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Refined crystal structure of an octanucleotide duplex with I.T. mismatched base pairs

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ABSTRACT

The structure of the synthetic deoxyoctamer d(GGIGCTCC) has been determined by single crystal X-ray diffraction techniques to a resolution of 1.7Å. The sequence crystallises in space group $P6_1$, with unit cell dimensions a = b = 45.07, c = 45.49Å. The refinement converged with a crystallographic residual R = 0.14 and the location of 81 solvent molecules. The octamer forms an A-DNA duplex with 6 Watson-Crick (G.C) base pairs and 2 inosine-thymine (I.T) pairs. Refinement of the structure shows it to be essentially isomorphous with that reported for d(GGGGCTCC) with the mispairs adopting a "wobble" conformation.

Conformational parameters and base stacking interactions are compared to those for the native duplex d(GGGGCCCC) and other similar sequences. A rationale for the apparent increased crystal packing efficiency and lattice stability of the I.T octamer is given.

INTRODUCTION

Deoxyinosine is effective as a universal base in synthetic hybridisation probes when cloning genes for proteins containing amino acids with degenerate codons (1). The use of inosine containing deoxyoligonucleotides for this purpose is now widespread. Inosine, despite the absence of the 2-amino group, is very similar to guanine and although it does not occur naturally in DNA, it is a very useful structural probe. It is reputed to pair with cytosine, adenine or thymine without significantly destabilising the double helix, even when several inosine containing base pairs occur in a short stretch of DNA. This implies that inosine forms stable mismatches, a feature that is not surprising when one considers the occurrence of inosine at the 5'-hydroxyl end, the wobble position, of some t-RNA anticodons where it is known to form hydrogen bonds with A, C or U at the m-RNA codon (2). Inosine is also able to occupy the

middle position of the anticodon and pair with A (3). The basis of the extra stability of inosine containing mismatches is not known so we undertook to examine a variety of these mismatches by X-ray crystallographic methods. We have previously reported the structure of the self-complementary B-DNA dodecamer d(CGCIAATTAGCG) which contains two I.A base pairs (4) and we now report the structure of d(GGIGCTCC), a self complementary A-DNA octamer containing two I.T base pairs.

EXPERIMENTAL

Synthesis, Crystallisation and Data Collection

The deoxyoctamer d(GGIGCTCC) was synthesised by the solid phase triester method (5) and purified by ion-exchange and reverse-phase HPLC. Crystals were grown in drops containing 5 μl of a 20 mg/ml solution of the octamer in sodium cacodylate, 50 mM, pH 6.5, 2 μl of 100 mM $MgCl_2$ and varying amounts of water. Initially, all wells yielded rhombohedral crystals which diffracted only to about 3.5Å. Analysis of these crystals showed them to have cell dimensions a = b = 41.2, c = 85.1Å. space group $P3_12_1$ or $P3_22_1$.

However these rhombohedra eventually redissolved and hexagonal needles formed. Precession photography showed these to diffract to much higher resolution. The intensities were measured on two crystals, one of dimensions 0.6 mm x 0.1 mm x 0.05 mm, which gave data to 2.25Å and one of size 1.0 mm x 0.15 mm x 0.15 mm, giving data to 1.66Å.

The crystals were mounted in glass capillaries and data was collected at room temperature on a SIEMENS AED2 4-circle diffractometer equipped with CuK_α radiation, a graphite monochromator, a long arm and a helium path. The reflections were measured using ω-scans with a scan width of 1° and scan speeds ranging from 2°/min to 0.17°/min. For the smaller crystal (d_o = 2.25Å) a total of 3437 reflections were collected, while for the larger crystal (d_o = 1.66Å) the total was 5795. In both cases three standard reflections were measured at two- hourly intervals to monitor crystal decay. All data were corrected for decomposition, Lorentz and polarisation effects and an empirical absorption correction was applied (6).

The two data sets were merged (first omitting all those reflections with $F < 1.0\sigma(F)$) to give a total of 3310 unique reflections (R_{merge} = 0.04) with $F \geq \sigma(F)$ representing 57% of the total number of observable reflections.

