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Extra thymidine stacks into the d(CTGGTGCGG)·d(CCGCCCAG) duplex. An NMR and model-building study

Yvonne Th.van den Hoogen, Aart A.van Beuzekom, Hans van den Elst, Gijs A.van der Marel, Jacques H.van Boom and Cornelis Altona*

Gorlaeus Laboratories, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands

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ABSTRACT

NMR and model-building studies were carried out on the duplex d(CTGGTGCGG)•d(CCGCCCAG), referred to as (9+8)-mer, which contains an unpaired thymidine residue. Resonances of the base and of several sugar protons of the (9+8)-mer were assigned by means of a NOESY experiment. Interresidue NOEs between dG(4) and dT(5) as well as between dT(5) and dG(6) provided evidence that the extra dT is stacked into the duplex. Thermodynamic analysis of the chemical shift vs temperature profiles yielded an average Tm_D value of 334 K and ΔH°_D of -289 kJmol^{-1} for the duplex $\rightleftarrows$ random-coil transition. The shapes of the shift profiles as well as the thermodynamic parameters obtained for the extra dT residue and its neighbours again indicate that the unpaired dT base is incorporated inside an otherwise intact duplex. This conclusion is further supported by (a) the observation of an imino-proton resonance of the unpaired dT; (b) the relatively small dispersion in ^{31}P chemical shifts (≈ 0.5 ppm) for the (9+8)-mer, which indicates the absence of t/g or g/t combinations for the phosphate diester torsion angles α/ζ. An energy-minimized model of the (9+8)-mer, which fits the present collection of experimental data, is presented.

INTRODUCTION [1,2]

During the past five years the results of several studies concerning the structure and dynamics of DNA duplexes containing an extrahelical base have been presented [3-12]. These investigations appear important because in mechanisms of frameshift mutagenesis an unpaired base plays an important role [13]. Some of the above-mentioned studies were directed towards the question whether the extrahelical base stacks into the helix or bulges out in solution. Patel *et al.* [3] showed, on the basis of chemical-shift and NOE data, that the extra dA in the otherwise self-complementary sequence d(CGCAGAATTCGCG) is stacked inside the duplex. Similarly the unpaired dA residue in d(CGCAGAGCTCGCG) resides in the interior of the helix [6]. Supporting observations were made by Roy *et al.* for the unpaired dA residue in d(CGCGAAATTTACGCG) [12] and by Woodson *et al.* concerning the duplex d(GATGGXGGAG)•d(CTC$_4$ATC) [10]. In case of a purine, X = dA or dG, the extra

base is found stacked in a B-type DNA helix. In contrast, Morden *et al.* [5] reported that the extrahelical dC bulges out in the d(CA_3CA_3G)•d(CT_6G) sequence. The stronger stacking proclivity of dA compared to dC was suggested to account for the observed differences in behaviour of extrahelical dA and dC residues. If such were the case, duplexes containing an extra dT are expected to display a bulged out pyrimidine. According to Evans and Morgan [14], poly[d(GAA)] and poly[d(TCTC)] form a duplex in which every other dT remains extrahelical. In poly[d(GA)].poly[d(TTC)] the existence of duplexes with bulged out dT residues is proposed in a similar vein [15].

In order to determine whether a single extrahelical dT favours to reside in the interior of a duplex or prefers to bulge out we have performed NMR and model-building studies on d(CTGGTGCGG)•d(CCGCCCAG), henceforth denoted (9+8)-mer. The choice of this particular (9+8)-mer is related to our study of the RNA analogue (CUGGUGCGG)•(CCGCCCAG) (van den Hoogen *et al.*, to be published). In the present report, chemical-shift and NOE data as well as information obtained from imino-proton and ^{31}P resonances are used to monitor the behaviour of the unpaired base in the (9+8)-mer. Chemical-shift data of the double-stranded (9+8)-mer, together with those of the single-stranded octamer and nonamer, are used to obtain thermodynamic parameters of the duplex $\rightleftarrows$ monomer transition. We present an energy-minimized model of the (9+8)-mer, which fits the accumulated experimental data.

MATERIALS AND METHODS

The octamer d(CCGCCCAG) and nonamer d(CTGGTGCGG) were synthesized via a solid-phase approach [16] on an automatic DNA synthesizer. After purification the fragments were treated with a Dowex cation-exchange resin (Na^+ form) to yield the sodium salt. NMR samples were lyophilized three times from 99.8 % D_2O. A trace of EDTA was added and the pH was adjusted to 7.0 - 7.5 (meter reading); Me_4NCl was used as internal reference. For most practical purposes the Me_4NCl scale can be converted to the DSS scale by setting $\delta_{Me_4NCl} - \delta_{DSS} = 3.18$ ppm. In order to observe imino-proton resonances of the (9+8)-mer a 3.5 mM sample was prepared in a H_2O/D_2O 90/10 mixture; in this case DSS was used as reference compound.

