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CONFORMATIONAL STUDIES OF d(CACGTG)2 AND d(CACCGTG).d(CACGGTG) BY TWO-DIMENSIONAL 1H, 31P NMR AND CIRCULAR DICHROISM SPECTRA+

K. N. Ganesh*
Division of Organic chemistry,
National Chemical Laboratory,
Pune, 411008, INDIA.
and
G. V. Rajendrakumar
Centre for Cellular and Molecular Biology,
Hyderabad, 500007, INDIA.

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ABSTRACT: *The conformational studies of synthetic oligonucleotides d(CACGTG)2 and d(CACCGTG).d(CACGGTG) by two-dimensional 1H NMR (COSY and NOESY), 31P NMR and CD spectroscopic methods are reported. The results suggest that these DNA duplexes adopt B-form globally and significant microheterogeneity persists in local conformation. In particular, the sugars of nucleotides C3 of the hexamer and C3, C10 of the heptamer exhibit 3'-endo conformations. These sequences fail to undergo B-Z transition in solution though, d(CACGTG)2 is reported to crystallise in a left handed form.*

INTRODUCTION

The current progress in our knowledge of solution conformation of nucleic acids has been largely due to application of NMR techniques, in particular two-dimensional NMR spectroscopy[1].The potential importance of structural studies on DNA need hardly be overemphasised as it is the most important macromolecule in the cell. The prime goal of most of the structural studies is to understand the sequence-specific local alteration of molecular structure at the level of individual nucleotidic components. DNA duplexes containing alternating purine-pyrimidine sequences such as d(CG)n, d(AC)n, d(GT)n and combinations thereof are interesting targets for studying sequence dependence of double helical conformation. d(CG)n derived sequences, though not widespread in nature, have been shown to undergo induced transitions to left handed structures[2]. On the otherhand, short sequences related to d(CA)n,d(GT)n series or their triplet subsets occur frequently in many regulatory regions on genes[3]. One such triplet CAC/GTG is a common feature that appears in a number of DNA operator sites specific for protein interaction[4]. In order to understand the sequence specific structural features adopted by these triplets, we

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herein report the conformational studies of the self-complementary hexamer d(CACGTG)2 and the related heptamer duplex d(CACCGTG).d(CACGGTG) using two-dimensional 1H NMR, 31P NMR and circular dichroic techniques. Interesting features are seen in the conformational details of d(CACGTG)2 in solution by NMR studies as compared to its crystal structure that was recently reported[5]. These results and the nature of asymmetrical perturbations caused by the insertion of a central C:G base pair into the hexamer duplex are discussed.

RESULTS AND DISCUSSION

The one-dimensional 500MHz 1H NMR spectrum of the self-complementary hexamer d(CACGTG)2 and the heptamer duplex d(CACCGTG).d(CACGGTG) are shown in figures 1(a), (b) respectively. These spectra depict non-exchangeable resonances from the base, sugar and thymine methyl protons in the regions 7.0-9.0, 2.0-6.5 and 1.5-2.0 ppm respectively. The chemical shifts for all these protons in both duplexes as assigned by two-dimensional NMR are shown in tables 1 and 2.

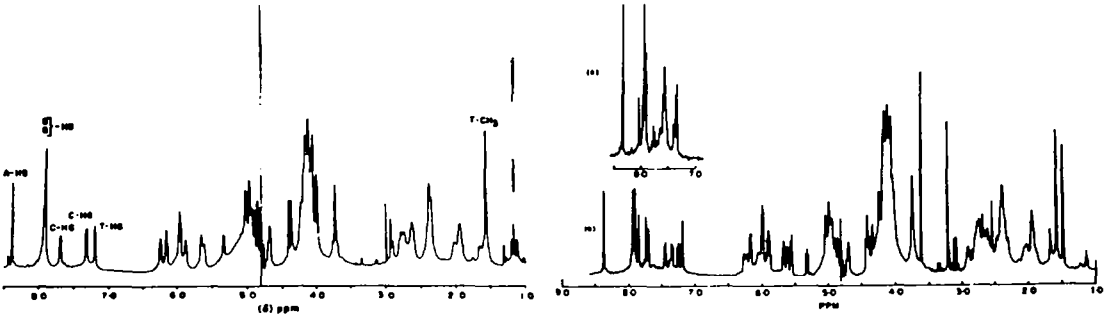


Figure 1. 500 MHz 1H NMR spectrum of (a) d(CACGTG)2 (b) d(CACCGTG).d(CACGGTG) and (c) partial spectrum of d(CACCGTG).