Structure Refinement

It was assumed that the crystal structure of this sequence would be quasi-isomorphous with that of d(GGGGCCCC) and so refined coordinates of that structure, based on synchrotron data (7), were used as a starting model. This model was refined against the experimental data as a rigid body using a modified version of SHELX (8). Initial refinement was carried out with data in the region 10-6Å. The resolution was gradually increased to include the data to 3Å and the refinement converged at R = 0.30. In these calculations the bases in the same positions as the mismatches were given low occupancy so that they would not contribute significantly to the structure factor calculations.

The refinement was then continued with the restrained least-squares method of Konnert and Hendrickson (9) using NUCLSQ (10), a program modified for DNA. In this part of the refinement the low occupancy of the base-pairs $C_3.C_{14}$ and $C_6.G_{11}$ was maintained and initial refinement was with data to 2.25A with $F \geq 2.0\sigma(F)$. From an initial R of 0.35 the refinement converged after four cycles, to R = 0.31.

Difference Fourier and $2F_O-F_C$ maps were generated and examined on an Evans and Sutherland PS300 graphics system using FRODO (11). The maps indicated a "wobble" type conformation for both mismatches and idealised I.T mispairs were fitted to map positions 3.14 and 6.11 of the model. (The nucleotides are numbered G1 to C8 in strand 1 and G9-C16 on strand 2 (5'-3' direction)).

Subsequent refinement was carried out using reflections to 1.7°A (2853 with $F \geq 2.0\sigma(F)$ and $d_o \geq 10$Å) by restrained least-squares calculations and manipulation of atomic positions on the graphics system. To avoid introducing bias into the conformations of the furanose rings, no torsional restraints were employed during the refinement.

Solvent molecules were located at different stages of the refinement from difference maps. Peaks with $\rho > 0.25 e/Å^3$ were

inspected on the graphics and accepted or rejected on the basis of a combination of good (spherical) density and plausible hydrogen bonding geometry. A minimum of 2.0Å between donor/acceptor atoms was used to allow for possible Mg^{2+} cations. All potential solvent molecules were refined as oxygen atoms with isotropic temperature factors and those with high B-values were removed. Despite careful scrutiny of $2F_o-F_c$ and the Fourier maps, we were unable to unambiguously assign any of the solvent peaks as cations probably because of the likelihood of interchange with other solvent atoms in the aqueous enviroment.

The refinement was terminated after the inclusion of 81 solvent molecules with B < 72Å^2. The final residual was 0.14 and peaks in the final difference map were generally less than 0.25e/Å^3.

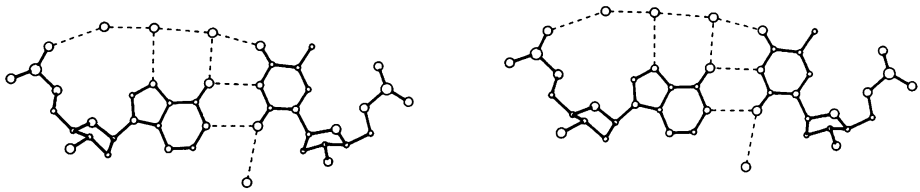
The refined coordinates will be deposited with the Cambridge Crystallographic Data Centre

RESULTS AND DISCUSSION

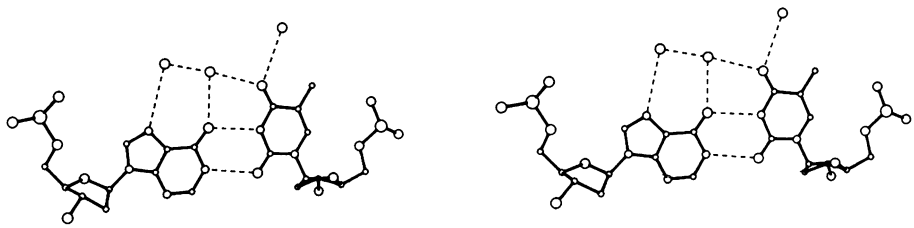
The deoxyoctanucleotide d(GGIGCTCC) crystallises as A-DNA. The asymmetric unit consists of two chemically equivalent strands which are coiled about each other to form an antiparallel double helix with six standard Watson-Crick base-pairs and two I.T mismatches at $I_3.T_{14}$ and $T_6.I_{11}$ (Fig. 1).