^1H NMR spectra were recorded on a Bruker WM-300 spectrometer equipped with an Aspect 2000 computer and on a Bruker AM-500 spectrometer interfaced with an Aspect 3000 computer. Spectra of the separate octamer and nonamer as well as of the (9+8)-mer 1:1 mixture were recorded at 3.5 mM nucleotide concentration. In order to obtain an 1:1 mixture of the nonamer + octamer,

the concentrations of the separate strands were determined spectrophotometrically and afterwards the two solutions were mixed in the correct ratio. Measurements were performed at several temperatures between 0 and 100 °C in order to obtain chemical-shift vs temperature profiles. In case of D_2O samples the residual HDO peak was suppressed by means of irradiation at the HDO frequency. The H_2O signal in the imino-spectra was minimized by means of a time-shared long pulse in combination with a data-shift accumulation routine [17,18]. A phase-sensitive NOESY experiment was recorded of the (9+8)-mer at 292 K. A mixing time of 0.5 s was used; 512 4K spectra were collected. Before phase-sensitive Fourier transformation, the t_2 and t_1 domain were apodized with a shifted sine-bell squared window and the t_1 domain was zero-filled to 2K.

^{31}P spectra (broad-band ^{1}H decoupled) were recorded on a Bruker WM-300 spectrometer at an operating frequency of 121.6 MHz.

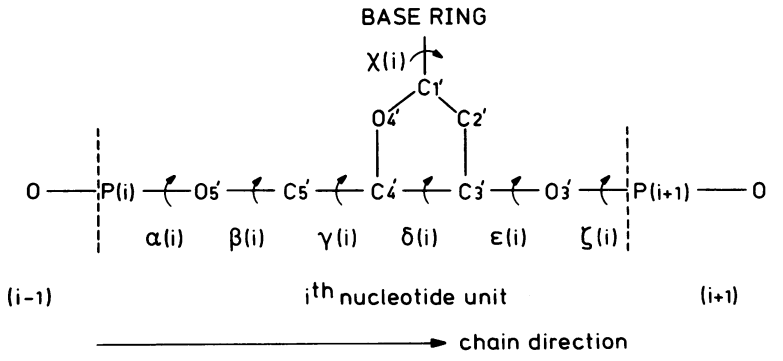
DNA models were generated with the aid of the program MacroModel on an Evans and Sutherland PS 350 computer-graphics system, interfaced to a VAX 11/750. The structures were energy-minimized by means of the molecular mechanics program AMBER [19].

The recommended IUPAC-IUB nomenclature is used throughout this work [20,21]. A schematic representation is displayed in Scheme I.

RESULTS AND DISCUSSION

Assignment of non-exchangeable protons

The assignment of all base and most of the H1', H2' and H2" proton resonances of the (9+8)-mer is based upon a close analysis of a NOESY



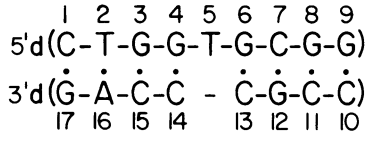
Scheme I. Conformational notation of oligonucleotides [20,21].

Table 1. Chemical shifts of base, H1, H2' and H2" protons of the (9+8)-mer duplex (3.5 mM, 292 K); shift reference Me_4NCl.

Residue	H8/H6	H2/H5/CH_3	H1'	H2'	H2"
	ppm				
C(1)	4.680	2.821	2.488	-0.611	-1.070
T(2)	4.321	-1.427	na	-0.909	-1.314
G(3)	4.704	---	2.457	<-0.472>[a]	
G(4)	4.614	---	2.830	<-0.578>[a]	
T(5)	4.063	-1.691	2.755	-0.918	-1.178
G(6)	4.497	---	2.619	-0.535	-0.635
C(7)	4.189	2.173	2.511	-0.907	-1.349
G(8)	4.683	---	2.477	-0.511	na
G(9)	4.649	---	3.011	-0.668	-0.860
C(10)	4.593	2.796	2.679	-0.822	-1.119
C(11)	4.427	2.570	na	na	na
G(12)	4.777	---	2.752	<-0.462>[a]	
C(13)	4.168	2.290	2.860	-0.782	-1.112
C(14)	4.361	2.529	na	na	na
C(15)	4.392	2.584	2.014	-0.941	-1.125
A(16)	5.043	4.741	2.884	-0.300	-0.433
G(17)	4.599	---	na	na	na

