xT base triads, shows a single cooperative transition. Although natural DNA oligomers composed of $dT_n{\cdot}dA_n{\cdot}dT_n$ strands are also known to form triple helices, such triplexes are not fully cooperative, giving separate melting transitions for the third strand and the remaining duplex.[22]

Notably, two examples of self-complementary strands that were designed to form xDNA duplexes did not show cooperative two-state behavior. The sequences were $(xA)_5T_5$ and $(xT)_5A_5$; these sequences did display hyperchromicity with increasing temperature (Figure S1, Supporting Information), but did not show an obvious inflection characteristic of two-state duplex melting.

Observation of Double and Triple Helices. The above studies indirectly implicated double- and triple-stranded structures for the various xDNA complexes. Thus, we sought new data that would give more direct evidence of stoichiometry. Although self-complementary sequence **1** is unsuitable for a Job plot analysis, our previous structural study by solution-phase NMR unambiguously showed 1:1 binding for that case.[23] Determination of the binding ratio between sequences **2** and **3** proved to be difficult. The optical methods of UV, CD, and fluorescence all gave too small a signal change to be useful in determining stoichiometry by the Job method. However, we were able to obtain a qualitative result from a native gel shift experiment (Figure S2, Supporting Information), which was most consistent with 1:1 binding between sequences **2** and **3**. Sequences **4** and **5** were determined to undergo 1:1 binding with no difficulty, by measuring hyperchromicity in the UV absorption spectrum at varied molar ratios of the strands (Figure 2).

Interestingly, sequences **6** and **7**, under varying ionic strength conditions, were assigned by UV absorption experiments, along with other data, to form either duplex or triplex, depending on ratios of the two strands and salt concentration. Job plot analysis (Figure 2) shows a clear complex at roughly 74 mol % of the $d(xT)_{10}$ strand; we assign this as most probably a triplex, which

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Table 2. Melting Temperatures and Estimated Free Energies Derived from van't Hoff Plots for Hybridization Directionality Studies[a]

sequences	T_m (°C)	$-\Delta G_{310K}°$ (kcal/mol)
2:3	35.7 ± 0.5	8.2 ± 0.2
b:c	16.3± 0.5	3.8 ± 0.5
8:3	12.1 ± 0.5	6.2 ± 0.2
2[b]	26.5 ± 0.5	5.0 ± 0.5
3[b]	27.5 ± 0.5	6.9 ± 0.7

[a] Conditions: 100 mM NaCl, 10 mM $MgCl_2$, 10mM Na·PIPES, pH = 7.0. DNA concentration was 5.0 μM for shown T_m. [b] Measured from single curve fitting. Errors were estimated at ±0.5 °C in T_m, ±0.10% in ΔG.

would ideally give a value of 67%. We attribute this difference to error in strand concentration measurements for the nonnatural xDNA strand. A four-stranded complex (3:1 in the two strands) cannot be completely ruled out, but a triplex is much more likely, based on literature precedent with DNAs of analogous sequence. In addition, we assigned a low-hyperchromicity break at ca. 53% molar ratio to a duplex between these strands. Although the change in slope is subtle, it appears to be present at all three wavelengths studied. The assignment of a two-stranded complex was also supported by the van't Hoff behavior, which fit well a bimolecular model. In addition, the bimodal ionic strength dependence for **6** and **7** clearly showed (see below) that two different complexes are formed.

Strand Orientation. Although natural nucleic acid duplexes most favorably form antiparallel structure, a few sequences are capable of forming parallel duplexes as well.[24] Thus, we asked the analogous question of which hybridization directionality xDNA would prefer. The new sequence **8** was prepared as a parallel complement for sequence **3**. Our results showed, at least for this example, that the xDNA strongly preferred the antiparallel complement over the parallel one (Table 2). Although the parallel strands did give an apparently cooperative complex that was distinct from the single strands alone, the T_m value and estimated free energy were considerably less favorable (by 23 °C and 2 kcal/mol, respectively) for the parallel orientation as compared to the antiparallel.

