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Effects of 5-fluorouracil/guanine wobble base pairs in Z-DNA: molecular and crystal structure of d(CGCGFG)

Miquel Coll, Daniel Saal, Christin A.Frederick, Juan Aymami, Alexander Rich and Andrew H.-J.Wang[1]*

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139 and [1]Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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ABSTRACT

The chemotherapeutic agent 5-fluorouracil is a DNA base analogue which is known to incorporate into DNA *in vivo*. We have solved the structure of the oligonucleotide d(CGCGFG), where F is 5-fluorouracil (5FU). The DNA hexamer crystallizes in the Z-DNA conformation at two pH values with the 5FU forming a wobble base pair with guanine in both crystal forms. No evidence of the enol or ionized form of 5FU is found under either condition. The crystals diffracted X-rays to a resolution of 1.5 Å and their structures have been refined to R-factors of 20.0% and 17.2%, respectively, for the pH=7.0 and pH=9.0 forms. By comparing this structure to that of d(CGCGCG) and d(CGCGTG), we were able to demonstrate that the backbone conformation of d(CGCGFG) is similar to that of the archetypal Z-DNA. The two F-G wobble base pairs in the duplex are structurally similar to the T-G base pairs both with respect to the DNA helix itself and its interactions with solvent molecules. In both cases water molecules associated with the wobble base pairs bridge between the bases and stabilize the structure. The fluorine in the 5FU base is hydrophobic and is not hydrogen bonded to any solvent molecules.

INTRODUCTION

Wobble base pairing has been used to explain the stability of various mismatched, non-Watson-Crick DNA and RNA structures. In 1966 Crick devised an alternate set of hydrogen bonding rules to explain how a limited number of tRNA molecules could recognize a wide range of codons. These alternative bonding schemes were termed wobble base pairs (1). Since then, many studies of mismatched structures have been carried out using crystallographic as well as spectroscopic techniques (2-9). Among these structures is the T-G mismatch in Z-DNA which has been found to form wobble base pairs (7,8). A notable aspect of the T-G wobble base pair is that it is fairly stable although its melting temperature is significantly lower than that of normal Watson-Crick GC or AT base pairs (9).

5-Fluorouracil, a base analog of thymine, is an antitumor drug which has been under investigation for clinical use for many years. There are several potential modes of action for the antitumor activity (10-13). One important cellular process affected by 5FU is the conversion of dUMP to TMP by thymidine synthetase. In this pathway the 5FU is converted into 5-fluoro-dUMP and acts as a competitive inhibitor of thymidine synthetase by binding to the active site of the enzyme but not allowing the reaction to proceed due to the fluorine

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atom on the C5 position of uridine. Another possible mode of action involves the incorporation of the drug into RNA. In this case several aspects of cellular activity involving RNA would be affected. For example, RNA processing for translation as well as incorporation into ribosomes could be impeded. In addition, modified mRNAs might not bind to ribosomes or be translated by the normal mechanisms (13).

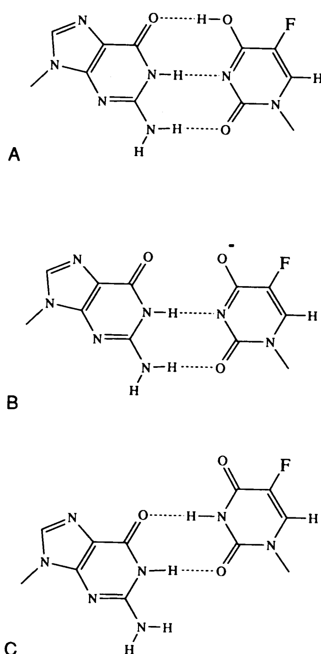
Alternative processes involve the drug being incorporated into DNA. Cheng et al. found that at concentrations of 1 and 10 μM ^{3}H labelled 5FU, DNA from cultured cells exposed to 5FU consisted of smaller average fragment lengths than the DNA of untreated cells (10). They suggested that 5FU was first inserted into DNA of cultured cells and then excised at a faster rate than ligation could occur. This resulted in fragmentation, and therefore altered the supercoiling of the DNA, leading to cell death. Finally it is possible that 5FU is incorporated into DNA and directly inhibits transcription and replication processes.

