Effect of Distortions in the Phosphate Backbone Conformation of Six Related Octanucleotide Duplexes on CD and ^{31}P NMR Spectra†

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ABSTRACT: We examined the structural properties of six octanucleotide duplexes, d(TGACGTCA), d(ACTGCAGT), d(CTTCGAAG), d(CATCGATG), d(GTACGTAC), and d(CATGCATG). Circular dichroism (CD) and 2D ^{31}P and ^{1}H NMR spectroscopies were used in conjunction. Although of the B-DNA type, it was possible to arrange CD spectra into two families, A and B. Family A resembled poly(dG–dC) with a positive signal at ~280 nm and a negative one at ~260 nm, while family B resembled poly(dA–dT) with a positive signal at ~270 nm and a negative one at ~250 nm. All ^{31}P resonances were assigned through constant-time heteronuclear ^{31}P–^{1}H correlated spectra. J(H3′–P) coupling constants related to dihedral angles ϵ (C4′–C3′–O3′–P) were determined from ^{1}H–^{31}P J-resolved selective proton-flip 2D experiments. A good correlation was observed between ^{31}P chemical shifts and coupling constants for all oligonucleotides. The patterns of these two parameters *vs* the base position along the sequences were almost similar. They were confronted with CD spectra. The results indicated that the position and magnitude of the signals were mainly affected by the CpG and ApT steps whose ^{31}P chemical shifts were the farthest away from the mean ^{31}P chemical shift value. This is in keeping with greater rigidity at these steps and should explain the influence of the local order on the shape of the CD spectra. Lastly, both UV absorption and ^{31}P chemical shifts *vs* temperature provided normal temperature melting (T_m) values for all of the octanucleotide duplexes except for d(CTTCGAAG), for which the T_m was ~10 °C lower compared to its counterpart d(CATCGATG). The decrease in the thermal stability of this octanucleotide duplex was imputed to its contained TT and AA repeats, which might be able to induce correlated base destacking and phosphate group distortion in the oligonucleotide and especially on the intermediate CpG. We demonstrate that the CpG step displayed ^{31}P NMR properties similar to those found in mismatched nucleotides exclusively in the d(CTTCGAAG) duplex.

Oligonucleotides have proven to be useful models for the analysis of structural and biological features found in natural DNAs (Saenger, 1984). On account of their functionality and complexity, they must be analyzed from as many points of view as possible: conformation (Dickerson & Drew, 1981; Calladine, 1982; Saenger, 1984; Wüthrich, 1986; Mauffret *et al.*, 1992) dynamics (Nilsson *et al.*, 1986; Ravishanker *et al.*, 1989; Nikonowicz *et al.*, 1990; Rao & Kollman, 1990), mechanism of melting (Breslauer *et al.*, 1986; Roongta *et al.*, 1990; Naristin *et al.*, 1991), interactions with proteins (Gross & Garrard, 1988; Travers, 1989; Freemont, 1991; Parraga & Klevit, 1991) and with small ligands (Monnot *et al.*, 1991; Mauffret *et al.*, 1991; Monnot *et al.*, 1992), as well as sensitivity to degradation by endonucleases (Martin & Schleif, 1986; McClarin *et al.*, 1986). Any type of variation due to substitution is useful, for it can improve our understanding of the precise origin of the effects which govern the recognition of DNA domains by proteins and other DNA ligands (Neidle & Abraham, 1984; Storms *et al.*, 1991).

A wide variety of structures that exhibit considerable backbone flexibility and local sequence dependence in DNA conformations have been revealed by X-ray crystallography (Dickerson, 1983; Saenger, 1984; Privé *et al.*, 1991; Yanagi *et al.*, 1991) and NMR (Feigon *et al.*, 1983; Frechet *et al.*, 1983; Hare *et al.*, 1983; Broido *et al.*, 1984; James, 1984; Kearns, 1984; Scheeck *et al.*, 1984; Wüthrich, 1986; Schroeder *et al.*, 1987; Patel *et al.*, 1987; Van de Ven & Hilbers, 1988; Gorenstein *et al.*, 1988), although crystal results are not always in good agreement with solution-state results (Assa-Munt & Kearns, 1984; Clore *et al.*, 1985; Rinkel *et al.*, 1987; Patel *et al.*, 1987; Gorenstein *et al.*, 1988; Nikonowicz *et al.*, 1989; Roongta *et al.*, 1990). In recent years, emphasis has mostly been placed upon the use of two-dimensional (2D) ^{1}H NMR methods to extract structural information about oligonucleotides in solution (Wüthrich, 1986; Nikonowicz & Gorenstein, 1990; Robinson & Wang, 1992; Ulyanov *et al.*, 1992). Only now it is clear that the structural information provided by NOESY[1] distances alone is insufficient and that more data from the backbone and the local base order are required to describe the entire conformation of the double helix (Gronenborn & Clore, 1989; Baleja *et al.*, 1990a,b; Metzler *et al.*, 1990; Nikonowicz & Gorenstein, 1990; Ulyanov *et al.*, 1992; Mauffret *et al.*, 1992).

Our laboratory has contributed to a wider use of circular dichroism (CD), a method capable of providing information on the global conformation of DNA fragments and also, to

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; COSY, 2D homonuclear shift correlated spectroscopy; NOESY, 2D homonuclear NOE correlated spectroscopy; TOCSY, total correlated spectroscopy; COLOC, X–^{1}H shift correlation by long-range coupling with ^{1}H–^{1}H decoupling; CRE, cAMP responsive element; EDTA, ethylenediaminetetraacetic acid; TMP, trimethyl phosphate.

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a certain extent, on sequence-dependent local structures (Mauffret *et al.*, 1989, 1991; Monnot *et al.*, 1992). We have noted that chain reversal in autocomplementary octanucleotide duplexes entails a drastic change causing the adoption of a completely different CD spectrum with differences in the magnitudes and positions of signals (Mauffret *et al.*, 1989; Monnot *et al.*, 1992). Confronted with ^{1}H NMR data, CD results suggest that there is a relationship between the base sequence and the local conformation, as expressed by the individual deoxyribose ring structures, although the global conformation of the octanucleotides continues to be of the B type (Saenger, 1984; Rinkel & Altona, 1987; Mauffret *et al.*, 1989).

In this article, we used CD in conjunction with 2D ^{1}H/^{31}P NMR techniques to conduct a comparative study of the conformations of six self-complementary octanucleotide duplexes in solution. Our objective was to identify complementary aspects of the two approaches. The octanucleotides either correspond to a promoter domain, such as the cAMP responsive element (CRE) which is specifically recognized by transcriptional factors controlled by cAMP (Montminy *et al.*, 1986; Ziff, 1990), or contain sites for restriction endonucleases (Kessler *et al.*, 1985; Roberts & Macelis, 1991). The CpG step is to be found in the center of four of them. It has revealed peculiar properties such as high malleability and base destacking from X-ray crystallographical studies (Yanagi *et al.*, 1991; Grzeskowiak *et al.*, 1991). Moreover, CpG is a hot spot for mutation in genes and accounts for about 25–45% of known point mutations which lead to human genetic disorders or cancers (Youssoufian *et al.*, 1986; Steinberg & Gorman, 1992).