Base Pairing Selectivity. One hallmark of natural DNA is its high base pairing selectivity. If xDNA were to be applied in hybridization, information storage, or amplification applications, then a high degree of discrimination between matched and mismatched bases would be an important feature. We therefore set out to study the selectivity of xDNA pairing for each size-expanded base and in two different sequence contexts. In one set of experiments, duplex **2**:**3** was employed as a starting point, and one base pair was replaced near the center of the 10mer sequence with various paired combinations (Table 3). High selectivity was observed; several sequences displayed no obvious sigmoidal transition above room temperature, indicating that they are quite destabilized by the mismatches (this was true also for some of the natural DNAs (see Tables 3 and 4). Our results showed that the xA base exhibited high pairing selectivity for T over other bases, and the magnitude was comparable to that observed for natural adenine in DNA.[25] However, the results for the neighboring xT base in this sequence were quite different, showing no significant selectivity regardless of partner. In a second set of experiments, we studied selectivity of xT again in the different context of duplex **6**:**7** (Table 4). In this experiment, sequence 6 had a 3′-phosphate group, which was employed to avoid having a natural nucleoside at the 3′ end. Interestingly, in this case the xT base exhibited very high selectivity (Table 4) that is much greater than reported for natural T in DNA.[25]

In a four-base genetic system composed of xA, xT, A, and T, the bases must not only show selectivity against bases that have incompatible hydrogen bonding; they must also display selectivity against size mismatches to function correctly. Examination of the data in Tables 3 and 4 shows that xA (in the **2**:**3** context) did prefer to pair with T over xT (the xA−xT pair is expected to be too large for the xDNA helix) and that T showed selective pairing with xA rather than A (presumably because the T−A pair is too short).

Ionic Strength Dependence. To a first approximation, the greater separation of strands in xDNA might be expected to lead to an altered dependence on cation screening in solution. To test this possibility, we conducted experiments to measure thermal stability changes of xDNA complexes at varied ionic strength. Interestingly, as concentration of NaCl increased from 100 mM to 1.00 M, sequences **2** and **3** exhibited similar slopes of T_m as a function of $[Na^+]$ as did control DNAs of the same sequence (Figure 3).

Interestingly, sequences **6** and **7** behaved differently under varied salt conditions, although samples were all prepared at 1:1 ratio. At relatively low NaCl concentrations, the complex, denoted **6**:**7** in Figure 2, presumably a duplex, showed low dependence on ionic strength. However, at higher NaCl concentration, an abrupt increase in slope was noted. On the basis of this and previous mixing data, this higher-slope behavior was assigned to a triplex structure, disproportionated from the original 1:1 stoichiometry.

Fluorescence and CD Spectra. Fluorescence and CD spectra were measured for the xDNA complexes (as well as for the separate strands) to characterize similarities and differences of the larger xDNA genetic form compared with DNA. We reported previously that both xT and xA bases are fluorescent, with emission maxima near 390 nm.[21] Interestingly, little fluorescence change was observed for xDNA sequences **2** and **3** on comparing the component single strands to the **2**:**3** duplex (Figure S3, Supporting Information). This may be a result of helicity in the single strands (see Discussion). The duplex emission spectrum appeared to be a simple addition of the spectra of single-stranded (ss) **2** and **3** separately. However, both ss and duplex (ds) states showed a fluorescence emission band at 510 nm, which was not observed for xT and xA mononucleosides. We also measured ss and ds spectra for sequences **4** and **5**, with similar results (Figure S4, Supporting Information). In contrast to these results, xDNA sequence **6** did show a significant change on complexation with $d(A)_{10}$, displaying a drop in emission intensity by a factor of approximately two. The quenching occurred in similar amounts in both the duplex and putative triplex contexts.