Recently, 5-fluorouracil has been found to be useful in making oligonucleotide probes. Habener et al. have found that probes made with 5FU showed sharper banding and better hybridization than mixed probes (14). This observation was attributed partly to the potential enol form of 5FU because of the presence of the extremely electronegative fluorine atom at the C5 position of uracil. This might facilitate and stabilize the pairing of 5FU to guanine.

In order to understand better the effects of 5FU on the properties of DNA into which it is incorporated, we are investigating the structure and interactions of DNA oligonucleotides containing 5FU. In this paper we examine the possibility of observing the enol form of 5FU by crystallizing a 5FU-containing DNA hexamer, d(CGCGFG) where F is 5FU, under two different pH (7.0 and 9.0) conditions. We found that the hexamer crystallized in the Z-DNA conformation with two F-G wobble base pairs in the double helix under both conditions. The structures of both crystal forms are nearly identical and very similar to that of d(CGCGTG).

MATERIALS AND METHODS

The DNA hexamer d(CGCGFG) was synthesized by the phosphoramidite method on an Applied Biosystems 380A DNA synthesizer and purified by high pressure liquid chromatography. 5-Fluorouridine was used to prepare the phosphoramidite precursor by the method described earlier (14). The hexamer was crystallized from a solution containing 1 mM DNA hexamer (single strand concentration), 25 mM sodium cacodylate buffer at pH 7.0, 100 mM magnesium chloride, 5.6 mM spermine tetrachloride and 12.5% 2-methyl-2,4-pentanediol (2-MPD), equilibrated against 50% 2-MPD by vapor diffusion technique. Under these conditions, small crystals formed in seven to ten days. Small, but clean single crystals were used as seeds to grow crystals large enough for data collection. These crystals have the same morphology as other Z-DNA hexamer crystals. A crystal having a size of 0.2x0.3x0.4 mm



Scheme 1

was mounted in the thin-walled capillary and sealed with a droplet of the crystallization mother liquor for data collection. The crystal was found to be in the orthorhombic space group $P2_12_12_1$ with unit cell dimensions of a=17.38 Å,b=31.06 Å and c=45.39 Å. The diffraction data were collected on a Nicolet P3 diffractometer using an ω-scan mode at 10 °C to 1.5 Å resolution with CuK_α radiation. A total of 3008 reflections were considered to be observable at a 2.0 σ(F) level above background and they were used in the refinement. Lp, empirical absorption and decay corrections were applied.

Since the pK_a of the N3 position of 5FU is approximately 7.8 (15), it is possible that 5FU in the hexamer might have a significant portion in the enol form at elevated pH, thereby forming a base pair with guanine having three hydrogen bonds. Thus a second set of conditions was used to produce a high pH form of the crystal instead. In this case a 25 mM Tris buffer with pH=9.0 was used instead of the cacodylate buffer used for the other crystallization. These crystals grew readily and became large enough for data collection without seeding after two to three weeks. A crystal of 0.2x0.3x0.4 mm in size was used for data collection on a Rigaku AFC-5R rotating anode diffractometer to 1.5 Å resolution at 20 °C. 2770 reflections were measured above the 2.0 σ(F) level and used in the refinement. The crystal belongs to the same space group as the pH 7 crystal with essentially the same unit cell dimensions, a=17.36 Å, b=31.16 Å and c=45.40 Å.

