Stabilization of Z-RNA by Chemical Bromination and Its Recognition by Anti-Z-DNA Antibodies†

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ABSTRACT: Limited chemical bromination of poly[r(C-G)] (32% br^{8}G, 26% br^{5}C) results in partial modification of guanine C8 and cytosine C5, producing a mixture of A- and Z-RNA forms. The Z conformation in the brominated polynucleotide is stabilized at much lower ionic strength than in the unmodified polynucleotide. More extensive bromination of poly[r(C-G)] (>49% br^{8}G, 43% br^{5}C) results in stabilization of a form of RNA having a Z-DNA-like (Z_D) CD spectrum in low-salt, pH 7.0–7.5 buffers. Raising the ionic strength to 6 M NaBr or $NaClO_4$ results in a transition in Br-poly[r(C-G)] to a Z-RNA (Z_R) conformation as judged by CD spectroscopy. At lower ionic strength Z-DNA-like (Z_D) and A-RNA conformations are also present. ^{1}H NMR data demonstrate a 1/1 mixture of A- and Z-RNAs in 110 mM NaBr buffer at 37 °C. Nuclear Overhauser effect (NOE) experiments permit complete assignments of GH8, CH6, CH5, GH1′, and CH1′ resonances in both the A- and Z-forms. GH8 ↔ GH1′ NOEs demonstrate the presence of both A- and Z-form GH8 resonances in slow exchange on the NMR time scale. The NMR results indicate that unbrominated guanine residues undergo transition to the syn conformation (Z-form). Raman scattering data are consistent with a mixture of A- and Z-RNAs in 110 mM NaCl buffer at 37 °C. Comparison with the spectrum of Z-DNA indicates that there may be different glycosidic torsion angles in Z-RNA and Z-DNA [Tinoco, I., Jr., Cruz, P., Davis, P., Hall, K., Hardin, C. C., Mathies, R. A., Puglisi, J. D., Trulson, M. O., Johnson, W. C., & Neilson, T. (1986) in *Structure and Dynamics of RNA*, pp 55–68, Plenum, New York]. ^{31}P NMR spectra show six to eight resonances spread over a 1.8 ppm range whose chemical shifts are also consistent with an equilibrium mixture of A- and Z-RNAs. Radioimmunoassay and nitrocellulose filter binding competition experiments were performed to determine the extent of recognition of Br-poly[r(C-G)] by anti-Z-DNA antibodies. The polyclonal rabbit anti-Br-poly[d(C-G)] IgG preparations T4 and Z6 [Zarling, D. A., Arndt-Jovin, D. J., Robert-Nicoud, M., McIntosh, L. P., Thomae, R., & Jovin, T. M. (1984a) *J. Mol. Biol. 176*, 369–415; Zarling, D. A., Arndt-Jovin, D. J., McIntosh, L. P., Robert-Nicoud, M., & Jovin, T. M. (1984b) *J. Biomol. Struct. Dyn. 1*, 1081–1107] specifically recognize the Z-form of Br-poly[r(C-G)], although the binding affinities are lower for Z-RNA than for various forms of Z-DNA. Competition RIA experiments verify the presence of a Z-DNA-like determinant in left-handed Br-poly[r(C-G)] at physiological NaCl concentration. The phosphate anion specifically inhibits recognition of Z-RNA by anti-Z-DNA IgGs, consistent with recognition of a phosphodiester backbone determinant. In summary, these spectroscopic and immunochemical studies demonstrate that under conditions of conformational stress (i.e., containing brominated nucleosides) left-handed Z-RNA is stable and is specifically recognized by proteins at physiological temperature and ionic strength.

The right-handed (A-form) to left-handed (Z-form) transition in linear double-stranded RNA was first described by Hall et al. (1984b) for the synthetic alternating copolymer poly[r(C-G)] in 6 M $NaClO_4$ buffer at 45 °C. Proton and ^{31}P NMR studies demonstrated that guanine residues were in a syn conformation and that there were very different Z-form CpG and GpC phosphodiester conformations in the polynucleotide under these conditions. The circular dichroism (CD)[1] bands centered at 282 and 230 nm were shown to undergo temperature- and salt-dependent inversion. Taken together, these data are consistent with the characteristics of Z-DNA (Rich et al., 1984; Jovin et al., 1983). The vacuum UV CD spectra of Z- and A-form poly[r(C-G)] match spectra for left- and right-handed forms of DNA at wavelengths below 230 nm (Riazance et al., 1985). Calculated spectra for A- and Z-RNA and A-, B-, and Z-DNA in this wavelength region agree well with experiment (Williams et al., 1986). This demonstrates that the intense vacuum UV CD bands are most descriptive of helix handedness. Raman scattering spectra of poly[r(C-G)] in 6 M $NaClO_4$ buffer are also consistent with the left-handed conformation (Tinoco et al., 1986). However, the spectroscopic data and the different salt dependences for the transition to Z-form in DNA and RNA point to significant structural, kinetic, and thermodynamic differences between the two types of left-handed nucleic acid (Cruz et al., 1986a).

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[1] Abbreviations: bp, base pairs; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; FID, free-induction decay; IgG, immunoglobulin G; NOE, nuclear Overhauser effect; RIA, radioimmunoassay; TMP, trimethyl phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TSP, 3-(trimethylsilyl)propionate.

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In 4 M $MgCl_2$, poly[r(C-G)] adopts a conformation with a Z-DNA-like CD spectrum. In 6 M NaBr or $NaClO_4$ buffers, poly[r(C-G)] has a CD spectrum in the 240–300-nm region distinctly different from that of the 4 M $MgCl_2$ form of the polynucleotide. On the basis of this difference, these conformations have been termed Z_D- and Z_R-RNA, respectively (Cruz et al., 1986b).

Stabilization of Z-DNA requires either high ionic strength, base or backbone modification, topological stress or protein binding, or binding of specific small molecules such as $[Co(NH_3)_6]^{3+}$ [reviewed by Leng (1985), Rich et al. (1984), and Jovin et al. (1983)]. Prior to the studies described here, only high ionic strength conditions have been shown to stabilize polymeric Z-RNA (Cruz et al., 1986a).

In this work we demonstrate that chemical bromination of poly[r(C-G)] has a profound effect on the conformation properties of the RNA duplex. Characterization of the solution spectral properties of the polynucleotide confirms that it undergoes a cooperative transition between right- and left-handed conformations. Most importantly, the left-handed Z-form is shown to be present under physiological conditions of temperature, pH, and ionic strength. Finally, the anti-Z-DNA polyclonal antibodies T4 and Z6 (Zarling et al., 1984a,b) are shown to specifically recognize the Z-form of RNA under physiological conditions, indicating the presence of a Z-DNA-like structural element in Z-RNA and demonstrating specific recognition of Z-RNA by a protein.

Experimental Procedures

Materials

RNA polymerase was kindly provided by Professor Michael Chamberlin (Department of Biochemistry, UC, Berkeley). Deoxyribonuclease I, mung bean nuclease, and polynucleotide kinase were from Pharmacia Biochemicals. Nuclease S1, 5S rRNA, and calf intestinal phosphatase (molecular biological grade) were Boehringer-Mannheim products. 5.8S rRNA was the kind gift of Dr. Harold Kammen (School of Public Health, UC, Berkeley). 16S/23S rRNA was from Boehringer-Mannheim. Poly[d(C-G)], poly[d(A)·d(T)], poly[d(C)·d(G)], poly[d(br^{5}C-G)], poly[d(m^{5}C-G)], poly[r(A)·r(U)], poly[r-(I-C)], and poly[r(A-U)] were from Sigma or Pharmacia Biochemicals or were synthesized enzymatically. Polynucleotides were ^{32}P 5′-end-labeled for RIA experiments with polynucleotide kinase (Silberklang et al., 1979). Adenosine [γ-^{32}P]triphosphate was from Amersham. Kinase reaction products were purified by NENsorb chromatography (New England Nuclear) as described by the manufacturer. Residual [γ-^{32}P]ATP and [^{32}P]P_i were removed be ethanol precipitation and dialysis into 10 mM Tris-HCl, pH 7.5, and 0.5 mM EDTA. Polynucleotides used in competition radioimmunoassay experiments were sonicated for 2 h essentially as described by Möller et al. (1984) to preclude nonspecific aggregation. Affinity-purified goat anti-rabbit IgG preparations were from Miles Labs or Jackson Immunoresearch. All other reagents were of analytical grade or higher.

