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Synthesis and Characterization of Poly[d(G-z^{5}C)]. B-Z Transition and Inhibition of DNA Methylase[†]

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ABSTRACT: Deoxy-5-azacytidine 5′-triphosphate was synthesized and used as a substrate for the enzymatic synthesis of the polynucleotide poly[d(G-z^{5}C)]. Whereas the triphosphate decomposes in solution, the azacytosine analogue incorporated into DNA is stable under conditions preserving the double-helical structure. Poly[d(G-z^{5}C)] undergoes the transition to the left-handed Z conformation at salt (NaCl and MgCl$_2$) concentrations approximately 30% higher than those required for unsubstituted poly[d(G-C)]. However, the incorporation of azacytidine potentiates the formation at room temperature of the Z helix stabilized by the transition metal Mn^{2+}; in the case of poly[d(G-C)], a heating step is required. The spectral properties of the two polymers in the B and Z forms are similar. Both left-handed forms are recognized by anti-Z DNA immunoglobulins, indicating that the DNAs bear common antigenic features. Poly[d(G-z^{5}C)] is not a substrate for the DNA cytosine 5-methyltransferase from human placenta. It is a potent inhibitor of the enzyme when tested in a competitive binding assay. These results are compatible with a very strong, possibly covalent, mode of interaction between methyltransferases and DNA containing 5-azacytosine.

Substitution of a nitrogen atom into position 5 of cytosine yields the nucleoside analogue 5-azacytidine (z^{5}C).[1] This drug exhibits potent antibacterial, antitumoral, mutagenic (Vesely & Cihak, 1978), and carcinogenic (A. D. Riggs, personal communication) activities. It is used clinically as an antileukemic agent (Saiki et al., 1978) as well as in the treatment of patients with severe β-thalassemia (Ley et al., 1982). The cytotoxic activity of 5-azacytidine may be related to the inhibition of protein synthesis, possibly at the level of tRNA. Studies have demonstrated that administration of z^{5}C to mice results in an inhibition of the enzymatic methylation of cytidine in tRNA, apparently without direct incorporation of the nucleoside into the ribopolymer (Lu et al., 1976). However, z^{5}CTP is a substrate for the yeast AMP (CMP):tRNA nucleotidyltransferase, leading to the incorporation of the nucleotide analogue into the 3′ terminus of tRNA^{Phe} (Zielinski & Sprinzl, 1984), and 5-azacytosine can replace cytosine residues in DNA (Jones & Taylor, 1981).

At subcytoxic levels, z^{5}C dramatically inhibits the C5 methylation of cytidine in DNA (Taylor & Jones, 1982; Pfeifer et al., 1983) and displays pronounced inductive effects on the expression of certain eukaryotic genes and viruses [for reviews, see Felsenfeld & McGhee (1982) and Riggs & Jones (1983)]. Such biochemical activity is compatible with the as yet unexplained inverse correlation between the degree of cytosine methylation in the region near a gene and the transcriptional expression of that gene (Felsenfeld & McGhee, 1982; Doerfler, 1983; Razin & Szyf, 1984).

The in vivo inhibitory effect of z^{5}C on DNA methylation requires the incorporation of this modified nucleoside into DNA (Jones & Taylor, 1980; Taylor & Jones, 1982; Adams et al., 1982). However, the pronounced reduction of the methylation of cytosine cannot be explained simply by the

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[1] Abbreviations: z^{5}C, 5-azacytidine; dz^{5}C, deoxy-5-azacytidine; DNase, deoxyribonuclease; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

inability of z^{5}C to serve as a methyl acceptor. Instead, dz^{5}C-containing DNA inhibits processive methylation (Drahovsky & Morris, 1971a,b) of hemimethylated DNA by eukaryotic DNA methyltransferase, possibly by the formation of a tight-binding complex with the enzyme (Taylor & Jones, 1982; Pfeifer et al., 1983). According to one proposal, 5-azacytosine incorporated into DNA forms stable covalent adducts with DNA methylases, thereby irreversibly inactivating the latter (Santi et al., 1983).

Although significant advances have been made toward an understanding of the biological effects of z^{5}C, several fundamental questions remain to be answered. First, what are the properties of dz^{5}CTP as a substrate for DNA polymerases and how is the analogue incorporated into DNA [see Bouchard & Momparler (1983)]? Second, since 5-azacytidine hydrolyzes rapidly in solution even at neutral pH (Notari & DeYoung, 1975), to what degree is the nucleotide stabilized upon incorporation into DNA and what are the biological effects of its ultimate decomposition? Third, what are the biochemical properties, e.g., nuclease susceptibilities, of dz^{5}C-containing DNA? Fourth, what are the mechanisms and consequences of dz^{5}C interaction with proteins, including eukaryotic DNA methylases?

A final question regarding the role of incorporated 5-deoxyazacytidine pertains to structural perturbations of the DNA helix. For instance, dz^{5}C may *directly* influence the local stability of DNA and alter the potential of given sequences to adopt right- and left-handed helical conformations. Alternatively, the incorporation of dz^{5}C may *indirectly* influence the structure of DNA by inhibiting further cytosine methylation.

In order to address the above questions, we have chemically synthesized dz^{5}CTP and enzymatically incorporated the modified nucleotide into the alternating purine–pyrimidine copolymer poly[d(G-z^{5}C)]. The physical, structural, and biochemical (substrate) properties of this polymer are the subjects of this report.

EXPERIMENTAL PROCEDURES

Materials. Poly[d(I-C)] (ϵ_{251} = 6900 M^{-1} cm^{-1}; Wells et al., 1970) and dGTP were obtained from P-L Biochemicals. [8-^{3}H]dGTP and *S*-adenosyl-L-[*methyl*-^{3}H]methionine were from New England Nuclear Corp. *Micrococcus luteus* DNA polymerase (EC 2.7.7.7) was purchased from P-L Biochemicals and Sigma Chemical Co. Pancreatic DNase I (EC 3.1.21.1), snake venom phosphodiesterase (EC 3.1.4.1), and nuclease P1 (EC 3.1.30.1) were supplied by Boehringer Mannheim and *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) and micrococcal nuclease (EC 3.1.31.1) by Sigma Chemical Co. *Hha*I restriction endonuclease (EC 3.1.23.19) was purchased from Bethesda Research Laboratories. Proteinase K was from Merck (Darmstadt). Hoechst 33342 was obtained from Hoechst and ethidium bromide from Sigma Chemical Co. Sephadex G-10 was from Pharmacia, and AG 50W-X2 and Dowex 1-X4 ion exchangers and Bio-Gel A-5M were from Bio-Rad. Polygram Cel 300 PEI/UV$_{254}$ thin-layer chromatography sheets and Cel 300-10/UV$_{254}$ precoated plates were obtained from Macherey Nagel. All other chemicals were from Merck or J. T. Baker Chemicals.