a) near isochronous H2'/H2" resonances.
na: not assigned due to severe overlap

experiment. The chemical shifts of the assigned protons are given in Table 1; the numbering of the various residues is indicated in Scheme II. The assignment followed established procedures [22,23,24]. Suffice it to say that NOE intensities depend, among other factors, upon the interproton distances ($1/r^6$), thus the NOESY experiment contains structural information. In case of the (9+8)-mer this information appears most useful to determine whether or not the duplex is distorted by the presence of the extra dT and, moreover, whether the unpaired pyrimidine is stacked into the duplex or bulged out.



Scheme II. Numbering of the residues in the (9+8)-mer duplex.

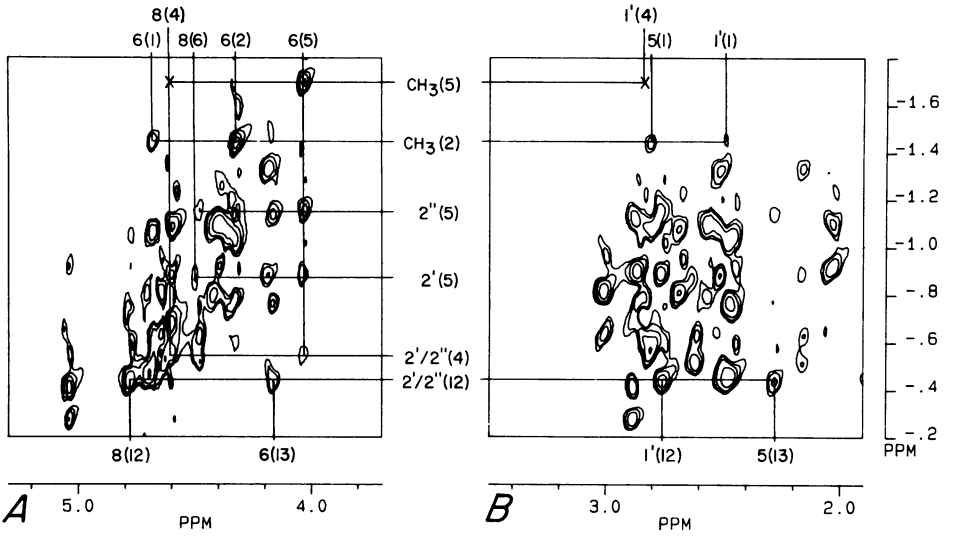


Figure 1. Contour plots of parts of the NOESY spectrum of the (9+8)-mer duplex; 3.5 mM, 292 K, pH = 7; (a) region in which H8/H6 - H2'/H2"/CH_3 connectivities are observed (b) region in which H1'/H5 - H2'/H2"/CH_3 connectivities are observed.

Parts of the NOESY spectrum of the (9+8)-mer are given in Fig. 1. The region of the spectrum in which one observes the connectivities between the H8/H6 and the H2'/H2" and the methyl resonances is shown in Fig. 1a. In Fig. 1b the region containing NOEs between the H1' and the H2'/H2" and methyl resonances is displayed. In the case of a regularly stacked oligonucleotide a sequential assignment usually rests upon the inter- and intraresidue NOEs observed in these regions.

For the purpose of the present discussion it appears convenient to start the sequential assignment of the (9+8)-mer from the observed interresidue NOE between H6(1) and CH_3(2) (Fig. 1a). A sequential assignment characteristic for right-handed B DNA based upon H8/H6(n) - H2'/H2"(n-1) connectivities can be continued up to dG(9). At this point we wish to stress that several internucleotide NOEs between dT(5) and its neighbours, dG(4) and dG(6), appear present, although some of them are very weak. For example, the NOEs between H8(4) and CH_3(5) as well as between H1'(4) and CH_3(5) are observed only at a lower level contour plot; their positions are indicated with a cross in Fig. 1a and Fig. 1b, respectively. Examination of the proton-proton distances expected for B DNA reveals a distance of 3.9 Å

