NMR Studies on Oligodeoxyribonucleotides Containing the *dam* Methylation Site GATC. Comparison between d(GGATCC) and d(GGm^{6}ATCC)

G. Victor Fazakerley,*,‡ Robert Têoule,§ Andrê Guy,§ Hartmut Fritzsche,‡,∥ and Wilhelm Guschlbauer‡

Service de Biochimie, Dêpartment de Biologie, Centre d'Etudes Nuclêaires de Saclay, F-91191 Gif-sur-Yvette Cedex, France, and Laboratoire de Chimie, Dêpartment de Recherche Fondamentale, Centre d'Etudes Nuclêaires de Grenoble, 85X, F-38041 Grenoble Cedex, France

Received October 17, 1984

ABSTRACT: The conformation of two hexanucleotides, d(GGATCC) and d(GGm^{6}ATCC), has been studied by proton nuclear magnetic resonance. Nuclear Overhauser effect (NOE) measurements on d(GGATCC) are in agreement with a normal B form right-handed helical structure. The single- and double-strand resonances are in fast exchange on a proton NMR time scale. The exchange is observed to be slow for d(GGm^{6}ATCC); up to the T_m, separate resonances are observed for each state, though above the T_m exchange becomes more rapid. The preferred orientation of the adenosine methylamino group (methyl cis to N^1) hinders base-pair formation. At 0 °C irradiation of the m^{6}A-T imino proton gives an NOE to AH^2, showing that base pairing is Watson–Crick. Intra- and interresidue NOEs show that the helix is right handed and in the B form. Comparing results on the two oligomers demonstrates that adenosine methylation induces little or no change in the conformation of the helix but reduces the T_m from 45 to 32 °C. All of the amino proton resonances, as well as the imino resonances, have been assigned. From NOE experiments on the unmethylated oligomer we have located the Watson–Crick and non-Watson–Crick adenosine amino protons. At 0 °C these resonances show broadening due to rotation of the amino group, and their rotation is slightly slower than for the adjacent guanosine amino group, though both these amino groups have lifetimes of less than 10 ms at 0 °C. The imino protons show normal behavior, disappearing from the spectra ca. 20 °C below the T_m. The methylated oligomer shows restricted rotation from the adenosine methylamino group. The imino protons are observed up to the T_m of the hexanucleotide. Not only is helix formation slow compared to the unmethylated oligomer but also individual base-pair opening is at least an order of magnitude slower at the same temperature. The different mechanisms contributing to the relaxation are discussed.

The role of methylation of DNA bases has received considerable attention in recent years [for review see Trautner (1984)]. Two kinds of methylations are known to occur: either methylation of the C^5 position of cytosine or methylation of the amino group of adenine. Both modifications have been found to be associated with restriction endonucleases in prokaryotes (Modrich, 1982; Trautner, 1984). It also appears that methylation of both A and C plays the role of a regulatory signal in eukaryotic cells [for review see Doerfler (1981)]. 5-Methylcytosine also favors the B–Z transition in poly(dG-dm^{5}C) (Behe & Felsenfeld, 1981).

The role and mechanism of adenine methylation appears to be more complex. Lacks & Greenberg (1975, 1977) have isolated two restriction nucleases from *Diplococcus pneumoniae* (DpnI and DpnII) which recognize the GATC sequence but which differ in an important aspect. While DpnII acts like most class II restriction nucleases, i.e., it is inhibited by adenine methylation, DpnI cleaves only Gm^{6}ATC sites (Lacks & Greenberg, 1975; 1977; Vovis & Lacks, 1975; Geier & Modrich, 1979).

Apart from its role in the restriction–modification system (Modrich, 1982), a methylase specific for the sequence GATC (*dam* methylase) (Radman et al., 1978) has been studied. This enzyme appears to act with a certain lag period behind replication on newly synthetized DNA daughter strands which are transiently unmethylated (Marinus & Morris, 1973). It is probably also involved in the repair of replication errors, since *dam* methylase acts primarily on hemimethylated GATC sequences (Pukkila et al., 1983). It appears that adenine methylation of GATC sequences directs strand discrimination of the mismatch repair system of *Escherichia coli* (Marinus & Morris, 1973; Radman et al., 1978; Pukkila et al., 1983).

The chemistry of N^6-methyladenine is unusual in so far as the methylamino group is coplanar with the purine ring and its rotation is hindered (Sternglanz & Bugg, 1973). The cis form (i.e., cis in respect to N^1 but trans to the C^5–C^6 bond) is the favored species (Sternglanz & Bugg, 1973; Engel & von Hippel, 1974). This form would be, however, unable to form a Watson–Crick base pair, although Hoogsteen pairing is possible. Thus, m^{6}A has a strong destabilizing effect on the polynucleotide complex with poly(dT) compared with poly(dA)·poly(dT) (Engel & von Hippel, 1978a). The preference for the cis form also strongly decreases the rate of the *pol*I DNA polymerase reaction (Engel & von Hippel, 1978b). This has been attributed to a rate-limiting base-pairing step which is slowed down by the required isomerization of the methylamino group.

From these remarks the question arises whether or not A-methylated sequences in DNA, and in particular the palindromic Gm^{6}ATC sequence, are conformationally different from the canonic B-DNA. If the sequence assumes the standard B form, the recognition by an enzyme should be reduced to recognition of the two methyl groups which are one on top of the other. In the alternative case where the methylamino group would induce a conformational change in the helical structure, a relatively large perturbation including for the neighboring G-C pairs could be expected. This would favor

‡ Centre d'Etudes Nuclêaires de Saclay.
§ Centre d'Etudes Nuclêaires de Grenoble.
∥ Present address: Zentralinstitut für Mikrobiologie und experimentelle Therapie der AdW der DDR, DDR-69, Jena, GDR.

0006-2960/85/0424-4540$01.50/0

recognition by an interacting enzyme (von Hippel & McGhee, 1972).

Nuclear magnetic resonance (NMR) can be used to establish the conformational state of a DNA, but also the factors governing the opening of a specific base pair or complete strand separation (Feigon et al., 1982). While in the helical state the imino protons are protected from exchange with solvent, under appropriate conditions exchange may take place each time a base pair opens. Depending upon the pH, the nature, and the concentration of the buffer, the same may be true for the amino protons. However, studies on the amino protons could lead to an understanding of processes in addition to that of helix opening, if these groups are involved in, or in the vicinity of, binding sites to antibiotics or proteins. In several reports exchangeable protons, which could only arise from amino groups, have been observed in the range 6–9 ppm and assigned to cytidine protons (Patel, 1976, 1977; Clore & Gronenborn, 1984). We have recently reported the assignment of all the exchangeable protons in d(CG)$_3$ (Fazakerley et al., 1984a).

The amino group of cytidine differs from those of guanosine and adenosine in that it shows restricted rotation about the carbon–nitrogen bond (McConnell & Seawell, 1972, 1973; Raszka & Kaplan, 1972; Raszka, 1974; McConnell, 1984). As a result, exchange with solvent is the primary influence on the spectra of these protons. We have shown that the guanosine amino protons are in intermediate exchange in d(CG)$_3$ (Fazakerley et al., 1984a) at 20 °C and as a consequence are not visible in the spectra. However, any interaction that would make the helix more or less rigid could push that exchange equilibrium into the slow or fast limit. Also the exchange with solvent of the amino protons as compared to the exchange of imino protons can differentiate between complete base-pair opening and a partial or scissor opening.