Methods

Preparation of Br-poly[r(C-G)]. Poly[r(C-G)] was prepared by transcription from the synthetic template poly[d(I-C)] as described by Hall et al. (1984b). The extinction coefficient for poly[r(C-G)] is ϵ_{260} = 6560 M^{-1} cm^{-1} nucleotide^{-1} (Gray et al., 1981). The purified polynucleotide was chemically brominated by modifications of the method of Möller et al. (1984). The polynucleotide (1 mM in nucleotides) was preequilibrated for 10 min at 45 or 55 °C in a reaction mixture containing 10 mM sodium phosphate (pH 7.0), 6 M NaBr or $NaClO_4$, and 1 mM EDTA in order to facilitate the A → Z transition. Bromine-saturated H_2O was prepared at room temperature and diluted 4-fold with 6 M NaBr or $NaClO_4$ buffer. The reaction volume was adjusted to 45 μL by rapid addition of Br_2/buffer reagent to achieve the desired Br_2/nucleotide ratio; e.g., 1.2 μL of reagent/43.8 μL of polynucleotide solution (v/v) = 1.5 Br_2/nucleotide. Incubation was continued at 45 or 55 °C for 10 min. The percent br^{5}C and br^{8}G values shown in parts B and C of Figure 1 and given in the text were obtained under a reaction temperature of 55 °C. Reactions were terminated by rapid addition of 755 μL of ice-cold 10 mM sodium phosphate (pH 7.0), 1 mM EDTA. Excess bromine was then removed by bubbling air through the reaction mixture on ice for 30 min followed by dialysis at 4 °C against excess 110 mM NaCl buffer, and finally 0.5 mM EDTA. Samples were lyophilized, resuspended into 0.5 mL of H_2O, dialyzed vs. several 500-mL changes of glass-distilled H_2O in a BRL 1200-MA microdialysis apparatus (M_r cutoff 12–14K), and resuspended into the appropriate buffers. Brominated polynucleotide samples were protected from light during all manipulations. Proton NMR samples were lyophilized twice from 99.8% D_2O (Stohler) and then resuspended into 99.998% D_2O (Stohler) in a glovebag under N_2. The polynucleotide size distribution ranged from 40 to 800 bp as determined by polyacrylamide gel electroporesis (Hall et al., 1984b). Approximately 80% of the polymer was in the 70–90-bp range as determined by agarose gel electrophoresis using *Escherichia coli* tRNA, 5S, 16S and 23S rRNA, and yeast 5.8S rRNA as size markers.

The percent br^{5}C and br^{8}G was determined by HPLC analysis of the neutralized perchloric acid hydrolysates of Br-poly[r(C-G)] on a Beckman liquid chromatography system using a Bondapak C18 reversed-phase column and methanol gradient as described by Möller et al. (1984). Molar percentages were obtained from HPLC peak integrals obtained at a detector wavelength of 254 nm and values for the base extinction coefficients under these conditions reported by Möller et al. (1984). We found reasonable agreement with the results of Möller et al. (1984) in analyses of Br-poly[d-(C-G)] prepared in this laboratory. These values were reproducible to within 5–15%. Proton NMR peak integrals were also compared, i.e., GH8 and CH6 (A and Z) vs. CH1′, GH1′, and CH5 (A and Z), and found to agree within 5–20% with the HPLC results. Comparing the integral of the 7.29 ppm peak from several samples with the percent br^{5}C leads us to the tentative assignment of this peak to br^{5}CH6. The extinction coefficient for Br-poly[r(C-G)] is 5770 M^{-1} cm^{-1} nucleotide^{-1}.

CD, Absorbance, and Raman Scattering Spectroscopies. CD spectra were recorded on a Jasco J500C spectropolarimeter in 1-cm path length quartz cuvettes surrounded by a temperature block controlled by a Zeiss Model P/N 00 80 thermoelectric unit. Absorbance spectra were recorded on a Cary 118 UV/vis scanning spectrophotometer. Raman samples were sealed in 1-mm-diameter capillaries and centrifuged for 10 min to remove suspended particles. Raman scattering spectra were obtained at a power output of 50 mW from the 514.5-nm line of a Spectra Physics 2020 argon ion laser. The spectra were accumulated on a Spex Model 1401 double monochrometer with photon counting detection.

^{1}H and ^{31}P NMR Spectroscopies. Proton NMR spectra were obtained at 500 MHz on a Bruker AM 500 spectrometer as described in the legend of Figure 5. Phosphorus NMR spectra were obtained at 202 MHz on the same spectrometer as detailed in Figure 6. Protons were broad-band decoupled

with a power of ca. 1 W during acquisition and with sufficient power to maintain the $^{31}P\{^1H\}$ NOE during the delay between pulses.

Radioimmunoassays. Radioimmunoprecipitation and nitrocellulose filter binding assays were performed in sterile 1.5-mL polyethylene (Eppendorf) tubes. The reaction mixtures (25-μL final volume) contained either 5, 10, or 50 ng (RIA) or 5 or 20 ng (filter binding assays) of RNA or DNA substrate in 50 mM Tris-HCl (pH 7.45) and 5 mM EDTA. Unless otherwise stated, samples containing either 110 mM NaCl (RIA) or 220 mM, 1.5 M, or 4 M NaCl (filter binding assays) were preequilibrated for 1 h at 37 °C. Next, 5 μL (0.5 mg/mL assuming 1.4 A_{280} unit = 1.0 mg/mL protein) of the purified rabbit polyclonal anti-Br-poly[d(C-G)] IgG preparation T4 or Z6 (Zarling et al., 1984a,b) was added. Purified normal rabbit IgG or buffer alone was used as negative control. Reactions between antibody and nucleic acid were incubated for 90 min at 37 °C. A second antibody (1 μL of goat anti-rabbit IgG) was then added, and incubation was continued for an additional 1 h at 37 °C. Immune complexes were collected by centrifugation at 13K rpm (20 °C), and most of the supernate was removed by aspiration. Immune complexes were then resuspended and washed three times as above with 200-μL aliquots of the appropriate buffer (including salt). The washed precipitates were resuspended in 0.2 mL of 0.2 N NaOH and counted in 5 mL of Aquasol 2 (New England Nuclear) in a Beckman 8000 liquid scintillation counter.

Nitrocellulose Filter Binding Assays. For filter binding experiments, immune complexes were prepared as above except that no second antibody was added. The immune complexes were diluted to 200 μL with the appropriate buffer and filtered through a Millipore Millititer-STHA09610 96-well filtration plate presoaked with buffer as described by the manufacturer. Filters were washed six times with buffer, dried, transferred to 5 mL of Aquasol 2 with a Millititer filter punch, and counted as above. The procedure was modified as described in the legend to Figure 8 for competition radioimmunoassay experiments.

