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An Alternative Nucleobase Code: Characterization of Purine–Purine DNA Double Helices Bearing Guanine–Isoguanine and Diaminopurine 7-Deaza-Xanthine Base Pairs

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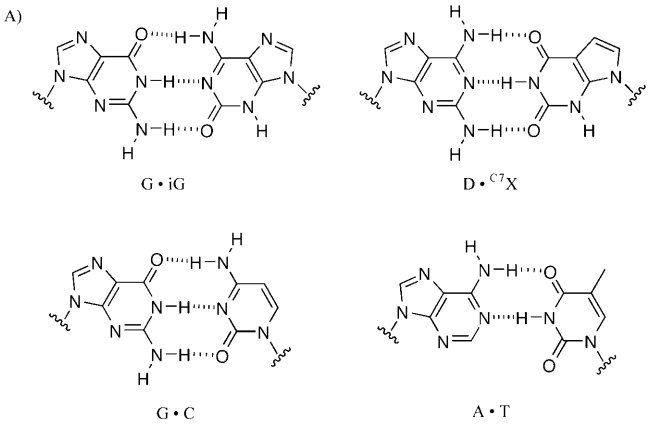
Non-natural nucleic acid frameworks have exhibited functional behavior as independent,[1] expanded,[2] and potentially prebiotic[3] genetic systems. While success has been achieved in these areas by replacing the native chemical functionality that is found in the sugar-phosphate backbone or bases with non-native groups, less-divergent structural alternatives to nucleic acids still remain to be fully explored. To date, nonstandard base pairs rely on alternate hydrogen-bonded motifs,[2] complementary hydrophobic surfaces,[4] dimensional homologues,[5] and metal coordination.[6] One minimally divergent alternative that is yet to be fully explored is an all-purine genetic system.

Crick proposed that a genetic system that incorporates only adenine and hypoxanthine might have preceded the modern genetic code.[7] In this connection, a wide array of helical structures have been reported for all-purine nucleic acids, including those that contain three,[8] four,[9] and five strands.[10] Double-stranded purine–purine structures are known in the context of both nonstandard[1a,11] and natural[12,13,14] nucleic acids; however, only two reports describe all-purine duplexes of DNA that display Watson–Crick[14] or reverse Watson–Crick[11] pairing for association. Whereas model systems have so far failed to demonstrate nonenzymatic oligomerization by purine–purine pairs,[15] insufficient information exists to date concerning the fitness of an all-purine double helix to assess its suitability as a precursor or independent genetic material. In an extension of our earlier work on iG self-pairing,[9c,10] we report the characterization of the G·iG and D·^{C7}X base-pairing system in DNA (Scheme 1).[16]

Several considerations guided purine nucleobase selection. To minimize potential mispairs (e.g., A·G[1a,17] or H·iG[1a]), diaminopurine and xanthine motifs were utilized in place of adenine and hypoxanthine. Further, 7-deazaxanthine was deemed a more-suitable complement to diaminopurine than xanthine because the lack of a 7-nitrogen atom leads to reduced susceptibility to depurination and an increased pK_a.[18] Additionally, the nitrogen substitution in 7-deazaxanthine removes a Hoogsteen-face hydrogen-bond acceptor, thereby inhibiting alternative modes of strand association.[8–10] Isoguanine, via its N3-H tautomer, was chosen to complement guanine in analogy with homo-DNA pairing.[1a,b] It is notable that both D·^{C7}X and iG·G base pairs benefit from the potential to form three hydrogen bonds via their Watson–Crick faces.