With these objectives in mind we have embarked on an investigation of DNA oligomers containing the GATC and related sequences and their N^6-methylated adenine analogues. In a previous communication (Fazakerley et al., 1984b) we reported that d(GGATCC) and d(GGm^{6}ATCC) show very different behavior in one respect: the former shows the normally observed fast exchange on an NMR time scale between the single- and double-stranded species, while the latter oligonucleotide shows slow exchange. We proposed that the preferred cis conformation of the adenine methylamino group was responsible for this unusual phenomenon.

Materials and Methods

DNA Syntheses. d(GGATCC) and d(GGm^{6}ATCC) were synthesized by a modified phosphate triester and phosphoamidite method on silica gel support (A. Guy, L. Wagrez, D. Molko, and R. Têoule, unpublished results). The oligonucleotide was 3 mM in strand dissolved in 10 mM phosphate buffer (pH 7.2), 150 mM NaCl, and 0.5 mM ethylenediaminetetraacetic acid (EDTA)[1] in D_2O or 90% H_2O/10% D_2O. Chemical shifts were measured relative to tetramethylammonium chloride and converted to the usual scale relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) by adding 3.18 ppm.

NMR Spectroscopy. NMR spectra at 500 MHz were recorded on a Bruker WM-500 spectrometer and for certain spectra at 400 MHz on a Bruker AM-400. The NOEs were observed by cycling 32 transients on-resonance, followed by 32 transients off-response. Irradiation was applied for 100 ms (above which spin diffusion effects were perceptible; see below) at a power level that gives 90% saturation of the irradiated resonance. The estimated error in NOE magnitudes is ≤1.0%. For the measurements of exchangeable protons the same buffer solution was used in 90% H_2O/10% D_2O. A time shared pulse sequence, θ_x–t–θ_{-x} (Plateau & Guéron, 1982), was used to suppress the water resonance. The carrier was placed on the water resonance, and the delay between the two pulses was 350 μs. In addition to the minimum at the water resonance this produces a null at ca. 5.6 ppm on either side of the water resonance. The imino protons are excited in the second envelope. Thus, an approximate 90° pulse can be applied both over the aromatic protons and the imino protons which facilitates NOE measurements in particular. A preirradiation time of 100 or 500 ms was employed. The power level was just sufficient to saturate the resonance.

Results

The 500-MHz spectra of the two hexanucleotides at 3 °C are shown in Figure 1. At this temperature both oligomers are ≥95% in the duplex state.

The assignment of the base, $H_{1'}$, $H_{2'}$, and $H_{2''}$ proton resonances was carried out according to the precedure that has been applied by using 1-D (Reid et al., 1983) or 2-D techniques (Hare et al., 1983; Scheek et al., 1983; Feigon et al., 1983; Frêchet et al., 1983). A base H^8 or H^6 proton will give rise to an NOE to its own $H_{1'}$ proton resonance and to the $H_{1'}$ resonance of the residue in the 5′ direction for a right-handed helix. Thus, with the exception of the 5′-terminal residue, NOEs to two $H_{1'}$ resonances will be observed. For a left-handed helix the interresidue NOEs will be in the 3′ direction. In addition the same base protons will give NOEs to the $H_{2'}$ and to a lesser extent to the $H_{2''}$ resonances of the same nucleoside, irrespective of the ribose conformation. However, the corresponding interresidue effects are very sensitive to the ribose conformation. For a B-form DNA the NOE between the H^8 and H^6 is much larger to $H_{2''}$ than to $H_{2'}$, whereas the order is reversed for an A-form DNA.

As the aim of this study is to compare as accurately as possible the helix conformation of two oligonucleotides differing only by methylation of the adenine, we carried out NOE measurements under conditions were effects of spin diffusion were rigorously absent (the sole exception being a small effect transmitted between $H_{2'}$ and $H_{2''}$). The preirradiation time employed was 100 ms.

For the fragment ApT in d(GGATCC) preirradiation of $^3AH^8$ gave an NOE to $^4TCH_3$ as expected for a B form helix, but no NOE to $^4TH^6$. At preirradiation times of ≥150 ms an NOE was observed on $^4TH^6$, which could only arise from spin diffusion.

A summary of the observed interresidue NOEs for both hexanucleotides is presented in Table I. In only one case, $^5CH^6$–$^6CH^5$ is an NOE observed for one oligonucleotide and not the other. This NOE is, however, only ca. 2% in d(GGm^{6}ATCC) and is perhaps not observed in d(GGATCC) because of greater mobility or fraying of the terminal base pair. The standard deviation between the interresidue NOEs observed on the two hexanucleotides is 1.8%, and the largest difference is 3%. As the experimental error is ca. 1%, we conclude that the helix conformation must be virtually unaltered by methylation of adenine.

All of the interresidue NOEs shown in Table I are consistent with a right-handed B form helix. While NOEs from base H^8 or H^6 protons to the $H_{2'}$ resonances of residues in the 5′

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; WC, Watson–Crick; nWC, non-Watson–Crick; EDTA, ethylenediaminetetraacetic acid; 1-D, one dimensional; 2-D, two dimensional.