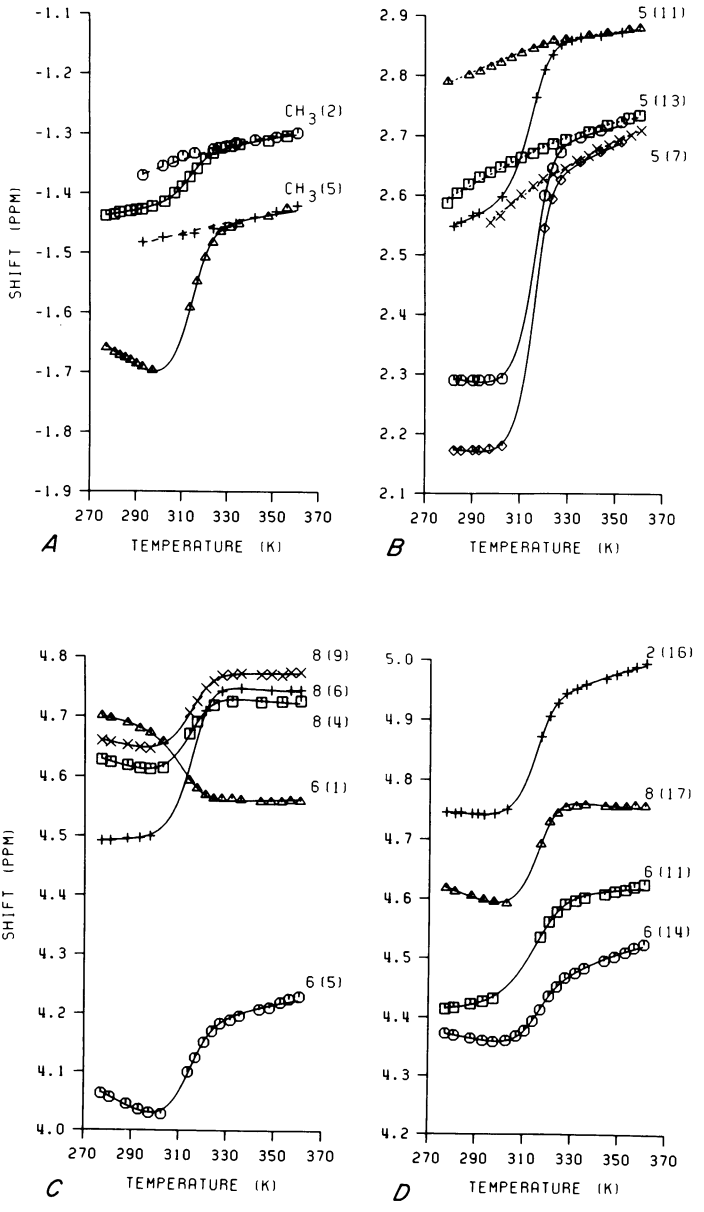


Figure 2. Chemical shift vs temperature profiles of some base protons in the separate single-strand octamer and nonamer and in the (9+8)-mer duplex; 3.5 mM. Points on the broken lines belong to the single strands; the broken line is calculated by means of the ASSTAK model (see text). Points on the solid lines belong to the duplex; the solid line is calculated by means of the DUPSTAK model (see text).

between H8(4) and CH_3(5) and 4.0 Å between H1'(4) and CH_3(5) [25], in line with the weak NOEs seen. Moreover, strong internucleotide NOEs between H6(5) and H2'/H2"(4) (near - isochronous resonances) and between H8(6) and H2'/H2"(5) are readily observed (Fig. 1a). This is in accordance with expectations for a B-type DNA structure. In summary, these data suggest that the unpaired dT is stacked between dG(4) and dG(6); in other words, the extra dT resides inside the duplex.

The assignment of the octamer strand is performed in an analogous fashion; some assignments (e.g. H6(13)-H2'/H2" (12) and H5(13)-H2'/H2"(12)) are indicated in Fig. 1. Unfortunately, severe overlap in the NOESY spectrum prevents an unambiguous decision whether or not interresidue NOEs appear between dC(13) and dC(14). This point remains of particular interest because these specific residues flank the unpaired dT in the opposite strand. At this point one can speculate that the helix could be locally distorted to accommodate the stacking of the unpaired pyrimidine; a distortion leading either to a larger distance between dC(13) and dC(14) in the opposite strand or to a disruption of some base pairs. In order to throw more light on these matters we carried out a chemical-shift and thermodynamic analysis.

Chemical-shift and thermodynamic analysis

Temperature vs shift profiles were measured for the separate single-stranded octamer and nonamer and for the (9+8)-mer duplex. In Figs. 2a,b the change in chemical shift with temperature of some resonances in the single strands and in the duplex are shown. The shift profiles of the resonances in the single strands reflect the change of the intramolecular base-base stacking equilibrium with the temperature. The shift profiles of the (9+8)-mer monitor the duplex $\rightleftarrows$ monomer transition from the dimer at low temperature to the random coil at high temperature. Thus, the plateau region in the shift profiles at low temperature corresponds to the intact duplex and the high-temperature plateau region to the monomer.