With the recent introduction of procedures to assign individual ^{31}P resonances and measure P–H3′ coupling constants of oligonucleotides, we are now able to give a better description of local structures through the analysis of the phosphodiester backbone conformation, since both the ^{31}P chemical shifts and the P–H3′ coupling constants are capable of providing detailed information on such a structural analysis (Gorenstein, 1984; Schroeder *et al.*, 1989; Karslake *et al.*, 1990; Roongta *et al.*, 1990; Mauffret *et al.*, 1992). We came to the conclusion that CD, which is more appreciated for its ability to provide information on the global structure of macromolecules, is also a useful tool for the analysis of oligonucleotide fine structure (Ivanov *et al.*, 1973; Cech & Tinoco, 1977; Baase & Johnson, 1979; Chan *et al.*, 1979; Cantor & Schimmel, 1980; Johnson *et al.*, 1981; Rizzo & Schellmann, 1984; Mauffret *et al.*, 1989; Monnot *et al.*, 1992).

EXPERIMENTAL PROCEDURES

Synthesis. The self-complementary octamer duplexes, d(TGACGTCA)$_2$ (OC1), d(ACTGCAGT)$_2$ (OC2), d(CTTCGAAG)$_2$ (OC3), d(CATCGATG)$_2$ (OC4), d(GTACGTAC)$_2$ (OC5), and d(CATGCATG)$_2$ (OC6), were synthesized using the solid-phase procedure on an Applied Biosystems 381 B automated apparatus. The resulting oligonucleotides were purified by reversed-phase HPLC on a DEAE column, followed by dialysis and lyophilization. Oligonucleotide concentrations were calculated using the following extinction coefficients (M^{-1} cm^{-1}/base at 260 nm): 8400 for OC1, OC2, and OC6; 8500 for OC3; 8200 for OC4, and 8700 for OC5 (Fasman, 1975).

UV Absorption and Circular Dichroism. Each oligonucleotide was dissolved at ~3 μM duplex concentration in a phosphate buffer containing 0.2 mM EDTA at pH 7 and ionic strength I = 0.1. UV absorption spectra and UV absorption melting profiles were recorded using a Uvikon 860 spectrophotometer. CD spectra between 200 and 320 nm were measured with a Jobin-Yvon Mark IV high-sensitivity dichrograph linked to a Digital Minc-11 miniprocessor, using a cell 1 cm in path length. The temperature of the samples was kept at ±0.1 °C during each spectral measurement using a thermostated cell holder linked to a Cole-Palmer thermistor.

Table I: Cleavage Sites on the Six Selected Octanucleotide Duplexes and the Corresponding Restriction Endonucleases

oligonucleotides	restriction endonucleases
TGA[1]/CGT[2]/CA (OC1)	[1]*Aat*II, *Aha*II; [2]*Mae*II
ACTGCA/GT (OC2)	*Pst*I
CTT[1]/C[2]/GAAG (OC3)	[1]*Taq*1, *Sfu*1, *Bst*BI; [2]*Fsp*II
CAT/CGATG (OC4)	*Taq*1, *Cla*I, *Tth*HhB8I
GT[2]/AC[1]/GT[2]/AC (OC5)	[1]*Sna*BI; [2]*Rsa*I
CATG[1]/CA[2]/TG (OC6)	[1]*Nla*III; [2]*Nsi*I

NMR. Each oligonucleotide was dissolved in 0.4 mL of phosphate buffer (the same as that used for CD) at ~3 mM duplex concentration. Samples were repeatedly lyophilized in increasing grades of D_2O and finally taken up in 0.4 mL of 99.99% D_2O.

COSY, TOCSY, and NOESY spectra used for the assignment of nonexchangeable protons of octanucleotides were recorded on a Bruker AM-500 NMR spectrometer. The 1D ^{31}P NMR spectra, ^{31}P melting profiles, 2D heteronuclear ^{1}H/^{31}P constant-time correlation (COLOC) (Kessler *et al.*, 1984) spectra, and 2D J-resolved spectra were recorded on a Bruker MSL-300 spectrometer. The ^{31}P spectra were referenced to external trimethyl phosphate (TMP) at 0 ppm. The Kessler–Griesinger long-range heteronuclear correlation (COLOC) experiments (Kessler *et al.*, 1984) were performed on the octamers at temperatures ranging from 20 to 80 °C. Data were collected with 1024 points in the ^{31}P dimension and 50 t_1 increments and zero-filled to 1024 × 512. The data sets were multiplied by a combination of an increasing exponential and a Gaussian function before Fourier transformation in both dimensions.

The Bax–Freeman selective 2D J-resolved correlation experiment with a "soft" pulse for the selective 180° pulse (Sklenar & Bax, 1987; Live & Greene, 1989) was performed in order to measure the ^{31}P–H3′ coupling constant in the octanucleotides. The data sets were collected with 1024 points in the ^{31}P dimension and 32 t_1 increments and then zero-filled to 1024 × 128. Gaussian-type resolution enhancements were applied before Fourier transformation. The final resolution in the F1 dimension was 0.3 Hz/point, and J values were measured from peak center to peak center. The measured three-bond coupling constants were then used through a proton–phosphorus Karplus-type relationship to extract the H3′–C3′–O–P torsional angle θ related to the C4′–C3′–O–P torsional angle ε. The selected relationship was as follows: $J = 15.3 \cos^2 \theta - 6.1 \cos \theta + 1.6$ (Lankhorst *et al.*, 1984).

RESULTS AND DISCUSSION

Choice of Oligonucleotides. The six self-complementary duplexes selected for this study are listed in Table I. The cleaved sites and corresponding restriction endonucleases are also indicated. It will be noted that all of the octanucleotide strands possess twice the same nucleotide A, C, G, and T. The oligonucleotides OC1, the cAMP responsive element (CRE) (Montminy *et al.*, 1988; Ziff, 1990), and OC2 present inverse sequences. The same is true for OC5 and OC6, while OC3 and OC4 are related by the simple permutation of their residues 2 and 7. Four oligonucleotides, OC1, OC3, OC4, and OC5,

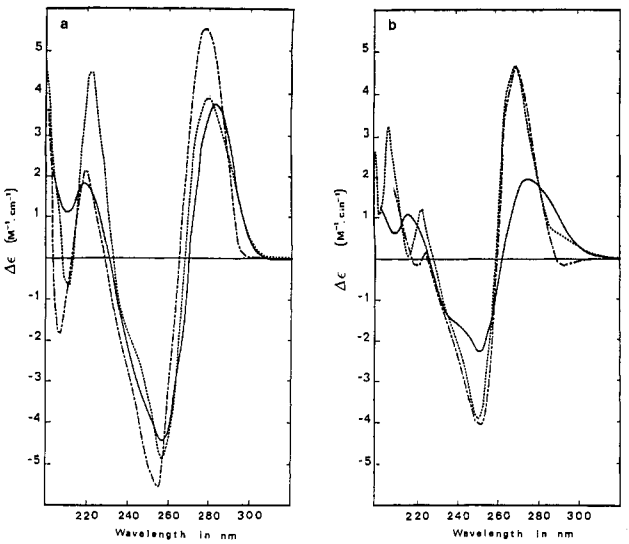

FIGURE 1: CD spectra in the far UV of the six octanucleotides classified in two families: (a) OC1 (—), OC3 (· – ·), OC5 (···); (b) OC2 (—), OC4 (· – ·), OC6 (···).

