The effects of N7-methylguanine on duplex DNA structure

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Background: Non-enzymatic methylation of DNA by endogenous and exogenous agents produces a variety of adducts, of which the predominant one is N7-methyl-2'-deoxyguanosine (m^7dG). Although it is known that living organisms counter the deleterious effects of m^7dG by producing adduct-specific DNA repair proteins, the molecular basis for specific recognition and catalysis by these proteins is poorly understood. In addition to its role as an endogenous DNA adduct, m^7dG is also widely used as an *in vitro* probe of protein–DNA interactions. We set out to examine whether incorporation of m^7dG into DNA affects duplex DNA structure.

Results: We carried out a large-scale synthesis of a dodecamer containing the m^7dG adduct at a single, defined position. Because the instability of m^7dG precludes its incorporation into oligonucleotides by standard solid-phase methods, a novel strategy employing chemical and enzymatic synthesis was used. Characterization of the m^7dG-containing dodecamer by NMR reveals no structural distortion; indeed, m^7dG appears to encourage a modest shift toward a more characteristically B-form duplex.

Conclusions: These results argue strongly against induced DNA distortion as a mechanism for specific recognition of m^7dG by adduct-specific repair proteins. The broad substrate specificity of these repair proteins disfavors a model involving direct recognition of aberrantly placed methyl groups; hence, it may be that m^7dG is recognized indirectly, perhaps by its effects on the dynamics of DNA. On the other hand, the evidence presented here suggests that m^7dG interferes directly with sequence-specific recognition by DNA-binding proteins by steric blockage or by masking of required contact functionalities. The synthetic methodology used here should be generally applicable to high-resolution structural studies of oligonucleotides bearing adducts that are unstable to the conditions of solid-phase DNA synthesis.

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Introduction

Nonenzymatic DNA methylation is believed to represent a persistent threat to the integrity of the genome [1]. The predominant adduct produced by both endogenous and exogenous methylating agents is N7-methyl-2'-deoxyguanosine (m^7dG; Fig. 1). Although m^7dG itself appears to be relatively innocuous from a biological standpoint, the products of its spontaneous breakdown are mutagenic; consequently, repair systems have evolved to remove m^7dG and the products of its decomposition from the genome [1–7]. At present little is known about the mechanism by which the proteins responsible for repair detect the presence of m^7dG amid the vast background of normal G residues in DNA, nor how they initiate repair of the lesion through catalyzing hydrolysis of the glycosidic bond. In addition to its role in genetic damage, m^7dG has found widespread use as a probe of guanine-specific protein contacts in the major groove [8,9]. A greater understanding of the recognition of m^7dG by repair proteins, and the inhibitory effects of the adduct on sequence-specific DNA binding by regulatory proteins, could be gained by elucidating the effects of m^7dG on duplex DNA structure. Structural studies on m^7dG in DNA have been hampered by the difficulty of producing homogeneous oligonucleotides containing the adduct at well-defined sites, however.

In a preliminary study, we reported a chemoenzymatic method for the site-specific incorporation of labile adducts such as m^7dG into DNA [10]. This initial report did not determine whether the synthesis could be scaled up to the levels required to support high resolution structural studies. Here we report the large-scale synthesis of a DNA dodecamer, 5'-d(CGC**G**AATTCGCG) [11], which spontaneously self-associates to form a duplex containing m^7dG (**G**) at two equivalent, symmetry-related positions (Fig. 2a). ^1H NMR studies on this m^7dG-containing dodecamer reveal that the adduct is accommodated into B-form duplex DNA with little structural adjustment. These findings rule out damage-induced DNA distortion as a mode of recognition by proteins that cleave m^7dG from the genome, in addition to validating the use of m^7dG as a probe of sequence-specific protein–DNA interactions.