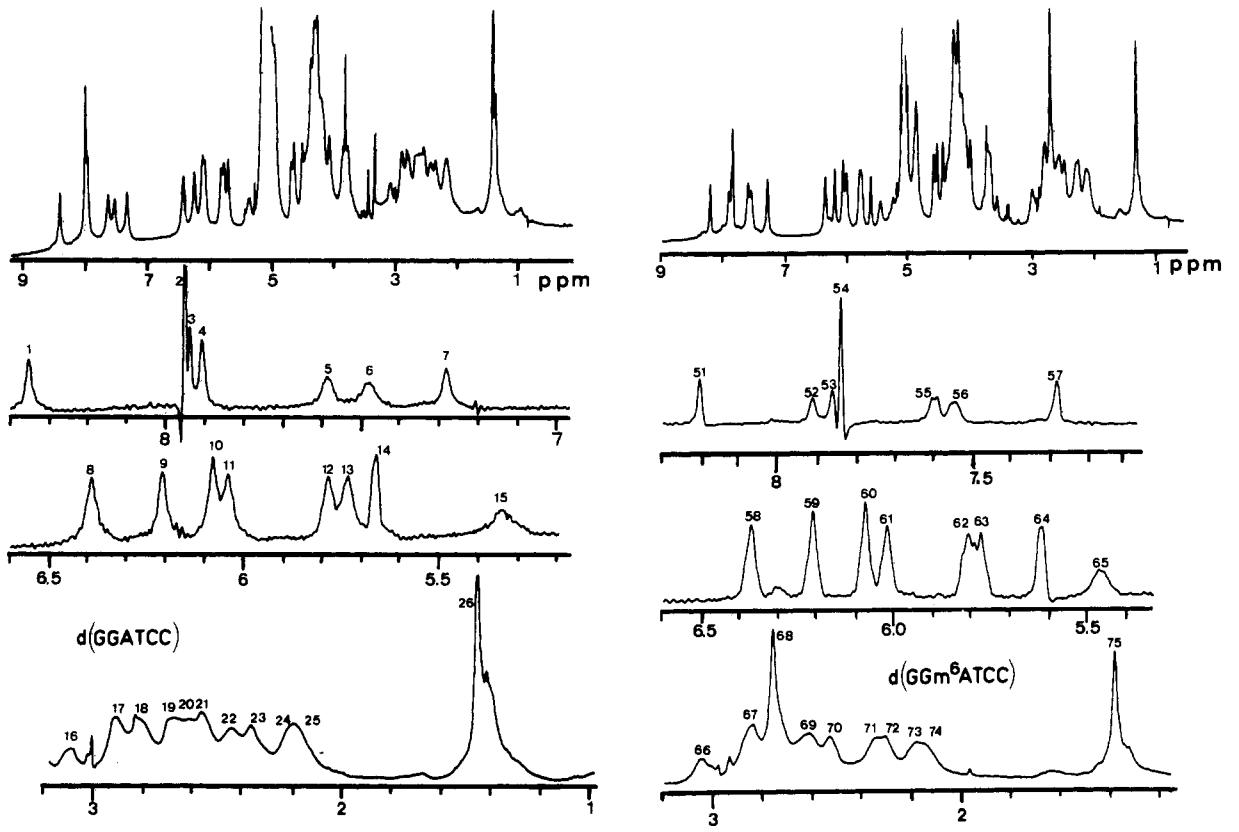

FIGURE 1: NMR spectra of d(GGATCC) (a) and d(GGm^{6}ATCC) (b) in D_2O. The numbering of the resonances is the same as in Table II. The expanded aromatic and anomeric regions are resolution enhanced.

Table I: Summary of Interresidue NOEs Observed for d(GGATCC) (●) and d(GGm^{6}ATCC) (○)

5' ⟶ 3'	^{1}G	^{2}G	^{3}A	^{4}T	^{5}C	^{6}C
$CH_{1'}$, $CH^8(CH^6)$	●○	●○	●○	●○	●○	●○
$CH_{2''}$, $CH^8(CH^6)$	●○	●○	●○	●○	●○	●○
$CH_{1'}$, CH_3			●○			
CH^8, CH_3			●○			
CH^6, CH^5				●○	●○	●○
CH_3, CH^5					●○	●○

direction were observed, these were generally 3 times smaller than to the $H_{2''}$ resonances which excludes a major contribution from an A type conformation. The sugar pucker may also be obtained from the $J_{1'2'}$ and $J_{1'2''}$ coupling constants (Guschlbauer, 1980). For both hexanucleotides these coupling constants are 7–7.5 Hz and show no significant changes for individual residues. From this the sugar pucker is ca. 75% 2'-endo in agreement with a B conformation for both oligomers.

Temperature Dependence of Nonexchangeable Protons. On raising the temperature the chemical shifts of the nonexchangeable protons of d(GGATCC) showed typical sigmoid curves (not shown) from which the T_m of the hexanucleotide is found to be 45 °C. The resonances broadened by at most 2 Hz during the melting process, showing that exchange is rapid on an NMR time scale. For d(GGm^{6}ATCC) we observe, what is to our knowledge, a unique case for oligonucleotides, in that on increasing the temperature the resonances of the duplex state decrease in intensity and new resonances appear corresponding to the single-strand species (Figure 2). Up to 35 °C the melting process can be followed by integration of the corresponding resonances of the duplex and single-strand states, where these are well resolved and readily assigned, as, for example, the $^4TCH_3$ (Figure 3). Above 30 °C, however, the system is no longer in the slow-exchange limit, and line broadening is observed on all resonances. The line width of $^4TCH_3$ for duplex and single-strand states are shown in Figure 3. The broadening is such that above 35 °C accurate integration of resonances of the duplex state is no longer possible. Nevertheless, the T_m is observed to be ca. 32 °C, or 13 °C below that of d(GGATCC). While line broadening has been observed during the melting process of oligonucleotides (Pardi et al., 1981), indicating intermediate exchange, d(GGm^{6}ATCC) is at or near the slow-exchange limit below 25 °C.

Finally, the connectivities may be established in the temperature region where both duplex and single strand are present. Preirradiation of a resonance for a short time will give rise not only to NOEs (in the case of irradiation of a resonance of the double strand) but also to transfer of saturation to the corresponding proton resonance of the single strand and vice versa. All the resonance assignments are shown in Table II.

Temperature Dependence of Exchangeable Protons of d(GGm^{6}ATCC). At 0 °C eight exchangeable protons (peaks a–h) are clearly visible in the spectrum (Figure 4). The peaks labeled d,e and g,h each integrate for two protons and become resolved at higher temperature. In addition two much broader peaks, i and j, around 6.7 ppm are observed, each of which probably integrates for one proton.

On raising the temperature to 3 °C all the resonances of exchangeable protons get narrower with the exception of peaks

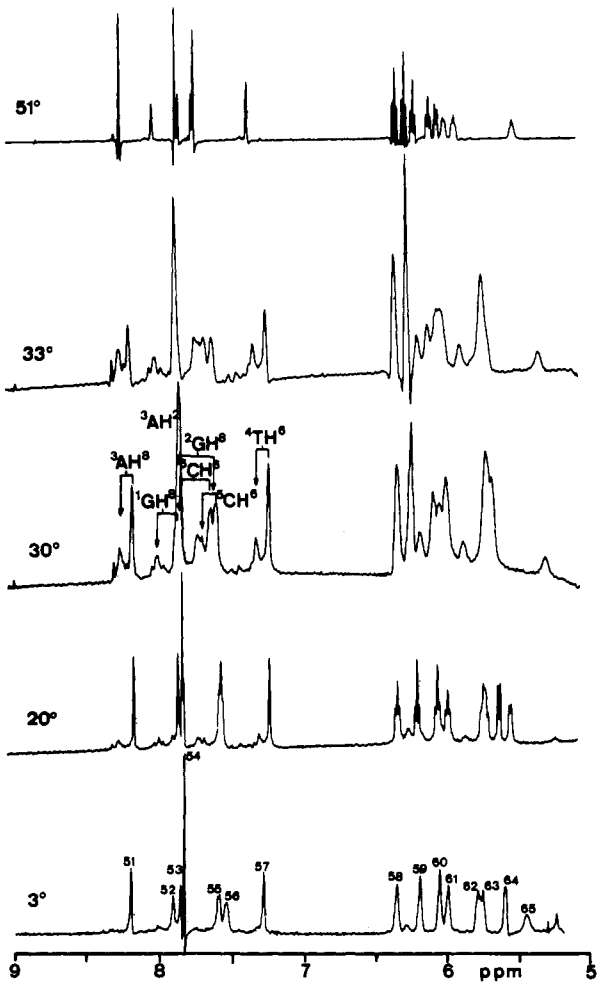

FIGURE 2: Partially resolution enhanced NMR spectra of d(GGm^{6}ATCC) in D_2O as a function of temperature. Note the connectivities between pairs of resonances at 30 °C. The arrows indicate the resonances of the single-stranded species.