An extrahelical residue would not be expected to display significant chemical-shift changes throughout the transition. In the present case, the shift profiles of the (9+8)-mer, including those of the resonances belonging to the dT(5) residue, display a roughly sigmoidal shape. Thus, the chemical-shift changes of circa 0.2 ppm of the H6(5) and CH_3(5) resonances during the duplex $\rightleftarrows$ monomer transition (Fig. 2) clearly indicate that the extra pyrimidine stacks into the duplex.

Fig. 2 also shows small but significant slopes of the shift profiles of most resonances in the high-temperature plateau region. These changes

correspond closely with the behaviour of these resonances in the respective single strands and are readily ascribed to the completion of the stack $\rightleftarrows$ random coil transition. The low-temperature plateau region also shows small changes in chemical-shifts for most resonances. This phenomenon normally occurs in oligonucleotide duplexes and is generally ascribed to small and local conformational transitions in the intact duplex [26,27].

The shift profiles of several base protons of the (9+8)-mer were used in order to obtain thermodynamic parameters for the duplex $\rightleftarrows$ random-coil equilibrium. The analyses of the shift profiles were carried out by means of a non-linear least-squares curve-fitting program in combination with the DUPSTAK model [Pieters J.M.L. *et al.* to be published]. In the DUPSTAK model, the relation between the observed chemical shift for a given proton, δ_{obs}, and the temperature is basically given by eqn. 1a:

$$\delta_{obs} = \delta_{free} + p_X \cdot \Delta_X + p_D \cdot \Delta_D \qquad (1a)$$

in which δ_{free} stands for the chemical shift of this proton in the random coil form; p_X and p_D represent the population of stacked species and duplex, respectively; Δ_X and Δ_D stand for the chemical-shift increments relative to the random coil in the stacked form and duplex, respectively. However, eqn. 1a does not account for the small chemical-shift changes that are observed in the low-temperature region. The latter changes occur in the intact duplex and the slope is often opposite to that of Δ_D. Since the chemical shift changes in this region appear to display a linear dependence upon temperature, Δ_D in eqn. 1a can be written as:

$$\Delta_D = \Delta_{D'} + T \cdot \Delta\Delta_D \qquad (2)$$

in which Δ_D' corresponds to the change in chemical shift between duplex and monomer and $\Delta\Delta_D$ represents the small temperature-dependent shift in the intact duplex. The description of the DUPSTAK model is then embodied in eqn. 1b:

$$\delta_{obs} = \delta_{free} + p_X \cdot \Delta_X + p_D \, (\Delta_{D'} + T \cdot \Delta\Delta_D) \qquad (1b)$$

The first step in the analysis consists of the determination of the parameters of the single-stranded stack $\rightleftarrows$ random coil equilibrium. The shift profiles of the separate octamer and nonamer were used for this purpose. As

explained earlier [28], the relative success of this approach hinges upon the presence of protons that display sufficiently large Δ_X (stacking-shift) values. Perhaps unfortunately, few such protons appear present in the single-stranded 8-mer and 9-mer, e.g. H8(16), H2(16), H5(7), H6(7) and H8(8). A way out of this dilemma was provided by one of our earlier studies on single-stranded DNA fragments [26]. More in particular, Tm_X values and entropy changes ΔS°_X have been found to occur in narrow ranges [26]: for R-R sequences ΔS°_X = -85 / -95 $Jmol^{-1}K^{-1}$ and Tm_X = 320 / 328 K, for R-Y and Y-R sequences ΔS°_X = -59 / -87 $Jmol^{-1}K^{-1}$ and Tm_X = 307 / 320 K and for Y-Y sequences ΔS°_X = -114 / -141 $Jmol^{-1}K^{-1}$ and Tm_X = 290 / 300 K. In preliminary evaluations of the shift profiles of the DNA fragments at hand Tm_X and ΔS°_X were varied within these established ranges in order to obtain the "best" Δ_X value. The calculated correspondence between observed and calculated shifts is shown in the shift profiles of the single-stranded octamer and nonamer, Fig. 2 a,b.

In the next step of the analysis, the determination of the parameters

Table 2. Thermodynamic data for the duplex $\rightleftarrows$ monomer transition of the (9+8)-mer. Standard deviations are given between brackets.