Chemical shifts of base H6/H8 protons: The presence of two cytidines (C1 and C3) in d(CACGTG)2 (figure 1a) is indicated by two doublets at 7.2 and 7.6 ppm for H6 protons. Similarly, the two guanines G4 and G6 are non-equivalent as seen by two singlets at 7.90 and 7.92 ppm. In case of the heptamer duplex (figure 1b) four doublets appear due to H6 of cytidines at 7.71, 7.45, 7.36 and 7.25 ppm. Thus introduction of a central C:G base pair into the hexamer generates asymmetry in the structure, leading to four non-equivalent (C1, C3, C4 and C10) and two apparently equivalent (C1 and C8) cytidine residues in the heptamer duplex. Similarly there are four non-equivalent (G5, G7, G11 and G12) and two equivalent (G7 and G14) guanines, the former set appearing at 7.95, 7.93, 7.86 and 7.75 ppm respectively. The two adenines (A2 and A9) seem

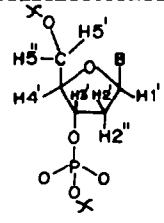
to be equivalent as shown by a single resonance line for AH8. Though a single base signal (H6) appears for two thymines T6 and T13, their non-equivalence is indicated by separate peaks for the 5CH3 at 1.48 and 1.60 ppm.

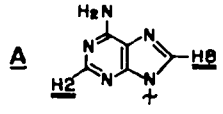
TABLE-1: d(CACGTG)2

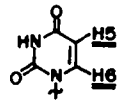
unit	H1'	H2'	H2''	H3'	H4'	H6/8	H2/H5/CH3
C1	5.64	1.92	2.33	4.69	4.04	7.68	5.92
A2	6.23	2.77	2.90	5.02	4.40	8.37	7.90
C3	5.58	2.00	2.37	4.65	4.17	7.29	5.32
G4	5.95	2.62	2.73	4.96	4.36	7.90	-
T5	5.85	1.89	2.36	4.69	4.04	7.17	1.48
G6	6.14	2.58	2.35	4.66	4.17	7.92	-

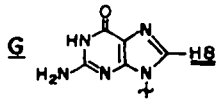
TABLE-2: d(CACCGTG):d(CACGGTG)

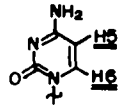
Unit	H1'	H2'	H2''	H3'	H4'	H6/H8	H2/H5/CH3
C1,C8	5.69	1.94	2.41	4.71	4.06	7.71	5.57
A2,A9	6.24	2.80	2.93	5.04	4.38	8.39	-
C3	5.93	2.04	2.45			7.36	5.31
C4	5.57	2.18	2.48	4.85		7.45	6.00
G5	5.62	2.67	2.75	4.98	4.41	7.95	-
T6,T13	5.91	1.92	2.42	4.87	4.15	7.19	1.60
G7,G14	6.18	2.60	2.37	4.98	4.41	7.93	-
C10	5.64	1.87	2.35			7.25	5.32
G11	5.62	2.67	2.75	4.98	4.41	7.86	-
G12	6.00	2.55	2.74	4.94	4.39	7.75	-











Chemical shifts of sugar protons: The pyrimidine nucleotides C3 and T5 in d(CACGTG)2 show their H1' upfield (table 1) as a result of higher ring current shifts from flanking purines. The large differences in H1' chemical shifts among cytosines C1 and C3 and guanosines G4 and G6 support an ordered duplex structure and rule out the possibilities of end to end aggregation. The H2' and H2'' protons of pyrimdine nucleotides (C1, C3 and T5) are shifted upfield compared to corresponding protons of purine nucleotides (A2, G4 and G6). The heptamer duplex shows similar patterns in chemical shift spread (table 2): the central base pairs C4:G11 and G5:C10 have the most sheilded H1' protons and the pyrimidines have lower chemical shifts for H2' and H2'' as compared to those of purines. Among the pyrimidines, C10 which is flanked by two purines A9 and G11 has the most highfield H2'(2'') protons. In both duplexes, the actual difference in chemical shifts between H2' nad H2'' are also larger than purines. All these results are consistent with the general chemical shift trends noticed in nucleic acids1.