B)
1: 5'-d^{C7}XGG^{C7}XG^{C7}X^{C7}XG-3'
2: 5'-diGDDiGDiGiGD-3'
3: 5'-dTGGTGTTG-3'
4: 5'-dCAACACCA-3'
5: 5'-dGDDGDGGD-3'
6: 5'-d^{C7}XiGiG^{C7}XiG^{C7}X^{C7}XiG-3'
7: 5'-dGAAGAGGA-3'
8: 5'-dTCCTCTTC-3'
9: 5'-diGG^{C7}XGDiGD^{C7}X-3'
10: 5'-dD^{C7}XG^{C7}XiGDiGG-3'
11: 5'-dCGTGACAT-3'
12: 5'-dATGTCACG-3'
13: 5'-diG^{C7}X^{C7}GiGDiG^{C7}X^{C7}G^{C7}XD^{C7}XG-3'
14: 5'-diGD^{C7}XDiGD^{C7}G^{C7}X^{C7}GiGDG-3'
15: 5'-diGA^{C7}XAiGA^{C7}G^{C7}X^{C7}GiGAG-3'
16: 5'-dCTGCACTGTATG-3'
17: 5'-dCATACAGTGCAG-3'
18: 5'-diGD^{C7}G^{C7}XD^{C7}XiGG-3'
19: 5'-diG^{C7}GD^{C7}XDiG^{C7}XG-3'
20: 5'-dCAGTATCG-3'
21: 5'-dCGATACTG-3'

Scheme 1. A) Purine–purine and purine–pyrimidine base-pair structures; B) oligonucleotide sequences.

The oligonucleotides displayed in Scheme 1 were synthesized from commercially available phosphoramidites on an Expedite 8909 DNA synthesizer. To assess the association of complementary strands, UV-monitored thermal denaturation experiments were performed on each of two strands individually, and then combined. Because the optimal wavelength at which to observe hyperchromicity is expected to be unique for a given nucleic acid complex, multiple wavelengths were monitored simultaneously during initial experiments. A clear melting transition was observed with maximal hyperchromicity at 250 nm for **1·2** (Figure 1 A). It can also be seen from Figure 1 that profiles for **1** and **2** alone do not sum to the denaturation profile of **1** and **2** combined. These results are consistent with duplex formation between **1** and **2**. Similarly, clear melting transitions are seen for **5·6** and **9·10** (Figure 1 C, E). Thermodynamic values for strand association were obtained by nonlinear regression of the melting curve traces[19] and a van't Hoff plot[20] (Table 1).

Whereas clear evidence is seen for association between oligomers **1** and **2**, thermal denaturation of the individual strands revealed that oligomer **2** self associates in the absence of its

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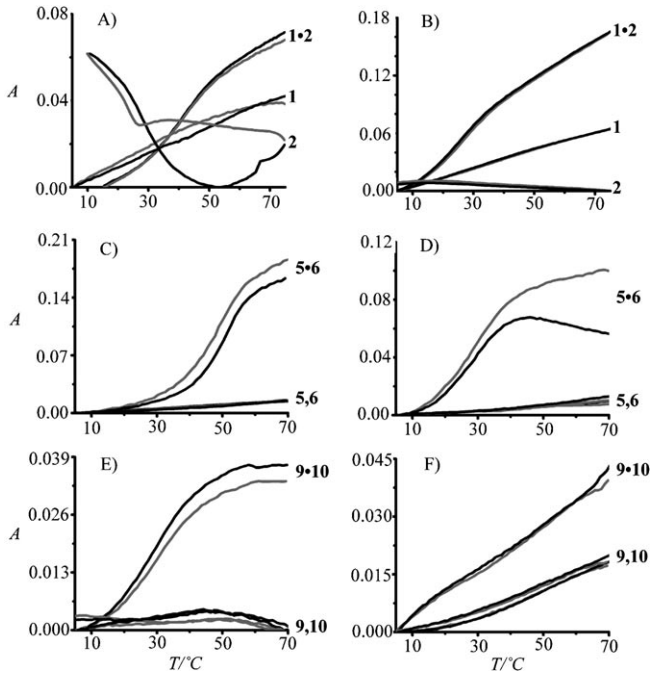


Figure 1. UV_{250} denaturation profiles: A) 1.0 M NaCl, 10 mM NaH_2PO_4, pH 7.0, 35 μM DNA; B) 10 mM NaH_2PO_4, pH 7.0, 25 μM DNA; C) 1.0 M NaCl, 10 mM NaH_2PO_4, pH 7.0, 25 μM DNA; D) 10 mM NaH_2PO_4, pH 7.0, 25 μM DNA; E) 1.0 M NaCl, 10 mM NaH_2PO_4, pH 7.0, 2.5 μM DNA; F) 10 mM NaH_2PO_4, pH 7.0, 2.5 μM DNA. Both heating and cooling curves are shown in all cases.