The structure is essentially isomorphous with those reported for d(GGGGCCCC) (12) and d(GGGGCTCC) (13). The mispairs form standard wobble-type base-pairs as shown in Fig. 2. The wobble pairs are linked by two hydrogen bonds: one between O6(I) and N3(T), the second joining N1(I) and O2(T). The duplex has a deep, extensively hydrated major groove, and a flattened minor groove. All but one of the furanose rings are in the C3'-endo conformation. As in the native sequence and the G.T analogue, the duplexes pack in infinite spirals about the 6-fold screw axis. The adjacent spirals, related by a 2-fold screw axis operation, are interleaved. The major groove of each duplex faces towards a fully solvated cylindrical channel approximately 22Å in diameter which is centred on and is parallel to each 6_1 axis (Table 1).

Figure 1. Stereo view of the dGGIGCTCC A-DNA duplex



I3.T14



I11.T6

Figure 2. The I.T wobble base pair. Hydration and hydrogen bonding are indicated.
above I13.T14
below I11.T6

Crystal Packing and Lattice Stability

Crystallisation and data collection for d(GGIGCTCC) and d(GGGGCCCC) were carried out at room temperature whereas for the G.T analogue this was done at 4°C. The I.T octamer crystals have higher thermal stability and diffracting power than the G.T analogue and this high lattice stability can be attributed to the efficient Van der Waals packing of the minor groove sides of both inosine bases into the surrounding hydrophobic environments. Such close Van der Waals contacts are not possible for octamers with guanine in position 3 due to the presence of the 2-amino group in the minor groove.

Solvent Environment

All solvent positions were assigned as water oxygen atoms even though Na^+ and Mg^{2+} cations were present in the crystallisation mixture. Mg^{2+} is expected to be octahedrally

Table 1. Intermolecular H-bonding between Duplexes (Å) for d(GGIGCTCC) and Analagous Octamers

(I) d(GGIGCTCC)	
O5'(G1)....O1P(C16)a	3.09
N2(G1).....N3(G12)b	3.34
N3(G1).....N2(G12)b	3.02
N2(G2).....O4'(G9)c	3.42
N2(G10)....O3'(G9)d	3.34
(II) d(GGGGCCCC)	
O5'(G1)....O2P(C16)a	3.36
N2(G2).....O2(C6)b	2.98
N2(G1).....N3(G12)b	3.37
N3(G1).....N2(G12)b	3.09
N2(G2).....O4'(G9)c	2.90
N2(G3).....N3(G9)c	2.78
N2(G11)....O2(C16)d	3.07
N2(G10)....O3'(C16)d	3.04
(III) d(GGGGCTCC)	
O5'(G1)....O1P(C16)a	2.83
N2(G1).....N3(G12)b	3.11
N3(G1).....N2(G12)b	2.91
N2(G2).....O4'(G9)c	2.75
N2(G3).....N3(G9)c	2.67
N2(G11)....O2(C16)d	3.02
N2(G10)....O3'(C16)d	3.19

Key to symmetry elements

(a) y, 1+y-x, -1/6+z
(b) 2-x, 1-y, -1/2+z
(c) 1+y, 1+y-x, -1/6+z
(d) 2-x, 1-y, 1/2+z

coordinated with cation-ligand distances in the region of 2.0-2.3Å. Na^+ coordination may be 5- or 6-fold with separation around 2.45Å. In cases where initial refinement led to short solvent-duplex or solvent-solvent interactions, we avoided repulsive restraints by placing the atoms in different symmetry-related positions. In spite of this there are only six contacts of less than 2.5Å and one less than 2.4Å in the final model.