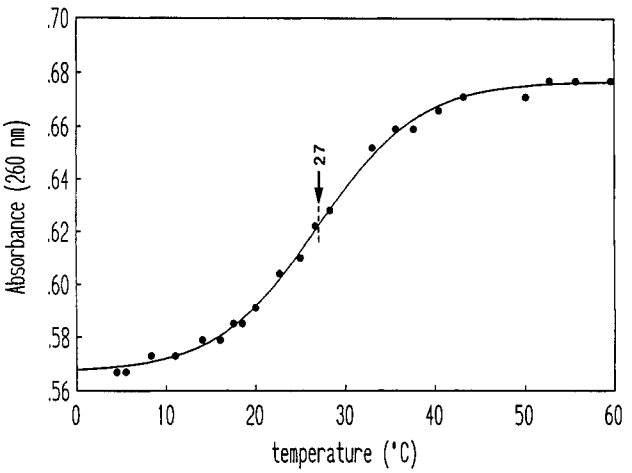

FIGURE 2: One representative UV melting curve (OC5) recorded from the absorption signals at 260 nm. T_m's of the other nucleotides are as follows: OC1, 31 °C; OC2, 29 °C; OC3, 19 °C; OC4, 32 °C; OC6, 25 °C.

have the CpG step in their center, whereas OC2 and OC6 have an inverted version of this step, GpC. Two oligonucleotides, OC1 and OC5, display the tetrad ACGT in their center, and two others, OC2 and OC6, display an inverted version TGCA, while two oligonucleotides OC3 and OC4 contain the TCGA tetrad. Moreover, each of the octanucleotide duplexes displays at least one site for restriction endonucleases (Roberts & Macelis, 1991).

With these six octamer duplexes, we hope to dispose of a palette that will enable us to accurately evaluate the positional and sequential effects in oligonucleotides by CD/NMR. Special emphasis will, however, be placed on the behavior of the CpG step, which has in many instances revealed very peculiar biological (Youssoufian *et al.*, 1986; Steinberg & Gorman, 1992) and structural (Grzeskowiak *et al.*, 1991) properties.

Circular Dichroism. (1) Shape of CD Spectra in the Far UV. Figure 1a,b shows the CD spectra in the nearest UV obtained for the six octanucleotides at a temperature of 5 °C. In all of them is noted a main positive signal pointing between 265 and 285 nm and a main negative signal pointing between 250 and 260 nm (Cantor & Schimmel, 1980; Saenger, 1984). The conservative nature of the positive and negative signals suggests an exciton splitting due to preferred orientations in neighboring base pairs similar to those found in a helix with a dominantly B-type conformation (Cech & Tinoco, 1977; Cantor & Schimmel, 1980; Johnson *et al.*, 1981; Rizzo & Schellmann, 1984). Noteworthy is the existence of more or less subtle differences in the CD spectra visible in the position, shape, and intensity of the two main signals. As the six oligonucleotides have the same base composition (Table I), we may assume that these differences are due to the dependence of conformations upon sequence, which generates local order variations in the helix.

(2) The Two Families of CD Spectra of the Six Octanucleotides. Several authors have observed that the positive signals are more reliable than their counterparts for tracking down conformational variations in DNA (Cantor & Schimmel, 1980; Johnson *et al.*, 1981; Monnot *et al.*, 1992). Johnson *et al.* (1981) have demonstrated that the electronic properties of bases expressed in the positive signal are related to their stacking and spatial organization. The magnitude and the exact position of the positive bands were then used to distinguish among the various DNA forms and to estimate the helical twist (Johnson *et al.*, 1981). However, to our knowledge, no attempt has been made until now to detect DNA local structure in relation to CD parameters and to determine to what extent an ordered pattern exists in oligonucleotides.

The CD spectra of the six oligonucleotides under study (Figure 1a,b) can be roughly divided into two families, hereafter termed A and B, according to the positions of their positive bands. In family A are placed the spectra of OC1, OC3, and OC5, whose maxima for the positive band are found at ~280 nm; family B includes spectra from oligomers OC2, OC4, and OC6, whose maxima for the positive band are located at ~270 nm, i.e., at a systematically lower wavelength compared to family A. Curiously, the CD spectra resemble those of either poly(dG–dC) (family A) or poly(dA–dT) (family B) genus of structures (Fasman, 1975; Cantor & Schimmel, 1980), although as already mentioned above the six oligonucleotides have the same base composition and vary only in the nature of their steps. We note however that within each family the intensity of the positive signal may vary consistently from one oligonucleotide to another: OC3 > OC1 ≈ OC5 for family A; OC6 ≈ OC4 ≫ OC2 for family B. We will see in the following that the influence of steps as CD contributors can be better understood in the light of the ^{31}P NMR results.

UV Melting Curves. The UV absorption has been used initially to follow melting and to determine T_m's in polynucleotides and DNAs (Cantor & Schimmel, 1980). Progress of the chemical synthesis of DNA has allowed the solution of thermostability problems on short oligonucleotide duplexes with known pecularities in the sequences. Most of the data has then been used to collect parameters linked to the structural effects of various base pairs, including nonstandard ones (Marky *et al.*, 1983; Marky & Breslauer, 1987; Naristin & Lyubchenko, 1991).

The UV melting curves for oligonucleotides dissolved at ~3 μM duplex concentration are presented in Figure 2. The T_m values determined from the corresponding derivative curves lie between 19 (OC3) and 32 °C (OC4), although most of the values are confined to the area between 25 and 32 °C. For similar duplex concentrations, the theoretical T_m's of oligonucleotides, which are obtained by using the nearest neighbor thermodynamic parameters established by Breslauer *et al.* (1986), range from 28 to 30 °C. Thus, the agreement between the experimental and calculated values appears good, except

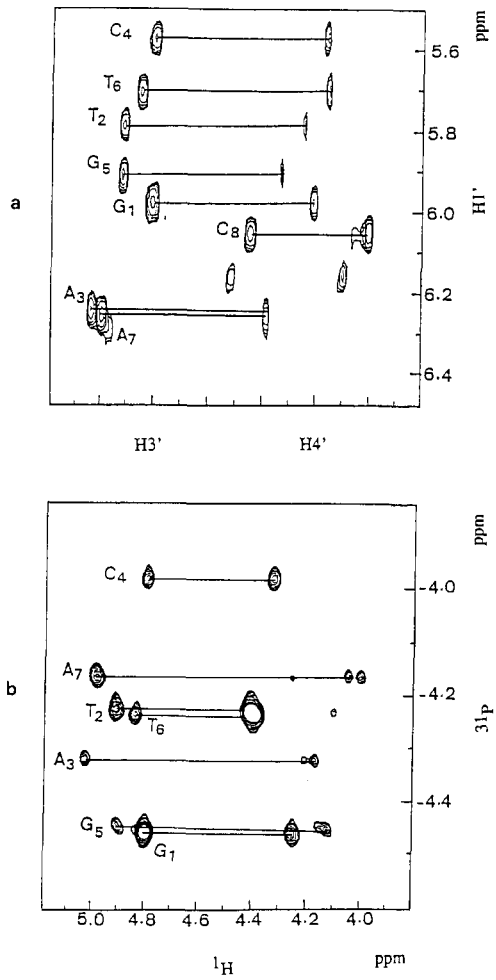

FIGURE 3: Spectral analysis for OC5. (a) Example of TOCSY spectrum corresponding to connectivities of the sugar proton systems H1′–H4′ and H1′–H3′. (b) Example of COLOC spectrum allowing the assignment of ^{31}P resonances through their connectivities to sugar H3′ and H4′ protons.