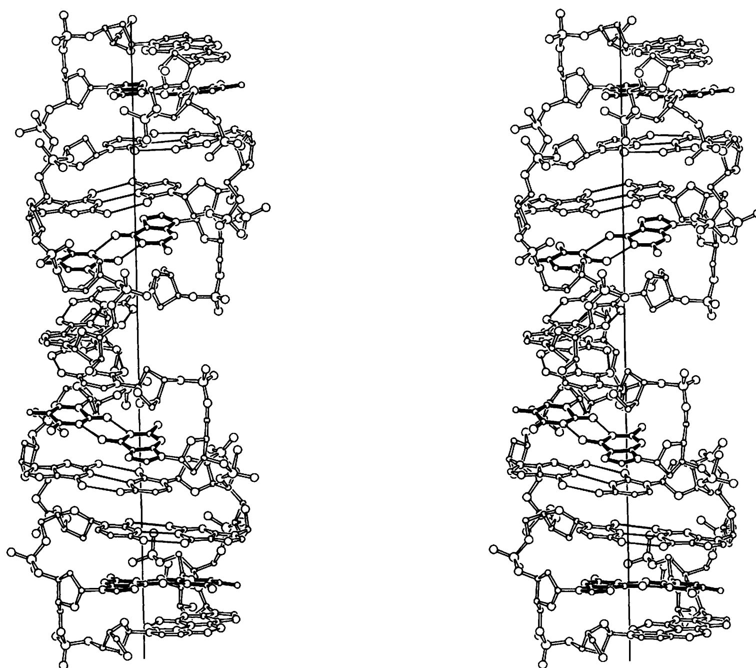


Figure 1. Stereoscopic views of the hexamer d(CGCGFG) Z-DNA double helix. Two symmetry-related hexamers stacked end-over-end are shown as they are in the crystal lattice . They are viewed looking perpendicular to the molecular two-fold axis which is horizontal in the plane of the paper. The helix axis is vertical. The F-G wobble base pairs are highlighted with solid bonds.

Due to the similarity of the diffraction patterns between the d(CGCGFG) and d(CGCGTG) crystals, the atomic coordinates of the latter structure were used as the starting model for the refinement of the structure of d(CGCGFG) for both neutral and high pH crystal forms. This model was independently refined against each data set using the Konnert-Hendrickson constrained refinement procedure (16).No hydrogen bond constraints were used initially for the F·G base pairs to avoid any bias toward a particular type of base pairing. These constraints were introduced later when it was obvious from the Fourier maps that wobble base pairs were present in both crystals in the F·G base pair. Solvent molecules located from difference Fourier maps were gradually included in the refinements. For the pH 7 structure, the final R-factor was 20.0% with one DNA duplex molecule, 64 water molecules and 1 Mg ion per asymmetric unit. For the pH 9 structure, the final R-factor was 17.2% with one DNA duplex molecule , 58 water molecules and 1 Mg ion per asymmetric unit, with a final root mean square deviation of bond lengths from the ideal value of 0.028 Å.

No spermine molecules could be identified unambiguously in either crystal form. The final atomic coordinates and structure factors of the complex have been deposited in the Brookhaven Protein Databank.

RESULTS

DNA conformation

The overall structure of the DNA d(CGCGFG) hexamer in the left-handed Z-DNA conformation is shown with a skeletal drawing viewed perpendicular to the helix axis in Figure 1A. Two symmetry related hexamer duplexes are stacked end-over-end along the helix axis which coincides with the two-fold screw rotation axis in the crystallographic c-axis direction. The two F-G base pairs in the hexamer helix are in the wobble geometry with two hydrogen bonds between the bases. In both structures, no evidence is seen of a three hydrogen bonds base pair involving the uncommon tautomeric form of the 5-FU base.

The overall structure of this hexamer is very similar to the atomic resolution Z-DNA structure of d(CGCGTG). The root mean square deviation between the two structures is 0.337 Å for the pH=9 form (0.341 Å for the pH=7 form). The average torsion angles along the backbone agree well with the standard Z-DNA conformation. As in the case of the structure of the d(CGCGTG) molecule, the two wobble base pairs cause some localized distortions in the backbone conformation (Table 1), especially near the 5-FU substituted nucleotides. For example, the α angles of both G6 and G12, which are associated with the phosphate groups at the FpG steps, are consistently higher (ave. 88°) than the rest of the corresponding CpG steps (ave. 68°). The wobble base pairs in the hexamer do not appear to cause any other significant alterations in the structure. All the deoxyguanosine residues adopt the *syn* glycosyl conformation and all the pyrimidine residues are in the *anti* conformation.