Synthesis of Deoxy-5-azacytidine 5′-Triphosphate. To a stirred suspension of dz^{5}C prepared according to Pliml & Sorm (1964) (55 mg, 0.24 mmol, in 1.4 mL of trimethyl phosphate) was added phosphorus oxychloride (37 μL, 0.4 mmol) at 0 °C. The mixture was stirred under exclusion of atmospheric moisture (under argon) for 4 h in a 5-mL microvial (Aldrich) and then added dropwise to an ice-cold solution of 0.2 M triethylammonium bicarbonate (6 mL). After 30 min the reaction solvent was evaporated, and the residue was taken up in 1 mL of water and applied to an AG 50W-X2 column (2 × 26 cm) in the H$^+$ form. The column was washed with water and the UV-absorbing material collected: yield of dz^{5}CMP, 1413 A_{245} units (pH 7.6), 89%; thin-layer chromatography (1 M sodium acetate, pH 6.0) R_f(dz^{5}CMP) 0.53, R_f(CMP) 0.48, R_f(dz^{5}C) 0.84; UV spectrum (100 mM Tris-HCl, pH 7.0) λ_{max} 243 nm, λ_{min} 228 nm. The molar extinction coefficient for phosphorylated dz^{5}C was assumed to be the same as for the nucleoside: ϵ_{255} = 2560 (pH 1, 0.1 N HCl); ϵ_{245} = 6560 (pH 7.6, 50 mM Tris-HCl).

Deoxy-5-azacytidine 5′-monophosphate (triethylammonium salt, 0.238 mmol) was dissolved in 5 mL of wet dimethylformamide, and 100 μL of tri-*n*-butylamine was added. The solution was evaporated and the residue dried by repeated coevaporation with anhydrous dimethylformamide (6 × 3 mL). Finally, the residue was dissolved in 4 mL of dry dimethylformamide, and 57 mg (0.352 mmol) of 1,1′-carbonyldiimidazole was added. After 45 min at room temperature, the solution was cooled on ice and a solution of tetrakis(tributylammonium) pyrophosphate (0.262 mmol) in 1 mL dimethylformamide was added under vigorous stirring. After 2 h at room temperature, the reaction mixture was cooled in an ice bath, treated with 30 μL of methanol, and after 15 min evaporated. The residue was dissolved in 2 mL of water and applied to a Dowex 1-X4 (formate) column (1 × 12 cm). The column was eluted with a linear gradient of ammonium formate (50 mM to 1.0 M, pH 4.2, 600 mL), and fractions containing the product eluting at 0.5 M buffer were pooled, concentrated, and desalted on Sephadex G-10 (2 × 54 cm): yield of deoxy-5-azacytidine 5′-triphosphate (dz^{5}CTP), 66 μmol, 28%; thin-layer electrophoresis (0.1 M citrate buffer, pH 5.6 at 23 V/cm), the mobilities relative to CMP, (dz^{5}CTP) 1.9, (CTP) 1.75, (dz^{5}CMP) 1.2; UV spectrum (100 mM Tris-HCl, pH 7.0) λ_{max} 242 nm. The triphosphate was stored lyophilized at −20 °C and used immediately after redissolving.

Preparation of Poly[d(G-z^{5}C)]. Poly[d(G-z^{5}C)] was synthesized in two separate reactions by a modification of procedures described elsewhere (L. P. McIntosh et al., unpublished results). The reaction mixtures for syntheses I and II (4.0 and 5.6 mL, respectively; 50 mM potassium phosphate, pH 7.0–7.5, 12 mM MgCl$_2$, 1 mM 2-mercaptoethanol, and 0.02% NaN$_3$) contained ca. 2 mM dz^{5}CTP, 3 mM dGTP, 25 μM poly[d(I-C)] template, and 5–12 units/mL *Micrococcus luteus* DNA polymerase. Additionally, [8-^{3}H]dGTP (150 μCi/mL) was included with a 0.6-mL aliquot from reaction mixture II for the preparation of radioactively labeled poly[d(G-z^{5}C)]. The reactions were incubated at 37 °C and the syntheses monitored by an assay based on the enhancement of ethidium bromide fluorescence or by measurement of the tritium label incorporated into DNA. After 24 h, synthesis I was quenched by the addition of excess EDTA followed by digestion (3 h, 37 °C) with 1 μg/mL proteinase K, phenol extraction, and purification by gel exclusion chromatography. Synthesis II was terminated by freezing followed by phenol extraction of the thawed sample. MnCl$_2$ was added to 15 mM, which resulted in the formation of a precipitate which was collected by centrifugation. The polymers in the supernatant and precipitate fractions (resuspended in excess EDTA) were isolated by gel exclusion chromatography (Bio-Gel A-5M). The logic for the MnCl$_2$ precipitation step was to separate poly[d(I-C)] and poly[d(G-z^{5}C)] by stabilizing the latter polymer in a sedimentable left-handed Z* state (van de Sande & Jovin, 1982; van de Sande et al., 1982; Jovin et al., 1983a).

The DNA samples were stored frozen in 1 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA.

Nucleoside Analysis of Poly[d(G-z^5C)]. The synthesized poly[d(G-z^5C)] samples were analyzed by HPLC after nuclease digestion in order to determine the nucleoside content of the polymer and to measure the degree of template contamination. In 50 μL of 10 mM Tris-HCl, pH 7.2, 0.6 mM $ZnCl_2$, and 4 mM $MgCl_2$, 0.05 A_{260} unit of DNA was incubated at 37 °C for 15 h with 20 μg/mL pancreatic DNase I, 100 μg/mL snake venom phosphodiesterase, and 100 μg/mL alkaline phosphatase. The product nucleosides were separated on a 0.46 × 25 cm Du Pont Sorbax ODS column (using a DuPont 850 LC HPLC system), eluted with 97.5% (v/v) 50 mM KH_2PO_4, pH 4.0 (buffer A), and 2.5% (v/v) methanol for 18 min followed by 92% buffer A and 8% methanol. The elution products were detected and quantitated on the basis of UV absorption using the following ϵ_{254} values (M^{-1} cm^{-1}): dG, 11 600; dI, 8200; dC, 7200. The dz^5C decomposed during the incubation of the DNA samples; the amount in DNA was assumed to be stoichiometric to dG. For the identification of the nucleosides in HPLC runs, the appropriate deoxynucleoside standards were employed. To verify that complete polymer digestion was achieved, aliquots from the above samples were further incubated with nuclease P1 and phosphatase or, alternatively, with DNase I, snake venom phosphodiesterase, and phosphatase. Similar results were obtained with each digestion procedure (data not shown).

Physical Properties of Poly[d(G-z^5C)]. The melting profile of poly[d(G-z^5C)] was measured according to Pörschke & Jung (1982). The polymer sizes were determined by gel electrophoresis using appropriate DNA restriction fragment standards. Ultraviolet absorption and circular dichroic (CD) spectra were recorded on a Kontron Uvikon 820 spectrophotometer and a Jobin-Yvon Mark IV dichrograph, respectively.

Nuclease Digestion Studies. Digestion with restriction endonuclease *Hha*I (1 unit) was carried out in reactions at 37 °C (60 μL) containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1 A_{260} of radiolabeled poly[d(G-z^5C)] (100 cpm/μL). The reaction was monitored by the radioactivity of aliquots augmented with 22 mM EDTA, frozen to inactivate the enzyme, spotted on 1 × 1 cm DE-81 (Whatman) paper squares, and washed successively with 0.35 M ammonium formate, 0.1 M ammonium bicarbonate, and ethanol. Micrococcal nuclease reactions were similar except that 2 units of enzyme was used in a buffer consisting of 10 mM Tris-HCl, pH 7.6, and 2 mM $CaCl_2$.