Of the 81 solvent atoms located, 10 are hydrogen bonded to the duplex in the minor groove, 21 hydrogen bond in the major groove and 29 interact directly with the phosphate oxygen atoms. Figure 3 shows the distribution of solvent atoms within 4Å of the duplex. Those illustrated twice form hydrogen bonding bridges between duplexes. Solvent molecules outside the 4Å limit

Figure 3. Distribution of solvent atoms within 4Å of the d(GGIGCTCC) duplex

generally lie on the major groove side of the duplex. Hydration in the minor groove is limited by the hydrophobic interactions described earlier.

In the major groove, there are chains of solvent atoms. These are mostly coplanar with the base-pairs and terminate with a hydrogen bond to the O1P atoms of the phosphate groups pointing into the major groove. In the central region of the duplex some chains, notably 17-27-32 and 30-6-10 are near-linear, suggesting potential sites for counter-ions. However as atoms with high B-values were excluded from the refinement procedure we see very few bridging sites outside the hydration shell and we are thus unable to assign any of these solvent atoms as counter-ions.

The major groove contains domains of fused circular hydrogen bonds similar to those observed in d(GGTATACC) (14). However in the present structure there are no regular patterns of fused pentagons.

The hydration of the central region of the major groove can be described in terms of regular patterns of solvent pairs (i.e. 33-X, 1-17, 9-38, 10-14, X-43 where X indicates a vacant site) terminated by the thymine methyl groups. This pattern is analogous to the uncollapsed spine of hydration of paired water molecules in the widened minor groove of B-DNA structure exhibiting alternating BI, BII conformations (15).

It has been shown (16) that the A-DNA conformation is stabilised by a continuous chain of water molecules linking O1P and O2P atoms of adjacent phosphate groups. A hint of this pattern can be seen in Figure 3, but if a cut-off of 3.4Å is used for H-bonded distances then we find only two such atoms W30 and W54.

The I.T Mismatch

Figure 2 shows the hydrogen bonding interactions around the wobble pairs. The bases pair by two strong H-bonds (d≈ 2.8Å) between N1(I) and O2(T) and O6(I) and N3(T). The O4 atoms of thymine, along with its neighbouring methyl group is displaced about 0.5Å towards the major groove and is thus more readily accessible to solvent molecules. There is a roughly equal but smaller displacement of the inosine base towards the minor groove.

Both mispairs are essentially identical and have similar primary solvation patterns on their major groove sides. There is

Table 2. Conformational Analysis for the Sugar-Phosphate Backbone of d(G-G-I-G-C-T-C-C)

Residue	Alpha	Beta	Gamma	Delta	Epsilon	Zeta	Chi	Pi..Pi+1	Tm	P
Strand 1										
G01	-	-	149	61	-152	-53	-175		52	7
G02	-87	191	72	81	-169	-57	-160	6.0	47	18
I03	-141	171	125	64	-160	-53	-180	6.3	56	26
G04	-89	183	69	83	-137	-86	-165	5.8	46	16
C05	-69	160	60	82	-141	-72	-165	6.0	44	21
T06	-67	170	55	82	-149	-95	-158	5.9	43	18
C07	-40	150	46	81	-161	-71	-153	6.0	44	17
C08	-61	178	44	82	-	-	-151		40	10
Strand 2										
G09	-	-	95	68	-141	-89	-184		45	11
G10	-53	163	53	79	-159	-75	-172	5.9	44	22
I11	-56	175	46	86	-158	-69	-156	5.9	44	9
G12	-71	172	56	73	-128	-93	-158	5.7	40	14
C13	-37	143	48	78	-154	-64	-164	6.2	52	8
T14	-68	158	58	77	-159	-79	-158	6.2	51	26
C15	-75	171	57	82	-160	-74	-159	6.1	51	22
C16	-82	174	87	110	-	-	-146		11	119
Average	-67	173	58*	78	-153	-70	-159	6.0		
Average values for d(G-G-G-G-C-T-C-C)										
	-76	174	71*	82	-154	-67	-160	6.0		
Average values for d(G-G-G-G-C-C-C-C)										
	-76	178	62	84	-156	-70	-158	6.0		
Average values for d(G-G-G-G-T-C-C-C)										
	-67	172	57	81	-150	-74	-161	5.9		