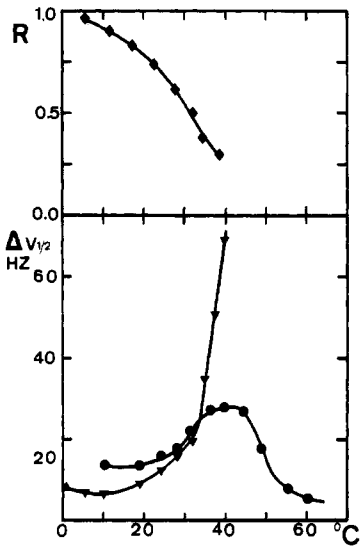

FIGURE 3: (Top) Fraction of double-strand form (R) of d(GGm^{6}ATCC) as a function of temperature. R was obtained by integration of the single- and double-stranded resonances of $^4TCH_3$. (Bottom) Line width of $^4TCH_3$ in the double-stranded (▼) and single-stranded species (●) of d(GGm^{6}ATCC) as a function of temperature.

i and j which broaden and disappear from the spectrum. In the temperature range 6–16 °C one of the imino protons, peak b, broadens rapidly, and two of the amino resonances, peaks

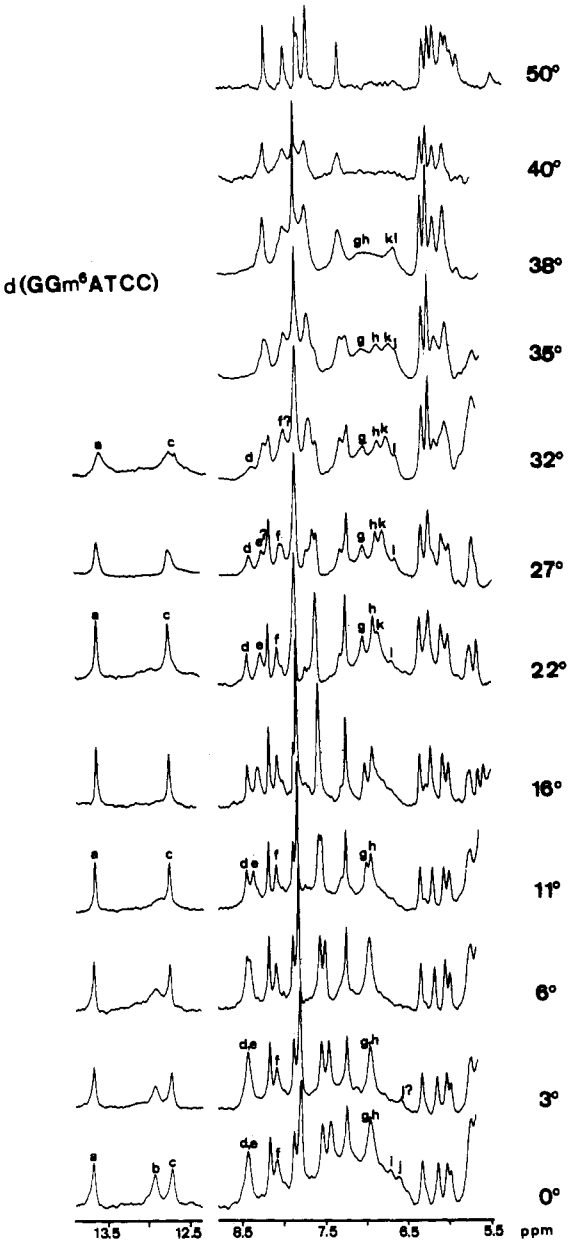

FIGURE 4: NMR spectra in 90% H_2O/10% D_2O of d(GGm^{6}ATCC) as a function of temperature. Only the exchangeable protons are labeled.

e and g, broaden, but also move, the former upfield and the latter downfield. At 22 °C a number of new resonances become visible: peaks k and l correspond to exchangeable protons (all other new resonances arise from nonexchangeable protons of the single-stranded species). At 28 °C peaks k and l exhibit their narrowest line widths. Peak e must be very broad as the sharp resonance observed at 8.28 ppm corresponds to the ^{3}A(H^8) resonance of the single strand. In the range 32–35 °C the two remaining imino protons and peak d broaden and disappear from the spectrum. It is not clear, however, whether peak f is still present. It would overlap with the GH^8 resonance of the single-stranded species, and in this temperature range the nonexchangeable proton resonances are broadened as the system is no longer in the slow-exchange limit. In the temperature range 35–38 °C the four remaining resonances, peaks g, h, k, and l broaden and then disappear at 40 °C. The effect of temperature on the chemical shifts of the amino protons is shown in Figure 5. The melting of the helix is still not complete at 40 °C as shown by the line width of the nonexchangeable protons which become much narrower when the

Table II: Proton Chemical Shifts of d(GGATCC) (Upper Line) and d(GGm^{6}ATCC) (Lower Line) at 3 °C and Difference in Chemical Shift between Resonances of Single and Double Strands of d(GGm^{6}ATCC) at 20 °C[a]

base	H^8 or H^6	$CH^5/TCH_3/H^2$	$H_{1'}$	$H_{2'}$	$H_{2''}$
^{1}G	7.94(3)		5.78(12)	2.64(20)	2.42(22)
	7.91 (+0.09)(52)		5.78 (+0.012)(63)	2.76(68)	2.31(72)
^{2}G	7.91(4)		5.73(13)	2.89(17)	2.79(18)
	7.86 (−0.20)(53)		5.81 (−0.47)(62)	2.86(67)	2.74(78)
^{3}A	8.35(1)	7.96(2)	6.39(8)	2.77(19)	3.07(16)
	8.20 (+0.10)(51)	7.84[b](54)	6.37[b](58)	2.73(68)	3.05(66)
^{4}T	7.28(7)	1.44(26)	6.04(11)	2.15(25)	2.59(20)
	7.29 (+0.07)(57)	1.40 (+0.24)(75)	6.02 (+0.05)(61)	2.15(74)	2.62(69)
^{5}C	7.59(5)	5.66(14)	6.08(10)	2.19(24)	2.54(21)
	7.60 (+0.14)(55)	5.63 (+0.37)(64)	6.08 (+0.10)(60)	2.18(73)	2.54(70)
^{6}C	7.48(6)	5.34(15)	6.21(9)	2.34(23)	2.42[c](22)
	7.55 (+0.24)(56)	5.48 (+0.48)(65)	6.21 (+0.06)(59)	2.31(72)	2.35[c](7)

[a] A positive shift means to low field of the double-strand resonance. [b] Too small to be measured. [c] The relative assignment of the 2′ and 2″ resonances is arbitrary. The resonances are numbered as in Figure 1.

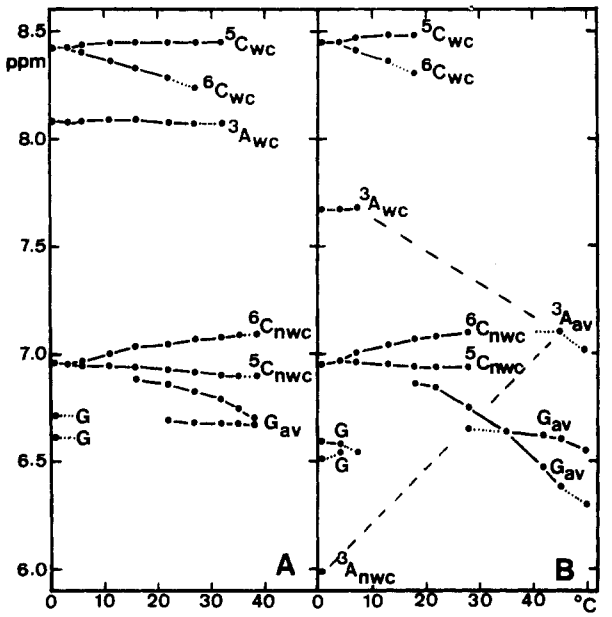

FIGURE 5: Effect of temperature on the chemical shifts of the amino protons of d(GGATCC) (A) and d(GGm^{6}ATCC) (B).

temperature is raised to 50 °C.

Assignment of the Exchangeable Protons of d(GGm^{6}ATCC). The three imino protons are readily assigned from their chemical shifts and the temperature dependence of their line width. The resonance at 13.66 ppm, peak a, is assigned to the m^{6}A-T thymidine imino proton of base pair (3, 4), while peaks b and c are the guanosine imino protons. Peak b rapidly broadens and disappears on raising the temperature from 3 °C. This is due to fraying (Patel & Hilbers, 1975; Kan et al., 1975) of the terminal base pair (1, 6); peak c is thus assigned to the guanosine imino proton of base pair (2, 5).