Drug Binding Studies. Fluorescence measurements of Hoechst 33342 (10 μM in 0.1 M NaCl and 10 mM Tris-HCl, pH 7.6) and ethidium bromide (0.5 μg/mL in 2 mM Tris-HCl, pH 8.5, and 2 mM EDTA) binding to DNA were performed with an SLM (Urbana, IL) 8000S photon counting spectrofluorometer using λ_{ex} 355 nm and λ_{em} 465 nm for Hoechst 33342 and λ_{ex} 525 nm and λ_{em} 600 nm for ethidium bromide.

B–Z Transition Analysis. The B–Z equilibria of poly[d(G-z^5C)] in NaCl and $MgCl_2$ were measured by a continuous dilution method using a motor-driven syringe to slowly add buffer to a mechanically stirred solution of DNA in a concentrated salt solution (McIntosh et al., 1983). The conformational states of the polymer were determined by the absorbance at 290 nm and by CD. The transition kinetics were monitored by the absorbance at 295 nm after adding the DNA to 10 mM Tris-HCl, pH 7.6, and 80 μM EDTA solutions also containing >3 M NaCl. The centrifugation assay for Z* DNA was carried out in an Eppendorf centrifuge operated at 12000*g* for 10 min.

DNA Methyltransferase Reactions. Methyl-accepting activities and binding properties of poly[d(G-z^5C)] were tested with highly purified DNA methylase (EC 2.1.1.37) from human placenta. The isolation of this enzyme and some of its properties have been described in detail previously (Pfeifer et al., 1983). The standard reaction mixture (0.1 mL) contained 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5 mM dithioerythritol, 2% glycerol, 10 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1.5 μCi), 0–1 μg of synthetic DNA polymer, and 0–25 units of DNA methylase (specific activity 1800 units/mg of protein; 1 unit of DNA methylase activity incorporates 1 pmol of methyl groups into double-stranded *M. luteus* DNA in 1 h under standard assay conditions). After incubation at 37 °C for 45 min DNA methylase activity was determined on the basis of incorporated radioactivity as described elsewhere (Pfeifer et al., 1983).

RESULTS

Synthesis and Stability of Deoxy-5-azacytidine 5'-Triphosphate. In order to avoid decomposition of the unstable triazine ring of dz^5C, a rapid phosphorylation procedure was employed. Extreme pH conditions during the synthesis were also avoided. The average yield of the phosphorylation from dz^5C to dz^5CTP was about 30%. Since no other UV-absorbing byproducts were detected in the reaction mixture, it is assumed that the low yield of triphosphate reflected the high susceptibility of the dz^5C triazine ring to nucleophilic attack, leading to decomposition of the base and loss of UV absorption. The dz^5CTP was characterized by chromatography, electrophoresis, and UV absorbance. No chromatographically detectable impurities were present. In aqueous solution, dz^5CTP decomposes readily but more slowly than the corresponding ribonucleoside triphosphate, z^5CTP (data not shown). The chemical synthesis of dz^5CTP offers advantages relative to enzymatic procedures [e.g., see Bouchard & Momparler (1983)], particularly in the case of large-scale preparations.

Synthesis of Poly[d(G-z^5C)]. The substrates dGTP and dz^5CTP were used for the synthesis of poly[d(G-z^5C)] by *Micrococcus luteus* DNA polymerase. Table I summarizes the results of these enzymatic polymerizations. HPLC analysis of the component nucleosides of the product DNAs indicated the expected composition of the products (Table II). Due to decomposition of the dz^5C during enzymatic digestion and the consequent formation of non-UV-absorbing products, this nucleoside was not identified in the chromatograms. However, the presence of dG confirmed that polymer synthesis had occurred; in the absence of dz^5CTP in the incubation mixture, no utilization of dGTP was detectable.

The compositional analyses also demonstrated the fact that the poly[d(I-C)] template constituted a significant fraction of the final isolated DNA preparations. This arose due to the low net yield of the deoxyazacytidine-containing polymer and the lack of an efficient means for selectively removing the template during the subsequent purification of the product DNA. An attractive strategy for the selective purification of the poly[d(G-z^5C)] product was based on the observation that, in the presence of millimolar $MnCl_2$, this polymer adopts a Z* conformation (see below), whereas poly[d(I-C)] remains right handed (Sutherland & Griffin, 1983). Previous studies with poly[d(G-C)] have demonstrated that self-associated Z* DNA may be selectively sedimented by centrifugation in the presence of B DNA (Jovin et al., 1983a). Accordingly, the synthesis II reaction mixture was separated into a supernatant and a $MnCl_2$-precipitated fraction. The latter contained 84% of the total DNA as judged by the absorbance at 250 nm (Table I). However, chromatographic analysis indicated that

Table I: Synthesis of Poly[d(G-z^{5}C)]

synthesis[a]	fraction[b]	A_{250} units[c]	template content[d] (%)	A_{250} units[c] (cor)	fold synthesis[e]	dz^{5}CTP utiliz[f]	T_m[g] (cor)
I		1.6	18	1.3_1	2.2	1.1_3	75
II	supern	0.3_4	41	0.2_0	0.2	0.1_6	
					1.2	1.0	
	pellet	1.6	34	1.0_6	1.0	0.8_5	76
^{3}H-II	pellet	0.3	(34)	0.2	1.6	1.3[h]	

[a] See Experimental Procedures for details. [b] Synthesis II employed a MnCl$_2$ precipitation procedure, yielding a supernatant (supern) and pellet fraction. For synthesis ^{3}H-II, only the pellet fraction was considered. [c] The A_{250} units represent the total poly[d(G-z^{5}C)] synthesized plus remaining template. The corrected values were determined from the nucleoside analysis of the samples, assuming a similar ϵ_{250} for the polymer, template, and possible hybrid species. [d] The poly[d(I-C)] template contents of the samples (% nucleosides) were taken from Table II. The ^{3}H-II sample was assumed to have the same template content as the synthesis II pellet. [e] The fold synthesis equals the corrected A_{250} units/initial template A_{250} units. [f] The percent utilization of dz^{5}CTP (nucleotide incorporated into the poly[d(G-z^{5}C)]) was calculated by using the corrected A_{250} values and assuming $\epsilon_{250} \sim 6.9$ mM^{-1} cm^{-1}. [g] Midpoint of the polymer melting transition in 1 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA; monitored at λ 280 nm. [h] The percentage utilization of [8-^{3}H]dGTP was 0.5%.

Table II: Analysis of the Base Composition of Poly[d(G-z^{5}C)] Preparations

		nucleoside content (%)[a]				template content (%)	
synthesis	fraction	dG	dz^{5}C	dI	dC	nucleoside analysis[b]	drug binding[c]
I		41	41	8	10	18	0.5
II	supern	30	30	23	18	41	18
	pellet	34	34	20	14	34	20

[a] Nucleoside content expressed as percent of total nucleotides in the polymer digest. The content of dz^{5}C was assumed to be equal to dG. [b] The template content from the nucleoside analysis is the percent nucleosides in the product attributable to the poly[(d(I-C)]. [c] Ethidium bromide was used to estimate the total base content of the polymer samples and Hoechst 33324 to determine the contribution due to poly[d(I-C)].