Results

Effect of Chemical Bromination on the A ↔ Z RNA Equilibrium: CD and UV Spectroscopy. The effects of chemical bromination on the solution structure of the alternating RNA copolymer poly[r(C-G)] was initially monitored by its effects on the optical spectra of the polynucleotide (Figures 1–3). The CD spectra of poly[r(C-G)] obtained in 6 M $NaClO_4$ buffer at 22 °C as a function of the Br_2/nucleotide molar ratio in a 45 °C bromination reaction mixture are shown in Figure 1A. Hall et al. (1984a) previously demonstrated that in 6 M $NaClO_4$ buffer temperatures above ca. 35 °C were required to induce the A → Z_R transition in unmodified poly[r(C-G)]. The data in Figure 1A show that at 22 °C in 6 M $NaClO_4$ buffer higher Br_2/nucleotide levels in the 45 °C reaction mixture result in polynucleotide CD spectra progressively more like that of Z_R-RNA.

The degree of modification of cytosine and guanine residues in Br-poly[r(C-G)] was determined by HPLC analysis of the neutralized perchloric acid hydrolysates. These data are shown in Figure 1B scaled to illustrate their relationship with the Br_2/nucleotide ratios in the reaction mixtures. The degree of modification is similar at each of these residues. However, there is a trend toward a slightly higher degree of modification at guanine residues. Similar results were obtained by Möller et al. (1984) with Br-poly[d(C-G)]; however, relative to guanine, cytosine residues are somewhat more effectively modified in the RNA. No other reaction products absorbing

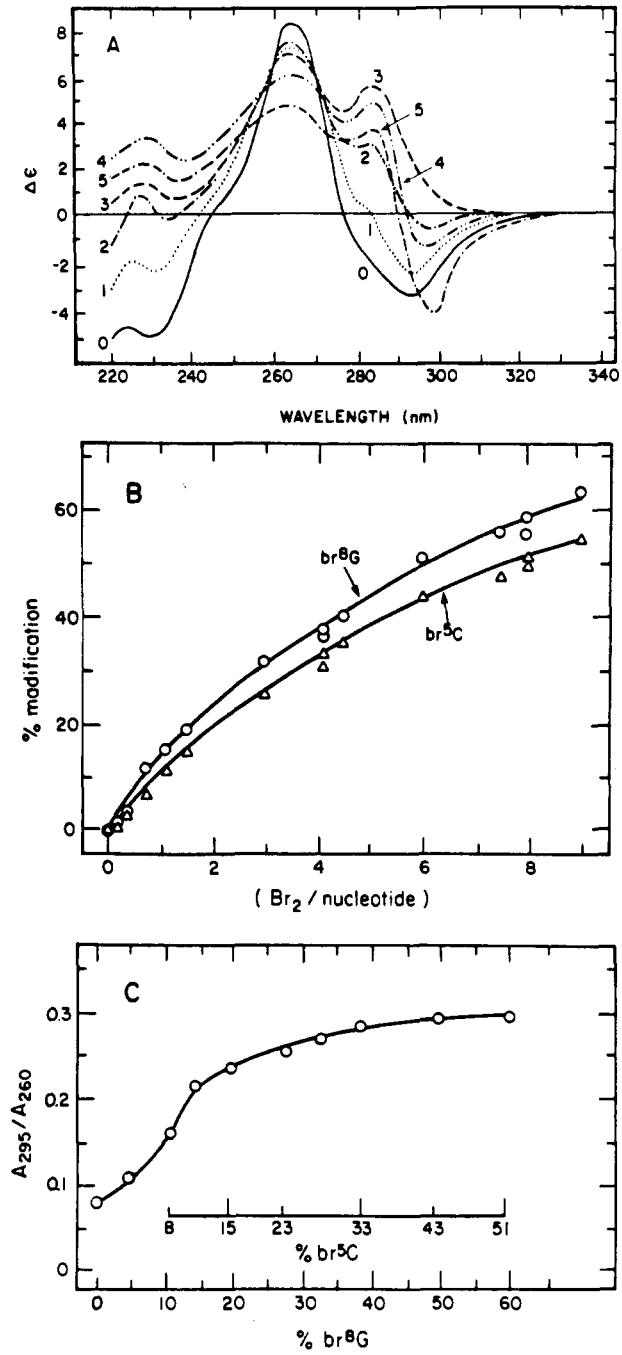

FIGURE 1: (A) Circular dichroism spectra of poly[r(C-G)] in the A-form (—) and brominated poly[r(C-G)] obtained using bromine/nucleotide ratios of 1 (···), 2 (·-·), 3 (---), 4 (·-·-), and 5 (·---), respectively, in 45 °C reaction mixtures. Spectra were obtained after resuspending the polynucleotides in 10 mM sodium phosphate (pH 7.0), 6 M $NaClO_4$ and 1 mM EDTA at 22 °C. (B) Percent modification of C (Δ) and G (O) residues in Br-poly[r(C-G)] as a function of the Br_2/nucleotide ratio in 55 °C reaction mixtures. (C) Absorbance ratios A_{295}/A_{260} plotted vs. the percent modification of C and G residues in Br-poly[r(C-G)] obtained in 55 °C mixtures. Conditions were the same as listed in (A).

at 254 nm were observed in the reaction. A comparison with the results of Möller et al. (1984) shows that higher Br_2/nucleotide ratios were required with poly[r(C-G)] (chain length ca. 80 bp) to achieve the same degree of modification obtained with poly[d(C-G)] (chain lengths 150 and 1500 bp). This result agrees with the trend toward a lower degree of modification with shorter chain length noted by those authors.

When poly[r(C-G)] undergoes the A → Z_R transition, there is an increase in the A_{295}/A_{260} UV absorbance ratio (Hall et

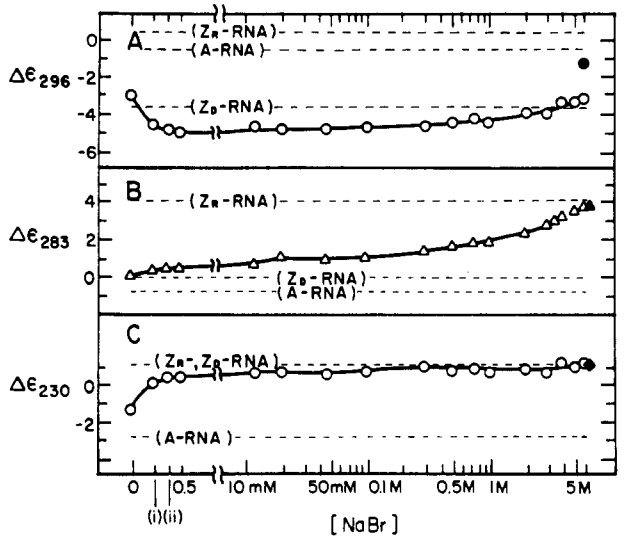

FIGURE 2: Effects of NaBr concentration on the CD spectrum of brominated poly[r(C-G)] (49% br^{8}G, 43% br^{5}C) at 22 °C. Conditions labeled on the abscissa are as follows: (0) following extensive dialysis against 0.1 mM EDTA and then into H_2O; after addition of concentrated sodium phosphate (pH 7.0)/EDTA solution to yield (i) 0.5 mM sodium phosphate (pH 7.0), 0.05 mM EDTA and (ii) 1 mM sodium phosphate (pH 7.0), 0.1 mM EDTA. Ellipticities were obtained as a function of NaBr concentration at 296 nm (A), 283 nm (B), and 230 nm (C) upon dissolving lyophilized aliquots from a 6 M NaBr solution in the sample. Darkened symbols denote ellipticities obtained upon raising the temperature to 60 °C in 1 mM sodium phosphate (pH 7.0), 6 M NaBr, and 0.1 mM EDTA. For reference, standard values obtained from A-RNA and Z_R-RNA (Hall et al., 1984a) and Z_D-RNA (Cruz et al., 1986a) are also plotted.