After preirradiation, at 0 °C, of the thymidine imino proton, a, we observe (Figure 6) an interresidue NOE to the imino proton, peak c, which further confirms this as being from base pairs (2, 5). In addition we observe a large NOE to $^3AH^2$ (which further confirms Watson–Crick hydrogen bonding) and an equally large one to exchangeable proton f. The only

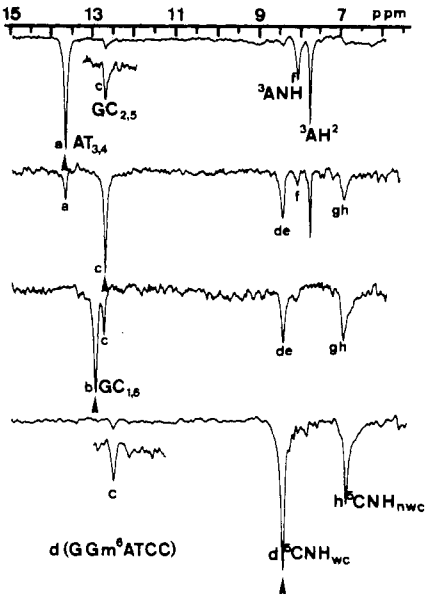

FIGURE 6: NOE difference spectra in 90% H_2O/10% D_2O of d(GGm^{6}ATCC) at 0 °C (except bottom spectrum which was recorded at 16 °C). Arrows indicate the resonances irradiated. The irradiation time was 0.5 s.

exchangeable proton close enough to the thymidine imino proton is the amino proton of the adenosine methyamino group. For short preirradiation times (less than 100 ms) no other NOEs are observed although when the irradiation time is long (≥0.5 s) an NOE to the adenine methyl group (not shown) is observed via spin diffusion. In addition a small NOE is found to peaks d,e.

Irradiation of the guanosine imino proton, peak c, of base pair (2, 5) gives the reverse interresidue NOE to the thymidine imino proton (Figure 6). Four NOEs are observed in the region 6–9 ppm, the largest of which is to peaks d,e, followed by those on peaks g,h. Smaller interresidue NOEs are observed on the adenosine H^2 and amino resonance, peak f. These experiments were done with a relatively long preirradiation time of 500 ms. When this is reduced to 100 ms, the NOE on peaks d,e is very much larger than on peaks g,h. The latter NOE arises, at least in part, from spin diffusion. Irradiation of the guanosine imino proton of base pair (1,6) gives an interresidue NOE to the adjacent imino proton and also to peaks d,e and g,h (Figure 6).

Upon irradiation of peak d at 16 °C a large NOE is observed on peak h and a small one on peak c. Peaks d and h must be geminal protons of base pair (2, 5). We have previously observed in d(C-G)$_3$ (Fazakerley et al., 1984a) that at low temperature the cytidine amino proton resonances were quite narrow, but we observed only one of the guanosine amino protons. This was due to the fact that the cytidine amino groups do not rotate, whereas the guanosine amino groups do, except for probably the central base pair at 0 °C. Thus, the strong NOE coupled pairs of resonances d,h and e,g must correspond to the cytidine amino groups of base pairs (2, 5) and (1, 6), respectively, with the first peaks, d and e, being the Watson–Crick hydrogen-bonded protons. Note that the hydrogen-bonded protons exchange more rapidly with solvent than the non-hydrogen-bonded protons of cytidine in the temperature range 27–38 °C. The same effect has been observed in d(C–G)$_2$ by saturation transfer experiments (McConnell, 1984).

In the 0 °C spectrum peaks i and j remain unassigned. They must be guanosine amino protons that are not yet completely in the limit of slow exchange. Irradiation of the two guanosine

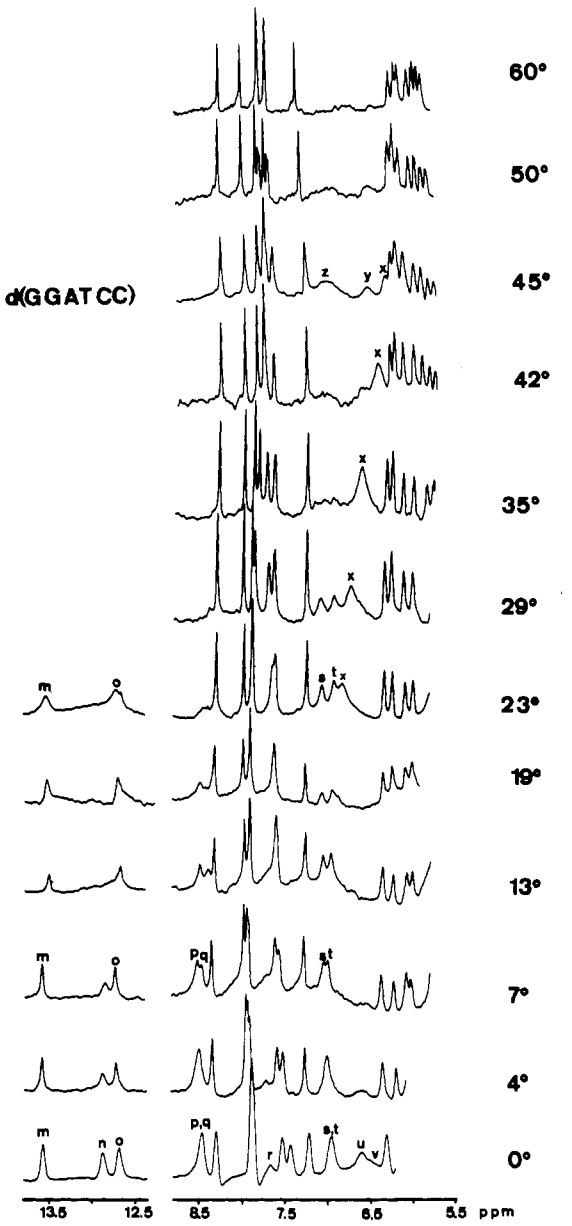

FIGURE 7: NMR spectra in 90% H_2O/10% D_2O of d(GGATCC) at different temperatures. Only the exchangeable protons are labeled.

imino protons did not produce significant NOEs which could be attributed to guanosine amino protons. In addition, irradiation of peaks i and j was unsuccessful in locating the corresponding geminal protons. The difference spectra showed nonselective effects, presumably due to partial irradiation of the solvent resonance. The absence of an NOE on guanosine amino protons upon irradiation of the guanosine imino protons show that the amino proton relaxation is still dominated by the rotation around the C^2 amino bond.

Above 10 °C, first peak k and the peak l appear. Rotation of the amino group is probably facilitated for the terminal base pair (1, 6) relative to base pair (2, 5). Also, as observed on the imino protons and cytidine amino protons solvent exchange is faster for the terminal base pair. Peak k appears before peak l and these protons, peak k, probably exchange slightly faster with solvent at 38 °C and can thus be tentatively assigned to base pair (1, 6).

Spectra of d(GGm^{6}ATCC) at pH 8 and at a variety of temperatures showed the same behavior as described above. The observed line widths were within 10% of those at pH 7.2 for the same temperature.