41% and 34% of the DNA nucleosides in the digested supernatant and pellet fractions, respectively, were due to contaminating template (Table II). Therefore, the MnCl$_2$ precipitation only slightly enhanced the product/template ratio.

To further characterize poly[d(G-z^{5}C)] and to estimate the poly[d(I-C)] content of the synthesized samples, the interactions of ethidium bromide and Hoechst 33342 with these DNAs were investigated. Ethidium bound to the synthesized poly[d(G-z^{5}C)] as evidenced by fluorescence enhancement, thereby providing evidence for the existence of a double-stranded helical structure. By comparison with a poly[d(G-C)] standard, an estimation of the total concentration of DNA was obtained. In order to determine the poly[d(I-C)] content in the final polymer samples, measurements of Hoechst 33342 fluorescence with poly[d(G-C)], poly[d(I-C)], and the poly[d(G-z^{5}C)] samples were also performed. Hoechst 33342 is an A-T (or I-C) specific minor-groove binding drug (Jorgenson and van de Sande, unpublished data) and displays a 22-fold greater fluorescence enhancement in the presence of poly[d(I-C)] as compared to poly[d(G-C)]. It was assumed that poly[d(G-z^{5}C)] is similar in this respect to poly[d(G-C)]. As summarized in Table II, the combined drug-binding measurements indicated that the products from syntheses I and II contained 0.5% and ca. 20% template (nucleotides), respectively. These values are lower than those obtained by direct chromatographic analysis, possibly due to differing fluorescent enhancement factors for the deoxycytidine- and deoxy-5-azacytidine-containing polymers and the possibility that hybrid poly[d(G-z^{5}C)·d(I-C)] binds Hoechst 33324 poorly or not at all.

In both syntheses (Table I), ca. 1% incorporation of dz^{5}CMP was observed. This result may primarily reflect the rapid decomposition of the 5-azacytosine base under the pH and ionic conditions employed for the synthesis reactions (Notari & DeYoung, 1975). The net fold synthesis of poly[d(G-z^{5}C)] relative to the initial template was slightly greater than 1, suggesting that a significant fraction of the final DNA may have consisted of a hybrid of poly[d(G-z^{5}C)] and poly[d(I-C)]. On the basis of the nucleoside analyses (Table II), the maximum possible hybrid content would have been 36% and 67–82% for preparations I and II, respectively.

By use of gel electrophoresis, the product polymers were found to be less than 250 base pairs in length.

Chemical Stability of Poly[d(G-z^{5}C)]. Poly[d(G-z^{5}C)] was stable in the double-stranded form, showing no apparent decomposition in the frozen state over a period of several months. The thermal stability of poly[d(G-z^{5}C)] was investigated by optical analysis of the helix–coil transition. As shown in Figure 1 and Table I, heating resulted in a cooperative hyperchromic effect at 280 and 260 nm with a transition midpoint T_m = 75–77 °C (using DNA from both syntheses). These results may be compared to the T_m = 72 °C recorded for poly[d(G-C)] under the identical ionic conditions. Such measurements demonstrate that the helical stability of poly[d(G-C)] is not markedly affected (possibly slightly increased) by the incorporation of deoxyazacytidine. Heating of the denatured polymer above the melting temperature resulted in an initial hypochromicity at 260 nm followed by a hyperchromic change near 90 °C. Upon subsequent cooling of the sample, reannealing of the polymer strands was not observed, particularly at the monitoring wavelength of 280 mM. On the basis of the complex absorbance profile at 260 nm, it can be concluded that decomposition of the azacytosine ring occurred upon denaturation to the single-stranded coil state. Although not fully understood, the hydrolysis of azacytosine appears to occur via the attack of a hydroxyl ion at the base C6 position, followed by ring opening and the formation of a UV-absorbing aldehyde intermediate; this intermediate then decomposes to non-UV-absorbing guanidine and deoxyribose (Notari & DeYoung, 1975).

Denaturation of poly[d(G-z^{5}C)] in alkali also resulted in the decomposition of the azacytosine ring. The supernatant fraction from synthesis II before and after exposure to 0.21 N KOH (37 °C, 3 h) demonstrated a 57% reduction in the fluorescence enhancement of added ethidium bromide. In other experiments, treatment with NH$_4$OH led to degradation of radiolabeled poly[d(G-z^{5}C)], as evidenced by loss of binding to DEAE paper.

Spectral Properties of Poly[d(G-z^{5}C)] at Low Ionic Strength. The UV absorbance and CD spectra of poly[d(G-

Table III: Absolute and Difference Spectral Properties of B- and Z-Form Poly[G-z^{5}C)]

		Ultraviolet Absorbance absolute spectra[a]				difference spectra (Z − B′)[b]		
polymer	form[a]	λ_{max} (nm)	A_{260}/A_{250}	A_{280}/A_{250}	A_{295}/A_{250}	λ_{max} (nm)	λ_0 (nm)	λ_{min} (nm)
poly[d(G-z^{5}C)]	B	250	0.85	0.36	0.13			
	B′	251	0.88	0.36	0.14	291, 261	245	230
	Z	252	0.93	0.40	0.19			
poly[d(I-C)][d]	B	251	0.84	0.61	0.14			

		Circular Dichroism absolute spectra			difference spectra (Z − B)[c]		
polymer	form[a]	λ_{max} (nm)	λ_0 (nm)	λ_{min} (nm)	λ_{max} (nm)	λ_0 (nm)	λ_{min} (nm)
poly[d(G-z^{5}C)]	B	288	273	254			
	B′	290	276	254	257	274	290
	Z	263	250, 273	237, 290			
poly[d(I-C)][d]	B	263	247, 254, 274	250, 284			

[a] Samples (synthesis I) in 10 mM Tris-HCl, pH 7.6 and 0.2 mM EDTA at 5 °C. The B sample had no added salt, the B′ sample was in 4.0 M NaCl (measured immediately), and the Z sample was in 4.0 M NaCl (after warming to 40 °C for 10 min). [b] The absolute absorbance spectra are described by the λ_{max} values and the listed absorbance ratios. The difference spectra are described by λ_{max}, λ_{min}, and λ_0 (isosbestic point). [c] The absolute CD spectra are described by λ_{max}, λ_{min}, and λ_0 (for which $\Delta\epsilon = 0$). The difference spectra are given as λ_{max}, λ_{min}, and λ_0 (isodichroic point). [d] Absorbance data from this laboratory; CD data from Mitsui et al. (1970).