Proton	Tm_D	$-\Delta S^\circ_D$	$-\Delta H^\circ_D$
	K	$Jmol^{-1}K^{-1}$	$kJmol^{-1}$
H6(1)	335.5 (1.3)	721 (32)	240 (11)
CH_3(2)	332.1 (1.3)	888 (37)	295 (12)
CH_3(5)	336.0 (1.3)	785 (28)	264 (10)
H8(6)	334.4 (0.8)	915 (28)	306 (9)
H5(7)	332.6 (0.8)	1053 (39)	350 (13)
H8(9)	337.3 (0.9)	797 (20)	269 (7)
mean 9-mer strand	334.6 (0.4)	837 (12)	282 (4)
H6(11)	335.7 (1.4)	765 (30)	257 (10)
H5(11)	332.1 (0.6)	926 (24)	308 (8)
H5(13)	332.6 (1.0)	1012 (47)	337 (16)
H6(14)	334.5 (0.9)	812 (21)	272 (7)
H2(16)	333.5 (0.7)	930 (22)	310 (7)
H8(17)	336.1 (1.1)	901 (30)	303 (10)
mean 8-mer strand	333.5 (0.3)	878 (11)	294 (4)
mean all	333.9 (0.2)	859 (8)	289 (3)

for the duplex $\rightleftarrows$ monomer transition, the obtained parameters of the monomers were used as constraints. Again, a successful analysis requires a substantial change in chemical shift, i.e. a large Δ_D' value in order to obtain reliable thermodynamic parameters. This condition indeed was met for several resonances in both the octamer and nonamer strand of the (9+8)-mer. The satisfactory correspondence between experimental data and shifts calculated by means of the DUPSTAK model is shown in Fig. 2. The thermodynamic parameters obtained for the duplex $\rightleftarrows$ monomer transition are listed in Table 2. A remarkable similarity between all Tm_D values obtained is noted. However, the ΔS°_D values display a considerable spread. The mean values obtained for the two strands, Tm_D = 334.6 ± 0.4 K and ΔS°_D = -837 ± 12 $Jmol^{-1}K^{-1}$ for the nonamer part and Tm_D = 333.5 ± 0.3 K and ΔS°_D = -878 ± 11 $Jmol^{-1}K^{-1}$ for the octamer part appear in mutual good agreement. Moreover, the Tm_D value obtained for the methyl resonance of the extra dT in the nonamer corresponds with these values. This again indicates that the extra dT residue behaves like the other residues, in other words: dT stacks inside the intact duplex. It is of interest to note that the resonances of the neighbouring and opposite residues of dT(5), *viz* H8(6), H5(13) and H6(14) display quite similar thermodynamic parameters. From this we conclude that the duplex structure in the neighbourhood of the extra dT seems to be relatively undisturbed by the presence of the unpaired base.

Now let us compare the present data on the (9+8)-mer with predictions of stability for the corresponding (8+8)-mer duplex which lacks the extra dT. From the nearest neighbour thermodynamic data given by Breslauer *et al.* [29], one predicts for the (8+8)-mer duplex at 1 M NaCl: ΔH° = -293 $kJmol^{-1}$; this value appears remarkably similar to the ΔH° value of the (9+8)-mer (-289 $kJmol^{-1}$). This observation is not without precedent: Miller *et al.* [9] found a good agreement between ΔH° values of duplexes with and without an extra base.

We conclude that, despite the presence of an unpaired dT residue, a stable duplex is formed between d(CTGGTGCGG) and d(CCGCCCAG). The extra dT residue is stacked inside the stable duplex and does not significantly disturb duplex formation of its neighbours.

Exchangeable protons

Fig. 3 shows the low-field part of the ^1H NMR spectrum of the (9+8)-mer duplex measured in H_2O/D_2O 90/10 at pH = 7. The signal at 14.3 ppm corresponds well with the position usually found for imino-protons of dA•dT base pairs, and is therefore assigned to the only dA•dT present. Between

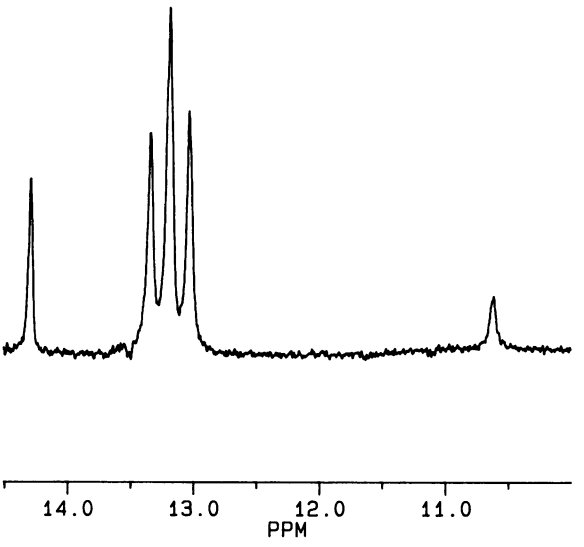


Figure 3. Imino-proton spectrum of the (9+8)-mer; 3.5 mM, 275 K, in H_2O/D_2O 90/10; shift reference DSS.