Table 1. Thermodynamic data for duplexes.

	Duplex [μM]	NaCl[a] [M]	$-\Delta H^\circ$ [kcal mol^{-1}]	$-\Delta S^\circ$ [cal mol^{-1}]	$-\Delta G^\circ_{37}$ [kcal mol^{-1}]	T_m [°C]
1·2	[b]	1.0	41.0	109	7.26	[b]
1·2	35	1.0	41.4	112	6.82	37.0
1·2	25	1.0	47.3	131	6.67	35.2
1·2	25	0	39.7	112	4.89	21.6
3·4	25	1.0	48.6	132	7.71	42.4
3·4	25	0	44.2	127	4.71	21.8
5·6	25	1.0	69.6	194	9.45	48.4
5·6	25	0	37.5	103	5.63	26.4
6·7	25	1.0	42.7	115	7.08	37.9
7·8	25	1.0	54.0	154	6.25	33.4
7·8	25	0	40.1	118	3.41	11.3
9·10	2.5	1.0	67.8	197	6.72	29.6
11·12	2.5	1.0	55.0	151	8.21	36.1
13·14	2.5	1.0	90.7	245	14.7	60.3
13·15	2.5	1.0	66.4	182	9.95	44.5
16·17	2.5	1.0	99.0	275	13.9	55.3
18·19	2.5	1.0	39.8	103	7.81	32.6
20·21	2.5	1.0	49.7	137	7.08	29.1

[a] All samples contained 10 mM NaH_2PO_4, pH 7.0, with DNA duplex and NaCl concentration as indicated. [b] van't Hoff analysis.

complement, oligomer **1**. The behavior of oligomer **2** was complex; it possesses both hypochromism with increasing temperature and hysteresis upon comparison of heating and cooling profiles (Figure 1A). The latter is indicative of a system that is out of equilibrium. Oligomer **2** contains isoguanine, which readily forms both quadruplexes[9b,c] and pentaplexes[10] in the presence of alkali metal cations. Interestingly, none of the remaining all-purine single strands in Figure 1A, C or E showed any tendency to self-associate, despite strand **6**, which contains four iG residues.

Thermal denaturation experiments were also performed in solutions of reduced ionic strength. In general, lowering the ionic strength was expected to disfavor any competitive higher-order association. In particular, removing excess Na^+ from the buffer was expected to eliminate self-association in the case of **2**. Figure 1B displays denaturation profiles for **1** and **2** under these conditions. It can be seen that reducing the Na^+ concentration avoided self-pairing by **2**, while at the same time it promoted the association of **1** and **2**. The T_m of **1·2** under low-salt conditions was 21.6 °C, which was effectively indistinguishable from the 21.8 °C T_m for the natural duplex analogue **3·4**.

To gain further information about purine–purine double-helix structure and to explore any differences under typical versus low-salt conditions, circular dichroism spectra were obtained. Figure 2 displays circular dichroism spectra in the presence and absence of 1.0 M NaCl. As anticipated, **2** gave a characteristic band at 314 nm that was representative of a high-order (quadruplex/pentaplex) structure in the presence of NaCl (Figure 2A).[21] This band diminished when **1** and **2** were combined. Circular dichroism spectra taken in 10 mM NaH_2PO_4 buffer in the absence of NaCl (Figure 2B) support a unique secondary structure for **1·2** due to spectral differences compared