Figure 2 shows the van der Waals stereo diagram of the double helix. As in the normal left-handed Z-DNA helix, the deep and narrow minor groove is evident with the phosphate groups lined up along the edges of the groove. It can be seen that the small fluorine atoms (black spheres) are tucked in the clefts on the surface of the Z-DNA helix. This is somewhat different from the structure of the T-G mismatched Z-DNA in which the bulkier methyl groups of the thymine bases protrude further out into the solvent region. This allows the 5-FU containing hexamers to come closer together in the crystal lattice resulting in a slightly shorter b-axis (31.06 Å in d(CGCGFG) vs 31.63 Å in d(CGCGTG)), as will be discussed later.

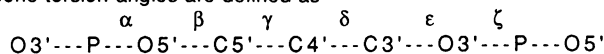
As observed in crystal structures of fluorine-containing nucleosides and nucleotides, fluorine atoms themselves have strong tendency to stack on nucleic acid bases(17). However, in this structure it appears that the presence of fluorine atoms does not grossly alter the stacking interaction in Z-DNA, although the wobble base pairs do perturb the base pair

Table 1

Conformational Torsion Angles of d(CGCGFG)*,**

Residue	α	β	γ	δ	ε	ζ	χ	*P*
C1	---	---	61	144	-93	75	-152	172
			40	149	-97	68	-143	168
G2	67	-165	-176	79	-124	-49	60	32
	72	-171	177	90	-124	-62	64	7
C3	-160	-120	57	136	-94	86	-152	159
	-159	-121	56	135	-91	77	-152	151
G4	66	-171	171	92	-124	-54	65	9
	56	-180	-175	93	-121	-62	63	34
F5	-154	-147	59	139	-94	69	-170	148
	-168	-137	70	130	-86	64	-168	148
G6	88	-176	-174	148	---	---	76	194
	87	176	-172	145			77	181
C7	---	---	56	140	-99	79	-154	148
			54	138	-91	71	-152	153
G8	71	-170	178	94	-121	-37	57	7
	70	-165	177	89	-142	-36	59	8
C9	-159	-148	53	148	-91	69	-144	157
	-175	-160	55	141	-98	76	-149	168
G10	68	-169	-180	86	-132	-61	66	48
	76	-172	170	85	-136	-63	63	31
F11	-158	-122	57	142	-95	63	-155	147
	-158	-123	61	137	-98	65	-163	161
G12	88	-175	-176	148	---	---	74	170
	87	-180	-178	159			65	170

* The backbone torsion angles are defined as



and χ is the glycosyl torsion angle. *P* is the sugar pseudorotation angle.

** The values in the upper row and in the lower row for each nucleotide are from the pH7 and pH9 forms respectively.

stacking pattern in the molecule as shown in Figure 3. In Figures 3(A-C) the successive nucleotides along the chain have a conformation *anti*-p-*syn* in the 5' to 3' direction. In this step, there is usually a moderate interstrand pyrimidine-pyrimidine overlap due to the sheared (large slide) geometry, as is evident in Figure 3B. The introduction of 5-FU into the molecule reduces this stacking interaction. The fluorine atom is not interacting with the cytosine base which is stacked below the 5-FU. In fact, the fluorine atom is situated at the