The sequential assignment of the 1H NMR resonances of oligonucleotides depends critically on the assumption that they exist as duplexes in solution. The UV absorption maximum (λ_{max}=260nm with no shoulder at 295nm), the 31P NMR (figure 2) and the CD spectrum (λ_{max} near 285nm with positive molar ellipticity, figure 3) indicate that both d(CACGTG)2 and d(CACCGTG).(GTG6CAC) exist as duplexes under the experimental conditions. This is further substantiated (i) by a good dispersion and nonequivalent signals for base protons in the region

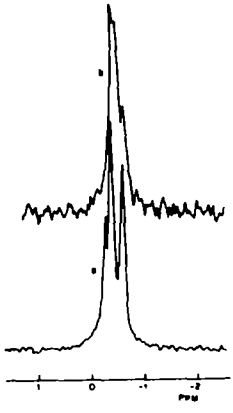


Figure 2

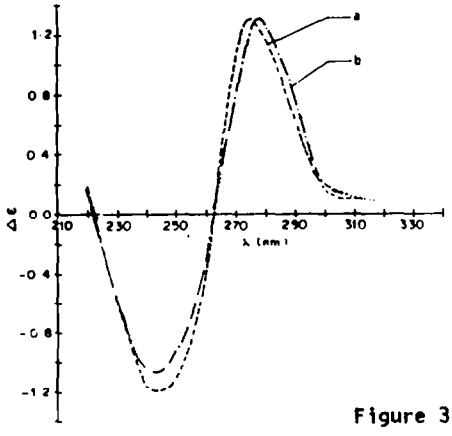


Figure 3

Left, Figure 2. 31P NMR (Reference: 85% H_3PO_4) of d(CACGTG)2 in 10 mM sodium cacodylate. Right, Figure 3. CD spectra of d(CACGTG)2 in 5 mM KH_2PO_4 (pH 7.2). (a) without and (b) with the addition of NaCl (5M)

7.0-8.4 ppm and (ii) observance of intrastrand specific base-base NOE (see later). Indirect confirmation for the heptamer duplex is provided by NMR of d(CACCGTG)2 (figure 1c) in which the possibility of a C:C mismatch destabilises the duplex resulting in a collapse of the non-degeneracy of signals for base protons.

Two-dimensional 1H NMR spectroscopy of nucleic acids: The individual resonance assignments were done by a combination of two-dimensional COSY and NOESY spectra (6, 7). In a COSY experiment cross peaks are seen for J-coupled protons and hence the spin system within the individual nucleotide units may be identified through *intra*nucleotide J-connectivity pathways: *H1'-[H2',H2'']-H3'-H4'-[H5',H5''], CH5-CH6 and TH6-TCH3.* Under low digital resolution the intensities of COSY cross peaks are approximately proportional to the magnitudes of coupling constants. Since the latter depend on the sugar conformation in a predictable way, the relative intensities of various COSY cross peaks can be qualitatively related to the sugar geometry[6].

The cross peaks in a NOESY spectrum arise by cross relaxation via dipole-dipole interactions between protons. In DNA, two categories of cross peaks are seen: (i) *intra*nucleotide cross peaks which establish base-sugar connectivity and (ii) *inter*nucleotide cross peaks which allow sequential connectivity. The assignment of individual sugar spin systems to particular nucleotide units along the chain is done by the use of following specific *intra* and *inter* nucleotidic NOE connectivities: *(H8/H6)n--(H8/H6/TCH3/H5)n+1 (H8/H6)n --(H2',H2'')n-1--(H8/H6)n-1*, where n, n-1, etc refer to sequence numbering from 3' end.