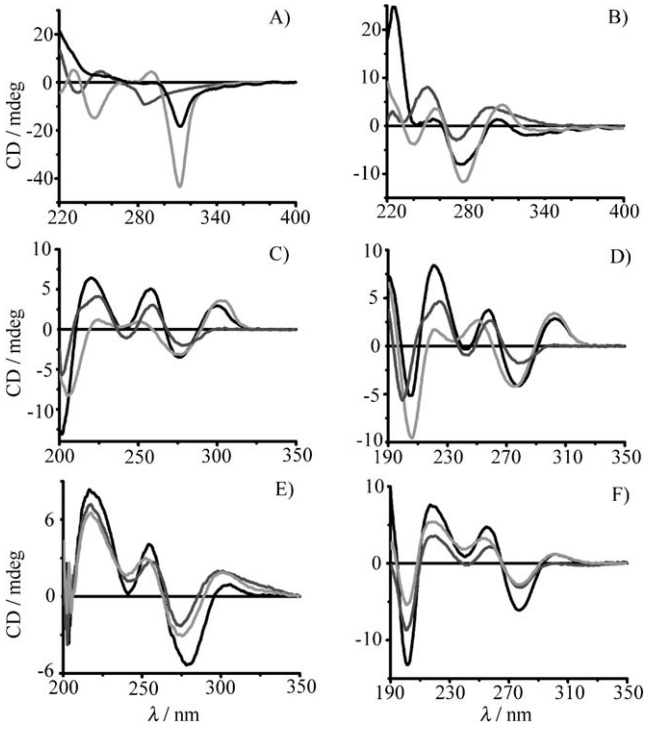


Figure 2. Circular dichroism spectra. A) 1.0 M NaCl, 10 mM NaH_2PO_4, pH 7.0, 25 μM **1**, **2**; B) 10 mM NaH_2PO_4, pH 7.0, 25 μM **1**, **2**; C) 1.0 M NaCl, 10 mM NaH_2PO_4, pH 7.0, 25 μM **5**, **6**; D) 10 mM NaH_2PO_4, pH 7.0, 25 μM **5**, **6**; E) 1.0 M NaCl, 10 mM NaH_2PO_4, pH 7.0, 2.5 μM **9**, **10**; F) 10 mM NaH_2PO_4, pH 7.0 2.5 μM **9**, **10**. Compounds **1**, **5**, **9**: medium gray; **2**, **6**, **10**: light gray; **1·2**, **5·6**, and **9·10** black.

to **1** and **2** alone. No distinguishable CD band at 314 nm was observed for **2** under these low-salt conditions. The remaining CD spectra support complementary strand association in the cases of Figure 2C–E, and no (or weak) association in the case of Figure 2F, which is consistent with the results of UV-monitored thermal denaturation. Higher oligonucleotide concentrations were used for initial experiments (shown in Figures 1 and 2, panels A–D) because these conditions would favor strand association, and the behavior of the system was unknown. With the association of **1·2** and **5·6** established, more typical strand concentrations were subsequently used (Figures 1 and 2, panels E, F; also vide infra).

Beyond the possible formation of four or five-stranded structures such as exhibited by **2** under high ionic strength conditions, there are also a plethora of possible triple-stranded structures available to oligomers **1**, **2**, **5**, **6**, **9**, and **10** given the propensity of purines to simultaneously use their Watson–Crick and Hoogsteen faces to bond.[8,18,22] Nevertheless, any tendency to form higher-order structures is balanced by D·^{C7}X and iG·G base pairs that bear Watson–Crick motifs with three hydrogen bonds, a feature that should help prevent disproportionation[8b] of a double helix to a triple helix plus a single strand. A further limitation on higher-order-structure formation derives from ^{C7}X, which lacks a standard Hoogsteen-pairing face; this base occurs in one strand of the **1·2** and **5·6** duplexes, and both strands of the **9·10** duplex.

To further assess the stoichiometry of strand association, two duplexes were prepared, **13·14** and **18·19**, which incorporate two 7-deaza bases, ^{C7}X and ^{C7}G. The introduction of both ^{C7}G and ^{C7}X leads to the disruption of 50% of all Hoogsteen-pairing faces (omitting consideration of the first or last nucleotide of the strands), and has unavoidable consequences for the stability of a hypothetical triplex or higher-order structure that is formed by formal disproportion of a duplex. UV-monitored thermal denaturation of **13·14**, **18·19**, and their component single strands (Figure 3A and 3E) supports robust double-helix formation in each case.