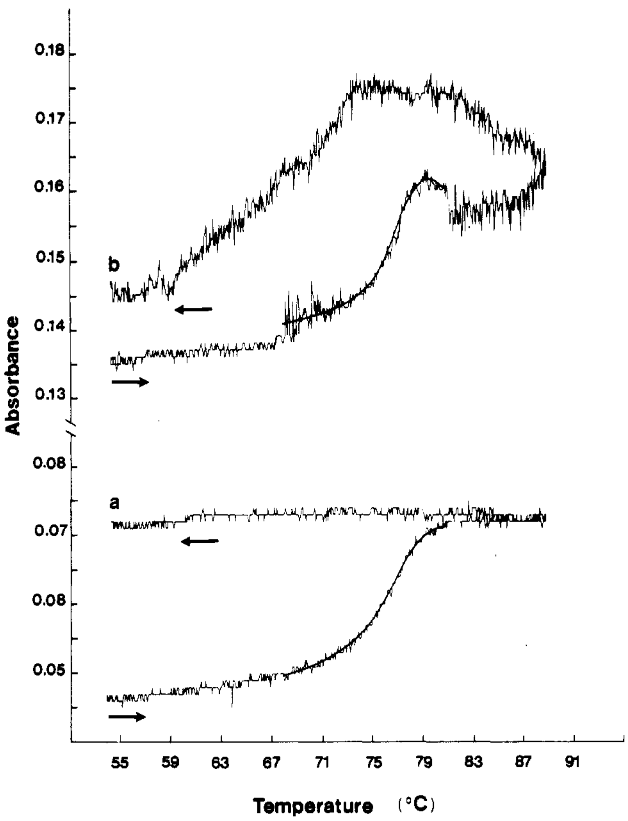

FIGURE 1: Thermal denaturation (and decomposition) of poly[d(G-z^{5}C)]. Preparation II (pellet; Table I) of poly[d(G-z^{5}C)] in 10 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA was heated from 52 to 92 °C (and back) in a 2 × 10 mm (width × path length) cuvette at a rate of 0.1 °C/min. The absorbance was recorded at 280 (a) and 260 nm (b). The transitions were fit according to the procedure of Pörschke & Jung (1982) in the regions with the solid curve segments yielding a T_m of 76.4 and 76.9 °C at 280 and 260 nm, respectively.

z^{5}C)] (synthesis I) are presented in Figure 2, and a summary of the spectral parameters for this sample is given in Table III. The spectra of poly[d(G-z^{5}C)] from synthesis II were similar to those of the polymer from synthesis I, although small differences in parameters such as the absorption ratios were noted (data not shown). Poly[d(G-z^{5}C)] in 10 mM Tris-HCl, pH 7.6, and 0.2 mM EDTA is characterized by an absorption λ_{max} of 250 nm. This blue-shifted absorbance spectra relative to poly[d(G-C)] (λ_{max} 256 nm) arises from the nitrogen substitution into the aromatic ring of cytosine (dC, λ_{max} 271 nm) to yield 5-azacytosine (dz^{5}C, λ_{max} 244 nm). In addition to the indirect chromatographic evidence, the UV spectrum gives further indication of the incorporation of dz^{5}C in the DNA. On the basis of the conditions employed and the appearance of the absorption and CD spectra of poly[d(G-z^{5}C)], we assign a right-handed B conformation to this polymer.

Although the absorption spectra of poly[d(I-C)] bears some qualitative resemblance to that recorded for the synthesized poly[d(G-z^{5}C)], the former DNA is characterized by a shoulder at 275 nm not seem with the latter sample (Gill et al., 1974). Additionally, a clear difference between the CD profiles of the template (Mitsui et al., 1970) and product DNAs exists. Therefore, the spectra presented in Figure 2 are due predominantly to poly[d(G-z^{5}C)]; as already discussed, contributions from up to 18% poly[d(I-C)] (or 36% hybrid) contamination may have been present.

B–Z Transition of Left-Handed Z Poly[d(G-z^{5}C)]. As demonstrated by solution (Pohl & Jovin, 1972) and crystallographic (Wang et al., 1979; 1981; Drew & Dickerson, 1981; Drew et al., 1980) studies, poly[d(G-C)] adopts a left-handed Z conformation under conditions such as elevated ionic strength. The B → Z transition of this polymer is characterized by a red shift in the UV absorption spectrum and a near inversion of the CD spectrum (Pohl & Jovin, 1972). In order to assess whether poly[d(G-z^{5}C)] adopts a similar left-handed conformation, the polymer spectroscopic parameters were measured in 4.0 M NaCl. Under these conditions, the UV and CD spectra of poly[d(G-z^{5}C)] bear a strong resemblance to those of Z form poly[d(G-C)] (Table III and Figure 2). The spectral changes occurring between the high- and low-salt forms of this polymer are presented in the form of difference UV and CD spectra (Figure 2). This difference spectroscopy serves to eliminate the contributions due to poly[d(I-C)], a polymer not capable of adopting a left-handed structure in concentrated NaCl solutions (Sutherland & Griffin, 1983). The spectral changes observed for poly[d(G-z^{5}C)] are quite similar to those exhibited by members of the poly[d(G-C)] and poly[d(A-C)·d(G-T)] polynucleotide families, which undergo a B → Z isomerization (Jovin et al., 1983a,b; McIntosh et al., 1983; McIntosh et al., unpublished results). However, some qualitative differences are evident. For example, the difference absorption spectrum of poly[d-

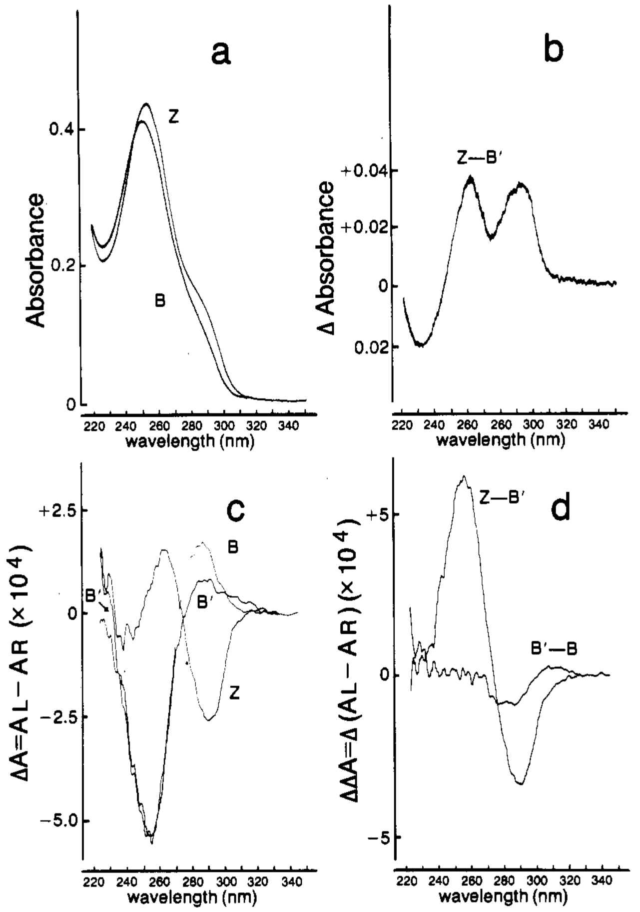

FIGURE 2: Spectral properties of poly[d(G-z^{5}C)] in the right- (B) and left-handed (Z) conformations. (a) Absorption spectra of the B and Z forms; (b) difference absorption spectra (Z − B′); (c) CD spectrum of B, B′, and Z forms; (d) difference cd spectra (Z − B′) and (B′ − B). The B form was measured at 5 °C in standard buffer: 10 mM Tris-HCl, pH 7.6, and 0.2 mM EDTA. The B′ form was generated immediately upon addition of NaCl to 4.0 M and brief centrifugation to eliminate a slight turbidity (possible due to the contaminating template poly[d(I-C)]). The Z form was seen after heating the B′ sample for 10 min at 40 °C, then cooling to 3.5–5 °C, and recording the spectra. Similar absolute and different spectra were observed in MnCl$_2$ solutions. Poly[d(G-z^{5}C)] preparation I (Table I) was used throughout. Panels a and c have been presented previously (Jovin et al., 1983b).