Main chain torsion angles are defined by:

P-α-O5'-β-C5'-γ-C4'-δ-C3'-ϵ-O3'-ζ-P-

Glycosyl torsion angle χ is defined by:
O4'-C1'-N1-C2 for pyrimidines
O4'-C1'-N9-C4 for purines

* Excluding values for G01, I03 and G09

a preferred hydration site which bridges O4(T) and O6(I). This site is particularly prominent in the final $2F_O-F_C$ maps both in this structure and in G.T mispair structures. The site is within H-bonding distance of a solvent molecule interacting with N7(I) and in the case of the I.T pairs this ligand is flanked by an additional solvent atom interacting with the O1P atom of the inosine phosphate group.

The solvation pattern on the minor groove side of the mispairs is less well-defined. This is probably because this

region is important in crystal packing and therefore is less accessible to solvent molecules. The only clearly defined interaction is with a single solvent atom 3.0Å from O2(T14).

The Sugar-Phosphate Backbone

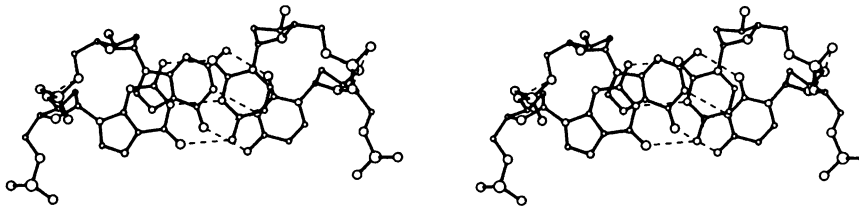
The distance between adjacent P atoms in each strand ranges from 6.30Å to 5.65Å with a mean value o´ 5.98Å. This is within 0.01Å of the average values found for the structures of the G.C and G.T analogues. The largest separation (between P2 and P3) in both this structure and that of the G.T analogue probably arises from the weak solvation of both phosphate groups. In contrast to the other phosphate groups in strand 1 there is no solvent bridge between P2 and P3. Accordingly, with reduced constraints on the orientations of these two groups, there should be increased flexibility (and potential disorder) in the immediate vicinity thus allowing the adjacent atoms C5' and O5' more vibrational freedom. This may help to explain the atypical values of α and γ for residue 3 (Table 2). Their association with the I.T mispair is probably incidental since the values for α and γ near T6 or I11 appear normal. Similar observations have been made for this region of the G.T analogue.

In this structure, the related G.T structure and the structure of d(GGGGTCCC) (17) the replacement of a Watson-Crick pair by a wobble pair is accommodated by small concerted adjustments of the backbone. The mean torsion angles for all four structures, including the native sequence, are very similar (Table 2). In the present structure 15 of the furanose rings have a C3'-endo conformation. Residue 16 has a pseudo-rotation angle indicating a C1'-exo conformation for the sugar ring, which is also somewhat flattened having a maximum puckering amplitude, T_m, of only 0.15Å. One might attribute this to the effects of disorder, but the average B-value for the furanose ring is quite reasonable and the final $2F_O-F_C$ map contains well-defined density. The position of the terminal O3' atom of C16 seems constrained by a strong interaction (d=2.8Å) with a bridging solvent atom, W25, which links O3'(C16) to O2P of residue 10 of a 2_1-related duplex.