The importance of the Watson–Crick faces in D and ^{C7}X for base pairing was addressed by replacing D with A in strands **5** and **14**, and then comparing the stability of duplexes with and without the 2-amino group. The difference in T_m and free energy values were found to be 10.5 °C/2.37 kcal mol^{-1} for **5·6** versus **6·7** and 15.8 °C/4.75 kcal mol^{-1} for **13·14** versus **13·15**, in favor of greater stability for D·^{C7}X over A·^{C7}X in both cases. Given six D·^{C7}X pairs in **13·14** and three internal D·^{C7}X pairs in **5·6**, there exists a $\Delta G^{\circ}_{37} = 0.79$ kcal mol^{-1} average stabilization per 2-amino substitution. The greater stability of D·^{C7}X over A·^{C7}X is consistent with Watson–Crick pairing. By extension, Watson–Crick pairing is expected to pertain for iG·G. Whereas X and ^{C7}X pairing opposite A in an otherwise standard purine–pyrimidine double helix has been investigated,[23] to our knowledge the D·(^{C7})X interaction has not been similarly explored. Previous work has reported that D·T or D·U base pairs contribute more to helix stabilization than A·T or A·U base pairs,[24] which is consistent with our findings.

The five duplexes incorporating D·^{C7}X and iG·G base pairs, namely, **1·2**, **5·6**, **9·10**, **13·14** and **18·19**, might be compared in

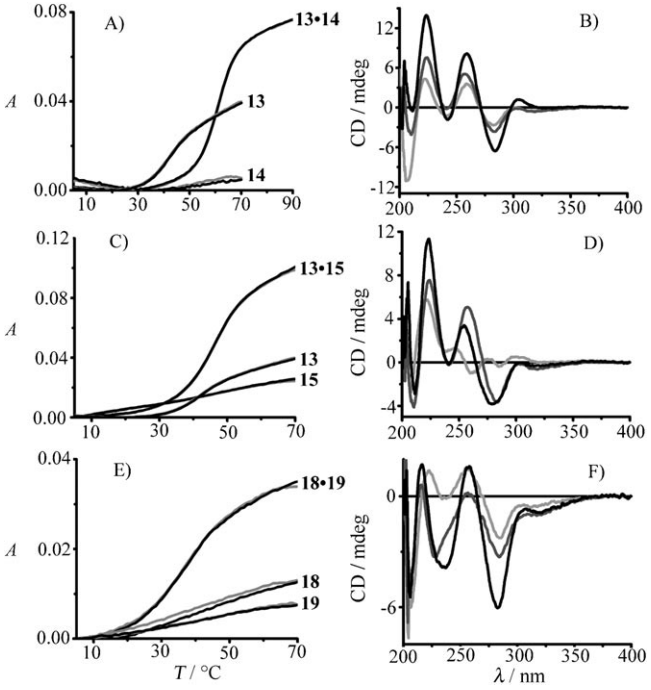
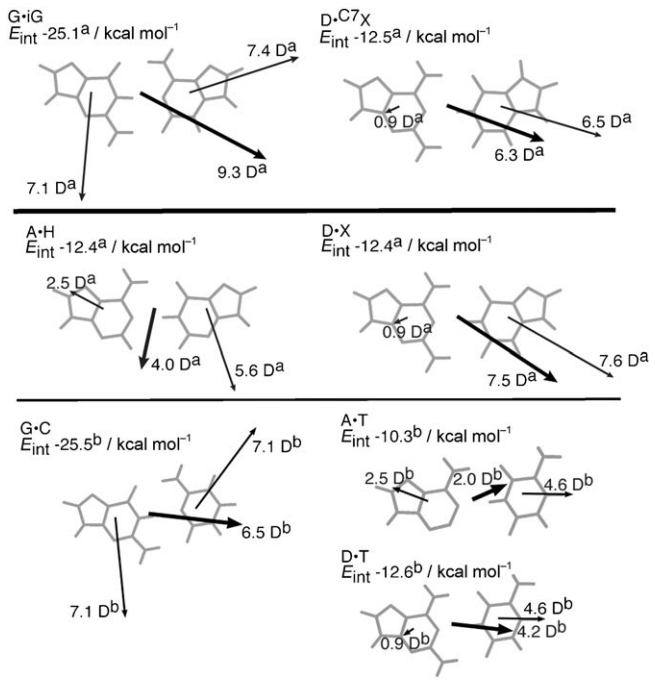