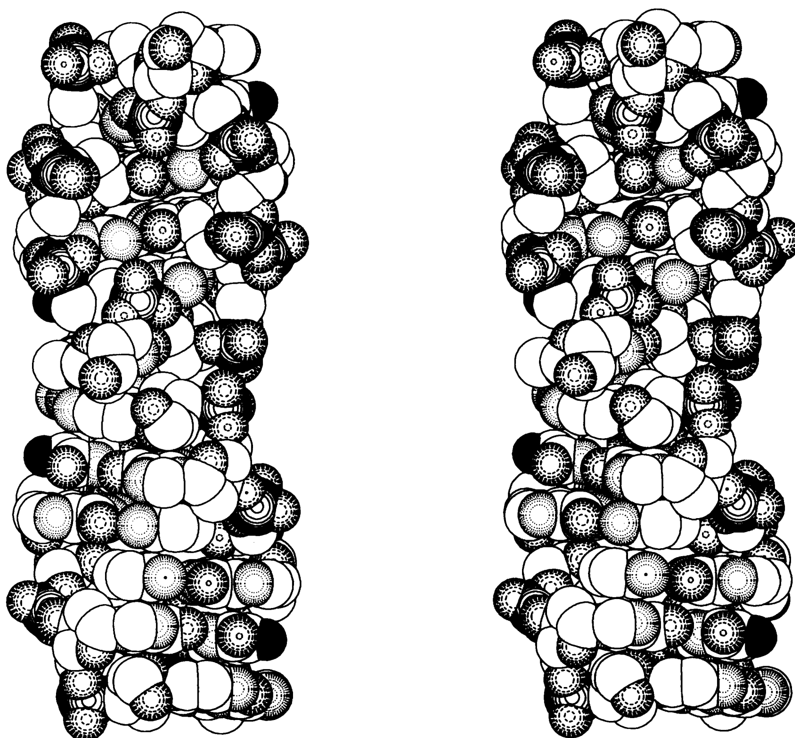


Figure 2. Van der Waals diagram of the hexamers. This view is 90° rotated relative to that in Figure 1 about the helix axis. The upper hexamer has the deep minor groove facing the reader, while the lower hexamer has the prominent surface facing toward the reader. The small fluorine atoms are drawn as solid black spheres.

perimeter of the helix on the concave outer surface. Figures 3(D-F) show the stacking pattern of the *syn*-p-*anti* steps in which a fairly good intra-strand purine-pyrimidine overlap is seen. Here the fluorine atom is near the N9 atom of the adjacent guanine, but still does not stack directly over the base.

Environment around fluorine atom

In this structure the organization of solvent molecules is influenced by both the wobble F-G base pairs and the hydrophobic fluorine atoms. Figure 4 shows the electron density maps of the solvent regions surrounding the two F-G base pairs in the crystal lattice. In Figure 4A, the magnesium ion cluster is clearly visible even at this resolution (1.5 Å). It occupies a well-defined position so that it bridges the F and G bases with coordinating water molecules. For example, one of the water molecules forms two hydrogen bonds to O4 of 5-FU (F5) and O6 of G8. This arrangement appears to be different from that seen in the structure