Correlation spectroscopy: The figures 4 and 5 show expansions of selected regions of the 2D COSY spectra of d(CACGTG)2 and d(CACCGTG).d(CACGGTG) respectively. The stereospecific assignment of H2' and H2'' protons are done by fact that in general H2' is upfield to 2'', the only exception being that of sugars at the 3'-end. All sugars in both duplexes show (i) identical intensities for H1'-H2' and H1'-H2'' cross peaks and (ii) presence of H2'-H3' cross peaks and (iii) absence of H2''-H3' cross peaks. These features are characteristic of *2'-endo* geometry for sugar rings. The only exceptions to this observation are the sugars C3 of the hexamer (figure 4b) and C3, C10 of the heptamer (figure 5). In these sugars,the intensities of H1'-H2'' cross peaks have higher intensities than H1'-H2'. More significantly, these do not show any cross peaks for H2'-H3' but distinctly exhibit H2''-H3' cross peaks. This has important structural implications and according to coupling constant- sugar conformation

correlations[6], this different behaviour of C3 and C10 are indicative of a *3'-endo* conformation for sugars in these nucleotides. The H4'- (H5'-H5'') cross peaks are not clearly seen as they are buried in the water signal and are saturated. Inspite of partial overlap of certain resonances, the resolution of the obtained COSY spectrum in both duplexes permit identification of all sugar spin networks.

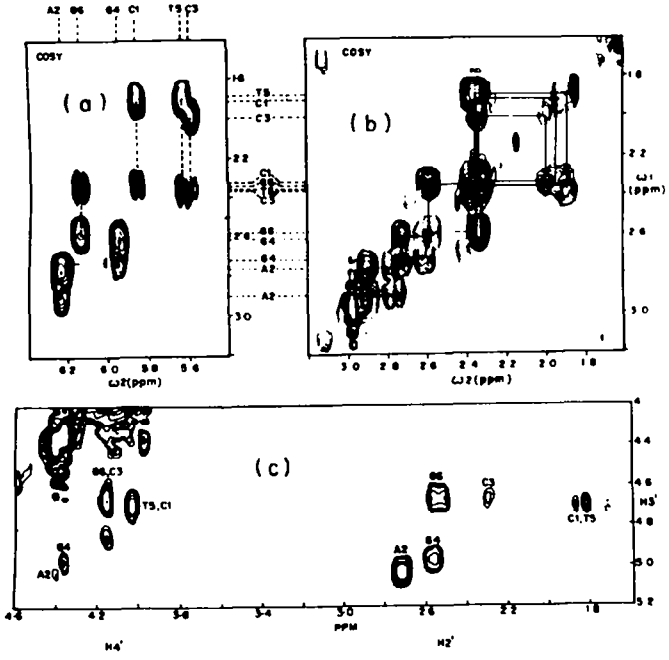


Figure 4

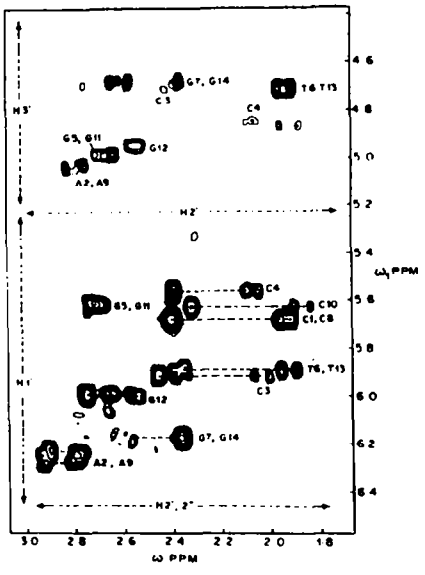


Figure 5

Left, **Figure 4.** Partial 500MHz COSY spectrum of d(CACGTG)2 with assignments and correlations for (a) H1'-H2'(2''), (b) H2'-H2'' (c) H2'-H3'-H4' cross peaks.
Right, **Figure 5.** Partial COSY spectrum of d(CACCGTG).d(CACGGTG), H1'-H2'(2'')-H3' cross peaks assignments.

Nuclear Overhauser enhancement spectroscopy: Figure 6 shows the observed $(H2'')_n$-$(H6/8)_n$-$(H2'')_{n-1}$-$(H6/8)_{n-1}$ NOE cross peaks for the hexamer duplex. Here the *intra*nucleotide H6/8-H2' NOESY cross peaks have higher intensities than the corresponding H6/8-H2'' peaks, whereas for the *inter*nucleotidic sequential connection, the reverse is observed: H6/8-H2'' cross peak intensities are higher than H6/8-H2' cross peaks. H2'(2'') have stronger *intra*nucleotides NOE's to base protons H6/8 than that due to H1' proton. While the NOESY

spectrum shows in case of pyrimidines, relatively stronger *intra*nucleotide NOE between H1'-H2'' compared to H1'-H2' protons, they are of almost equal intensity for purines. These NOE patterns can only be accounted for by an *anti* conformation of base residues with sugars in a *2'-endo* geometry 1,6,7. Figure 7 shows intra and internucleotide through-space connectivities employed for sequencing of the heptamer duplex. Due to the asymmetry induced by the central C:G base pair, the two strands are non-equivalent and both are separately sequenced as shown in figure 7. The NOE pathway shown for the DNA heptamer