(G-z^{5}C)] has two positive peaks at 291 and 261 nm, whereas poly[d(G-C)] has a single major peak at 294 nm. On the basis of these spectroscopic properties, we conclude that poly[d(G-z^{5}C)] in concentrated NaCl adopts a left-handed Z or Z-like conformation.

Figure 2 and Table III also summarize the UV absorbance and CD data for poly[d(G-z^{5}C)] in 4.0 M NaCl at 5 °C measured *before* the B → Z transition. As shown by Pohl & Jovin (1972), and B → Z isomerization of poly[d(G-C)] is very slow at low temperatures. Although no pronounced absorbance changes were noted for right-handed poly[d(G-z^{5}C)] in 4.0 M NaCl, a reduction in the CD position peak intensity, yielding the nonconservative B′ spectrum, was seen. The spectral alterations observed for this metastable species appear to be due to a generalized salt effect on the CD spectra of DNA (Tunis-Schneider & Maestre, 1970; Zimmerman & Pheiffer, 1980; McIntosh et al., 1983; McIntosh et al., unpublished results). After the solution was warmed to allow the B → Z transition, the pronounced spectral changes described above resulted. Cooling to 5 °C did not reverse the transition, indicating the lack of a significant temperature dependence for the conformational equilibria under the salt conditions employed. Similar results have been reported for poly[d(G-C)] (Pohl & Jovin, 1972).

Table IV: B → Z Transition Conditions

polymer	[salt]$_{1/2}$[a] (M) NaCl	MgCl$_2$	MnCl$_2$
poly[d(G-z^{5}C)]	3.0[b]	0.9	<0.00025
poly[d(G-C)][c]	2.3	0.67	>0.0005
polu[d(G-m^{5}C)][d]	0.7	0.0006	d

[a] The midpoints of the B → Z transition. The polymers were in ca. 10 mM Tris-HCl, pH 7.6, at 25–31 °C. [b] 3.0 ± 0.1 M for both preparations I and II (pellet) (Table I). [c] Data from Pohl & Jovin (1972), van de Sande et al. (1982), and McIntosh et al. (unpublished results). [d] Data from Behe & Felsenfeld (1982), van de Sande et al. (1982), and Jovin et al. (1983). In MnCl$_2$, the Z conformation is stable at substoichiometric concentrations of the bulk cation relative to DNA phosphate.

Chemical modifications of poly[d(G-C)] dramatically influence the conditions under which the left-handed conformation is stable. For example, methylation of cytidine to yield poly[d(G-m^{5}C)] reduces the transition midpoints for the B–Z transition of poly[d(G-C)] from 2.3 M NaCl or 0.7 M MgCl$_2$ to 0.7 M NaCl and <0.001 M MgCl$_2$, respectively (Table IV). Halogenation in this position renders the polymer left-handed under all practical ionic conditions (Malfoy et al., 1982; McIntosh et al., unpublished results). In order to determine the effect of the nitrogen substitution into the cytidine ring on the ionic requirements for the B–Z isomerization, continuous titrations of poly[d(G-z^{5}C)] in NaCl and MgCl$_2$ were performed. The conformational state of the polymer was assessed by the absorbance at 290 nm. Cooperative transitions were observed with decreasing ionic strength, attesting to a conformational change in the sense of the DNA helix. CD spectra confirmed that the B form was present at the end of the titrations. Note that the titrations represented Z → B isomerizations, thus demonstrating the reversible nature of the equilibria. Application of a two-state model to the NaCl and MgCl$_2$ titration curves yielded the transition midpoints listed in Table IV. Comparison with the data tabulated for poly[d(G-C)] and poly[d(G-m^{5}C)] demonstrates that the dz^{5}C substitution has a small inhibitory effect on the B → Z transition, relative to poly[d(G-C)]. This is, the transition midpoints in NaCl and MgCl$_2$ are approximately 30% higher.

The B → Z transition kinetics of poly[d(G-z^{5}C)] in 3.7 M NaCl at 26 °C was first order with a $t_{1/2}$ = 790 s. In comparison, the B → Z transition of poly[d(G-C)] in 3.3 M NaCl at 26 °C had a $t_{1/2}$ = 740 s. These results emphasize the relatively small effect of the 5-deoxyazacytidine substitution on the conformational properties of poly[d(G-C)].

Previous studies have demonstrated that the Z conformation of poly[d(G-C)] may be stabilized by submillimolar concentrations of transition metals such as MnCl$_2$, NiCl$_2$, and CoCl$_2$ (van de Sande & Jovin, 1982; van de Sande et al., 1982; Zacharias et al., 1982; Jovin et al., 1983a). Generally, large activation barriers exist for the metal-induced transitions, such that transient heating (van de Sande et al., 1982) or the presence of dehydrating solvents (Zacharias et al., 1982) is required for the formation of the Z polymers. In <0.25 mM MnCl$_2$, poly[d(G-z^{5}C)] was observed to be in a Z conformation as determined by CD and UV absorbance measurements. As in the case of poly[d(G-C)] (Zacharias et al., 1982; van de Sande et al., 1982), the CD spectra of Z poly[d(G-z^{5}C)] in NaCl and MnCl$_2$ differ (data not shown), possibly due to metal–base interactions or to polymorphism of the left-handed conformation. Interestingly, the MnCl$_2$-induced B → Z transition of poly[d(G-z^{5}C)] proceeded even at 9 °C, indicating that the dz^{5}C substitution serves to reduce the kinetic barrier for the inversion of the polymer helical sense.

In the presence of divalent cations poly[d(G-C)] exists in a self-associated left-handed state, termed Z* (van de Sande & Jovin, 1982; van de Sande et al., 1983; Jovin et al., 1983a,b). Although failing to display visible turbidity, Z* DNA may be sedimented out of solution by using moderate centrifugation. This sedimentation is selective for Z* DNA, even in the presence of B DNA (Jovin et al., 1983a). When a similar centrifugation assay was used, poly[d(G-z^{5}C)] in the Z state stabilized by MgCl$_2$ or MnCl$_2$ was also sedimentable as evidenced by a reduction of the supernatant absorbance at 250 nm. Thus, poly[d(G-z^{5}C)] forms Z* DNA in the presence of divalent cations.