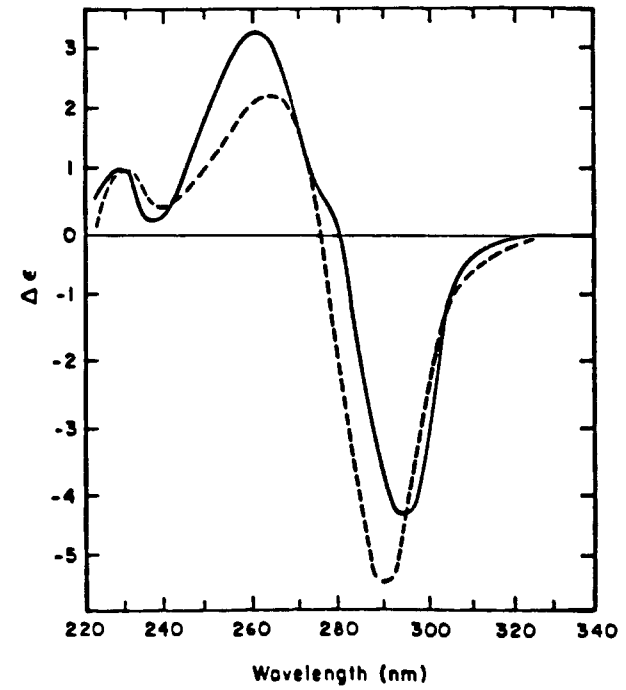

FIGURE 3: Circular dichroism spectra of extensively brominated poly[r(C-G)] (—) (49% br^{8}G, 43% br^{5}C) and poly[d(C-G)] (---) (35% br^{8}G, 19% br^{5}C) in 10 mM sodium phosphate (pH 7.0), 110 mM NaCl, and 1 mM EDTA at 22 °C. The brominated DNA is known to adopt the Z-conformation under these conditions (Möller et al., 1984).

al., 1984a). The increase in the A_{295}/A_{260} ratio parallels the A → Z transition induced by chemical bromination as monitored by CD (compare parts A and C in Figure 1). Circular dichroism spectra corresponding to polynucleotides obtained from reactions containing 2–2.5 Br_2/nucleotide (T = 45 °C; 14% br^{8}G, 11% br^{5}C) are nearly identical with the Z_R-RNA spectra of Hall et al. (1984b). Using levels >3 Br_2/nucleotide at 45 °C or >2 Br_2/nucleotide at 55 °C (23% br^{8}G, 19% br^{5}C) resulted in spectra with negative CD at 295 nm in 6 M NaBr buffer at 22 °C (Figure 2A). Brominated poly[r(C-G)] prepared using 3 Br_2/nucleotide at 45 °C retains a Z_R-form CD spectrum on lowering the ionic strength to 2 M $NaClO_4$ (Cruz et al., 1986a). Characteristic Z_R-form positive CDs are observed in these spectra at 230 and 280 nm (see Figure 1A, 2–4 Br_2/nucleotide spectra). A survey of chemically synthesized modified and unmodified tetranucleotide duplexes (Uesugi et al., 1984) shows that the positive CD at 230 nm is well correlated with the Z-conformation. Thus, bromination of poly[r(C-G)] stabilizes the Z_R-form at much lower ionic strengths than is necessary for the unmodified polynucleotide. Moreover, polynucleotides with a higher degree of bromination yield CD spectra progressively more like that of Z-DNA (Figure 1A, 4–5 Br_2/nucleotide). Based on a resemblance between the CD spectra of poly[r(C-G)] in 4 M $MgCl_2$ (pH 5.3) and several Z-DNAs, the conformation of left-handed RNA present in high $MgCl_2$ has been termed Z_D-RNA (Cruz et al., 1986b). The differences between Z_R- and Z_D-RNA are not clear at present but may include alternative base-stacking conformations, hydration states, or ionic interactions.

Lowering the ionic strength below 2 M $NaClO_4$ or NaBr resulted in a CD spectrum with characteristics resembling that of Z_R-, Z_D-, and A-RNA (Figures 2 and 3). Raising the temperature does not completely convert the polymer to Z_R-form at these salt concentrations. For example, in 500 mM $NaClO_4$ or NaBr this polymer is ca. 20% Z_R-form as judged by the positive CD at 283 nm (Figure 2B). Raising the temperature to 60 °C only partially converts the spectrum to Z_R-form (ca. 50%). In contrast, if any indication of inversion is seen at 280 nm in the A-form spectrum of unmodified poly[r(C-G)] in 6 M $NaClO_4$, it is readily converted to Z_R-RNA by a 10–15 °C increase in temperature (Cruz et al., 1986a). Thus, bromination of poly[r(C-G)] apparently stabilizes intermediate states in the A ↔ Z equilibria (Z_R- and/or Z_D-RNA) at lower ionic strengths. Lowering the ionic strength to 110 mM NaBr or NaCl for polynucleotides containing 49% br^{8}G and 43% br^{5}C (Figures 2 and 3) results in CD spectra very much like that of Z-DNA (Figure 3). A significant difference can be seen between the CD spectra of Br-poly[r(C-G)] and Br-poly[d(C-G)] at 283 nm (Figure 3). The inflection in the RNA spectrum at 283 nm is indicative of the presence of some Z_R-RNA even at this low ionic strength. These data show that the CD spectrum of Br-poly[r(C-G)] (>49% br^{8}G, 43% br^{5}C) at 110 mM NaCl has features in common with the spectra of both Z_R- and Z_D-RNA. Finally, at very low ionic strength, the CD parameters approach those corresponding to A-RNA (Figure 2). This discussion serves to emphasize the condition dependence of the forms adopted by both brominated and unmodified polynucleotides.

^1H Nuclear Magnetic Resonance Experiments. Proton NMR studies provide useful insights into the types of conformational states present in Br-poly[r(C-G)] in low ionic strength solution (Figure 4). The 500-MHz ^1H NMR spectrum of the polynucleotide in 110 mM NaBr/D_2O buffer is shown in Figure 4A. The assignments shown in Figure 5 are those most consistent with the nuclear Overhauser effect (NOE) data (Table I) and previous assignments made by Hall et al. (1984a). Samples were chemically brominated with a molar ratio of 3 Br_2/nucleotide in a 55 °C reaction, yielding polynucleotides containing 32% br^{8}G, 26% br^{5}C. Downfield

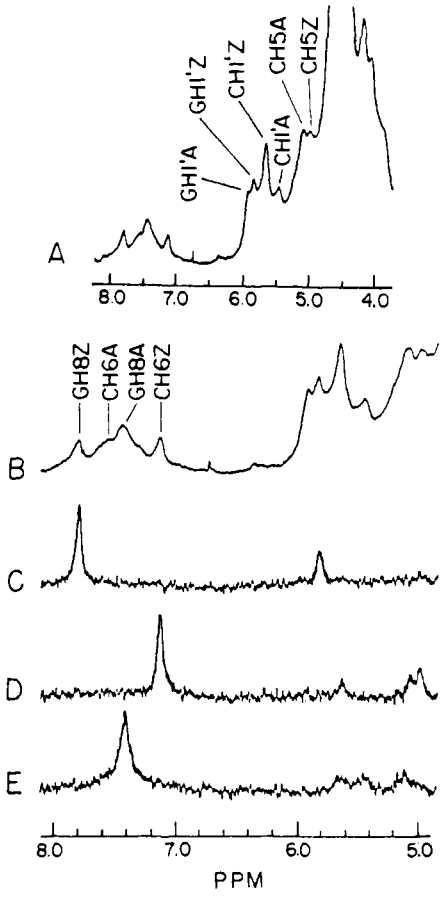

FIGURE 4: (A) and (B) 500-MHz ^{1}H NMR spectra of extensively brominated poly[r(C-G)] (32% br^{8}G, 26% br^{5}C) in 10 mM sodium phosphate (pH 7.0), 110 mM NaBr, and 0.5 mM EDTA at 37 °C. The concentration was approximately 55 mM in nucleotides. ^{1}H NOE difference spectra obtained on preirradiating resonances at (C) 7.79 ppm, (D) 7.13 ppm, and (E) 7.42 ppm. The spectra were obtained in an interleaved experiment (256 scans, 8 scans/cycle) included an off-resonance preirradiation control (ν_{irrad} = 6.79 ppm). A digital line broadening of 2 Hz was applied prior to Fourier transforming the NOE difference FIDs. Chemical shifts are reported relative to internal standard TSP.