13.3 and 13.0 ppm several overlapping resonances are observed. The positions of these resonances are in line with those reported for G•C base pairs and are collectively ascribed to the seven dG•dC base pairs in the (9+8)-mer duplex. The most remarkable imino-proton resonance, however, is found at 10.6 ppm and is tentatively ascribed to the N_3H of dT(5). Unfortunately, the relatively high exchange rate with the solvent protons prevents detection of NOEs for this proton. Note that the "free" (non-hydrogen-bonded) imino-proton of a thymidine residue resonates at 11.2 ppm and displays a very broad signal at pH = 7 [30]. Thus, the observation of the N_3H resonance of dT(5) at neutral pH indicates that this imino-proton is protected against rapid exchange with the solvent. This finding strengthens our conclusion above that the unpaired dT is stacked in the interior of the duplex. Moreover, the upfield chemical shift of 0.6 ppm of the latter imino-proton signal relative to that of a "free" dT accords with some kind of stacking of the dT residue between its neighbouring dG residues.

^{31}P chemical shifts

In d(CGCAGAATTCGCG), a duplex containing an extra stacked adenosine, one ^{31}P resonance shifted downfield by 0.5 ppm from the main cluster of resonances was observed [3]. This resonance was ascribed to the extended phosphodiester linkage opposite the extra dA residue. A downfield shifted

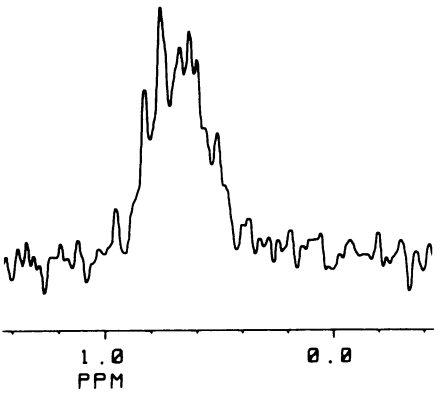


Figure 4. ^{31}P spectrum of the (9+8)-mer, 3.5 mM, 300 K. Shifts are relative to 3'5'-cAMP.

^{31}P resonance was also observed for the pentadecamer with an unpaired dA(11) residue, d(CGCGAAATTTACGCG), studied by Roy *et al.* [12]. Using a 2D ^{1}H - ^{31}P correlation technique, they assigned this particular resonance to the phosphorus nucleus located between the residues 12 and 13.

In contrast, in the (9+8)-mer under investigation all ^{31}P resonances are found clustered in a relatively narrow spectral region, 0.5 ppm (Fig. 4). This is taken to mean that no extended phosphodiester linkage, i.e. no t/g or g/t combinations for α/ζ are present in this duplex. Perhaps this difference in behaviour between a duplex containing an extra dA and a duplex with an extra dT residue can be accounted for by the difference in size between a purine base on the one hand and a pyrimidine base on the other hand. An extra dT base may be small enough to fit into an intact duplex without causing an extension of a phosphodiester linkage, whereas an extra dA fits less well.

Model building

Thus far, only a few models have been reported for a duplex with an extra base stacked inside the double helix [3,11]. Moreover, in these particular models the extra residue was represented by a purine (adenosine). As already mentioned above, effects like extension of a phosphodiester linkage are possibly related to the chemical nature of the extra base. Therefore, it appeared desirable to construct a model for the (9+8)-mer duplex which contains an extra pyrimidine. The features of this model should at least agree with the structural information obtained from the present NMR studies. The most important criteria which should be met are: (a) the extra

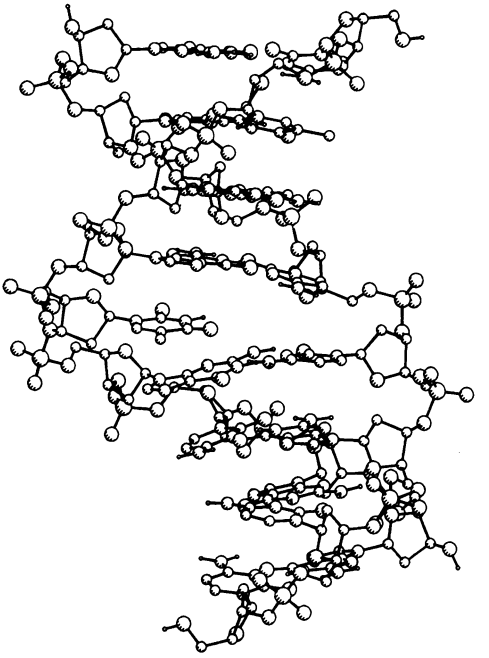


Figure 5. Energy-minimized structure of the (9+8)-mer duplex.

dT is stacked in an otherwise right-handed duplex; (b) all base pairs remain intact; (c) no t/g or g/t combinations of torsion angles around α/ζ are present.