for OC3 whose experimental T_m appears atypically low for an octamer duplex at ~3 μM concentration. Especially striking is the difference between the T_m of OC3 (~19 °C) and that of OC4 (~32 °C) since OC3 and OC4 differ by the simple permutation of T2 with A7, and this difference persists for UV–temperature experiments performed at higher oligonucleotide concentrations (not shown). We will see in the forthcoming section that the melting curves provided by ^{31}P NMR experiments at the high ~3 mM duplex concentration lead to the same conclusions (Figure 8). We thus suspect a particular destabilizing influence in the OC3 duplex of the TT and AA repeats flanking the CpG step. Such repeats, although small, have been studied for their potential to act as destabilizing elements of the B-DNA helix, especially by inducing less favorable stacking interactions within their own bases and those adjacent in the sequence (Kawase *et al.*, 1986; Crothers *et al.*, 1990; Taylor *et al.*, 1990; Balendiran & Sundaralingam, 1991; Trifonov, 1991; Zieba *et al.*, 1991).

NMR. (1) Assignment of H3′ and H4′ Proton and ^{31}P Chemical Shifts of the Six Octamers. The nonexchangeable proton signals of the six octanucleotide duplexes are assigned through the analysis of COSY and NOESY spectra *via* a sequential assignment methodology (Wüthrich, 1986). The connectivities of the entire intrasugar proton systems, H1′–H4′, are obtained in the TOCSY spectra. Figure 3a illustrates such an assignment for protons H3′ and H4′ *vs* H1′ in case of OC5. For the NOE spectra of the six octamers, the

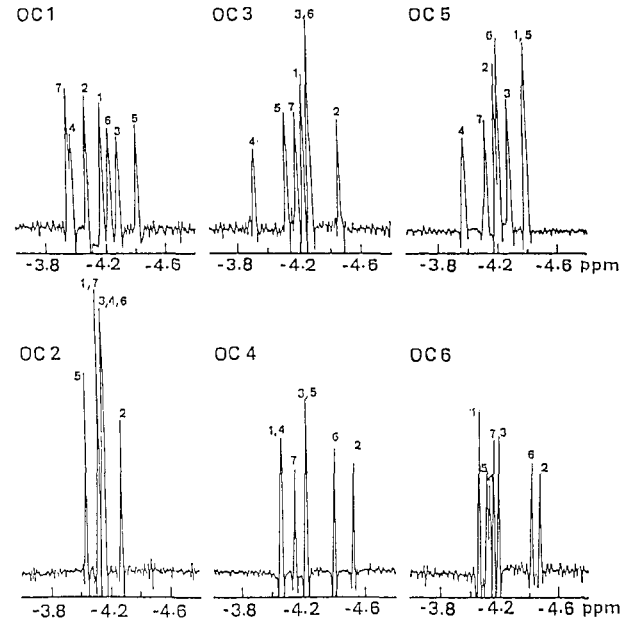

FIGURE 4: Comparison of the six octanucleotide ^{31}P NMR spectra recorded at 300 MHz (^{1}H). Assignments are indicated by numbering phosphates from the 5′ end to the 3′ end of the duplex sequences. Chemical shifts are referenced to TMP (trimethyl phosphate).

assignment pathway follows that expected for a right-handed DNA duplex where the H8/H6 base protons lead to characteristic cross peaks with their own and the $i - 1$ residue deoxyribose ring protons (Wüthrich, 1986). With this procedure, the assignment of the sugar ring protons H1′, H2′, and H2″ and H3′ and H4′ is straightforward for the six octamers.

The characterization of protons H3′ and H4′ is a prerequisite for the assignment of the ^{31}P resonances. The latter is achieved *via* a long-range heteronuclear correlation experiment termed COLOC (Kessler *et al.*, 1984), where cross peaks are obtained between the ^{31}P and the H3′ and H4′ resonances. The COLOC and TOCSY spectra are shown together in Figure 3a,b in order to illustrate the interest of their joint use for ^{31}P assignments. The ^{31}P signals of the six octamer spectra shown in Figure 4 are assigned according to the COLOC procedure, and Table II lists the corresponding ^{31}P chemical shifts measured at 30 °C.

(2) Effects Responsible for the ^{31}P Chemical Shifts. There is still a need for more data on chemical shifts which can be correlated to specific local and also long-range structural changes within the double helix, although it can already be concluded that spectral dispersion generally reflects the combined effects of the nucleotide position (relative to 5′ and 3′ extremities) and sequence on conformation. Most of the effects reported earlier are visualized in the plots of the ^{31}P chemical shift *vs* phosphorus position in the six sequences reported in Figure 5, in which the mean chemical shift (ca. ~4.2 ppm) determined from the 42 available chemical shift values is also indicated. In fact, this one changes only a little when the chemical shift values for residues at the 5′ and 3′ ends are excluded from calculations. Noteworthy is the dispersion of values, which strongly depends upon the sequence, *i.e.*, the constitutive Pu–Py steps of the octamer considered. For the four possible steps we find the following chemical shift gradation: Pu–Py (AC, AT, GC, GT) –4.30 ppm ≥ Py–Py (TT, CT, TC) –4.28 ppm > Pu–Pu (AA, AG, GA) –4.17 ppm > Py–Pu (CA, CG, TA, TG) –4.08 ppm. The hierarchy remains unchanged when the data for the 5′ and 3′ residues are not taken into account in the calculation of the above mean values.

Table II: ^{31}P Chemical Shifts, J(H3′–P) Coupling Constants, and ϵ and ζ Torsion Angle Values of the Six Octanucleotides at 30 °C

		δ (ppm)[a]	J (Hz)[b]	ϵ (deg)[c]	ζ (deg)[d]
OC1	TpG	−4.17	3.00	−176	−101
	GpA	−4.07	5.00	−165	−114
	ApC	−4.28	3.75	−171	−106
	CpG	−3.97	5.60	−162	−118
	GpT	−4.40	4.00	−170	−108
	TpC	−4.22	5.00	−165	−114
	CpA	−3.95	6.25	−158	−122
OC2	ApC	−4.16	3.90	−171	−106
	CpT	−4.26	4.70	−166	−112
	TpG	−4.13	3.90	−171	−106
	GpC	−4.14	3.90	−171	−106
	CpA	−4.00	4.80	−166	−113
	ApG	−4.16	3.90	−171	−106
	GpT	−4.11	3.90	−171	−106
OC3	CpT	−4.24	4.40	−168	−111
	TpT	−4.46	3.90	−171	−107
	TpC	−4.26	3.90	−171	−107
	CpG	−3.92	5.5	−162	−118
	GpA	−4.12	4.10	−169	−109
	ApA	−4.26	3.90	−171	−107
	ApG	−4.18	3.90	−171	−107
OC4	CpA	−4.06	6.25	−158	−122
	ApT	−4.51	3.10	−175	−102
	TpC	−4.21	4.30	−168	−110
	CpG	−4.05	6.25	−158	−122
	GpA	−4.21	4.30	−168	−110
	ApT	−4.39	3.90	−171	−107
	TpG	−4.14	5.10	−164	−115
OC5	GpT	−4.40	4.00	−170	−108
	TpA	−4.19	4.10	−169	−109
	ApC	−4.28	4.10	−169	−109
	CpG	−3.98	6.05	−159	−121
	GpT	−4.40	4.30	−168	−110
	TpA	−4.21	4.30	−168	−110
	ApC	−4.13	5.10	−164	−115
OC6	CpA	−4.05	5.30	−163	−116
	ApT	−4.47	3.50	−173	−104
	TpG	−4.18	4.90	−165	−114
	GpC	−4.15	4.70	−166	−112
	CpA	−4.10	4.90	−165	−114
	ApT	−4.41	3.90	−171	−107
	TpG	−4.12	4.70	−166	−112