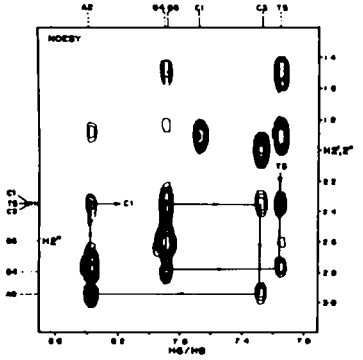


Figure 6. Expansions of 500MHz NOESY spectrum (symmetrised) of $d(CACGTG)_2$ showing sequential connectivities using H6/8 and H2'' protons. Vertical lines indicate $(H6/8)_n$--$(H2'')_{n-1}$ connection and horizontal lines represent $(H2'')_n$--$(H6/8)_n$--$(H2'')_{n-1}$ connections.

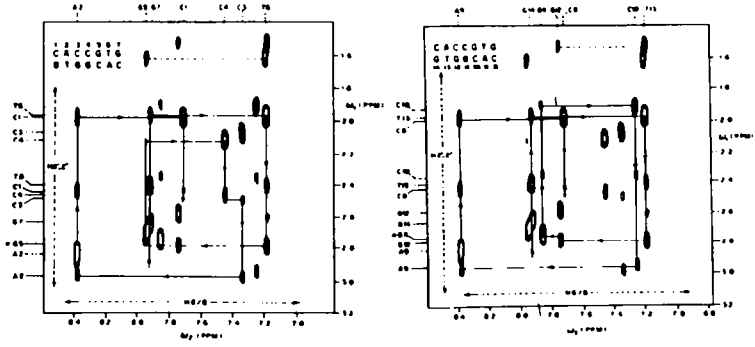


Figure 7. NOE sequential connectivities in (a) d(CACCGTG) and (b) d(CACGTGG). Vertical lines indicate $(H6/8)_n$--$(H2'')_{n-1}$--$(H2')_{n-1}$--$(H6/8)_{n-1}$ and horizontal lines represent $(H2'')_n$- -$(H2')_n$--$(H6/8)_n$--$H2'')_{n-1}$ connectivities.

duplex sequencing involves both H2' and H2'' protons: $(H6/8)_n$--$(H2'')_{n-1}$--$(H2')_{n-1}(H6/8)_{n-1}$--$(H2'')_{n-2}$. The appearance of the cross peaks conform to the general pattern observed in case of the hexamer: stronger NOE's for intranucleotide H6/8-H2',2'' protons compared to H6/8-H1', higher intensities for intranucleotide H8/6-H2' cross peaks compared to H8/6-H2'' and the converse for corresponding internucleotide connectivities. All H6/8-H2'/2'' NOESY peaks fall into sequential connectivities expected for right handed DNA and none of the cross peaks are left unassigned.

In case of the *3'-endo* nucleotides (C3 of hexamer and C3, C10 of heptam-

er) the intranuclear H6-H2' and H6-H2'' NOE's have widely different intensities , the former being much stronger than the latter. This is indicative of a high anti conformation for these bases[6]. The only significant base-base NOE relation seen is that due to intrastrand G4 H8-T5 CH_3 in case of hexamer (figure 6) and G5 H8-T6 CH_3 and G12 H8-T13 CH_3 in case of heptamer (figure 7). In both duplexes, no interstrand NOE's were seen. The absence of certain expected cross peaks for terminal nucleotides in COSY and NOESY spectra is due to the mobility of strands associated with the duplex ends.