DNAs such as poly[d(G-m^{5}C)] which are left handed under physiological conditions are immunogenic and may be used for the production of anti-Z DNA antibodies (Lafer et al., 1980; Zarling et al., 1984a,b). In turn, these antibodies serve as probes for the identification of left-handed Z DNA. The high-salt form of poly[d(G-z^{5}C)] was found to interact with a polyclonal antibody raised against chemically brominated poly[d(G-C)], but not with immunoglobulins produced upon immunization with the enzymatically synthesized polymer poly[d(G-br^{5}C)] which is constitutively in the Z conformation [the data are reported in Zarling et al. (1984b)].

Nuclease Susceptibility of Poly[d(G-z^{5}C)]. The nuclease sensitivity of a given DNA sequence may be altered due to chemical modification of base or backbone sites. For example, methylation (Behe & Felsenfeld, 1981) or halogenation (Malfoy et al., 1982; McIntosh et al., unpublished observations) of deoxycytidine in the C5 position renders the sequence d(G-C)$_n$ resistant to *Hha*I digestion. Thus, the nuclease sensitivity of the synthesized poly[d(G-z^{5}C)] was of interest particularly with respect to the possible biological effects of the incorporation of 5-azacytosine into DNA. *Hha*I and micrococcal nuclease degraded tritiated poly[d(G-z^{5}C)] at a rate 60–80% that observed with B-form poly[d(G-C)]. When excess enzyme concentrations were used, up to 81% of both polymers were digested to fragments no longer scored by the filter binding assay. Additionally, the complete digestion of poly[d(G-z^{5}C)] to its component nucleosides by DNase I, snake venom phosphodiesterase, and alkaline phosphatase which were used for the compositional analysis (Table II) demonstrates that the polymer exhibits sensitivty to a range of nucleases.

Substrate Properties of Poly[d(G-z^{5}C)] for DNA Cytosine 5-Methyltransferase. Highly purified DNA methyltransferase from human placenta was employed to test the substrate properties of poly[d(G-z^{5}C)] polymers. No methyl group transfers were observed to poly[d(G-z^{5}C)] in contrast to the normal activity seen with the substrate poly[d(G-C)]. However, DNA methylase showed a high binding affinity to the 5-azacytosine-containing polymer. In a competitive binding experiment, in which a limited amount of the enzyme was added to mixtures of poly[d(G-z^{5}C)] and poly[d(G-C)], the methylation reaction was observed only in the presence of more than a 10-fold excess of poly[d(G-C)] (Figure 3A). In the same system no inhibitory effect of poly[d(I-C)] on the enzymatic methylation of poly[d(G-C)] was observed (data not shown). Since the enzyme, after initial binding to the DNA substrate, is capable of accomplishing a series of methyl group transfers without being detached, i.e., acts processively (Drahovsky & Morris, 1971a), the data from the competitive binding experiment indicate that in the initial binding reaction the DNA methylase had a more than 10-fold higher affinity for poly[d(G-z^{5}C)] than for poly[d(G-C)]. A tight binding of the enzyme to the 5-azacytosine-substituted polymers is also implied by an experiment in which an increasing amount of DNA methylase was added to the mixture of poly[d(G-C)] and poly[d(G-z^{5}C)] (molar ratio 1:0.07). The methylation reaction was observed only at high enzyme concentrations (Figure 3B). Apparently, at low enzyme concentrations all the DNA methylase molecules were bound to poly[d(G-z^{5}C)] and the methylation reaction was totally inhibited. From the data in Figure 3B, we have calculated that 70 ng of poly[d(G-z^{5}C)] bound approximately 12.5 units of DNA methyltransferase. After the binding sites on poly[d(G-z^{5}C)] molecules were saturated, additional enzyme molecules were able to methylate poly[d(G-C)] substrate molecules at a rate that increased with the enzyme concentration in parallel to the reactions without poly[d(G-z^{5}C)].

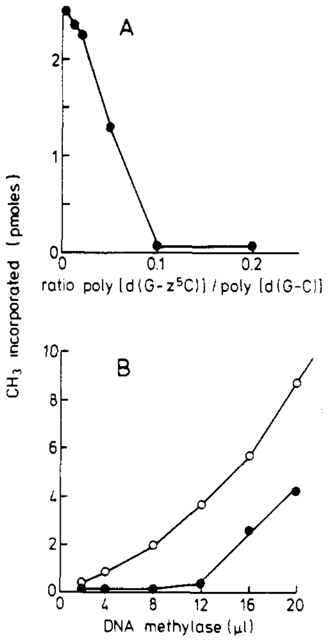

FIGURE 3: Effects of poly[d(G-z^{5}C)] on human placental DNA methylase. (A) Competition between poly[d(G-C)] and poly[d(G-z^{5}C)] for the DNA binding site of DNA methylase. To mixtures of poly[d(G-C)] (1 μg) and poly[d(G-z^{5}C)] (0–0.2 μg) was added 12.5 units of DNA methylase from human placenta and *S*-adenosyl-L-[*Me*-^{3}H]methionine. After incubation for 45 min at 37 °C the DNA methylation was measured as described under Experimental Procedures. (B) Methylation of poly[d(G-C)] and a mixture of poly[d(G-C)] and poly[d(G-z^{5}C)], with DNA methylase from human placenta. Increasing amounts of enzyme were used to methylate poly[d(G-C)] (1 μg) alone (○) and in a mixture with poly[d(G-z^{5}C)] (0.07 μg) (●).

DISCUSSION

Poly[d(G-z^{5}C)] Synthesis. As is evident from the syntheses of poly[d(G-z^{5}C)], deoxy-5-azacytidine triphosphate is a substrate for *Micrococcus luteus* DNA polymerase. Although not described here, incorporation of dz^{5}CMP into poly[d(I-z^{5}C)] and poly[d(A-z^{5}C)·d(G-T)] was also achieved. However, poor yields were observed in all cases, as exemplified by the average 1% utilization of dz^{5}CTP in the case of poly[d(G-z^{5}C)]. This is in striking contrast to the 20–40% utilization of dCTP in the synthesis of unmodified poly[d(G-C)] under similar conditions. In vivo, dz^{5}C is incorporated into DNA by eukaryotic DNA polymerases, although low yields have been reported (Taylor & Jones, 1982; Jones & Taylor, 1980; Adams et al., 1982).

The reduced polymerization yields with dz^{5}CTP certainly reflect the rapid hydrolysis of the triazine ring under the synthesis conditions employed (Notari & Young, 1975). The low incorporation of dz^{5}CMP into DNA may also indicate that the degradation products of the triphosphate are poor substrates or perhaps even inhibitors of DNA polymerase. The latter possibility has been invoked as an explanation for the reduced in vivo synthesis of DNA in the presence of z^{5}C (Taylor & Jones, 1980). However, similar K_m values for dCTP and dz^{5}CTP have been reported in reactions with calf thymus DNA polymerase α (Bouchard & Momparler, 1982). In addition, the low net fold syntheses of polymer relative to the initial poly[d(I-C)] may suggest that poly[d(G-z^{5}C)] is itself a poor template.