Results and discussion

Site-specific incorporation of the m^7dG moiety into a dodecamer was carried out using a previously reported method that combines chemical and enzymatic DNA synthesis [10]. The use of enzymatic steps in the synthesis is necessitated by the extreme instability of m^7dG toward the acidic and basic conditions encountered during solid-phase DNA synthesis. In the present study, we increased the scale of this synthesis by ~50-fold, to generate quantities sufficient for high-resolution structural studies. Briefly, the method involves use of solid-phase methods to produce a 5'-phosphorylated 11-base DNA sequence, which spontaneously self-assembles into a polymeric repeat structure having regularly spaced gaps (Fig. 2a). These gaps have all of the functionality required for enzymatic insertion of a single nucleotide that can pair with a

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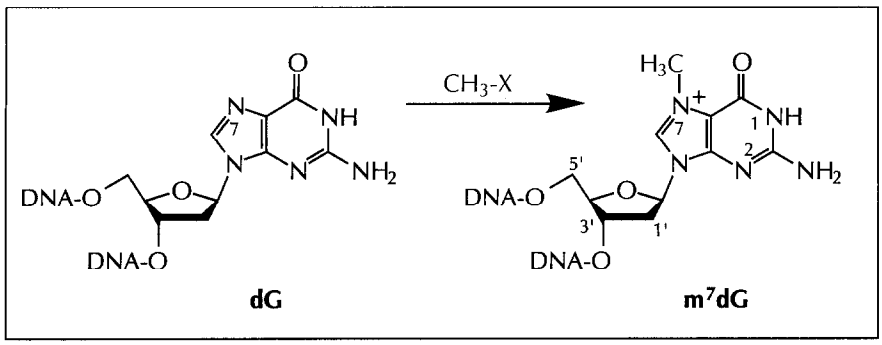

Fig. 1. Production of m^7dG in DNA by attack of endogenous or exogenous methylating agents.

C residue on the opposite strand (Fig. 2b). Covalent insertion of m^7dG into the gap, using the corresponding 5'-triphosphate as a source of the modified nucleoside, is catalyzed by a DNA polymerase (Sequenase II) together with T4 DNA ligase. Finally, cleavage of the regularly-repeating array using a restriction endonuclease liberates the C_2-symmetric dodecamer.

Two dimensional ^1H-^1H nuclear Overhauser effect spectroscopy (NOESY) [12,13] and double-quantum-filtered correlation spectroscopy (DQF-COSY) [14,15] spectra were used to assign the proton resonances of the methylated dodecamer and its unmodified counterpart [16] (further details available in the Supplementary materials). Preliminary measurements indicated that the m^7dG-containing duplex is C_2 symmetric on the NMR time-scale, exhibiting identical spectra for each of the two equivalent strands. The spectra were assigned through sequential nuclear Overhauser effects (NOEs) from the base protons (H6 or H8) to the H1' and H2'/H2" protons of the same