^{31}P NMR: The ^{31}P chemical shifts are very sensitive to changes in phosphodiester backbone conformation in nucleic acids. Figure 2a shows the ^{31}P NMR of $d(CACGTG)_2$ in 10mM sodium cacodylate where three distinct peaks are seen at -0.30, -0.42 and -0.64 ppm. This is ^{31}P chemical shift region for phosphates in a B-DNA geometry and from the present work it is not possible to make assignments of these peaks to any specific phosphate residues. This pattern suggests significant microheterogeneity in phosphate backbone structure, arising as a consequence of different conformations related to B-DNA geometry. The addition of 5M salt does not alter the chemical shifts but is accompanied by line broadening (figure 3b). This line broadening which is also observed in ^1H NMR at higher salt concentration may be ascribed partly to the presence of more conformational types and partly due to viscosity effects. However no peaks were seen due to any left handed Z-DNA form, either in the absence or presence of added salt. The heptamer duplex also showed a similar ^{31}P NMR spectra consisting of three resonances unaltered by addition of added salt.

Circular dichroic studies: Circular dichroism is very sensitive to conformational changes in nucleic acids, especially to alterations of base tilt, twist, stacking and handedness[9]. The CD spectra of the hexamer (figure 3) shows positive ellipticity at 280nm and a negative ellipticity at 245nm which are characteristics of B-DNA. The addition of NaCl (5M), ethanol (60%) along with addition of $NiCl_2$ (upto 200mM) unchanged the CD profile indicating the absence of inducible conformational transitions in solution. For regular B-DNA structures, the negative ellipticity is higher in magnitude than the positive ellipticity. The near equal magnitude of positive and negative ellipticities noticed for the hexamer suggests possible conformational deviations from the standard B-DNA geometry. The origin of a similar situation in CD profile of $d(CDCGTG)_2$ (D= 2-amino adenine) under high salt conditions was interpreted as due to the presence of 20-25% of Z-DNA population in solution[10]. Even though the CD of the hexamer without salt addition was similar to that of $d(CDCGTG)_2$ under comparable conditions both ^1H and ^{31}P NMR ruled out any Z-form.

Conformation of d(CACGTG)2 and d(CACCGTG).d(CACGGTG): From temperature dependent one-dimensional studies on d(CACGTG)2 it has been previously shown[11] that this duplex adopts a B-helical conformation, with *2'-endo* geometry largely predominant for all sugar residues. The coupling constants used to derive this information were measured at 70°-80°C, at which the hexamer would be present mostly as single strands (t_m for helix-coil transition is 47°C). Further, the overlap of certain resonances in one-dimensional NMR gives rise to uncertainities in assignments and hence coupling constant estimations. Two-dimensional ^1H NMR (COSY and NOESY), with its inherently superior resolution not only permits unambiguous assignments but also provides adequate cross checks for internal consistencies.

The pattern of two-dimensional NOESY connectivities observed for both duplexes strongly indicate that these exist in solution in an overall right handed B-DNA. However the observance of H2''-H3' COSY cross peaks instead of H2'-H3' cross peaks for sugars of C3 of hexamer and C3, C10 of heptamer strongly point to a *3'-endo* conformation for these sugars. This inference is further substantiated by differences in intensities of H1'-H2' and H1'-H2'' COSY cross peaks for these sugars. The bases attached to them are perhaps in an *high anti* orientation as suggested by NOESY spectra in which the *intra*nucleotide H6-H2'' cross peaks for these sugars have significantly stronger intensities than corresponding H6-H2'' cross peaks. In the limits of experimental conditions employed for two-dimensional experiments, such inferences on sugar conformation and glycosidic geometry from the cross peak intensity patterns are valid and supported by theoretical correlation of coupling constants with pseudorotation phase angles[6,7]. Thus although the hexamer adopts B-form globally, there are significant local variations in its conformation in solution, particularly at cytidines C3 and C10. ^{31}P NMR also indicates a conformational microheterogeneity in the phosphate backbone structure. The CD spectra show an unusual pattern related to B-DNA, with both duplexes reluctant to undergo transitions to any other forms even under forcing conditions.