[a] ^{31}P chemical shifts are referenced to TMP (trimethyl phosphate). [b] Estimated error is ±0.3 Hz. [c] The torsional angle ϵ is given by the relationships of Lankhorst *et al.* (1984). Only the conformationally most probable value is presented. [d] The torsional angle ζ is given by the relation of Dickerson (1983).

Comparison of oligonucleotides shows additional more or less subtle effects. Obviously, the strongest effects are caused by the switch of OC1 and OC5 sequences into their inverse sequences OC2 and OC6, respectively. Yet, the double permutation G1 with T2 and A7 with C8 at each end of OC5, leading to OC1, does not disturb the resonance of the innermost CpG (−3.98 *vs* −3.97 ppm), while more individual modifications, such as the permutation of T2 with A7 switching OC3 into OC4, affect the central CpG pattern through long-range effects (−3.92 *vs* −4.05 ppm). We may note that the CpG resonance of OC3 has the most downfield shift within all of the series, although the difference of its chemical shift from that of OC1 does not exceed 0.05 ppm.

The weak dispersion of ^{31}P chemical shifts displayed by OC2 appears striking, especially in comparison with that of OC6 which contains the same central TGCA tetrad. Both oligonucleotides differ through the sole permutation of one residue at both the 5′ and 3′ ends (residue 1 with residue 2 and residue 7 with residue 8). However, unlike OC2, OC6 contains two ApT steps at positions 2 and 6 of its sequence, and in such Pu–T steps, the phosphorus resonance is found to be strongly upfield shifted from the mean value. Illustrative examples are ApT at positions 2 and 6 of OC4 (−4.51 and −4.39 ppm, respectively) and of OC6 (−4.47 and −4.41 ppm, respectively) and also GpT at position 5 of OC1 and OC5 (−4.40 ppm). The peculiar influence of T is also observed in the Py–Py TpT repeat of OC3 (−4.46 ppm), but not in the other Py–Py CpT step (−4.29 ppm in OC2). We also note that, among the three T-containing steps, ApT, TpT (ApA), and TpA (each formed of two A·T base pairs), it is the TpA step that exhibits the smallest deviation from the mean ^{31}P chemical shift (−4.19 and −4.21 ppm for positions 2 and 6, respectively, of OC5), while its reverse version ApT shows the largest deviation.

In summary, the phosphorus resonance of the Py–Pu CpG step is found to be the most downfield shifted in the present series, while in contrast, the most upfield chemical shifts are found for the Pu–T steps with, however, ApT > GpT. Regardless of what is Pu at 5′, in a step T induces a stronger upfield effect than C (Pu–T ≫ ApC ≫ GpC); this effect is also found for the Py–Py TpT repeat but not for the CpT step, a feature which, once again, suggests a particular structure for the TpT repeat.

(*3*) *J-Resolved NMR.* The dihedral angle ϵ (C4′–C3′–O–P) can be obtained from the ^{31}P–H3′ coupling constant, which is accessible through a J-resolved experiment (Figure 6). Here, we apply the heteronuclear proton-flip experiment (Bax & Freeman, 1982) adapted to J(H3′–P) couplings (Sklenar & Bax, 1987), without reverse detection. The experiment is repeated at several temperatures between 20 and 70 °C. Above the latter temperature, once the strands are separated there is only little variation in the coupling constants, as is also the case for the chemical shifts.

The plots of J(H3′–P) coupling constant values measured at 30 °C *vs* the position of phosphorus along the sequence are presented in Figure 5; these can then be compared to the plots of ^{31}P chemical shifts also given in Figure 5. Overall, the quality of the correlation between the two NMR parameters confirms the sensitivity of the ^{31}P chemical shifts to the ϵ angle changes and thus their suitability for the conformational study of oligonucleotides (Gorenstein *et al.*, 1988; Nikonowicz *et al.*, 1990; Roongta *et al.*, 1990). Table II presents those coupling constant values measured at 30 °C for the six oligomers. The mean coupling constant determined from the 42 measured coupling constant values indicated in Table II is 4.48 Hz, while for the four possible Pu and Py combinations the mean values are as follows: Py–Pu, 5.05 Hz > Py–Py, 4.37 Hz > Pu–Pu, 4.18 Hz > Pu–Py, 4.00 Hz. As observed above for the chemical shifts, the mean coupling constant values are only weakly modified by the withdrawal of the data corresponding to the 5′ and 3′ end residues from calculations. However, it can be noted that the chemical shifts and coupling constants do not yield the same hierarchies as the positions of the Py–Py and Pu–Pu steps are found to be reversed, despite the observation that these two parameters appear linearly linked (not shown). Probably, more samples are required to know whether this apparent discrepancy is due to the number of collected data being insufficient to afford a statistical value (there are 6 Py–Py and 6 Pu–Pu steps *vs* 14 Pu–Py and 16 Py–Pu steps) or due to conformational reasons; the influence of the ζ and α angles and even of the O–P–O bond angle upon ^{31}P chemical shifts is larger compared to that of the ϵ angle in the two Py–Py and Pu–Pu steps.

The values of ϵ are derived from the proton–phosphorus Karplus relationship of Lankhorst *et al.* (1984). Due to the