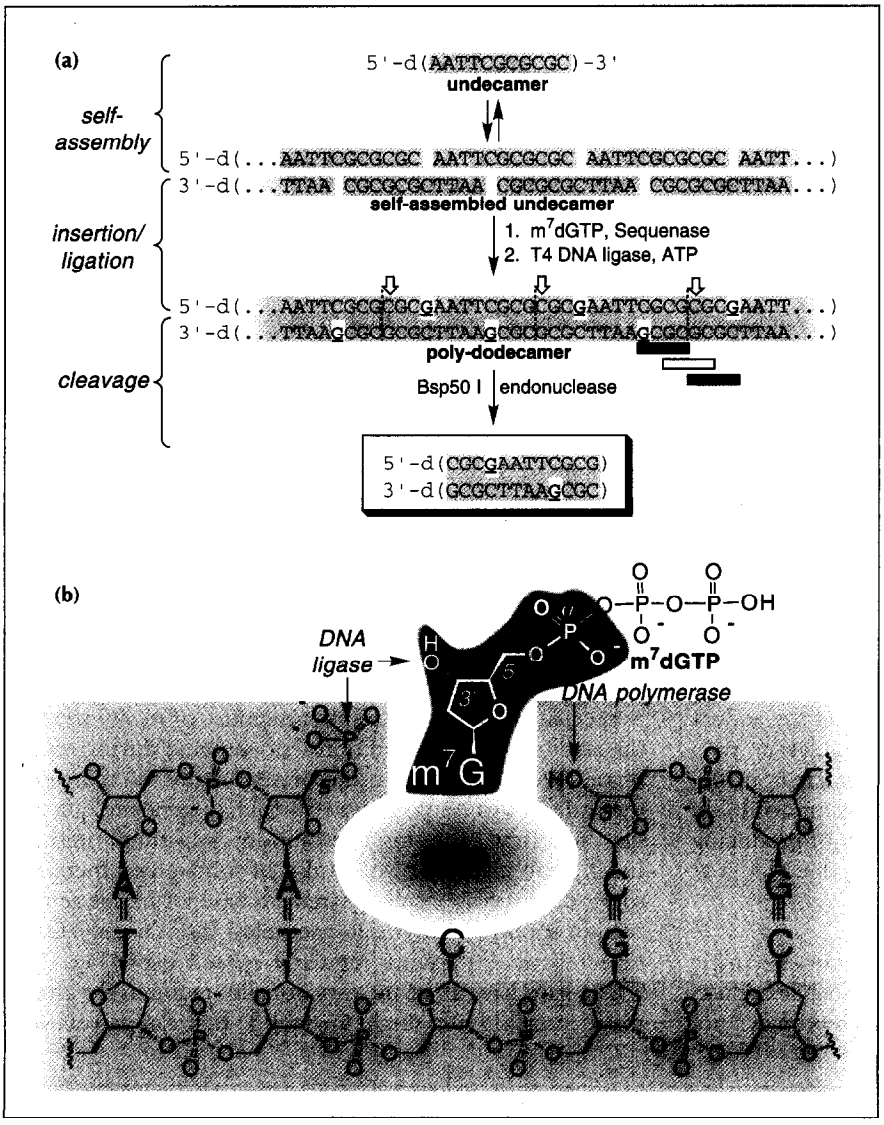

Fig. 2. Synthesis of a dodecamer specifically modified with m^7dG. **(a)** Reaction scheme. Arrows denote sites at which blunt-ended cleavage yields the desired product. Boxes directly under sequence denote overlapping *Bsp50*I sites: unmodified site (unshaded); m^7dG-containing sites (shaded). *Bsp50*I cleaves only at the unmodified site. All oligonucleotides shown in this scheme and elsewhere in the text possess a 5'-phosphoryl substituent; 3'-phosphoryl substituents, if any, are shown explicitly. **(b)** Structure of the single-nucleotide gaps into which an m^7dG nucleotide unit (pink) is inserted enzymatically. The orientation of the strands is reversed from that shown in (a). Arrows denote atoms that are joined by the enzymes indicated. Adapted from [10].