The CD spectra of the xDNAs showed marked differences from those of DNA, no doubt because of the large electronic

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Table 3. Thermal Melting Temperatures and Estimated Free Energies for Singly Mismatched xDNA Duplexes in the 2:3 Duplex Context[a,b]

5' - xT T xA A **X** xA T xA T A
3' - A xA T xT **Y** T xA T xA xT

Y	X = xA		xT		A		T	
	$-\Delta G_{310K}^{\circ}$ (kcal/mol)	T_m (°C)	$-\Delta G_{310K}^{\circ}$ (kcal/mol)	T_m (°C)	$-\Delta G_{310K}^{\circ}$ (kcal/mol)	T_m (°C)	$-\Delta G_{310K}^{\circ}$ (kcal/mol)	T_m (°C)
xT	6.1 ± 0.5	13.0	6.2 ± 0.3	18.0	6.1 ± 0.2	15.7	nt	nt
xA	5.3 ± 1.5	12.2	nt	nt	nt	nt	nt	nt
A	nt	nt	5.8± 0.2	19.4	nt	nt	nt	nt
G	5.8 ± 0.4	15.0	5.7 ± 0.1	13.9	nt	nt	nt	nt
C	5.7 ± 0.6	12.3	nt	nt	nt	nt	nt	nt
T	8.4 ± 0.3	35.6	6.1	12.8	6.0	16.9	5.6	15.2

[a] Conditions: 100 mM NaCl, 10 mM $MgCl_2$, 10 mM sodium PIPES, pH = 7.0 DNA concentration was 5.0 μM for shown T_m. Standard deviations for free engergies are shown, and errors in T_m were estimated at ±0.5 °C. [b] No cooperative melting transition was observed for cases marked with "nt".

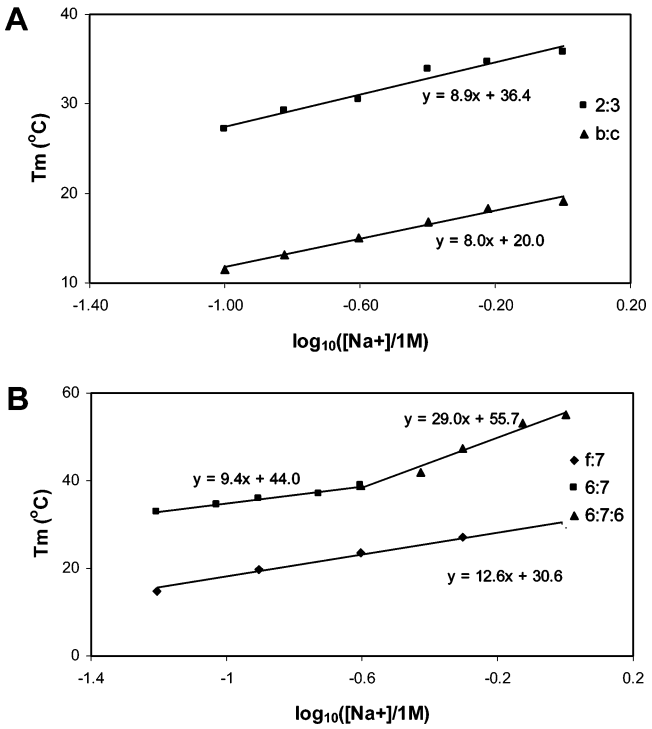

Figure 3. Ionic strength dependence studies for sequences **2** and **3** (A) and sequences **6** and **7** (B).

Table 4. Thermal Melting Temperatures and Estimated Free Energies for Singly Mismatched xDNA Duplexes in the 6:7 Duplex Context[a,b]

5' - xT xT xT xT **xT** xT xT xT xT xT
3' - A A A A **Y** A A A A A

Y	T_m(°C)	$-\Delta G_{310K}^{\circ}$ (kcal/mol)
A	38.1 ± 0.5	8.6 ± 0.9
G	9.0 ± 0.5	2.5 ± 0.3
T	17.1 ± 0.5	3.7 ± 0.4
C	18.8 ± 0.5	4.6 ± 0.5

[a] Conditions: 125mM NaCl, 3.0 mM Tris-HCl, 1.0 mM EDTA, pH = 7.0, 10.0 μM DNA. Errors were estimated at ±0.5 °C in T_m and ±10% in ΔG. [b] Low salt conditions were used to favor duplex structures.