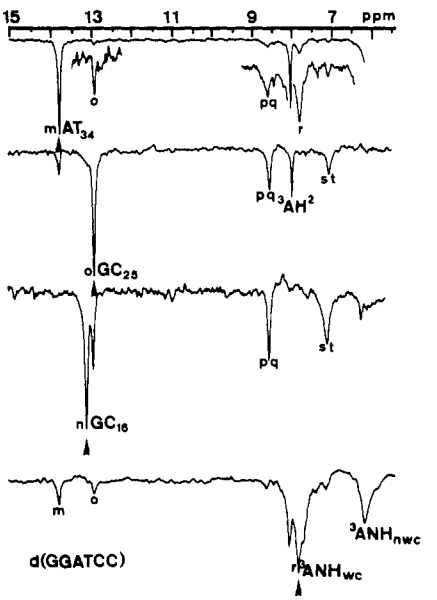

FIGURE 8: NOE difference spectra in 90% H_2O/10% D_2O of d(GGATCC) at 0 °C. Arrows indicate the resonances irradiated. The irradiation time was 0.5 s.

Temperature Dependence of the Exchangeable Protons of d(GGATCC). Figure 7 shows the spectra of d(GGATCC) observed in 90% H_2O as a function of temperature. In the slightly resolution-enhanced spectrum at 0 °C, 10 exchangeable protons can be distinguished (m to v) in which the resonances at 8.45 and 6.95 ppm integrate for two protons. The resonance that we have assigned to the amino proton of adenosine in the methylated hexamer is absent, and a peak r at 7.66 ppm is observed. Peaks u and v are shifted ca. 0.15 ppm upfield from their probable counterparts, peaks i and j in Figure 4. On raising the temperature to 7 °C the spectrum simplifies in that peaks r, u, and v broaden and disappear. Only above 20 °C do new resonances appear in the spectrum. First, a peak x at 6.8 ppm shifts rapidly to high field on further raising the temperature. At 35 °C peak x integrates for more than two protons, and at 42 °C a new resonance, peak y, appears, which is more clearly visible at 45 °C where peak z can also be seen. Spectra with different acquisition parameters (delay between the two pulses etc.) always gave a broad resonance at 7.1 ppm which does not seem to be a base-line artifact, even though peak z is seen only over a very narrow temperature range. The remainder of the exchangeable protons shows a behavior with respect to temperature similar to that observed for the methylated hexamer (Figure 4). Results on the temperature dependence of the imino protons are presented below.

Assignment of the Exchangeable Resonances of d(GGATCC). The exchangeable protons were assigned in the same way as described above for d(GGm^{6}ATCC). Preirradirradiation of peak o, the imino resonance of base pair (2, 5), gives intraresidue NOEs to the cytidine amino protons p,q (WC) > s,t (nWC) and interesidue NOEs to the imino (peak m) and adenosine H^2 resonances of base pair (3, 4) (Figure 8). The only major difference between the two oligonucleotides is observed upon preirradiation of the thymidine imino proton which gives an NOE on peak r which must arise from one or both of the protons of the adenosine amino group. At 0 °C its line width is ca. 60 Hz, and it is not possible to say whether it integrates for one or two protons. Saturation of peak r at 0 °C (Figure 8) gives three NOEs. As expected an NOE is observed to the thymidine imino proton, peak m, but a much larger one is observed to a resonance at 6.0 ppm.

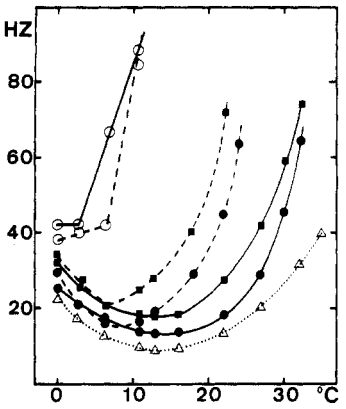

FIGURE 9: Line widths (in Hz) of imino proton resonances as a function of temperature: (○) G-C (1, 6) imino proton; (■) G-C (2, 5) imino proton; (●) A-T (3, 4) imino proton. (Broken lines) d(GGATCC); (solid lines) d(GGm^{6}ATCC). The line width of ^{3}A(H^8) of d(GGm^{6}ATCC) (△) is also shown.

In addition a small NOE is observed to a guanosine imino proton of base pair (2, 5), peak o. Care was taken not to irradiate the cytidine H^6 resonance just to high field of peak r. Totally specific irradiation of such a broad resonance, while maintaining an acceptable signal to noise level in the difference spectra, is difficult. The adenosine H^2 was about 10% saturated during this experiment. Only geminal protons could be expected to yield such a large NOE as that observed at 6.0 ppm; we thus conclude that peak r corresponds to the Watson–Crick adenosine amino proton and the resonance at 6.0 ppm is the non-hydrogen-bonded one.

As observed with the methylated hexamer, raising the pH of the solution to pH 8.0 did not change the observed line widths of the exchangeable protons.

Effect of Temperature on the Imino Proton Resonances of d(GGm^{6}ATCC) and d(GGATCC). We shall exclude discussion of the terminal base pairs as the relaxation of their imino protons is dominated by fraying of the helix. The imino protons of d(GGATCC) show the narrowest line widths at 6–8 °C (Figure 9). At lower temperatures broadening is observed due in part to the greater viscosity of the solution. Above 8 °C the relaxation rates (as shown by the changes in line width) rise due to an increasing contribution from exchange with solvent. Above 20 °C the rates increase very rapidly, and at 27 °C the imino resonances are no longer observed. The thymidine imino proton is less influenced than the adjacent guanosine imino proton by exchange, indicating that the fraying process may also contribute to the relaxation of the latter. That the imino protons disappear from the spectrum well below the T_m of the helix is a generally observed phenomenon for an "open limited" case where proton exchange occurs each time a base pair opens.

For the internal imino protons of d(GGm^{6}ATCC) the narrowest line widths are observed at ca. 15 °C (Figure 9). Above this temperature the relaxation rates increase, but these protons are still observed up to the T_m of oligonucleotide. We find these protons at higher temperatures than for d(GGATCC), even though the T_m of the methylated hexamer is 13 °C lower. In the range 27–32 °C we find not only broadening of the imino resonances but also a decrease in their intensities. The ratio of the intensity of each imino proton to a resonance of a nonexchangeable proton of the helix state remains unity. While above 20 °C line broadening is observed, a significant part of this is due to a shift in the exchange rate, helix $\rightleftharpoons$ coil, from the slow-exchange limit at low temperature toward intermediate exchange. This broadening is observed on all resonances of the double strand, and the line width of ^{3}A(H^8) is shown in Figure 9 for comparison.

Discussion

The comparison between d(GGATCC) and d(GGm^{6}ATCC) shows that at low temperature both hexanucleotides assume the same B-form geometry. Inter- and intraresidue NOEs (Table I) clearly establish that both hexanucleotides do not differ significantly from each other. Irradiation of H^8 or H^6 resonances give only small NOEs to the corresponding H$_{1'}$ protons which excludes syn conformations and makes a left-handed helix or Hoogsteen pairing unlikely. Also irradiation of the thymidine imino protons yields NOEs on the H^2 of adenosine which proves the Watson–Crick pairing even in the presence of the methylamino group.

From the results presented above on the nonexchangeable protons the major difference between the methylated and unmethylated hexanucleotide is in the rate of helix formation or rupture which led us to examine the exchangeable proton resonances. The adenosine amino resonances have not previously been assigned unambiguously in oligonucleotides.