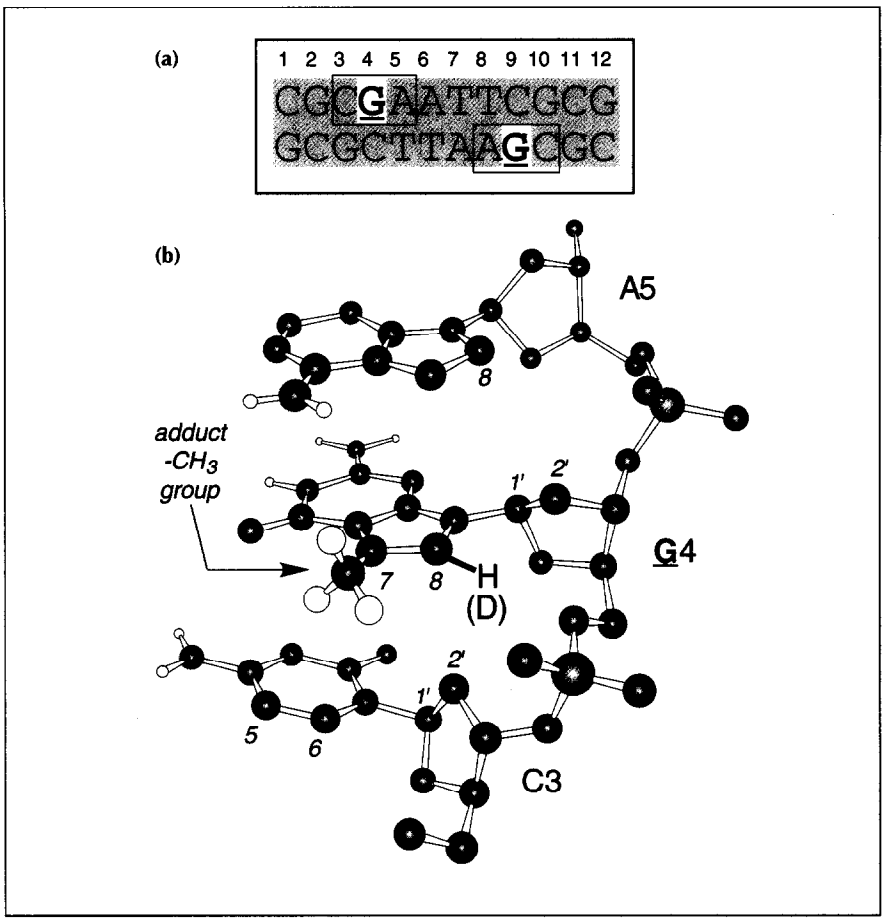

Fig. 3. Schematic of DNA containing m^7dG. **(a)** Sequence of the m^7dG-containing dodecamer studied in this work (**G** = m^7dG). The top and bottom strands are identical on the NMR timescale, hence equivalent positions on the two strands are indistinguishable. Boxed triplet is the sequence depicted in (b). **(b)** Model of a three base-pair section of the dodecamer containing a central m^7dG residue. Only one strand of the duplex is shown and only one of the hydrogen atoms is labeled, to simplify the picture. In D_2O, sequential NOE connectivity along the chain is lost at the H8 position of **G**4, owing to exchange with solvent. The model was constructed by addition of a methyl group to G4 of the Dickerson/Drew dodecamer, using the crystallographic coordinates [11] as starting point. Only the bond lengths and angles involving the newly added methyl group were minimized. This is not intended as a depiction of the fine details of the structure, but should be used for comparison with the NOE data. Atoms are color-coded as follows: white, H; black, C; blue, N; red, O; green, P. H atoms are named by the atom to which they are attached; H2' and H2'' are both attached to C2'.

residue and its neighbor on the 5' side [17]. For spectra recorded in D_2O, sequential NOE connectivity was lost at the position of the m^7dG (**G**4) due to the lack of NOEs to its H8 (Fig. 3). This is consistent with rapid proton–deuterium exchange at H8, a characteristic feature of N7,N9-dialkylguanines [18]. Sequential connectivity was reestablished by recording the spectra in 90 % H_2O/10 % D_2O. In addition to the rapid exchange of H8, its unusual chemical shift (9.41, compared with 7.87 ppm for the corresponding proton in the unmodified dodecamer) is in the range observed for dG residues in DNA containing more highly functionalized adducts at N7 [19–21].

The methyl group of **G**4 shows intense NOEs to the H5 and H6 of C3 (Fig. 3b), with the latter being stronger. Weaker cross-peaks are also observed between the **G**4 methyl group and the H2' and H2'' protons of C3. These NOEs establish that **G**4 possesses the usual (*anti*) orientation about its glycosidic bond [17]. The relative intensities of inter-residue NOEs throughout the helix are characteristic of a right-handed (B-form) helix [17]. Overall, the DQF-COSY and NOESY spectra of the m^7dG-containing oligonucleotide closely resemble those of the control, suggesting the adduct is accommodated in the duplex with only minor structural adjustments (details available in the Supplementary materials).

The puckers of the 2'-deoxyribose sugars were deduced from the intensity of H1' to H4' NOEs, taken together with the magnitude of the COSY cross-peaks for several spin systems within the sugar [22]. The sugar puckers of the modified dodecamer closely paralleled those of the native control, though they were slightly closer to the pucker characteristic of the prototypical B-form 2'-*endo* configuration at many positions throughout the helix.

The base-pairing properties of the m^7dG-containing dodecamer were investigated through examination of its imino proton spectrum (Fig. 4). At low temperature, imino proton peaks for all six non-equivalent base-pairs were visible in the spectra of both the modified and control dodecamers. NOESY experiments in 90 % H_2O were used to assign the imino proton peaks. Two of the peaks in the spectra of the modified dodecamer were broadened by exchange with solvent; one belonged to the terminal C1–G base-pair (Fig. 4, signal 1), which exchanges rapidly because of fraying at the helix termini. The other was assigned to the modified guanine in the **G**4–C base-pair (signal 4). The rapid exchange of this proton is consistent with the presence of NOE cross-peaks between the **G**4 imino proton and the H_2O resonance, and between the imino and C8 protons of **G**4 (resulting from spin diffusion; see, for example [23,24]). *N*7-alkylation is known to lower the pK_a of the dG imino proton to a value of ~7, although undoubtedly this pK_a is somewhat higher in duplex DNA [25,26]. However, breathing of the helix may transiently acidify the *N*7-H and thereby allow rapid exchange. Hence, the broadening of the peak for the **G**4