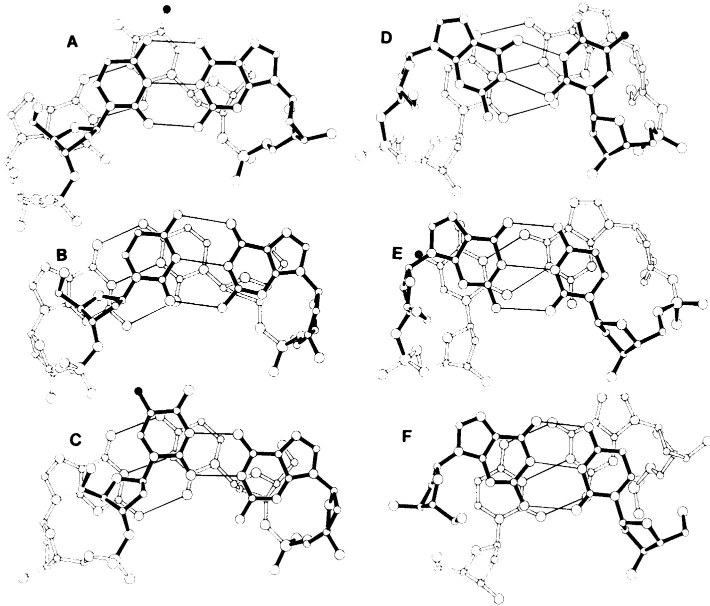


Figure 3. Base pair stacking diagrams in the hexamer. Successive base pairs are shown with the upper base pair in solid bonds and the lower base pairs in open bonds. The dinucleotide steps in the left column (a-c) have *anti-p-syn* glycosyl conformation, while in the right column (d-f) they have *syn-p-anti* conformation going from the 5' to 3' direction. The fluorine atoms are shown with filled circles and it can be seen they do not stack with the base rings.

of d(CGCGTG) in which two water molecules from the same magnesium cluster form two separate hydrogen bonds to O4 of T5 and O6 of G8. This difference may be related to the distinct solvent organization in the vicinity of the fluorine atom on 5-FU of d(CGCGFG) versus that near the methyl group of the thymine base of d(CGCGTG). Other than this difference, all the first shell hydration water molecules occupy very similar locations in these two structures of d(CGCGFG) and d(CGCGTG). However, these two crystal forms diffract to different resolutions, with the later diffracting to 1.0 Å atomic resolution while the former diffracts to only 1.5 Å resolution. This may be partly related to the difference in intermolecular packing interactions involving fluorine atoms of 5-FU and the methyl group of thymines. Both of these interactions represent hydrophobic interactions.

The hydrophobic nature of the fluorine atoms of 5-FU can be easily visualized in Figures 4(A-B). In Figure 4A, the fluorine atoms can be seen approaching the flat surface of the sugar ring of the symmetry related G8 residue (using symmetry operator 2-x, -0.5+y,

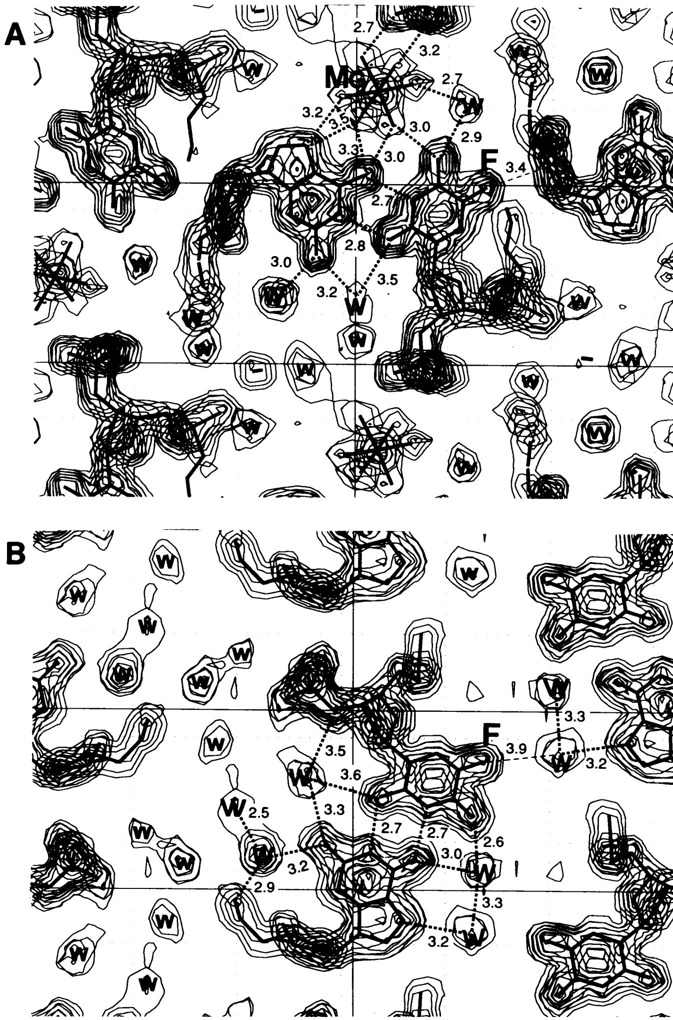


Figure 4. Electron density maps of the two F-G wobble base pairs showing the detailed geometry around the base pairs. The fluorine atoms (F) of the 5-FU are hydrophobic and they are not involved in any hydrogen bonding interactions. **Mg** indicates an octahedral magnesium-water cluster; solvent water molecules are indicated as **W**.