Amino Protons. The methylated oligomer d(GGm^{6}ATCC) was chosen as a starting point in searching for the adenosine amino protons as the methylamino group would not be able to rotate in the way that the guanosine amino group has been observed to rotate in d(C–G)$_3$ (Fazakerley et al., 1984a). Studies on monomers have shown that rotation of the cytidine amino group is slow on a NMR time scale (Raszka & Kaplan, 1972; McConnell & Seawell, 1973; Raszka, 1974) while rotation of the amino group in guanosine and adenosine is rapid (McConnell & Seawell, 1972; Raszka & Kaplan, 1972; Raszka, 1974; McConnell, 1984). Between 6 and 11 °C the line width of the methylamino proton (peak f, Figure 4) is only 18 Hz which is no greater than that of other exchangeable protons. Above 16 °C the resonances of the single-stranded species are clearly visible in the spectra. At 27 °C one of these, a G(H^8) resonance, overlaps with peak f, and above 27 °C it is not clear if peak f is still present. Between 30 and 40 °C all the nonexchangeable protons are broadened as the signal- and double-stranded forms are no longer in the slow-exchange limit.

The two Watson–Crick (WC) and similarly the two non-Watson–Crick (nWC) cytidine amino protons are coincident at 0 °C. Above 4 °C the resonances that we have assigned to the terminal base pair move toward each other and broaden, presumably due to fraying at the ends of the helix.

The cytidine amino protons of base pair (2, 5) neither shift (Figure 5) nor broaden until the temperature is within 5 °C of the T_m. The WC proton, peak d, then rapidly broadens and is lost from the spectrum at 35 °C. At 32 °C peak d is not only broadened but also reduced in intensity of about 50%. The intensity of peak d, like all the resonances of the nonexchangeable protons of the double helix, decreases as melting takes place. The same decrease in intensity is evident for the imino protons while for the remainder of the exchangeable protons accurate integration is impossible. Comparing the WC and nWC cytidine protons, we observe a significant preferential exchange of the WC protons.

The major difference between the spectra of the two oligomers at 0 °C is, as expected, the chemical shift of the WC adenosine amino proton (peaks f and r). In addition, the line width of peak f is similar to that of the cytidine amino protons, whereas that of peak r is similar to that of the guanosine amino protons. The difference illustrates that at 0 °C exchange broadening from rotation is still observed for the adenosine and guanosine amino protons. The excess line width is 40–70 Hz from which a lifetime of ca. 5 ms can be calculated for

the adenosine (peak r) and internal guanosine (peak u) amino groups. The terminal guanosine amino group has a somewhat shorter lifetime.

That both the adenosine and guanosine amino protons for base pairs (3, 4) and 2, 5) exhibit a similar line broadening due to rotation indicates a common mechanism for both. This is most probably a movement in the plane of the base pairs about the helix axis in which the amino proton hydrogen bonds lengthen and shorten. Transient weakening of the hydrogen bonds could allow the amino groups to rotate.

Having located the nWC adenosine amino proton at 6.0 ppm at 0 °C, we would expect that at higher temperature we would observe a resonance at ca. 7 ppm, corresponding to the two adenosine amino protons, if the amino group rotates rapidly. In d(C-G)$_3$ (Fazakerley et al., 1984a) we observed that the terminal guanosine amino group appeared at 18 °C and in d(GGm^{6}ATCC) the first guanosine amino group resonance appeared at 16 °C (Figure 4). Via fraying the guanosine amino group of the terminal G-C base pairs will rotate more freely than those in the center of the helix, and this fraying will be largely independent of the T_m of the oligomer. Note that there is a 50 °C difference in T_m between d(GGm^{6}ATCC) and d(C-G)$_3$. If this argument can be generalized, then peak x can be assigned to the guanosine amino protons of the terminal base pairs of d(GGATCC).

In d(GGATCC), peak z (Figure 7) is only observed over a narrow temperature range, 44–50 °C, and is a very broad resonance. In these experiments the base line slopes considerably toward the still intense residual water resonance. However, experiments with different acquisition parameters showed that peak z is not an artifact but a genuine resonance. From chemical shift considerations, we tentatively assign peak z to the adenosine amino group as it is about halfway between the WC and nWC resonances at 0 °C. This is, however, an extrapolation over 30 °C which may not be valid. With the exception of the adenosine amino (or methylamino) group all the other exchangeable resonances show very similar chemical shifts for the two hexamers, which would support the assignment of peak y as the guanosine amino group of base pair (2, 5) and thus peak z as the adenosine amino protons.

As observed for the methylated oligomer we find that the nWC cytidine amino protons of d(GGATCC) exchange more slowly with solvent than the corresponding WC amino protons. Between 15 and 28 °C only the nWC protons are visible in the spectrum. While base protonation would seem to be a prerequisite for amino proton exchange (McConnell & Seawell, 1973; McConnell, 1974, 1984), it is not clear how this would preferentially weaken the N–H bond which is cis to the protonation site.

Imino Protons. When a base pair in a helix opens, the imino protons may exchange with solvent protons, and this will shorten both the T_1 and T_2 values for that imino proton (Hilbers, 1979). In the open limited case, exchange takes place each time a base pair opens. The absence of effect upon T_2 of the exchangeable protons on raising the pH shows that their exchange with solvent is open limited. The exchange contribution to the observed relaxation gives an approximate measure of the lifetime of a base pair in the closed state. Thus, when the average lifetime of a base pair drops below 3 ms, the imino protons of oligonucleotides will show line widths of greater than 100 Hz.

The temperature dependence of the line widths of the imino protons of d(GGATCC) (Figure 9) is typical for the open limited case which has already been observed in many studies. Below 8 °C some broadening is observed due, at least in part, to increased viscosity of the solution. Above 8 °C proton exchange with solvent decreases the T_2 values. At 10 °C this exchange contribution is small, but at 23 °C the line widths have increased to ca. 70 Hz. The average lifetime of a base pair is ca. 6 ms at a temperature 22 °C below the T_m where the single-strand concentration is still quite small.

The imino (and amino) proton resonances of d(GGm^{6}ATCC) display a unique behavior with respect to their temperature dependence. Below 8 °C the observed line widths are very similar to those observed for the unmethylated hexamer, a minimum is found at ca. 14 °C, and exchange only dominates relaxation above 25 °C or close to the T_m (32 °C).

Above 14 °C three exchange processes are operating: (1) Even at 14 °C the concentration of single-stranded species is not negligible; the imino (and amino) protons of the single strand exchange very rapidly with solvent protons, but this exchange is not reflected in our spectra. The increasing concentration of single strand on raising the temperature is seen by a decrease in intensity of the exchangeable protons. (2) Exchange of imino protons may occur via transient base-pair opening, leading to a decreased T_2 for these protons. (3) An exchange contribution will come from a shortened lifetime of the helix state as the system moves out of the slow exchange limit. This exchange will equally be observed on the line widths of the nonexchangeable protons. The line widths of the nonexchangeable protons increase above 20 °C (Figure 3).

The imino proton resonances will reflect the sum of exchange processes 2 and 3. Between 8 and 23 °C the change in line width of the m^{6}A-T imino proton resonance corresponds to that observed for the adenosine H^8 resonance (Figure 9). The difference in their line widths is 6 ± 1 Hz throughout this temperature range. Thus, proton exchange with solvent (process 2) must be very slow. Transient opening must be a rare event, and a lower limit of 200 ms can be calculated for the average life time of the base pair 9 °C below the T_m of the oligomer. Between 20 and 23 °C the adjacent G-C imino proton resonance shows a slightly faster decrease in relaxation rate, suggesting that transient opening or fraying contributes to its relaxation.