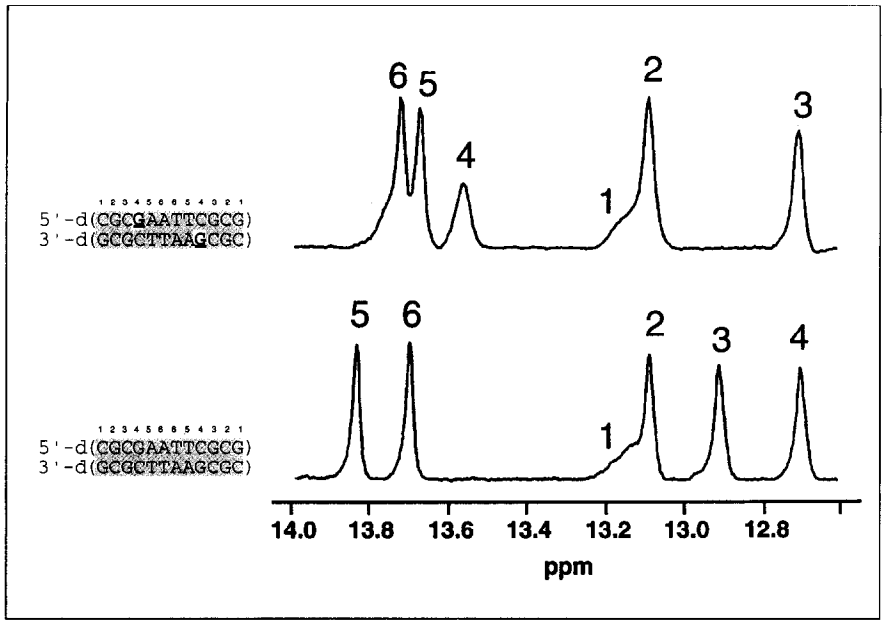

Fig. 4. Imino proton spectra of the m^{7}dG-containing dodecamer (top), and the unmodified control (bottom). Signal 1 is from the terminal C1–G base pair, signal 4 from the G4–C base pair. The absence of additional imino proton signals in this and other NMR spectra (not shown) illustrates the homogeneity of DNA produced by this method.

imino proton does not necessarily reflect weakened base-pairing; it may instead indicate lability of the proton when unpaired. Indeed, evidence based on thermodynamic measurements of duplex denaturation suggests m^{7}dG forms a stronger base-pair than does dG [10], most likely because N7-methylation increases the strength of base stacking interactions [27–29]. The lack of a correlation (for internal base pairs) between base-pair strength and proton exchange kinetics has been experimentally demonstrated in other systems [30]. In comparing the native and modified dodecamers, the most pronounced changes involve the modified base-pair and those immediately flanking it (see Figs 3b and 4). In the absence of any evidence for all but minor structural changes, it is likely that the chemical shift changes at the flanking base-pairs may arise simply from deshielding effects caused by the delocalized positive charge on G4 [31].

To assess changes in the backbone conformation, we assigned the ^{31}P resonances of the m^{7}dG and control dodecamers using ^{1}H–^{31}P hetero-total correlation spectroscopy (TOCSY) [32,33]. Since ^{31}P signals in oligonucleotides are strongly affected by even minor changes in structure [34–36], these data provide a sensitive measure of the local versus global influence of m^{7}dG on duplex structure. Many of the ^{31}P resonances were slightly different in the two dodecamers, as they would be for DNA molecules differing in one base pair. Somewhat larger changes, however, were observed for the phosphates immediately 5' and 3' to the G residue (see Fig. 3b; details available in the Supplementary materials). The upfield shift of these signals in the methylated dodecamer is consistent with an increased tendency to adopt the *gauche, gauche* (g-,g-) conformation, which is representative of canonical B-form DNA [17].