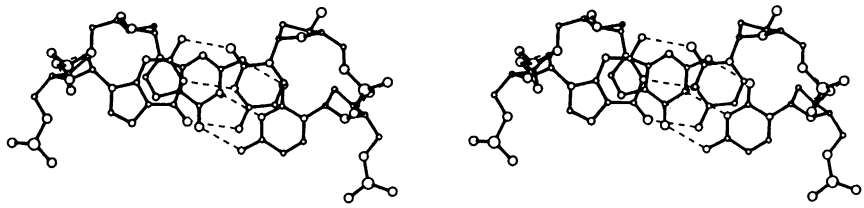
Helical Properties and Base-Stacking

The formation of a wobble pair requires that the paired bases

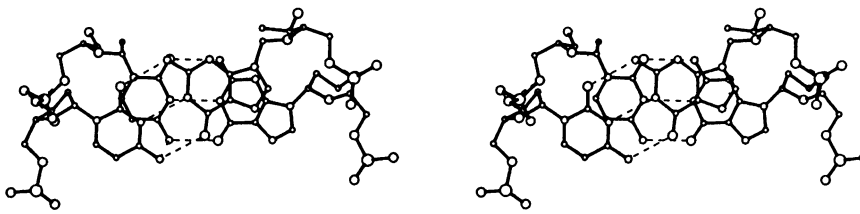
a



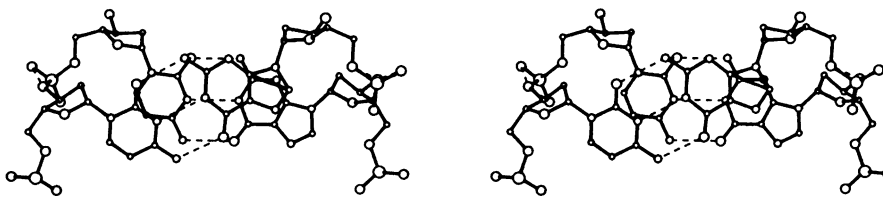
G2.C15
G3.C14



G3.C14
G4.C13

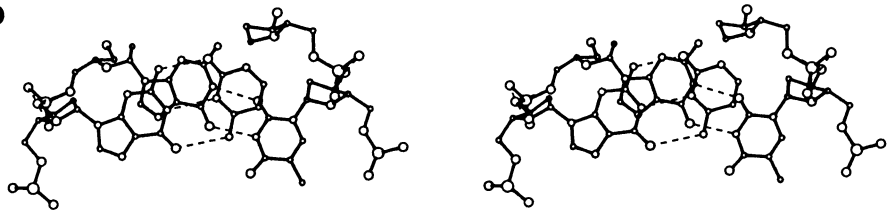


C5.G12
C6.G11

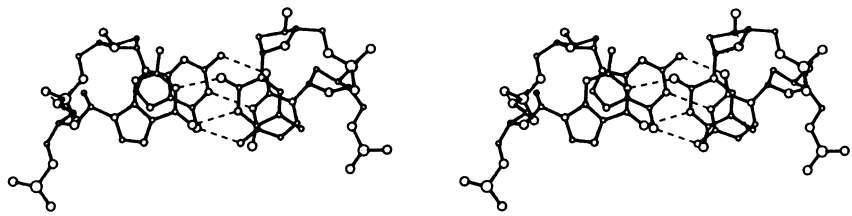


C6.G11
C7.G10

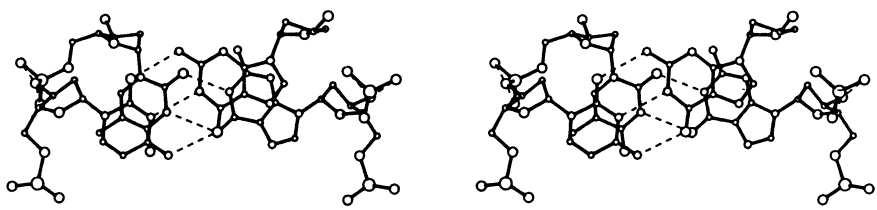
b



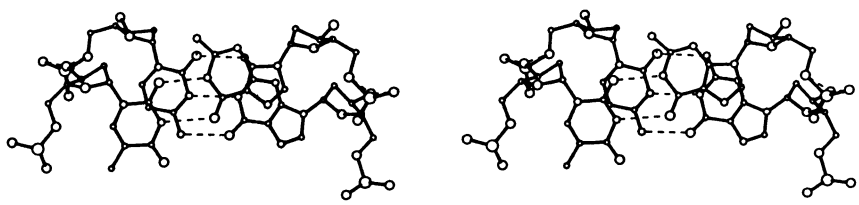
G2.C15
G3.T14



G3.T14
G4.C13

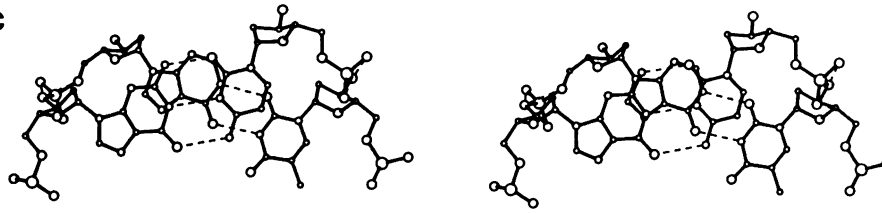


C5.G12
T6.G11

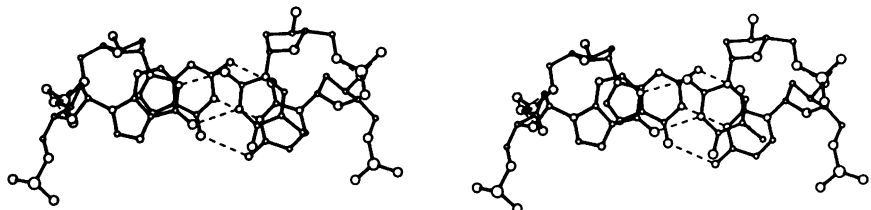


T6.G11
C7.G10

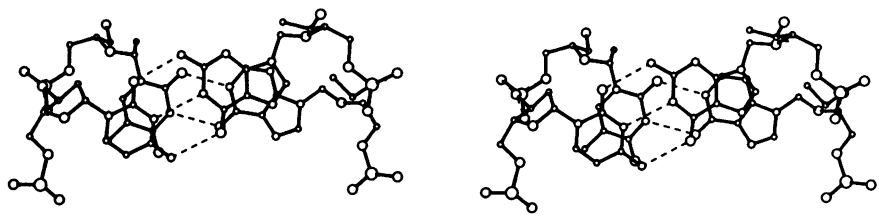
c



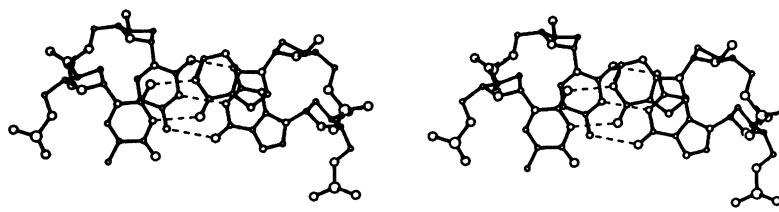
G2.C15
I3.T14



I3.T14
G4.C13



C5.G12
T6.I11



T6.I11
C7.G10

must be displaced relative to each other by roughly 2.5Å normal to the direction of the hydrogen bonding in the plane of the base pairs. This leads to a modification of the base stacking above and below each I.T pair. Figure 4 shows a comparison of base stacking involving the 3.14 and 6.11 pairs in the present structure, the native sequence and the G.T analogue.

For steps 2 and 5 the extent of the base overlap is similar for all three structures, with perhaps marginally more overlap for the native sequence. At steps 3 and 6 the extent of the overlap is similar for both the mismatch structures with the native sequence having less overlap especially at the pyrimidine-pyrimidine step.

From these observations it would seem that incorporation of purine-pyrimidine wobble base-pairs into sequences of poly(dG)-poly(dC) with an A-DNA conformation leads to increased intra-strand pyrimidine-pyrimidine overlap on the 3' side of the wobble base-pair and slightly decreased overlap on the 5' side.