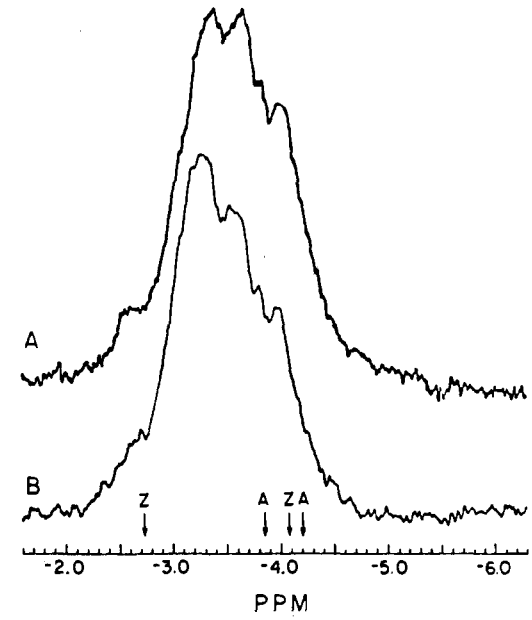

FIGURE 5: 202-MHz ^{31}P NMR spectrum of Br-poly[r(C-G)] containing (A) 23% br^{8}G, 19% br^{5}C and (B) 32% br^{8}G, 26% br^{5}C in 10 mM sodium phosphate (pH 7.0), 110 mM NaBr, and 0.5 mM EDTA at 37 °C. A digital line broadening of 8 Hz was applied prior to Fourier transforming the FID. Chemical shifts are reported relative to internal standard TMP. A- and Z_R-form ^{31}P chemical shifts for poly[r(C-G)] (Hall et al., 1984) are also shown.

Table I: NOEs Observed in the ^{1}H NMR Spectrum of Br-poly[r(C-G)]

preirradiation frequency (ppm)	NOE frequency (ppm)	% NOE[a] 500-ms preirradiation	% NOE[a] 200-ms preirradiation	NOE[b] contact
7.79	7.13	<5	<5	GH8Z → CH6Z
(GH8Z)	5.83	30	25	→ GH1'Z
	5.65	<15	<10	→ CH1'Z
7.59	5.65	20	<10	CH6A → GH1'Z[c]
(CH6A)	5.44	15		→ CH1'A
	5.07	30	30	→ CH5A
7.42	5.65	15	<10	GH8A → CH1'Z[c]
(GH8A)	5.44	15	<10	→ CH1'A
	5.07	20	<15	→ CH5A
7.13	5.92	<10	<5	CH6Z → GH1'A[c]
(CH6Z)	5.83	<10	<5	→ GH1'Z
	5.65	20	<15	→ CH1'Z
	5.07	20	<15	→ CH5A[c]
	4.98	30	30	→ CH5Z

[a] Percentages accurate to ±5%. [b] Proposed NOE-contacted atom. [c] NOEs that were inconsistent with the other assigned NOE contacts. It is not known whether these effects are physically significant (i.e., exchange NOEs) or are due to subtraction errors (most are small percent NOEs).

peaks (7.1–7.9 ppm) are present with chemical shifts clearly corresponding to those of GH8 and CH6 resonances in both the A- and Z_R-forms of poly[r(C-G)] (Hall et al., 1984a). Peaks are also present in the 5.1–6.0 ppm region corresponding to CH5 and H1′ resonances for both cytosine and guanine in the A- and Z-forms. The integral of the 7.29 ppm peak is correlated with the extent of C5 modification, suggesting that this peak corresponds to br^{5}CH6. Resonances upfield from HDO (3.8–4.5 ppm) correspond to aliphatic protons on the ribose moieties of the nucleotides in Br-poly[r(C-G)] (Uesugi et al., 1984). Thus, according to chemical shift and integral data, it is apparent that the ^{1}H NMR spectrum of Br-poly[r(C-G)] in 110 mM NaBr shows a ca. 1/1 equilibrium mixture of normal A- and Z-form RNA nucleotide units. This conclusion was verified by NOE experiments.

The NOE results from a transfer of dipolar spin energy between two or more spatially proximal nuclei (Solomon, 1955; Noggle & Schirmer, 1971). One-dimensional NOE connectivity patterns were studied in the A- and Z_R-conformations of poly[r(C-G)] by Hall et al. (1984a). In order to verify the A ⇌ Z RNA conformational equilibrium in Br-poly[r(C-G)] at 120 mM Na^+ concentration, NOE experiments were performed using 200- and 500-ms irradiation times prior to acquiring the FIDs (Figure 4C–E; Table I). With a 500-ms irradiation time, spin diffusion effects [domino-like NOEs; see Kalk and Berendsen (1976)] will be effective for Br-poly[r(C-G)] (average molecular weight ca. 50K; approximately 80 bp). The Z-form GH8 ↔ GH1′ and CH5 ↔ CH6 reciprocal NOEs are visible at 5.83 and 4.98 ppm (Figure 4C,D). The guanine NOE intensities (25–30%) are in good agreement with the expected syn glycosidic torsional conformation. In contrast, much lower NOE intensities are consistent with the expected anti conformation in A-form guanine residues (7.42 ↔ 5.44 ppm; Figure 5E; Table I).

These data show that brominating poly[r(C-G)] produces a transition to a polynucleotide that includes G8-protonated Z-form guanine residues. Peak integrals show that approximately 50% of the unmodified residues in the polynucleotide

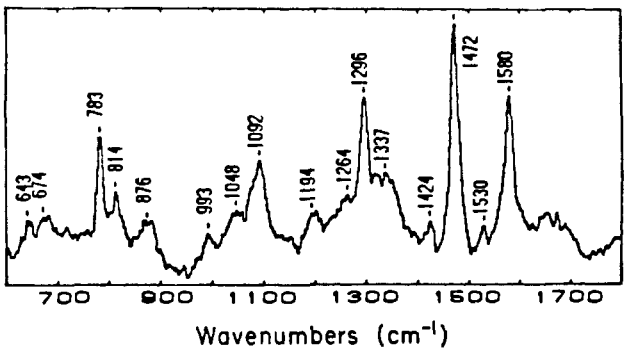

FIGURE 6: Raman spectrum of Br-poly[r(C-G)] (32% br^{8}G, 26% br^{5}C) in 10 mM sodium phosphate (pH 7.0), 100 mM NaCl. The spectral resolution was 6 cm^{-1}; the spectrum was the average of ca. 10–12 h of accumulation.

are in the Z-RNA conformation at physiological ionic strength and temperature (compare CH6 and GH8 A- and Z-form peak integrals in Figure 5).