differences between xDNA and DNA bases. For example, the **2**:**3** duplex showed a spectrum quite distinct from that of the control DNA (Figure S5, Supporting Information). Interestingly, as with the above fluorescence result, the spectra of sequences **2** and **3** showed little difference between the duplex and the single strands alone, which again may suggest a strongly organized and helical structure for the single xDNA strands (see Discussion). Sequences **4** and **5** showed some difference between ss and ds states. A weak positive band at 240 nm was observed for the ss state, but it was absent when **4** and **5** formed a duplex. However, a negative band appeared at 225 nm instead. The strand xT_{10} gave somewhat different results: the xT_{10} strand alone showed an absorption maximum at 235 nm, and the duplex gave a spectrum similar to this but quite different from the **2**:**3** complex. Finally, sequence **6** in complexes with $d(A)_{10}$ showed similar CD spectra when present in either 1:1 or 2:1 stoichiometry.

Discussion

The current data yield new insights into xDNA as a novel designed genetic form. Note that we use the term "xDNA" here to denote "size-expanded DNA"; thus, any duplex with all of its base pairs having this specific geometric expansion would be referred to as xDNA, even though all the bases on each component strand are not necessarily expanded in size. Of course, one possible configuration of xDNA is to segregate all the expanded bases onto one strand and use this to target naturally sized DNA strands (see examples in Table 1).

Overall, the data show that many of the helix-forming properties of xDNA strands are surprisingly similar to those of natural DNA. At least for the sequences tested, this large helix is most favorably antiparallel, as is natural DNA. In addition, it forms two-stranded complexes with apparently similar helicity and backbone conformation. We surmise that this similarity is partly because the modification to natural DNA's structure, a ca. 20% expansion of base pair length, is reasonably conservative. Our previous NMR studies of one xDNA duplex showed that this stretching of base pair length requires little if any change in sugar or phosphodiester conformation and that the main adaptation is a greater number of base pairs per turn, which results from a smaller base step twist in response to the larger circumference of the cylinder traced by the backbone.[23]

The high thermal stabilities of the xDNA helices apparently arise from the exceptionally strong stacking of the base on one another, which is apparently enhanced in the ds form as compared with the ss state.[19] The preliminary data herein suggest

significant increases in thermodynamic stability. This might arise in two possible ways: one of these would be strong prestacking of the ss state, which might lower the entropic cost to duplex formation. We note that helical single strands were observed long ago for poly(A),[26] and previous experiments have shown that the xA and xT bases stack considerably more strongly than A does.[19] A second influence might be stabilization by a more favorable enthalpy in the duplex form because of more extensive stacking interactions there. The van't Hoff method used here introduces significant uncertainties in the present case because it assumes that ΔC_p is zero in the ss and ds states.[27] While this is usually considered to be a reasonable assumption in polar natural DNA, this is not necessarily the case with the larger, presumably more hydrophobic, xDNA bases. Thus, we do not report entropy and enthalpy data here, and a definitive study of thermodynamic effects will await calorimetric experiments.

As for the sequence generality of xDNA double helix formation, our experiments revealed several sequences that did form well-behaved complexes, but also showed two sequences that did not. The data showed that these latter two did yield increasing absorbance with temperature, suggesting that helical-stacked complexes might be formed, but the absence of clear sigmoidal transitions suggests that either the duplexes, if formed, did not behave in a two-state fashion or that other folded structures (such as hairpins) were in competition with the duplex structures. Such behavior is not uncommon in self-complementary natural DNAs as well.