In Table 3 we present the stepwise variation of helical parameters as defined by Calladine and Drew (18). The average values for propellor twist, roll, slide, helical twist and rise in the present structure are the same as in all analogous structures. The C1'-C1' distances average 10.4°A with no significant changes at the mismatch sites. Also shown in Table 3 are λ_1 and λ_2, where for (say) the G1.C16 pair λ_1 is the angle N9(G1)---C1'(G1)---C1'(C16) and λ_2 is N1(C16)---C1'(C16)---C1'(G1). For the Watson-Crick pairs the values of λ_1 and λ_2 are quite similar, the largest difference being 9°. However for the mispairs $\Delta\lambda$ is 30° for I3.T14 and 24° for T6.I11. In the structure of the G.T analogue, the same pattern is observed with $(\Delta\lambda)_{max}$ = 6.5° for the G.C pairs and $\Delta\lambda$ of 27° for G4.T14 and 22° for T6.G11. Clearly the I.T and G.T mispairs are much less symmetrical than the Watson-Crick pairs. The same type of asymmetry has been shown for A.C mispairs in the structure of d(CGCAAATTCGCG) (19) and for the G.A mismatch (20) in a B-DNA dodecamer. In the latter case the mismatch is of the form

Figure 4. Base Stacking Interactions
(a) d(GGGGCCCC), (b) d(GGGGCTCC), (c) dGGIGCTCC

Table 3 Geometrical properties of base-pairs and base-pair steps in d(G-G-I-G-C-T-C-C)

Base Pair	C1'...C1' (Å)	λ1(°)	λ2(°)	Propellor Twist (°)	Step	Roll (°)	Slide (Å)	Helical Twist (°)	Rise (Å)
G1.C16	10.7	55	59	9.8					
					1	6.0	2.0	30.8	3.2
G2.C15	10.3	57	59	11.0					
					2	7.4	2.2	35.3	2.7
I3.T.14	10.3	40	70	13.4					
					3	0.8	1.4	28.5	2.7
G4.C13	10.4	59	59	6.0					
					4	10.0	1.0	30.7	3.2
C5.G12	10.7	58	54	14.5					
					5	2.9	0.9	29.1	3.2
T.6.I11	10.3	72	48	13.5					
					6	7.8	1.7	35.4	2.7
C7.G10	10.4	58	53	16.2					
					7	7.6	1.7	34.4	3.2
C8.G9	10.5	57	48	6.8					
Averages for									
d(G-G-I-G-C-T-C-C)				11.7		5.7	1.55	31.6	3.0
d(G-G-G-G-C-T-C-C)				12.1		5.5	1.55	31.5	3.0
d(G-G-G-G-T-C-C-C)				12.1		6.0	1.51	32.2	2.9
D(G-G-G-G-C-C-C-C)				10.3		6.2	1.54	31.6	2.9

G(anti)-A(syn). A recent crystallographic analysis of Pol1 from E. coli has shown that the minor groove contacts between the polymerase and DNA may play an important role in the recognition of mismatches at the proof reading domain of the enzyme (21).

We were unable to measure the relative duplex stability of d(GGIGCTCC) and d(GGGGCTCC) in solution by UV melting techniques, as the sequences tend to aggregate on heating. However the self-complementary dodecamers d(CGCGAATTTGCG) and d(CGCIAATTTGCG) have identical duplex melting temperatues (22). Hence I.T and G.T mismatches have similar effects on duplex stability. This is in accordance with the observation that I.T and G.T mismatches both occur in mRNA-tRNA codon-anticodon interactions (2). Inosine has been described as a "universal base" (1) able to form very stable mismatches but in terms of structure and stability the I.T base pair is very similar to the G.T base pair.

CONCLUSIONS

Although inosine is not a naturally occurring base in DNA, being prevented from misinsertion by the enzyme hypoxanthine glycosylase which hydrolyses the glydosidic linkage, there are interesting parallels between the use of the base as a probe in gene sequences corresponding to proteins with redundant codons and as a probe for the 3-dimensional structure of DNA. We have

shown how a very small substituent change, namely the absence of an amino group on each strand can lead to increased lattice stabilisation by subtle changes in the crystal packing.

Detailed analysis of d(GGIGCTCC) has shown that its structure closely parallels that of its guanine analogue d(GGGGCTCC) which was refined at lower resolution. If this is the case for other guanine-containing mispairs, it may be possible to analyse mismatch sequences which have so far failed to yield good quality crystals by substituting inosine for guanine.

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