1.5-z). This is better illustrated by the close up view of the van der Waals drawing in Figure 5A where the fluorine atom abuts the sugar ring, almost touching the hydrogen atoms of the sugar. In comparison, the methyl group from the T5 residue in the d(CGCGTG) crystal structure clearly is in close van der Waals contacts with the sugar ring (Figure 5B).

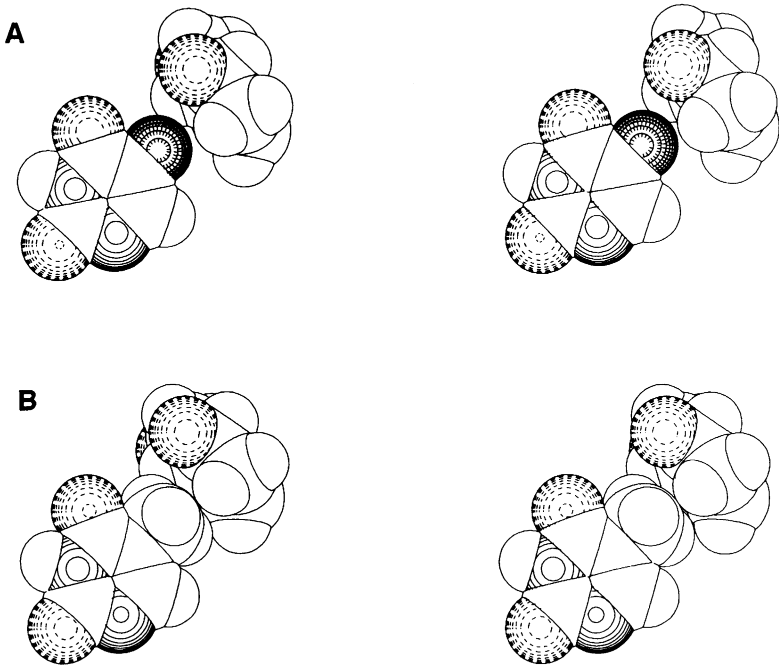


Figure 5. Stereoscopic van der Waals packing diagrams of the 5-FU base with the neighboring sugar ring (A) in the d(CGCGFG) structure and the corresponding interactions between the thymine and the sugar ring (B) in the d(CGCGTG) crystal lattice. Notice that the methyl group from thymine base nudges the sugar ring very tightly forming strong hydrophobic interactions, while the fluorine atom does not make contacts with any of the atoms of the sugar ring leaving a small empty space.

DISCUSSION

Certain chemical modifications of DNA have profound effects on the properties of DNA molecules. For example, the introduction of an iodine or bromine atom at the C5 position of each cytosine base in the poly(dG-dC) molecule strongly favors the formation of the left-handed Z-DNA conformation (18). The physico-chemical basis of this dramatic shift in the B-Z equilibrium contributed by the bromination of cytosine is not clear. Substitution of another halogen atom, i.e., chlorine may have a somewhat less pronounced effect on the B-Z equilibrium. Other chemical modifications have less pronounced effects on the overall conformation of DNA. For example, the restriction methylations at the N6 position of adenine or C5 position of cytosine produce only minor changes in the DNA structure (19,20). In addition, a number of 5-bromocytosine and 5-bromouracil substituted DNA oligomers have been used as heavy atom derivatives for the structure determinations of native unmodified DNA molecules, and in general, no adverse effects on conformation were seen.