^{31}P NMR and Raman Scattering Experiments. Additional evidence for a left-handed Z-form of Br-poly[r(C-G)] at 120 mM Na^+ concentration was obtained from ^{31}P NMR spectroscopy. ^{31}P resonances corresponding to the A- and Z_R-form phosphodiesters of poly[r(C-G)] were assigned by Hall et al. (1984a) as follows: Z-form, −2.74 and −4.07 ppm; A-form, −3.64 and −4.14 ppm. The 202-MHz ^{31}P NMR spectra of Br-poly[r(C-G)] in 110 mM NaBr buffer corresponding to 23% br^{8}G, 19% br^{5}C and 32% br^{8}G, 26% br^{5}C are shown in Figure 5, parts A and B, respectively. The CD spectra in 100 mM NaBr and NaCl buffers are almost identical; NaBr was used since Br-poly[r(C-G)] is more soluble in this salt. Similar chemical shift patterns in the ^{31}P NMR spectra of r(C-br^{8}G-C-br^{8}G) and r(C-m^{8}G-C-m^{8}G) in 110 Na^+ mM buffer were assigned by Uesugi et al. (1984) to left-handed (presumably Z) conformations. Möller et al. (1984) previously noted in ^{31}P NMR studies of brominated poly[d(C-G)] that peaks corresponding to CpG and GpC in brominated tracts of the polymer resonate in the −3.6 to −3.9 ppm region [also see Uesugi et al. (1984)]. The −4.07 ppm Z-form peak is obscured by the complex signal in the spectrum of Br-poly[r(C-G)]; however, the −2.74 ppm peak is reproducibly visible just downfield from the main cluster of peaks in the −3 to −4.3 ppm range. Thus, the ^{31}P NMR data shown in Figure 5 are consistent with the presence of at least one of the two types of Z-form phosphodiester groups in Br-poly[r(C-G)]. The remaining signal probably represents phosphodiester groups within brominated tracts, as observed by Möller et al. (1984), and phosphodiesters in unmodified A- and Z-form tracts.

Raman scattering spectroscopy showed that the Z-DNA conformation observed in single-crystal X-ray crystallography studies (Wang et al., 1979; Drew & Dickerson, 1981) was the same as that observed in solution (Thamann et al., 1981). The Raman spectrum of Br-poly[r(C-G)] in 110 mM NaCl buffer (32% br^{8}G, 26% br^{5}C; concentration ~45 mM) is shown in Figure 6. CD spectra of Br-poly[r(C-G)] in 110 mM NaCl and NaBr buffers (concentration ~2 mM) are essentially identical (Figures 2B and 3). Thus, if the increase in concentration does not greatly perturb the A ↔ Z equilibrium, the Raman spectrum should represent a ca. 1/1 mixture of A- and Z-form RNAs.

The most characteristic change seen in the Raman spectrum of poly[r(C-G)] due to the A → Z transition is the loss of intensity at 813 cm^{-1} due to loss of the antisymmetric phosphodiester stretch mode (Tinoco et al., 1986). The intensities of the peak at 814 cm^{-1} and the shoulder at 807 cm^{-1} are consistent with equal amounts of A- and Z-form RNA. The peaks at 674 and 643 cm^{-1} correspond to guanine imidazole ring-breathing modes in unmodified A- and Z-forms, respectively. Their intensities are considerably lower than those of the corresponding peaks in the spectra of normal A- and Z_R-RNA. The narrower distribution and smaller intensity in the Z-form band at 642 cm^{-1} resemble the Z_D-RNA Raman spectrum (Tinoco et al., 1986). The frequency of this band is sensitive to the glycosidic torsional angle. As observed for Z_R-RNA (Cruz et al., 1986a,b), this band is at a frequency intermediate between the right-handed B-DNA (682 cm^{-1}) and A-RNA (671 cm^{-1}) and the left-handed Z-DNA (625 cm^{-1}) peaks, suggesting the presence of different glycosidic torsion angles in Z-RNA and Z-DNA. No indication of Z-DNA intensity at 625 cm^{-1} can be seen in Figure 6. Bands at 1194 and 1264 cm^{-1} and in the 1337–1363-cm^{-1} range are consistent with both Z_R- and Z_D-RNA Raman spectra. A higher relative intensity at 1296 cm^{-1} is peculiar to the brominated RNA. This band probably corresponds to the strong base-stacking-sensitive guanine mode at 1320 cm^{-1} in unbrominated Z-RNA (Chinsky et al., 1978). Broadening of the band at 783 cm^{-1} toward lower wavenumbers and the presence of the 719-cm^{-1} peaks in Figure 6 are consistent with an equal representation of normal A- and Z-RNAs. Although a complete analysis of Raman assignments has not been made, the spectrum obtained is entirely consistent with a mixture of A- and Z-RNAs. Characteristics of both Z_R- and Z_D-RNA are observed, and the Raman evidence is consistent with a Z-RNA guanine glycosidic torsional conformation different from that of Z-DNA. As pointed out by Cruz et al. (1986a,b) this may be an important difference between the two left-handed forms of nucleic acid.

Recognition of Z-RNA by Anti-Z-DNA Antibodies. Figure 7A shows results from RIA and filter binding studies, which demonstrate the recognition of Br-poly[r(C-G)] (49% br^{8}G, 43% br^{5}C) by the purified rabbit polyclonal anti-Br-poly[d-(C-G)] IgG preparation T4 (Zarling et al., 1984a,b) in 110 mM NaCl/Tris buffer. Unmodified A-form or single-stranded RNAs poly[r(C-G)], poly[r(A)·r(U)], poly[r(A-U)], poly[r-(I-C)], and 16S/23S rRNA were not recognized under these conditions in direct binding assays. Similar data were obtained in both RIA and nitrocellulose filter binding experiments; the amount of Z-RNA bound depended on both the antibody and substrate concentrations (Figure 7A).

Figure 7B shows the results of a more extensive characterization of the recognition of Br-poly[r(C-G)] by two rabbit polyclonal anti-Br-poly[d(C-G)] IgGs, T4 and Z6, in 220 mM NaCl/Tris buffer using a second antibody-independent nitrocellulose filter binding assay. For comparison, data obtained from parallel reactions with the Z-DNAs poly[d(br^{5}C-G)] in 220 mM NaCl, poly[d(m^{5}C-G)] in 1.5 M NaCl, and poly-[d(C-G)] in 3 M NaCl are also shown. These data show that T4 IgG has about a 50% higher affinity for Br-poly[r(C-G)] than does Z6 IgG and that these antibodies bind with a higher affinity to Z-DNA than to Br-poly[r(C-G)]. Note that these antibodies do not require C5 or G8 bromination in order to recognize the antigenic determinant on Z-DNA.

Figure 8A shows the specificity in the binding of T4 IgG to Br-poly[r(C-G)] as determined by competitive filter binding assay. Since Z-DNA in the form of poly[d(br^{5}C-G)] can successfully compete with radiolabeled Br-poly[r(C-G)] in binding to T4 IgG, the same antibody populations in the polyclonal preparation are probably binding to the brominated Z-RNA. The data shown in Figure 8B demonstrate that this is the same population that binds to unmodified Z-DNA in