When the two hexamers are compared at 23 °C, the contribution from exchange with solvent is small for the G-C base pair (2, 5) or neglibible for the m^{6}A-T base pairs of d(GGm^{6}ATCC), whereas it is very large for the unmethylated oligomer. This is the reverse order to that which could be expected from their relative thermal stabilities as measured by their T_m values.

Above 23 °C the imino proton resonances of d(GGm^{6}ATCC) broaden rapidly, more rapidly than does the adenosine H^8 resonance. Relaxation of the latter is dominated by the exchange helix $\rightleftharpoons$ coil. From our data it is not possible to separate quantitatively the contribution that this process makes to the imino proton relaxation from that of proton exchange with solvent. The total exchange contribution to the m^{6}A-T imino proton line width is ca. 50 Hz at the T_m (32 °C). Thus, the lifetime of the base pair is 5–10 ms. This is at least an order of magnitude longer than that for the A-T base pair in d(GGATCC) at the same temperature.

Conclusion. By comparing two oligonucleotides for which the sole difference in sequence is in the replacement of adenine by N^6-methyladenine, we have studied the effect of this methylation upon the conformation of the duplex state. Any difference in conformation must be quite small. Both hexanucleotides adopt a right-handed B-form helix with Watson–Crick base pairing. We could therefore conclude that enzymes

recognize specifically the two methyl groups which are very close to each other. However, our results suggest another possibility. We have observed a unique feature in the study of these oligonucleotides, that of slow exchange between the duplex and single-strand states. We have proposed (Fazakerley et al., 1984b) that the formation of the m^6A-T base pair is retarded by the unfavorable orientation of the adenine methylamino group which would inhibit base-pair formation when cis to N^1. We do not know what the equilibrium ratio of cis to trans for the methyl group is in the oligonucleotide single strand. On the monomer level (Engel & von Hippel, 1974) a 20-fold preference for the cis conformation was found, and if the same were to hold for the hexanucleotide, this would mean that only in 1 out of 400 collisions would the two methyl groups be both in favorable position for duplex formation. Another possibility is that only one methyl group is trans, that partial duplex formation occurs, and that the second methyl group turns around and the helix closes. Either way, helix formation would be slowed down and lead to the observed decreased T_m. The observation of slow exchange is not due to a significantly greater separation of the proton resonances for the two states. For the thymidine methyl group the separation between the resonances of the single strand and duplex is 120 Hz in d(GGm^6ATCC) and 110 Hz in d(GGATCC). Nor is this slow exchange due to a steric interaction between the two close adenosine methyl groups, as we observed the same phenomenon in the pseudohemimethylated octamer d(GGm^6ATATCC) (G. V. Fazakerley et al., unpublished results).

ACKNOWLEDGMENTS

We express our gratitude to Prof. J. M. Lhoste for permitting us to measure certain NOE difference spectra on the 400-MHz spectrometer at the Institut du Radium in Orsay.

Registry No. m^6A, 1867-73-8; d(GGm^6ATCC), 94040-20-7; d(GGATCC), 75605-61-7.

REFERENCES

Behe, M., & Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1619–1623.

Clore, G. M., & Gronenborn, A. M. (1984) *Eur. J. Biochem. 141*, 119–129.

Doerfler, W. (1981) *J. Gen. Virol. 57*, 1–20.

Engel, J. D., & von Hippel, P. H. (1974) *Biochemistry 13*, 4143–4158.

Engel, J. D., & von Hippel, P. H. (1978a) *J. Biol. Chem. 253*, 927–934.

Engel, J. D., & von Hippel, P. H. (1978b) *J. Biol. Chem. 253*, 935–939.

Fazakerley, G. V., Guschlbauer, W., van der Marel, G., & van Boom, J. H. (1984a) *Nucleic Acids Res. 12*, 8269–8279.

Fazakerley, G. V., Guy, A., Têoule, R., & Guschlbauer, W. (1984b) *FEBS Lett. 176*, 449–452.

Feigon, J., Wright, J. M., Denny, W. A., Leupin, W., & Kearns, D. R. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 207–217.

Feigon, J., Leupin, W., Denny, W. A., & Kearns, D. R. (1983) *Biochemistry 22*, 5943–5951.

Frêchet, D., Cheng, D. M., Kan, L.-S., & Ts'o, P. O. P. (1983) *Biochemistry 22*, 5194–5200.

Geier, G. E., & Modrich, P. (1979) *J. Biol. Chem. 254*, 1408–1413.

Guschlbauer, W. (1980) *Biochim. Biophys. Acta 610*, 47–55.

Hare, D. R., Wemmer, D. E., Chou, S.-H., Drobny, G., & Reid, B. R. (1983) *J. Mol. Biol. 181*, 319–336.

Hilbers, C. W. (1979) in *Biological Applications of Magnetic Resonance*, pp 1–43, Academic Press, New York.

Kan, L.-S., Borer, P. N., & Ts'o, P. O. P. (1975) *Biochemistry 14*, 4864–4875.

Lacks, S., & Greenberg, B. (1975) *J. Biol. Chem. 250*, 4060–4068.

Lacks, S., & Greenberg, B. (1977) *J. Mol. Biol. 114*, 153–168.

Marinus, M. G., & Morris, N. R. (1973) *J. Bacteriol. 114*, 1143–1150.

McConnell, B. (1974) *Biochemistry 13*, 4516–4523.

McConnell, B. (1984) *J. Biol. Struct. Dyn. 1*, 1407–1421.

McConnell, B., & Seawell, P. C. (1972) *Biochemistry 11*, 4382–4392.

McConnell, B., & Seawell, P. C. (1973) *Biochemistry 12*, 4426–4434.

Modrich, P. (1982) *CRC Crit. Rev. Biochem. 13*, 287–323.

Pardi, A., Martin, F. H., & Tinoco, I., Jr. (1981) *Biochemistry 20*, 3986–3996.

Patel, D. J. (1976) *Biopolymers 15*, 533–558.

Patel, D. J. (1977) *Biopolymers 16*, 1635–1656.

Patel, D. J., & Hilbers, C. W. (1975) *Biochemistry 14*, 2651–2660.

Plateau, P., & Guêron, M. (1982) *J. Am. Chem. Soc. 104*, 7310–7311.

Pukkila, P. J., Peterson, P., Herman, G., Modrich, P., & Meselson, M. (1983) *Genetics 104*, 571–582.

Radman, M., van der Elsen, P., & Glickman, B. (1978) *Mol. Gen. Genet. 163*, 307–312.

Raszka, M. (1974) *Biochemistry 13*, 4616–4622.

Raszka, M., & Kaplan, N. O. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2025–2029.

Reid, D. G., Salisbury, S. A., Bellard, S., Shakked, Z., & Williams, D. H. (1983) *Biochemistry 22*, 2019–2025.

Sheek, R. M., Boelens, R., Russo, N., van Boom, J. H., & Kaptein, R. (1984) *Biochemistry 23*, 1371–1376.

Sternglanz, H., & Bugg, C. E. (1973) *Science (Washington, D.C.) 182*, 833–834.

Trautner, T. A. (1984) *Curr. Top. Microbiol. Immunol. 108*, 176.

von Hippel, P. H., & McGhee, J. D. (1972) *Annu. Rev. Biochem. 41*, 233.

Vovis, G. F., & Lacks, S. (1977) *J. Mol. Biol. 115*, 525–538.