Although dz^{5}CTP readily hydrolyzes in aqueous solution, incorporation of the nucleotide into double-stranded DNA resulted in a dramatic stabilization of the triazine base. No evidence was seen for the hydrolysis of the analogue in the native form of poly[d(G-z^{5}C)], but rapid decomposition ensued upon denaturation of the DNA by heat or alkali. The stabilization of z^{5}C upon incorporation into the 3′ terminus of tRNA has also been observed (Zielinski & Sprinzl, 1984). We attribute the enhanced stability of polymerized 5-azacytosine to the geometrical constraints imposed by base stacking and the helical structure of DNA. That is, the nucleophilic attack at the triazine ring C6 position and the subsequent formation of a nonplanar reaction intermediate are relatively hindered due to steric constraints and base-stacking interactions. The stabilization of dz^{5}C in DNA has some biological implications. For example, there may not be a significant decomposition of the base analogue which would otherwise lead to enzymatic DNA repair or to mutagenic effects. These conclusions may require reevaluation if accelerated decomposition during transient single-stranded states of DNA can occur.

B- and Z-Helical Structures of Poly[d(G-z^{5}C)]. Under low ionic strength conditions, poly[d(G-z^{5}C)] displays optical spectra and biochemical properties consistent with a right-handed B conformation. Thus, the dG·dz^{5}C base pair is stable. As in the case of poly[d(G-C)] (Pohl & Jovin, 1972), a dramatic inversion in the CD spectrum and an accompanying red shift in the UV absorption spectrum of poly[d(G-z^{5}C)] occur in concentrated NaCl solutions. These spectroscopic phenomena, the observed cooperativity and kinetics of the salt-induced transitions, and the demonstrated ability to bind anti-Z DNA antibodies lead to the conclusion that the high-salt form of poly[d(G-z^{5}C)] is left handed, i.e., Z-like. Through the use of difference spectroscopy, the spectral contributions of poly[d(I-C)], which does not adopt a left-handed conformation under these salt conditions (we assume that any template–product hybrid would disproportionate at equilibrium), may be largely eliminated. An interesting point to note is that the substitution of a nitrogen atom into the aromatic ring of cytosine significantly alters the absorption properties of the pyrimidine base. This feature may account for the qualitative differences in the spectral changes accompanying the B → Z isomerization poly[d(G-C)] and poly[d(G-z^{5}C)]. However, the similarities are even more striking and imply strong structural homologies between the two polymers.

In addition to NaCl, $MgCl_2$ and $MnCl_2$ were also effective at stabilizing the left-handed conformation of poly[d(G-z^{5}C)]. As observed with poly[d(G-C)], the Z conformation induced by divalent cations is in the associated, sedimentable form, previously designated as the Z* state. Interestingly, the B → Z* transition of poly[d(G-z^{5}C)] in $MnCl_2$ is not characterized by the large kinetic barrier observed with poly[d(G-C)] (van de Sande et al., 1982), possibility due to altered metal ion–dz^{5}C interactions. A similar finding has been reported for the corresponding DNA bearing a backbone modification in the form of a phosphorothioate linkage between G and C (Jovin et al., 1983a,b). It has been suggested that the dramatic effect of transition metal ions on stabilization of the Z conformation of poly[d(G-C)] arises partially due to site-specific interactions of these cations with the N7 position of guanine (van de Sande et al., 1982; Jovin et al., 1983a,b). As demonstrated by crystallographic studies, guanine N7 is exposed on the surface of the Z helix, allowing the direct coordination of a Mg^{2+} ion to this site in $d(C\text{-}G)_3$ (Wang et al., 1981). The N5 position of 5-azadeoxycytosine is similarly exposed and thus may also serve as a locus for metal ion interactions.

A comparison of the ionic conditions (Table IV) required for the B → Z transition of poly[d(G-C)] and poly[d(G-z^{5}C)] suggests that the incorporation of dz^{5}C into poly[d(G-C)] is a fairly "neutral" substitution with respect to the conformational polymorphism exhibited by this DNA sequence. However, since the transition midpoints in NaCl and $MgCl_2$ increased about 30%, we conclude that dz^{5}C in general leads to a slight destabilization of the left-handed conformation relative to the B form. Previous studies have demonstrated a hierarchy in the effect of pyrimidine C5 substituents on the stability of the Z conformation of alternating purine–pyrimidine copolymers (Jovin et al., 1983b; McIntosh et al., unpublished results; see Results). The results presented for poly[d(G-z^{5}C)], a polymer lacking any exocyclic group in the 5-position of cytosine, extend this series to iodo > bromo > methyl > hydrogen > aza.

Poly[d(G-z^{5}C)], a Potent Inhibitor of DNA Methylase. Because of an inverse correlation between gene expression and the methylation of certain sequences within or in the vicinity of some genes (Doerfler, 1983; Riggs & Jones, 1983; Razin & Szyf, 1984), it has been surmised that the biological effects of 5-azacytidine–X chromosome activation, activation of various genes, and the induction of cell differentiation (Felsenfeld & McGhee, 1982; Riggs & Jones, 1983) are related to a direct inhibition of maintenance DNA methylation by the analogue. The highly purified DNA methylase from human placenta used in this study carries out both maintenance and de novo DNA methyltransferase activities (Pfeifer et al., 1983). The high binding affinity of the enzyme to poly[d(G-z^{5}C)] we have demonstrated is compatible with the observed in vivo effects of 5-azacytosine. The phenomenon is not restricted to this alternating sequence since similar results were obtained with the polymer poly[d(A-z^{5}C)·d(G-T)] (data not shown). In another study (Bouchard & Momparler, 1983), dz^{5}CMP was incorporated by the in vitro nick translation of hemimethylated *M. luteus* DNA. A very significant inhibition of DNA methylase was observed, which increased with the extent of incorporation of the analogue. It remains to be established whether covalent and/or noncovalent interactions of the enzyme with the dz^5-containing polymers and DNAs are responsible for the inhibition or inactivation.

The mechanism(s) by which DNA methylation modulates gene expression is (are) unknown. Some functional proteins may exist in chromatin capable to discriminating between m^{5}C-containing and m^{5}C-free DNA sequences in analogy to the distinction between methylated and unmethylated sequences demonstrated by the procaryotic restriction and modification enzymes. Indeed, a protein showing high-binding affinity to m^{5}C-rich DNA sequences was recently isolated from human placenta (Huang et al., 1984). Another possibility is that enzymatic methylation may have a direct effect on DNA structure. Since 5-methylcytosine potentiates the right-to-left transition of alternating purine–pyrimidine sequences (Behe & Felsenfeld, 1981), dz^{5}C, through the inhibition of DNA methylation, could indirectly affect chromatin structure by influencing the occurrence of potential Z DNA structures and, as a consequence, gene expression. For example, due to the relationship between superhelix density and the handedness of a DNA helix (Peck & Wang, 1983), the local and global topological structural features of DNA and the regulatory processes dependent upon them would be affected.

In conclusion, one would infer from this study that the deoxy-5-azaycytidine substitution leads to relatively minor alterations in the physical attributes of DNA, at least as can be assessed with the synthetic polynucleotide poly[d(G-z^{5}C)]. The most dramatic effect of biochemical consequence is the potent inhibition of DNA methyltransferase by direct interaction(s) with the enzyme.

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