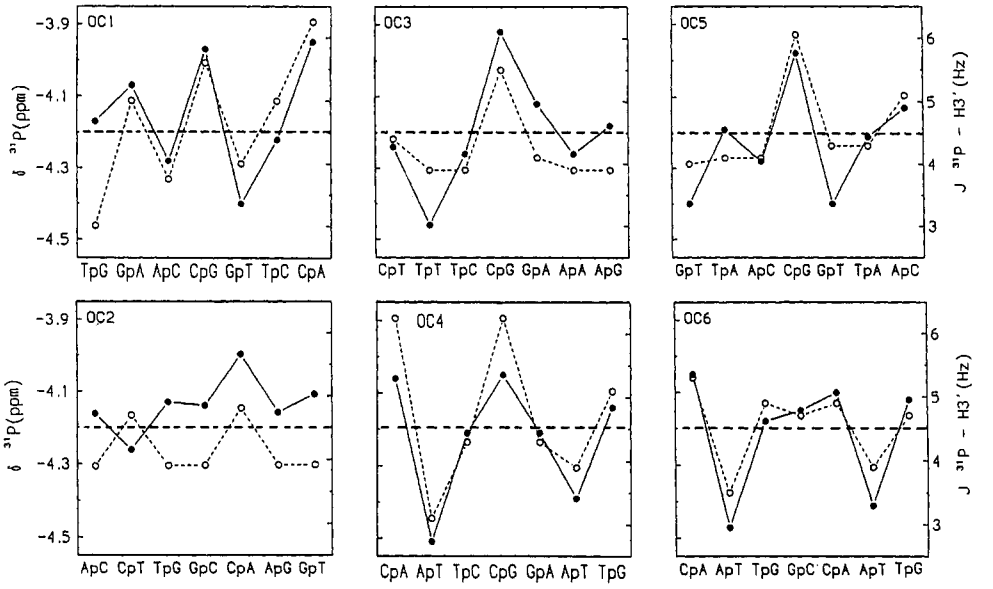

FIGURE 5: Plots of the ^{31}P chemical shifts (—) and J(H3′–^{31}P) coupling constants (- - -) *vs* phosphorus position along the 5′ to 3′ direction for the six duplexes. Values concern experiments performed at 30 °C. The mean chemical shift values (ca. –4.2 ppm) is also indicated (– –)

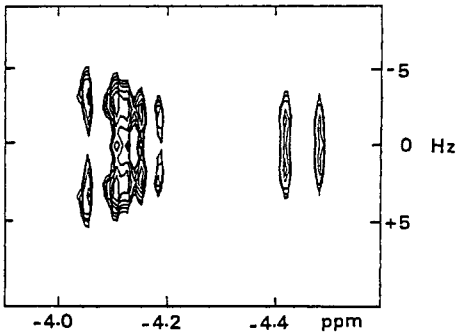

FIGURE 6: Example of 2D J-resolved $^{31}P/^{1}H$ spectrum of OC6 allowing determination of J(H3′–P) coupling constants related to the ϵ torsion angle.

shape of this curve, a given coupling constant provides four different ϵ angles between 0° and 360°. For the values presented in Table II, we assume, as did Nikonowicz *et al.* (1990), that the most common crystallographical ϵ value, –169° (Saenger, 1984), is the correct one. It is noteworthy that the mean coupling constant value of 4.50 Hz found in this study fits well with this crystallographical ϵ value within the curve of Lankhorst *et al.* (1984), reported by Nikonowicz *et al.* (1990). The same occurs when the mean chemical shift value (–4.2 ppm) is used within the curve where ϵ is given *vs* the chemical shift (Nikonowicz *et al.*, 1990).

As found by Dickerson and Drew (1981) and Dickerson (1983) for crystals, and then used extensively by Nikonowicz and Gorenstein (1990) for solutions, ϵ (C4′–C3′–O–P) and ζ (C3′–O3′–P–O5′) are related by the equation: $\zeta = -317 - 1.23\epsilon$. The values so obtained are given in Table II as well. For crystals, in the most common backbone structure, termed B_I, the ϵ conformation is *t* (*trans*, ca. ±180°) and the ζ conformation is g^- (*gauche*$^-$, ca. –60°), while in another common structure, termed B_{II}, the values of ϵ and ζ are reversed (g^- and *t*, respectively) (Dickerson & Drew, 1981; Dickerson, 1983). In solution, according to Nikonowicz and Gorenstein (1990) there is a rapid "crankshaft" motion that interconverts B_I and B_{II}. The ^{31}P chemical shift and ^{31}P–H3′ coupling constant values might then largely depend upon this backbone property, although other possibilities cannot be discarded.

^{31}P Melting Curves. Some illustrative examples of ^{31}P spectra recording at increasing temperatures are given in Figure 7. The temperature dependence curves derived for the six oligonucleotide chemical shifts are given in Figure 8. With increasing temperature we observe a global downfield shift of the resonances, except in one case where the resonance follows an upfield shift in the temperature range ~35–55 °C. Such an effect is not unique since it has already been reported (Nikonowicz *et al.*, 1989; Nikonowicz & Gorenstein, 1990; Roongta *et al.*, 1990) for phosphate groups involved in mismatch structures. The melting curves obtained from the six octamers have various shapes and magnitudes, and the T_m's measured from the inflection points are thus not very precise. Yet, they clearly indicate differences between the thermal stabilities of the various duplexes. A noteworthy fact is the persistence at the NMR concentration (3 mM *vs* 3 μM for UV absorption) of a large difference between the T_m's of OC3 and OC4 (~45 *vs* ~55 °C). It is the CpG step of OC3 which at 30 °C exhibits both the largest downfield shift (–3.92 ppm) (Table II) and the peculiar upfield shift in the temperature range 35–55 °C mentioned above. The CpG resonance of OC3 even broadens and disappears with increasing temperature to reemerge beyond 40 °C (Figure 7) and then show an upfield shift to 55 °C, a temperature where it begins to move downfield with the remaining peaks. Also noticeable is the TpT resonance in OC3, which with regard to its shape variation mimicks to a certain degree that of CpG (Figure 7). The whole process suggests that the CpG step and TpT repeat of OC3 are involved in the same premelting transition, implying a chemical exchange between two conformational states. Tentatively, these should correspond either to the B_I and B_{II} structures assumed by some mismatched base pairs (Nikonowicz *et al.*, 1989; Roongta *et al.*, 1990) or to the B′ and B forms that TT repeats and their neighbors are sometimes able to display in particular oligonucleotide sequences [Saenger (1984) and references cited therein].

Correlation between CD and ^{31}P NMR. Two major features seem to emerge: firstly, the octanucleotides yielding the largest CD positive signal are also those found to contain the steps displaying the largest deviations from the mean ^{31}P chemical shift, a good example being OC3 (compare Figures 1a and 5). A corollary is that to the weakest positive CD signal corresponds the weakest dispersed ^{31}P NMR spectrum, as illustrated by OC2 (Figures 1b and 5). Secondly, the position of the CD positive signal is governed by the base pair

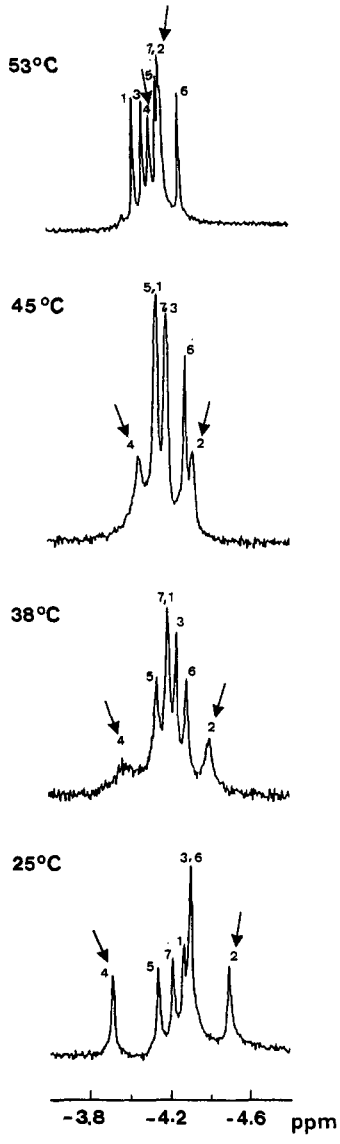

FIGURE 7: 1D ^{31}P NMR spectra of OC3 recorded at various temperatures. In order to follow the shape of the resonance, no window function is used in these spectra. Signals of interest are indicated by arrows.

composition of the dinucleotide step(s) displaying the largest deviation from the mean ^{31}P chemical shift. Thus, the largest ^{31}P chemical shift deviation exhibited by the two G·C base pair containing CpG steps and the poly(dG–dC)-like CD spectra are correlatively due to the particular conformations of CpG in family A (Fasman, 1975; Cantor & Schimmel, 1980). Likewise, the largest chemical shift deviations of the two A·T base pair containing ApT steps and poly(dA–dT)-like CD spectra are related to the particular conformation of ApT in family B (Fasman, 1975; Cantor & Schimmel, 1980). We will note, however, that despite the presence of a CpG step in the OC4 sequence the CD spectrum of this compound belongs to the B family. This can be explained by the strong CD contribution of the two ApT steps contained in the OC4 sequence, which display large chemical shift deviations from the mean chemical shift (Figures 1b and 5).