Taken together, the results of NMR analysis rule out induction by m^{7}dG of pronounced helical aberrations such as bending, kinking or abnormal base-pairing; they do not, however, preclude modest adjustments such as gentle bending or changes in propeller-twisting. Indeed, the observation of minor differences in sugar puckers between the m^{7}dG-containing oligonucleotide and the native dodecamer indicates that some modest adjustments do occur. Remarkably, however, the m^{7}dG nucleotide evidently confers upon the dodecamer a more canonical B-form structure than that adopted by its unmodified counterpart.

Significance

Aberrant methylation of DNA produces a variety of adducts, of which the most prevalent is m^{7}dG. DNA repair proteins have evolved to recognize and excise m^{7}dG from the genome. Attempts to understand the molecular basis for adduct-specific recognition and repair by these proteins have been hampered by the paucity of structural information on the adducts and on their cognate repair proteins. In general, it has not been feasible to prepare oligonucleotides containing chemically unstable adducts such as m^{7}dG for structural studies, because the adducts decompose when subjected to the harsh conditions of chemical DNA synthesis. To overcome this problem, we have turned to the development of a method that employs chemical synthesis to produce the native portion of the oligonucleotide, and enzymatic synthesis to introduce the unstable adduct under mild conditions. The quantities of m^{7}dG-containing oligonucleotide produced here are sufficient to support complete characterization by NMR or X-ray. This method should be generally applicable to the synthesis of DNA containing a variety of unstable lesions.

Characterization of an m^{7}dG-containing dodecamer by multidimensional NMR spectroscopy

reveals that the adduct causes little if any remodeling of duplex DNA structure. Indeed, the changes in NOE intensities observed between the native and unmodified dodecamers are sufficiently subtle that they are unable to produce statistically significant different structures from distance-refined molecular dynamics calculations (data not shown). These results suggest that DNA repair proteins recognize m^7dG by some mechanism other than adduct-induced DNA distortion, perhaps by detecting the effect of the adduct on DNA dynamics. Another possibility, direct recognition of the methyl group, is unlikely in light of the broad substrate specificity of these enzymes, which recognize adducts bearing a variety of alkyl substituents located in both the major and minor groove [2,7,37]. Finally, the lack of structural perturbation caused by m^7dG provides the first direct validation for its long-standing use as a local probe of sequence-specific contacts in protein–DNA complexes. In other words, m^7dG appears to interfere with the sequence-specific recognition by proteins through direct steric blockage or masking of required contact functionalities rather than through perturbation of duplex DNA structure.

Materials and methods

Oligonucleotide synthesis

The 11-base DNA sequence 5'-d(AATTCGCGCGC) was synthesized using the four conventional phosphoramidites (Milligen) and 5'-phosphorylated while resin-bound, using the Phosphate-ON™ reagent (Clontech). Following standard ammonia deprotection, the oligonucleotide was purified by denaturing polyacrylamide gel electrophoresis and recovered by a crush-and-soak procedure (Supplementary material).

Large scale synthesis of 5'-d(CGCGAATTCGCG)

The 5'-phosphorylated 11-mer 5'-d(AATTCGCGCGC) (500 nmol) was incubated with 500 units Sequenase II (generous gift of Dr. Stanley Tabor) in 2 ml aqueous buffer containing 50 mM TRIS-HCl, pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM m^7dGTP (Sigma). After 2 h at 25 °C, the reaction mixture was ethanol-precipitated three times and the pellet redissolved in 1.6 ml aqueous ligase buffer (10 mM TRIS-HOAc, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM ATP). T4 DNA ligase (800 units; gift of Richard Kolodner) was added and the ligation allowed to proceed for 12 h at 16 °C. The ligated DNA was ethanol-precipitated and redissolved in 50 µl TE buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8.0). The ligated oligonucleotide (500 nmol based on the initial 11-base sequence) was then incubated at 25 °C with the restriction endonuclease *Bsp50*I (1500 units; Stratagene) in a buffer containing 25 mM TRIS-HOAc, pH 7.6, 10 mM magnesium acetate, 0.5 mM 2-mercaptoethanol, 10 µg ml^{-1} bovine serum albumin. The 5'-phosphate was then removed to allow direct comparison with prior studies of unmodified and modified 5'-d(CGCGAATTCGCG), which used the 5'-OH form. Calf intestinal alkaline phosphatase (500 units; Boehringer Mannheim) was added directly to the restriction digestion mixture and the reaction allowed to proceed 2 h at 25 °C. After ethanol precipitation, the 12-mer 5'-d(CGCGAATTCGCG) was dissolved in 100 µl formamide loading dye, heated at 50 °C for 5 min and then loaded directly onto a 20 % denaturing PAGE gel. Following visualization and excision of the desired product band by UV-shadowing, the DNA was recovered by electroelution into 10 M NH_4OAc, pH 7.5 (80 volts, 30 min). The electroelution was done twice to ensure completion. The combined NH_4OAc fractions were ethanol-precipitated. This procedure, which typically yields 300–400 nmol of 5'-d(CGCGAATTCGCG), was repeated four times to obtain quantities sufficient for NMR measurements. The DNA generated by this procedure is typically greater than 95% pure as determined by denaturing polyacrylamide gel electrophoresis, NMR spectroscopy and nucleotide composition analysis.