Figure 3. UV_{250} denaturation profiles A), C), E) and the corresponding circular dichroism spectra B), D), F) in 1.0 M NaCl, 10 mM NaH_2PO_4, pH 7.0. Duplex and single-strand concentrations were 2.5 μM. A), C), E) Heating and cooling curves shown in all cases; B), D), F) **13**, **19** medium gray; **14**, **15**, **18** light gray, **13·14**, **13·15**, **18·19**: black.

stability to their natural DNA counterparts by using the data given in Table 1. In the presence of 1.0 M NaCl, which is commonly used for thermodynamic measurements,[19,20] the relative changes in free energy of duplex formation ($-\Delta G^{\circ}_{37}$) are, respectively, −13, +51, −18, +6, and +10%. Thus, three of the five purine–purine duplexes are more stable than comparable duplexes of natural DNA. Also of interest is a comparison of the stability of the two duplexes bearing A·^{C7}X in place of D·^{C7}X to natural DNA. In these cases, the number of donor/acceptor groups involved in base-pairing map between the nonstandard and standard duplex (i.e., G·iG/G·C = 3 H-bonds and A·^{C7}X/A·T = 2 H-bonds). Here the relative stabilities are +13% and −28% for **6·7** and **13·15**, respectively.

To gain a better understanding of the forces that govern the stability of purine–purine duplexes, ab initio computations were performed on the base pairs that are shown in Scheme 1 and two additional base pairs: D·X and A·H. The results are summarized in Scheme 2. A striking trend is that the interaction energies fall in two categories independent of their purine–purine or purine–pyrimidine composition. Thus, base pairs with an A·D·D (acceptor–donor–donor) pattern (G·iG, G·C) exhibit an $E_{int} \approx 25$ kcal, and base pairs with a D·A(·D) pattern have E_{int} values of approximately half of the A·D·D value, or 10–12 kcal. Significantly, the two purine–purine base pairs that were in the present study, G·iG and D·^{C7}X, have nearly identical E_{int} values in comparison to their purine–pyrimidine analogues G·C and D·T, respectively. A similar comparison of the dipole moments for the latter base pairs reveals that they also share nearly identical orientations, although the magnitude of mo-



Scheme 2. Summary of HF/6-31G**//HF/6-31G** dipole moments and interaction energies. Bold arrows correspond to dipole moments for base pairs, whereas nonbold arrows correspond to dipole moments for the isolated bases. a) This work; b) ref. [38].

ments for the purine–purine pairs is approximately 50% greater than that of their purine–pyrimidine counterparts. The similarity in dipole moment orientation also extends to D•X (the parent of D•^{C7}X) in comparison to D•T, but does not extend to A•H when compared to its counterpart, A•T. The close parallel in interaction energies and electronic properties calculated for D•^{C7}X and D•X provides a basis for anticipating similar thermodynamic stabilities for a duplex incorporating G•iG/D•X, beyond the presently studied G•iG/D•^{C7}X; this analysis omits consideration of the difference in pKa between ^{C7}X and X.

The purine–purine DNA duplexes characterized herein have thermal stabilities and free energies that, on average, parallel the analogous purine–pyrimidine DNA duplexes. Recently, Battersby et al.[14] described a mixed sequence all-purine DNA duplex with A•H pairs in contrast to the D•^{C7}X pairs used here. Their purine duplex displayed diminished stability ($T_m = -15$–$20\,^{\circ}C$; $\Delta G^{\circ} = -40\%$) relative to the analogous natural DNA duplex. While the sequences used in the two studies were different, D•^{C7}X appears to contribute considerable stability to a double helix over A•H. Interestingly, the two reverse Watson–Crick (parallel-stranded) purine duplexes reported by Seela[11] with guanine–5-aza-7-deazaguanine base pairs appear to have higher stability than that which may be predicted[25] for the analogous natural (antiparallel stranded) DNA duplexes; this is consistent with the results from three of the purine duplexes studied here.