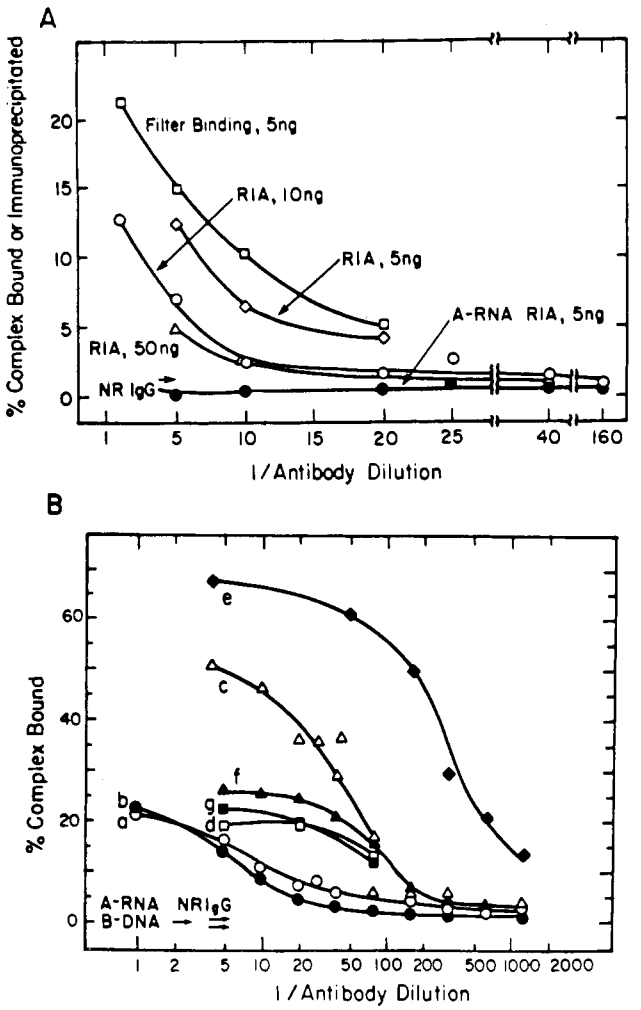

FIGURE 7: Binding of anti-Br-poly[d(C-G)] polyclonal IgGs to [^{32}P]Br-poly[r(C-G)] (49% br^{8}G, 43% br^{5}C) in 40 mM Tris-HCl (pH 7.5), 110 mM NaCl, and 4 mM EDTA. (A) T4 IgG dilutions were tested by RIA using goat anti-rabbit IgG as second antibody as described under Methods. Second antibody-independent nitrocellulose filter binding assay results demonstrating recognition of Br-poly[r(C-G)] by T4 IgG are also shown. Poly[r(C-G)] (A-RNA) and poly[d(C-G)] (B-DNA) were not recognized under these conditions. Specific anti-Z-RNA IgG recognition was verified in both types of assay using the IgG fraction of normal rabbit serum (NR IgG→) as first antibody. Background levels were obtained by adding 5 μL of buffer to the preincubated polynucleotide solutions in place of antibody (→). (B) Filter binding assay results comparing the recognition of Z-RNA and Z-DNA by anti-Br-poly[d(C-G)] IgGs T4 and Z6. Dilution curves are labeled as follows: recognition of Br-poly[r(C-G)] in 220 mM NaCl/buffer by (a) T4 and (b) Z6 IgGs; (c) poly[d(br^{5}C-G)] in 220 mM NaCl/buffer, (d) poly[d(m^{5}C-G)] in 1.5 M NaCl/buffer, and (e) poly[d(C-G)] in 3 M NaCl/buffer by T4 IgG; (f) poly[d(br^{5}C-G)] in 220 mM NaCl/buffer and (g) poly[d(m^{5}C-G)] in 1.5 M NaCl/buffer by Z6 IgG.

4 M NaCl/Tris buffer. Thus, both T4 and Z6 IgG populations bind to unmodified Z-DNA-like determinants present in Br-poly[r(C-G)]. Since the competition is >80% complete, the conclusions regarding the relative affinities of these antibodies for Z-RNA and Z-DNA noted above are valid and are not merely due to reaction with different populations in the polyclonal antibody preparations. This provides complementary immunochemical support for the conclusion drawn from the spectroscopic analysis that chemical bromination of poly[r(C-G)] stabilizes a Z-DNA-like conformation under physiological conditions.

Figure 9 shows the effects of NaCl (A) and sodium phosphate (B) on the binding of anti-Br-poly[d(C-G)] IgGs T4 and Z6 to Br-poly[r(C-G)]. Higher NaCl concentrations

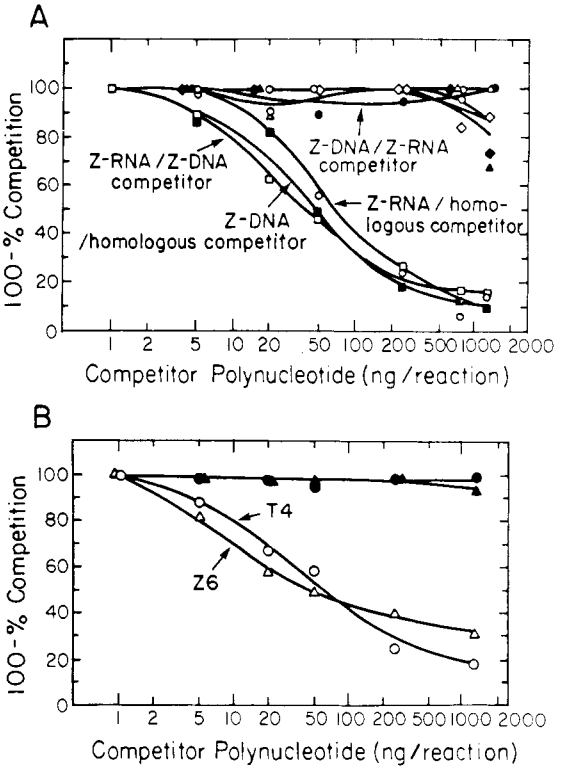

FIGURE 8: Specificity in the binding of anti-Br-poly[d(C-G)] IgGs T4 and Z6 to [^{32}P]Br-poly[r(C-G)] (Z-RNA) in 220 mM NaCl and [^{32}P]poly[d(C-G)] (Z-DNA) in 4 M NaCl. (A) Filter binding competition assay results obtained by preincubating 2.5 ng of ^{32}P-labeled and 17.5 ng of unlabeled polynucleotide in 40 mM Tris-HCl (pH 7.5), 4 mM EDTA, with the specified amounts of unlabeled competitor polynucleotide prior to addition of T4 IgG. Polynucleotides were as follows: Z-RNA, Br-poly[r(C-G)]; Z-DNA, poly[d(br^{5}C-G)]; A-RNA, poly[r(C-G)]; B-DNA, poly[d(C-G)]. A-RNA (◇, ◆) and B-DNA (△, ●) did not compete with Z-RNA or Z-DNA in these assays. Due to the considerably reduced affinity of T4 IgG for Z-RNA, Br-poly[r(C-G)] does not compete with Z-DNA at the competitor levels tested in these assays (▲). (B) Results obtained by preincubating Br-poly[r(C-G)] (2.5 ng of ^{32}P-labeled and 17.5 ng of unlabeled) with unlabeled, unmodified poly[d(C-G)] (Z-DNA) competitor (○, △) and the B-DNA negative control poly[d(C)·d(G)] (●, ▲) in 4 M NaCl/buffer prior to adding T4 or Z6 IgGs.

reduced the binding levels significantly. For example, at 1 M NaCl, the binding levels were reduced to about 80% of those obtained in the 50–100 mM NaCl range (Figure 9A). In contrast, at 220 mM NaCl, raising the sodium phosphate concentration above 10 mM reduced the binding levels to 40% of those obtained in the absence of the phosphate anion (Figure 9B). A direct comparison between the NaCl and sodium phosphate data at 1 M Na^+ concentration indicates that the phosphate anion is at least 2–4-fold more effective in inhibiting the binding reaction than the Cl^- ion. This general dependence on ionic strength and the specific inhibition by the phosphate anion strongly suggest the recognition of a phosphodiester backbone determinant common to the Z-forms of RNA and DNA. In addition, binding of Z6 IgG to Br-poly[r(C-G)] is sensitive to much lower phosphate ion concentrations than T4 IgG (Figure 9B). These data suggest that Z6 IgG binding is more dependent on recognition of phosphodiester backbone determinants.