In contrast to these results, crystal structure of d(CACGTG)2 reported recently[4] shows the hexamer to be in a left handed Z-form, in which peculiarities in the stacking of bases at A2/C3 steps induces an asymmetry in the crystal structure. In our solution studies, such asymmetry has not been noticed for this hexamer duplex. A direct comparison of the details of base stacking in solid state and the solution conformational results is not truly valid as the geometry of stacking of base pairs differs widely among B and Z

forms of DNA. However, the fact that structural peculiarities are noticed both in crystal and solution at A2/C3 steps should not be construed as purely coincidental. We suggest that a non-B, pseudo-Z type of 'local' conformation seen for C3 nucleotide in d(CACGTG)2 could possibly be an intermediate state necessary for transition from B to Z form. As these transitions are co-operatively induced, in solution this hexamer fails to switch forms due to energetic barriers whereas in crystals the packing effects and other stabilizing forces such as hydration network, may promote the transition to Z-form.

The introduction of a C:G base pair at the centre of the hexamer though it disrupts the purine-pyrimidine alternation, does not alter the conformation significantly. However it induces subtle asymmetric perturbations leading to non-equivalence of near neighbour base pairs as reflected in the chemical shift dispersions in 1H NMR (table 2). The two strands of the heptamer become non-equivalent by virtue of the central base and the NOE sequentially connectivity pathway traced for both strands separately confirm the duplex structures. Such total assignment of all resonances by 2D NMR techniques as shown here aids identify specific minor conformational variations as in case of C3 and C10 nucleotides.

Conclusions: The conformational analysis of d(CACGTG)2 and d(CACCGTG).d(CACGGTG) indicate that though these adopt B-form globally, significant microheterogeneity persists in local structure of base, sugar and phosphate backbone conformation. These local variations are centred around C3 of the hexamer andf C3, C10 of the heptamer sequences. In solution, B to Z transition could not be induced although d(CACGTG)2 is known to crystallise in Z-form. As CAC/GTG sites are present in multiple copies in regulatory regions on DNA, the present studies may have relevance in understanding the sequence dependent effects on nucleic acid structure and possible role of these triplets in DNA-protein recognition. The heptamer duplex constitutes the central site of P22 *mnt* operator DNA and the subtle structural perturbations in this sequence induced by point asymmetry may have importance in understanding the molecular mechanisms of symmetry based nucleic acid-protein interactions.

EXPERIMENTAL PROCEDURES

The oligomers d(CACGTG)2, d(CACCGTG) and d(CACGGTG) were synthesized by solution phase phosphotriester chemistry[12] and purified by preparative fast protein liquid chromatography (FPLC, Pharmacia) over MonoQ (10x10) anion exchange FPLC column as described elsewhere[12]. All NMR experiments were car-

ried out on a Bruker AM500 FTNMR spectrometer operating at 500MHz frequency for 1H and 202.46 MHz for 31P. The CD spectra of DNA between 210nm and 310nm were recorded on a JASCO J-20 spectropolarimeter.

Sample preparation for NMR studies: The hexamer d(CACGTG)2 (2.7mg, 3mM) was dissolved in 0.5ml of phosphate buffer (0.02M, pH7.2), lyophilised, redissolved in D2O and relyophilised (3 times) and finally made upto 0.5ml with D2O. In case of the heptamer duplex, equimolar amounts of the two strands d(CACCGTG) (76 A260units) d(GTGGCAC) (79 A260units) were dissolved in 0.5ml phosphate buffer (0.02M, pH7.0), heated to 90oC and then allowed to cool to ambient temperature over 30 minutes and the sample was prepared for NMR as above. For 31P NMR, the oligomer d(CACGTG)2 (3.1mg, 3mM) was dissolved in sodium cacodylate buffer (10mM, pH7.0) containing 10% D2O and the spectra recorded using 85% phosphoric acid as external reference.

Two-dimensional 1H NMR experiments: Two-dimensional COSY experiment for d(CACGTG)2 was carried out with 2048 and 260 datapoints along *t2* and *t1* axis respectively using the pulse sequence (90o-t1-90o-t2), modified by insertion of a fixed delay millisecs after each 90o pulse (15). The NOESY experiments were carried out with 1024 and 170 data points along t2 and t1 directions respectively, with a mixing time of 300 millisecs. The HOD signal was suppressed with low power continuous irradiation to avoid dynamic range problems. A relaxation delay of 1sec. was permitted in all two-dimensional experiments. The time domain data were multiplied by sine square bell and sine bell window functions prior to Fourier transformation along t2 and t1 directions respectively. The chemical shifts are expressed with respect to sodium-3-trimethylsilyl-[2,2,3,3-2H]-propionate (TSP).

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