It is interesting that the ionic strength dependences of xDNA duplexes were not markedly different from those of natural DNAs. Our results showed that in one context the dependence (as measured by slope of $\log[Na^+]$ vs T_m) was slightly greater than that of DNA, while in a second context it was slightly less. At first glance, one might expect that a greater separation of polyanionic strands might lead to lower dependence of stability on concentration of cations. While the increase in interstrand separation for xDNA phosphates may be 2.4 Å within one base pair (an increase of ca. 15%), such an increase may be nonexistent at closest anion−anion approach. The closest approach of interstrand phosphates in DNA depends on the helical twist, which structural studies suggest is different for xDNA.[23] Moreover, the *intrastrand* phosphate−phosphate distance for xDNA in the helical form may well be less than that of DNA, given the lower rise per pair. In addition, some of the existing evidence suggests that xDNA single strands remain helical, which would suggest similar intrastrand phosphate−phosphate distances upon melting. Thus, change of the overall concentration of phosphate anions on denaturation may not be greatly different for duplex xDNA than for natural DNA. By contrast, it is not surprising that the current studies indicated that the triple helical form of xDNA has greater ionic strength dependence, since the anion concentration is roughly 50% higher. This greater dependence on ionic strength is also found for triple helices formed from natural DNA as well.[28]

It is also quite interesting that xDNA can, at least for some sequences, form triplex structures as well as duplexes. It has long been known that oligomers of T in DNA can form 2:1 complexes with oligomers of A by forming a duplex and then fitting a third T-rich strand into the major groove to form Hoogsteen hydrogen bonds.[22] A simple examination of models suggests no reason xDNA strands of xT could not also form such structures with strands of A-rich DNA; the hydrogen-bonding pattern is likely very similar, and the groove of xDNA is at least as wide and probably deeper than that of DNA.[23] Future studies will be needed to evaluate whether other triple helical complexes can be formed with other sequences and bases of xDNA and whether the central purine-rich strand can also be composed of xDNA. It is conceivable that one might extend the present results to design clamp-type or circular oligomers composed of xDNA that would be expected to bind a central strand composed of natural DNA (or, conceivably, RNA) with exceptional affinity.[29,30]

In two of the three contexts tested here, sequence specificity of xDNA pairing was high, with a magnitude similar to, or even greater than, that of natural DNA oligonucleotides at similar ionic strengths. For example, a previous study of the mismatches of T in short (12mer) DNA duplexes showed mismatch selectivity of 12−20 °C in T_m.[25] By comparison, mismatches of xA in xDNA result in a drop in T_m of 21−23 °C. In a second context, mismatches of xT in the $(dxT)_{10}\cdot(dA)_{10}$ context were selected against by a larger 20−37 °C. Surprisingly, however, xT in a different context was observed to give little selectivity at all. This is more surprising when one considers that this is the same context that yielded the aforementioned selectivity of xA. It is not yet clear what the origin of this low selectivity is. We hypothesize that the geometry of xA−T pairs may be slightly different from those of xT−A pairs and that the change from one to the next may require some free energy cost. We have noted in the design of these pairs that the vector of extension differs by ca. 12−16°.[21] Placing two slightly different geometries adjacent to one another could change either the energy or geometry of matched and mismatched pairs in this context. Detailed structural studies will likely be required to clarify this issue.

Taken together, the current results suggest that this new size-expanded form of DNA has many, if not all, of the properties necessary for a viable information-encoding genetic system. It encodes four bases of sequence information, and it forms antiparallel double helices of high stability and (generally) high selectivity. Clearly, the current results show that natural DNA base pairs are not unique in enabling stable helix formation and sequence-specific recognition, suggesting that the natural four-base genetic system may have evolved for reasons other than these. Future experiments in the synthetic biology of xDNA will address whether the new, nonnatural genetic system can encode yet more information than it currently does and whether it can be replicated, another requirement of a functioning genetic system.

Some of the properties of xDNA oligomers make them attractive as possible tools for detecting and analyzing RNA and DNA. First is the high affinity for complementary strands, which is useful for outcompeting and displacing folded and duplex structures in natural nucleic acids. The data show that

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segregating all expanded bases on one oligomeric strand allowed the strand to recognize a complementary strand having only natural bases. However, the current results only demonstrated this for xA and xT bases, whereas practical applications would require the development and testing of xG and xC bases as well. Moreover, we have shown strong and selective binding to DNA targets, but binding of RNA has not yet been tested. Future work will address these issues.

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Supporting Information Available: Sample melting curves, strand mixing data, and fluorescence spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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