Our findings indicate that a four-letter purine–purine code for DNA might be viable for information storage. Whereas the G•iG/D•^{C7}X DNA system studied does not exhibit stronger base pairing than G•C/A•T DNA in all cases studied, and, therefore, does not necessarily undergo sequence-independent, nonenzymatic replication,[26] a purine-only genetic code notably simplifies prebiotic synthesis by requiring only one type of heterocycle. Purines have proven to be more readily accessed than pyrimidines in prebiotic model reactions;[27] benefiting from this phenomenon in the present context requires successful substitution of ^{C7}X by its parent, X, which the current study only addresses computationally.

Relationships between purine–purine and purine–pyrimidine coding systems might impact molecular evolution as originally proposed by Crick.[7] Thus, a two-letter genetic system that is comprised of A•H might evolve to the current four-letter code of A•T(U)/G•C via the intermediacy of A•T(U)/H•C, given seamless hydrogen-bond and size complementarity of the bases. A similar transition can be imagined by beginning from G•iG/D•X; however, this four-letter purine–purine system might be constrained in its evolution to the current four-letter code by a requirement for segregation of G/D and iG/X in separate strands if the conservation of base-pair dimensions in a helix is required for fitness. An N-7-G•iG/D•X origin of the genetic code is reminiscent of the proposed replacement of C and U with N-3-iG and N-3-X.[28] Given that pyrimidine complements are known for iG and X in addition to G and D, the evolution of this four-letter all-purine system to an eight-letter purine–pyrimidine system (comprised of G•C, D•T(U), iG•iC[29] and X•K[30]) is a formal possibility. The four-letter purine–purine-pairing system investigated here is directly, or indirectly, relevant to terrean, exo,[31] and synthetic[32] biology. Independent of whether a purine–purine genetic system could support life, our findings inform the function/fitness landscape of (secondary) structures that are related to natural DNA and RNA.[1c,14,29,33,34] While iG presents challenges as a component of a genetic code due to its tendency to populate more than one tautomeric form,[1a,35] a recent demonstration of faithful copying[36] and replication[37] suggests that they are surmountable.

Experimental Section

Oligonucleotide synthesis: Oligonucleotides **1**--**21** were synthesized from commercially available reagents and phosphoramidites (Glen Research, Virginia, USA) on an Expedite 8909 DNA synthesizer by using the standard (1 μmol) DNA synthesis protocol and the manufacturer's recommendations. A 16-minute coupling time and two additional phosphoramidite reagent pulse cycles were used with unnatural phosphoramidites. Stepwise coupling yields were 98–99%. Cleavage from the universal CPG support and deprotection of the oligomers followed the manufacturer's guidelines for unnatural nucleosides, and in most cases was achieved by heating in concentrated NH_4OH at 55 °C for 16 h. The crude oligonucleotides were purified by 20% (29:1) denaturing PAGE, and UV_{260} quantified. dG $\varepsilon_{260} = 12010\,M^{-1}cm^{-1}$, diG $\varepsilon_{260} = 5500\,M^{-1}cm^{-1}$, dD$\varepsilon_{260} = 8500\,M^{-1}cm^{-1}$, d^{C7}X $\varepsilon_{260} = 8440\,M^{-1}cm^{-1}$. Buffer 1: 1.0 M NaCl and 10 mM NaH_2PO_4, pH 7.0. Buffer 2: 10 mM NaH_2PO_4, pH 7.0.

Thermal denaturation: Experiments were performed on a Varian Cary 500 UV-Vis spectrophotometer. A_{250} was recorded at 1 °C inter-

vals while the temperature continuously ramped from 75→5→75 °C at a rate of 1 °C min^{-1}.

Circular dichroism spectra: Spectra were collected on a JASCO J-815 CD spectrometer at 20 °C, and corrected for buffer.

Abbreviations: ^{C7}G, 7-deaza-guanine; ^{C7}X, 7-deaza-xanthine; CD, circular dichroism; CPG, controlled pore glass; D, diaminopurine; H, hypoxanthine; iG, isoguanine; X, xanthine.

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Keywords: artificial nucleic acids · DNA structures · isoguanine · purines

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