Obviously, the other constitutive steps of the six octanucleotides also contribute to the CD spectra, but more modestly. Their effects may however, be responsible for the differences observed within a family. A better idea about their CD activity can be deduced from their respective ^{31}P chemical shift deviations shown in the pattern of Figure 5. An example is provided by OC2, which has been placed in family B although the position of its broad and weak positive CD signal lying between 270 and 280 nm remains to be well-defined (Figure 1b). Once more, we will mention the good agreement which exists for OC2 between the weakness and breadth of the positive CD signal and the feeble dispersion of its ^{31}P chemical shifts (Figures 1b and 5). The most remote resonances concern CpT (ApG) and CpA (TpG), and their contribution to the CD spectrum of OC2 must be stronger compared to remaining steps, whose chemical shifts are found to be closer to the mean chemical shift (Figure 5). However, the CD signal of OC2 appears modest for the chemical shift deviations of CpT (ApG) and CpA (TpG) and is weak compared to that of the five other oligomers. A comparison of OC2 with OC6 may also help to illustrate the CD contribution of individual steps. Using the preceding considerations the shoulder observed at the right side of the main positive signal of OC6 would mainly arise from the C·G base pair belonging to the CpA step found at position 5 in both the OC6 and OC2 sequences. Of particular interest is also the difference in behavior shown by the A·T base pair within the three steps TpA, ApT, and TpT (ApA) encountered in our six oligonucleotides. For instance, OC4 contains twice ApT and OC5 contains twice TpA, with these steps flanking the same central CpG step. As mentioned repeatedly, the two ApT steps of OC4 show a large deviation from the mean ^{31}P chemical shift, and their two A·T base pairs may then confer upon OC4 a poly(dA–dT)-like spectrum, especially since the deviation of the CpG resonance remains weak in comparison (Figures 1b and 5). In contrast, the ^{31}P resonance of the TpA version in OC5 does not, or only weakly, deviate from the mean chemical shift value, while the ^{31}P resonance of CpG is largely remote. The consecutive domination of the C·G base pair activity of CpG over that of A·T base pairs of TpA results in a poly(dC–dG) like CD spectrum for OC5.

Thus, previous results have shown that the CD spectra of polynucleotides can usually be calculated quite closely using the summation of CD contributions of all nearest-neighbor base pairs (Gray & Tinoco, 1970; Allen *et al.*, 1972; Gray *et al.*, 1978; Gudibande *et al.*, 1988). Here, the results suggest that, for short and mixed-sequence oligonucleotides, there is a significant impact of the individual conformations of the steps upon the CD patterns. We conclude that the achievement of a correct CD analysis for such DNA fragments requires a good knowledge of their step conformations, as provided by ^{31}P NMR.

CD and ^{31}P NMR Properties and Conformational Aspects. We then addressed the question of whether CD and ^{31}P NMR demonstrate correlated conformational features. On the one hand the ^{31}P chemical shifts are connected to the conformation of the phosphodiester backbone mainly through both the ϵ angle [which is assessed by the $J(H3'–^{31}P)$ coupling] and the ζ angle (Gorenstein *et al.*, 1988; Nikonowicz *et al.*, 1990; Roongta *et al.*, 1990). On the other hand, the magnitude and position of CD signals are singularly sensitive to helix global geometry (Johnson *et al.*, 1981) and likely also to local order because of their sensitivity to base stacking and local conformational motions (Fasman, 1975; Cantor & Schimmel, 1980). This latter point appears interesting since NMR studies have shown that the degree of conformational freedom within the helix is an important factor affecting ^{31}P chemical shifts (Gorenstein, 1981; Patel *et al.*, 1982; Conolly & Eckstein, 1984; Ott & Eckstein, 1985a,b; Roongta *et al.*, 1990). Base "fraying" at the ends of the helix and conformational flexibility in the phosphodiester backbone, which is higher at the ends

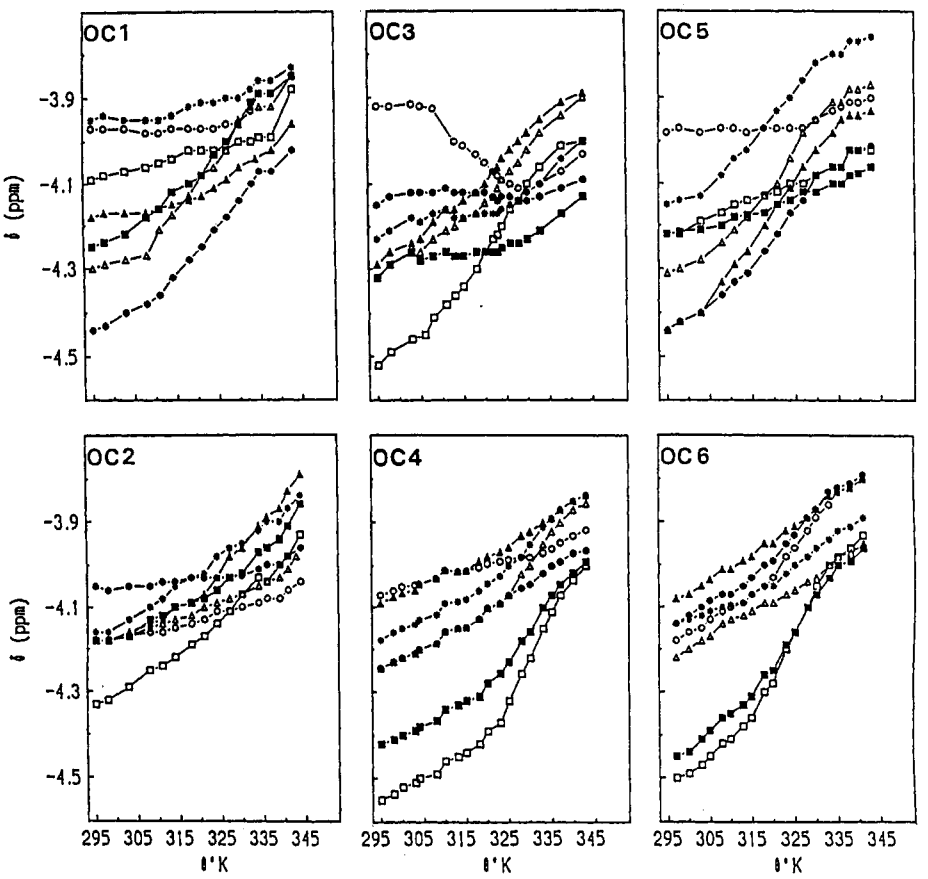

FIGURE 8: Temperature dependence of ^{31}P chemical shifts of the six octanucleotide duplexes. The phosphate assignments are indicated by numbering from the 5′ end to the 3′ end of the duplexes: P1 (△); P2 (□); P3 (▲); P4 (○); P5 (●); P6 (■); P7 (*).