Preparation of 5'-d(CGCGAATTCGCG) for NMR measurements

The oligonucleotide samples from four synthetic runs were combined together in 2 ml TE buffer containing 1 M NaCl, transferred to a Centricon 3 microconcentrator (3 kDa cut-off; Amicon), and centrifuged at 7000 x g (4 °C) until the volume of the solution was reduced to ~400 µl. The counterions were then exchanged by washing the DNA in the Centricon 3 with 1 ml of 1 M NaCl, 1 mM sodium phosphate, pH 8.0, which was carried out four times in succession. The DNA was desalted by washing five times with 99 % D_2O containing 1 mM sodium phosphate, pH 8.0. The sample (400 µl) was transferred to an Eppendorf tube and the Centricon 3 membrane was washed twice with 0.5 ml D_2O. To the combined DNA-containing fractions were added 40 µl 1 M NaCl and 36 µl 0.1 M sodium phosphate, pH 8.0. This solution was lyophilized to dryness. For spectra obtained in 10 % D_2O the sample was redissolved in 360 µl H_2O and 40 µl D_2O. For all other spectra the DNA was lyophilized three times after dissolution in 400 µl 99.96 % D_2O, then finally dissolved in 400 µl 99.96 % D_2O and transferred directly to an oven-dried NMR tube. The final concentration of the oligonucleotide was ~2 mM and the final buffer concentration was 0.1 M NaCl, 10 mM sodium phosphate, pH 8.0.

NMR spectroscopy

All spectra were recorded at 287 °K. ^1H NMR spectra were referenced with respect to internal standard 3-trimethylsilyl(2,2,3,3-d_4)propionate at 0.0 ppm, and ^{31}P spectra were referenced to external standard trimethyl phosphate at 0.0 ppm. The data were processed using the FELIX software package (Hare Research, Inc.). For NOESY spectra taken in 90 % H_2O/10 % D_2O, a 1-3-3-1 acquisition pulse was used to suppress the H_2O signal. A total of 320 t_1 increments were accumulated. The mixing time was set to 200 ms. Phase-sensitive NOESY spectra in 99.998 % D_2O were performed using time-proportional phase increments (TPPI). A total of 256 t_1 increments were accumulated. DQF-COSY spectra were recorded (in 99.96 % D_2O) in the phase-sensitive mode (using TPPI) with presaturation of the solvent. A total of 512 t_1 increments were accumulated. ^{31}P–^1H hetero-TOCSY spectra were taken in the inverse mode as described [32]. The decoupler and transmitter were used for the proton and ^{31}P pulses respectively. A total of 128 t_1 increments were accumulated. The mixing and evolution time were set to 50 msec and 3 msec, respectively. The ^{31}P 90° pulse width was determined using trimethyl phosphate as a standard.

Supplementary material available

Supplementary materials are available directly from the authors by fax (617-495-8755). These describe the oligonucleotide purification, NOESY and COSY methods, ^{31}P–^1H hetero-TOCSY and NMR methods, the sequential assignment of

NOEs, the determination of the identity of exchangeable protons, and the assignment of sugar pucker.

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