A structure was generated with the aid of the program MacroModel. The model thus obtained was energy-minimized with the all-atom version of the molecular-mechanics program AMBER [19]. The energy-minimized structure, which fits all our experimental criteria, is shown in Fig. 5. The backbone torsion angles, sugar-ring pseudorotation parameters and glycosyl torsion angles are listed in Table 3. From both Fig. 5 and Table 3 it is clearly seen that this structure hardly deviates from a regular B DNA helix. The torsion angles in all residues of the (9+8)-mer appear within the ranges normally observed (i.e. S-type sugar, α^-, β^t, γ^+, ϵ^t, ζ^- and χ anti). Some internucleotide proton-proton distances between dT(5) and its neighbours, dG(4) and dG(6), are: H8(4)-CH_3(5) 3.6 Å; H1'(4)-CH_3(5) 4.3 Å; H6(5)-H2"(4) 2.3 Å; H8(6)-H2'(5) 3.2 Å and H8(6)-H2"(5) 2.2 Å. These distances are in reasonable agreement with the intensities of the observed NOEs, *vide supra*. In addition, all base pairs, including those neighbouring the unpaired dT,

Table 3. Torsion angles of the model of the (9+8)-mer duplex after energy-minimization (ϵ = 1/R). For notation see scheme I and [20,21].

Strand	Residue	α	β	γ	δ	ϵ	ζ	χ	P	ϕ_m
		degrees								
I	C(1)			61	135	189	269	-124	148	37
	T(2)	283	171	63	116	187	269	-125	123	44
	G(3)	286	165	65	118	188	247	-122	125	42
	G(4)	287	170	66	117	192	259	-148	126	31
	T(5)	286	165	68	114	185	265	-127	117	42
	G(6)	288	169	64	126	182	255	-115	138	39
	C(7)	291	169	68	130	191	266	-122	142	34
	G(8)	279	170	62	128	181	254	-117	138	40
	G(9)	291	179	57	140			-116	149	38
II	C(10)			61	135	191	272	-126	148	37
	C(11)	284	168	64	111	185	268	-128	117	42
	G(12)	290	169	62	128	186	250	-119	138	43
	C(13)	286	173	65	143	181	261	-119	155	42
	C(14)	286	184	59	137	185	257	-130	157	36
	C(15)	290	176	65	140	201	237	-117	155	37
	A(16)	282	169	64	136	183	269	-115	156	34
	G(17)	288	175	53	129			-117	134	42

remain intact. In summary, an extra dT seems to be incorporated easily in an intact B-DNA helix without disruption of base pairs or dramatic changes of torsion angles.

CONCLUSIONS

Previous studies on duplexes containing an unpaired base revealed that in case of an extra purine this base is stacked in the helix [3,6,10,12]; in case of an extra cytosine this base appears bulged-out [5]. The reason for this difference in behaviour was ascribed to the stronger stacking proclivity of purine residues compared to dC residues.

In the present study the duplex between d(CTGGTGCGG)·d(CCGCCCAG), which contains an extra dT, was investigated. Surprisingly, all data from NMR studies (e.g. NOEs, chemical-shift profiles and imino-proton resonances) indicate that the extra dT residue is stacked in the duplex. For the insertion of this unpaired dT in the duplex no major changes of the B-DNA

duplex appear necessary; all base pairs remain intact and no important deviations from normal B-DNA torsion angles are present. This finding remains in contrast to the suggestion of an extended phosphodiester linkage in case of an extra intrahelical adenosine [3,12].

It seems hard to imagine that the difference in behaviour between an unpaired dT and an unpaired dC rests upon a difference in stacking proclivity between these pyrimidines. Perhaps, the behaviour of a particular unpaired residue is related somehow to local structural characteristics and base sequence of the duplex. Further work appears necessary in order to settle these important points.

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* to whom correspondence should be addressed.

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2. Abbreviations used: Me_4NCl, tetramethylammoniumchloride; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulphonate; NOESY, two-dimensional nuclear Overhauser spectroscopy; COSY, correlated spectroscopy.
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