Discussion

Since RNA was shown to adopt a left-handed conformation under the extreme conditions of 6 M $NaClO_4$ (Hall et al., 1984a), a prime question has been whether this phenomenon is biologically significant (Tinoco et al., 1986; Usher et al., 1984; Zwieb & Ullu, 1986). It is possible that Z-RNA may

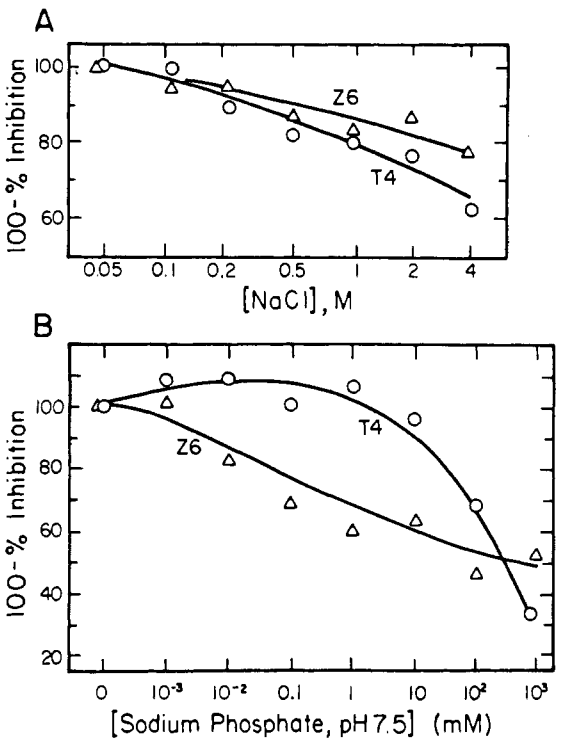

FIGURE 9: Effects of sodium and phosphate ions on the binding of anti-Br-poly[d(C-G)] antibodies to Br-poly[r(C-G)] (Z-RNA) in 40 mM Tris-HCl (pH 7.5), 4 mM EDTA. 2.5 ng of ^{32}P-labeled and 17.5 ng of unlabeled Br-poly[r(C-G)] were incubated with 5 μL of T4 (○) or Z6 (△) IgG at the specified NaCl (A) or sodium phosphate (B) concentrations. Filter binding assays were performed as described under Methods. The initial NaCl concentration was 220 mM in the phosphate inhibition experiments.

have a functional role as a molecular switch in the ribosome (Tinoco et al., 1986) or in other ribonucleoprotein complexes [e.g., the spliceosome; see Grabowski et al. (1985)]. The first fact to be established in investigating this possibility is whether Z-RNA can be stabilized at physiological ionic strength.

Topological stress (i.e., negative supercoiling) can effectively induce the B → Z transition in DNA [reviewed by Rich et al. (1984)]. Chemical bromination (Möller et al., 1984) and high ionic strength also favor the Z-form of DNA. These conditions effectively serve to alter the energetics of the polymer: bromination of guanine residues at C8 favors transition to the normally higher energy syn glycosidic conformation (Jordan, 1973), while increased ionic strength neutralizes the unfavorable ionic repulsion of backbone phosphate moieties in the Z-form. Biologically relevant ligands contacted by the polynucleotide in its natural environment [see Rich et al. (1984), pp 828–840] may thus temper the conformation of the polynucleotides. Presumably a knowledge of the effects produced by stressed conditions on the A ↔ Z transition will help us to better understand the possible functional roles of this transition in native RNA (Zarling et al., 1987).

The most important result presented in this study is that bromination of guanine residues, which alters the energetics of the syn ↔ anti glycosidic equilibrium, can stabilize the Z-RNA conformation in 110 mM NaCl buffer at sites other than the sites of modification (Figures 5–8). As noted with Z-DNA [reviewed by Rich et al. (1984)], altering the energetics in one portion of the polymer can induce structural transitions in adjacent regions of the polynucleotide.

Möller et al. (1984) showed that 38% bromination of guanine residues and 18% bromination of cytosine residues in poly[r(C-G)] results in an inversion in the CD spectrum from B- to Z-form [compare with data of Pohl and Jovin (1972)] and in an increase in the absorbance ratio A_{295}/A_{260}. From ^{1}H NMR GH8 integration data (such as in Figure 4A for a 1/1 mixture of A- and Z-forms) and HPLC analyses (Figure 1B) we estimate that 50–55% modification of the guanine and cytosine residues in Br-poly[r(C-G)] is required to produce a polynucleotide containing >80% Z-RNA in 110 mM NaCl buffer. At 32–35% br^{8}G and 26–30% br^{5}C, a 1/1 mixture of unmodified A- and Z-form nucleotide units is present (Figure 4). Thus, the studies reported in this paper show that a higher degree of chemical modification is required to stabilize Z-RNA than Z-DNA under physiological conditions. Again, as shown by Hall et al. (1984a) for the salt dependence of the A ↔ Z_R transition in RNA, more rigorous conditions are required to stabilize Z-RNA than Z-DNA.

Three clues regarding the structural differences between Z-RNA and Z-DNA were noted in these studies [see also Cruz et al. (1986a), Tables II and III]. (1) Raman scattering evidence indicates that Z-RNA may have a different guanine glycosidic torsional angle than Z-DNA [see also Cruz et al. (1986b)]; CD evidence is consistent with different base-stacking conformations, hydration states, or ionic interactions for Z_R-RNA and Z-DNA. (2) CD data indicate that in 4 M $MgCl_2$, a Z-DNA-like (Z_D) structure is present in poly[r(C-G)] (Tinoco et al., 1986). Since both Z_R- and Z_D-RNA-like characteristics were seen in the CD spectra of Br-poly[r(C-G)] in low-salt buffers (Figures 2 and 3), these data may suggest heterogeneity in the population of Z-RNA forms in this polynucleotide under these conditions. (3) Finally, it was noted that relative to guanine residues cytidine residues are more effectively modified in poly[r(C-G)] than in poly[d(C-G)]. As noted by Möller et al. (1984), this probably reflects the relative reactivities as attenuated by the degree of accessibility. Thus, these data indicate different degrees of solvent accessibility at G8 and C5 positions in Z-RNA when compared to Z-DNA.

Due to their inherent specificity and sensitivity, immunoglobulins have been used as indirect probes for DNA conformations, both in solution and in cytological preparations (Stollar, 1980). This was shown to be a useful approach in the case of Z-DNA by several investigators (Lafer et al., 1981; Malfoy & Leng, 1981; Nordheim et al., 1981; Zarling et al., 1984a,b). The constitutive Z-DNA Br-poly[d(C-G)] was used as the immunogen in eliciting the rabbit polyclonal anti-Z-DNA IgGs T4 and Z6 (Zarling et al., 1984a,b). A variety of control experiments demonstrated that anti-Br-poly[d(C-G)] antibodies are directed against left-handed conformation determinants and not solely the brominated nucleoside (Zarling et al., 1984a; Nordheim et al., 1981). Thus, the extent of recognition of Z-RNA by T4 and Z6 IgGs is an indirect measure of the conformational relatedness between Z-DNA and left-handed RNA characterized in this study.

The anti-Br-poly[d(C-G)] IgG-binding studies (Figures 7 and 8) demonstrate that some immunological (and therefore protein-binding) properties of Z-RNA are similar to those of Z-DNA under physiological conditions. Inhibition of [anti-Z-DNA IgG–Z-RNA] complex formation by phosphate and high ionic strength (Figure 9) strongly suggests the presence of a common phosphodiester determinant on the left-handed forms of RNA and DNA. ^{31}P NMR (Figure 5) and Raman scattering data (Figure 6) are also consistent with a zigzag backbone conformation.

If Z-RNA has a structure similar to the crystal structure of Z-DNA [see Rich et al. (1984)], the bromine atom at C5 closely contacts the guanine imidazole ring, and the edge of

the hydrophobic convex surface of the polynucleotide is composed of the following group of substituents (...G2′-hydroxyl, GpC-phosphate, G8-bromine, C5-bromine...). Thus, the most obvious difference between Z-RNA and Z-DNA, the 2′-OH, is certainly at least partially responsible for the substantially lower binding affinities of the anti-Z-DNA IgGs T4 and Z6 for Z-RNA (Br-poly[r(C-G)]) compared to Z-DNA.

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Registry No. Poly[r(C-G)], 49846-05-1.

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