than in the interior of the helix, are responsible for the progressive upfield shift of the ^{31}P resonances observed toward the center of the sequence (Connolly & Eckstein, 1984; Nikonowicz & Gorenstein, 1990). The other distinctive information is the downfield ^{31}P chemical shift which has been associated with a possible minor groove clash step, namely, Py–Pu (Powers *et al.*, 1989), while the loss of correlation in mismatch-containing sequences would reflect backbone distortions even at steps remote from the unpaired bases (Nikonowicz *et al.*, 1989; Nikonowicz & Gorenstein, 1990).

We will hereafter attempt to better understand the implications of local conformation on CD and NMR parameters in order to improve their use in the study of oligonucleotides. In a recent joint 2D ^{1}H and ^{31}P NMR and molecular simulation study carried out on two 12-mers, one of them containing OC1, *i.e.*, the cAMP responsive element sequence (CRE), and the other its inverse sequence OC2, we have concluded that the strong impact of sequence explains both the structural variations occurring along each helix and the differences between the two helices (Mauffret *et al.*, 1992). In summary, the two 12-mers belong to the B-DNA family, the OC1-containing compound is characterized by a structurally heterogeneous helix, and the second 12-mer displays a more homogeneous B-standard helix conformation (Mauffret *et al.*, 1992).

The agreement between the results relative to 12-mers and the present spectroscopic data on the two octamers OC1 and OC2 is encouraging, especially with regard to the particular behavior of their common steps CpG (Py–Pu) and ApT (Pu–Py). Both the high CD activity and the mean chemical shift deviations suggest that within octamers these two steps are confined in more rigid conformations as compared to all of the remaining steps. However, since both the chemical shifts and the coupling constants of CpG and ApT demonstrate opposite tendencies, these two steps must have completely different conformations; systematically, chemical shifts are found at high field for CpG and low field for ApT, while the coupling constants are large for CpG and small for ApT. Considering the available data using NMR distances in conjunction with molecular and molecular dynamics simulations, we will note for tetranucleotide sequences that the central twist angle value equals 30° for CApTG (Baleja *et al.*, 1990a; Mauffret *et al.*, 1992) and 43° for ACpGT (Baleja *et al.*, 1990a; Mauffret *et al.*, 1992). Thus, for the step examples ApT and CpG, the twist angle and the NMR parameters seem well correlated: the larger the ^{31}P chemical shift and coupling constant, the larger the twist angle. This also holds for the twist angle of the inverse versions TpA (Py–Pu) and GpC (Pu–Py): 41° (Mauffret *et al.*, 1992) and 38–45° (Clore *et al.*, 1985a; Lane, 1990) for GTpAC and 37° (Mauffret *et al.*, 1992) and 39° (Baleja *et al.*, 1990a) for TGpCA.

Obviously, anticipation on the basis of a generalization requires that we unravel those helical parameters which, among the twist, roll, propeller twist and also rise and cup, most affect the phosphodiester backbone conformation (Grzeskowiak *et al.*, 1991). For instance, the high twist and high positive roll have been shown to modify the phosphodiester backbone and decrease the base stacking interactions in TpA, compared to its inverse version ApT (Drew *et al.*, 1981; Dickerson *et al.*, 1989; Balendiran & Sundaralingam, 1991; Grzeskowiak *et al.*, 1991; Poncin *et al.*, 1992; Zakrzewska, 1992). The same likely applies to the singular CpG whose propensity to destack its bases and modify its backbone, especially under the influence of its flanking steps, is high compared to that of its inverse version GpC (Saenger, 1984; Grzeskowiak *et al.*, 1991;

Poncin *et al.*, 1992; Mauffret *et al.*, 1992).

A last point again concerns the influence in the sequence of the neighbor steps upon the step under consideration. It has become apparent during this work that the step behaves as the functional sequence unit in studying local structures for B-DNA molecules in solution. The conclusions are thus remarkably similar to those reached by crystallography (Grzeskowiak *et al.*, 1991). We note, however, that the structure of a given step may be influenced by its flanking steps, which once again is in agreement with the crystallographical data (Grzeskowiak *et al.*, 1991). Namely, for the CpG step that is central in the four sequences OC1, OC3, OC4, and OC5 (Table I), while it belongs to the ACGT sequence its chemical shift is remarkably constant (−3.97 and −3.98 ppm in OC1 and OC5, respectively; Table II) and thus independent of the preceding and succeeding steps in the sequences (GpA and TpC, respectively, in OC1; TpA in OC5). A switch of ACGT into TCGA (reversal of A and T) modifies the CpG chemical shift value either by a slight increase to −3.92 ppm (OC3) or a slight decrease to −4.05 ppm (OC4) (see Table II and Figure 5). A line of comparisons across the chemical shift data again points out the particular role exerted by the TT and AA repeats (compared to ApT) which flank CpG in OC3. The effects can be compared to those observed by Grzeskowiak *et al.* (1991) in their X-ray analysis of the AACGTT and ATCGAT sequences (the latter is similar to that found in OC4). While the authors expected only minor changes, they found that the switch of the first sequence into the second entails drastic differences between the two helix structures. Namely, the following changes are observed in the central step: the twist angle decreases from 44.8° to 29.3°; the roll (slightly) increases from 6.3° to 8.5°; the cup decreases from 21.9° to −4.4°; and the rise increases from 3.05 to 4.08 Å.

CONCLUSION

In this article we have described the joint use of CD and ^{31}P NMR experiments to determine and compare some of the structural features of six functional octanucleotide duplexes. Results from CD data and NMR demonstrate that B-DNA is the major conformation of these molecules in solution, and this is further confirmed by the NOESY spectra performed in the course of our 2D ^{1}H and ^{31}P NMR studies. However, individual ^{31}P NMR parameter values are shown to differ considerably from the mean values of standard B-DNA, and CD spectra were also able to illustrate local order variations in the double helix. ^{31}P NMR data imply that the larger the constraint in a dinucleotide step, the more intense its contribution toward CD spectra. The step emerges as the active sequence unit in the DNA backbone structure. It may even happen that the optical activity of a particular step dominates that of the other steps and then governs the shape and position of the CD signals. The conformation of a step is, however, submitted to the influence of its flanking steps. When these correspond to TT and AA repeats, as is the case for OC3, the intermediate CpG step may even show mismatch-like properties characterized by backbone distortions and an anomalous decrease in the duplex thermal stability.

Finally, we believe that the results of such studies on the local structure of functional DNA segments are biologically significant as they provide a valuable basis for the growing number of investigations on DNA recognition by proteins and ligands in general. They might help, for instance, to better understand the cleavage specificity of restriction endonucleases, the preference shown by anticancer drugs for particular steps, and finally the effects that base mutations can produce in genes.

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