Fluorine, another halogen atom, is the most electronegative atom. When it is bonded to an aromatic uracil ring at the C5 position, its effect on the stability of the DNA double helix poses an interesting question. Recently, it has been shown by UV spectroscopic melting experiments that an adenine/5-FU base pair is slightly more stable than an AT base pair (14). Similarly the G/5FU base pair is also more stable than the GT base pair. The increased stability of these 5FU-containing base pairs may be due to several factors associated with the unique properties (eletronegativity, hydrophobicity and small size) of the fluorine atom.

The pK_a value of a free 5-FU base is estimated to be about 7.8 by titration experiments monitored by the NMR resonance of the N3 imino proton of 5FU (15). At pH=9.0, a significant portion of the 5-FU molecules in solution may exist in their deprotonated (ionized) form which can pair with guanine using two hydrogen bonds in the Watson-Crick geometry (Scheme 1c). If this were the case, the d(CGCGFG) hexamer would form a more regular Z-DNA double helix with no wobble base pairs. However, our results did not show any ionized 5FU. As described above, 5-FU forms only wobble base pairs with guanine even under basic conditions (pH=9.0) in the crystal lattice. One can not rule out the possibility that the pK_a of 5FU is different in the Z-DNA structure. The conformational dependence of the pK_a value of a base is not uncommon. For example, the pK_a of a free cytosine base is 4.3, but it increases to about 6.0 in a polynucleotide such as poly(C) (21). Therefore it would be of interest to determine the actual pK_a value of 5FU in the Z-DNA conformation.

Our structural analysis of the 5-FU containing oligonucleotide molecules provides some insight on the questions related to the chemotherapeutic activity of 5-FU. The results clearly show that 5-FU can be easily incorporated into a DNA double helix without any significant effect on the DNA conformation. Furthermore, earlier melting experiments have shown that 5-FU can pair with both adenine and guanine with added stability relative to thymine (14). This difference in base pair stability may affect the rate of transcription in an AT-rich region if thymine is replaced by 5FU in this region. Alternatively, the more stable FG base pair may escape the cellular repair mechanisms more often, causing more frequent mutations and leading ultimately to the death of the rapidly growing cancer cells.

There are several unique properties of the fluorine atom on 5-FU which might have important effects on the manner in which the DNA double helix is recognized by proteins. The hydrophobic nature of the fluorine atom would cause some rearrangements of the solvent structure around the helix near the 5FU residue. Its size is substantially smaller than the methyl group of thymine. Therefore proteins, such as a repair enzyme, which are designed to detect the bulky hydrophobic methyl group of thymine might not be able to interact with the smaller fluorine atom with the same specificity. This effect of size difference is well illustrated in Figure 5 where the small fluorine atom of 5FU of d(CGCGFG) can not make good van der Waals contacts with the neighboring sugar ring (Figure 5A), while the methyl group

of thymine from d(CGCGTG) tightly nudges against the sugar ring. This could be viewed as an analogous model system for protein-DNA interactions in which certain van der Waals interactions between thymine methyl groups and hydrophobic amino acid side chains exist. The importance of these hydrophobic interactions in protein-DNA recognition has recently been inferred from the crystal structures of protein-DNA complexes (22). In our case, when a bulky methyl group is replaced by a smaller fluorine atom, potential van der Waals interactions will be weakened. Alternatively, some conformational changes in the protein may occur in order to regain the van der Waals stability which in turn may lead to altered enzymatic activity.

The three-dimensional structure of several anticancer and antitumor drugs complexed with DNA oligonucleotides have been solved by X-ray diffraction analysis recently (for reviews, see 23,24; see also 25-27 for more recent structures). However, this structure is the first one of an oligonucleotide that incorporates a nucleoside anticancer drug into the molecule, therefore providing an opportunity to visualize its effects on DNA conformation and the solvent organization around the double helix. Oligonucleotides containing other type of nucleoside anticancer drugs are being synthesized for structural studies in order to better understand the molecular basis of their biological activities.

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*To whom correspondence should be addressed

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