Characterization of Anti-Z-RNA Polyclonal Antibodies: Epitope Properties and Recognition of Z-DNA†

Charles C. Hardin,*,‡ David A. Zarling,§ Steven K. Wolk,‡ Wilson S. Ross,‡ and Ignacio Tinoco, Jr.‡

Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, California 94720, and Molecular Biology Department, SRI International, Menlo Park, California 94025

Received September 18, 1987; Revised Manuscript Received January 21, 1988

ABSTRACT: Chemically brominated poly[r(C-G)] [Br-poly[r(C-G)]] containing 32% br^{8}G and 26% br^{5}C was recently shown to contain a 1:1 mixture of A- and Z-form unmodified nucleotides under physiological conditions of temperature, pH, and ionic strength [Hardin, C. C., Zarling, D. A., Puglisi, J. D., Trulson, M. O., Davis, P. W., & Tinoco, I., Jr. (1987) *Biochemistry 26*, 5191–5199]. Proton NMR results show that more extensive bromination of poly[r(C-G)] (49% br^{8}G, 43% br^{5}C) produces polynucleotides containing >80% unmodified Z-form nucleotides. Using these polynucleotides as antigens, polyclonal antibodies were elicited in rabbits and mice specific for the Z-form of RNA. IgG fractions were purified from rabbit anti-Br-poly[r(C-G)] sera and characterized by immunoprecipitation, nitrocellulose filter binding, and ELISA. Two different anti-Z-RNA IgG specificities were observed. Decreased levels of brominated nucleotides in the immunogen correlated with an increased extent of specific cross-reactivity with Z-DNA. Inoculation of rabbits with polynucleotide immunogens containing 49% br^{8}G and 43% of br^{5}C produced specific anti-Z-RNA IgGs that do not recognize Z-DNA determinants. This suggests that the 2′-OH group is part of the anti-Z-RNA IgG determinant. In contrast, Br-poly[r(C-G)] immunogens containing 32% br^{8}G and 26% br^{5}C produced IgGs that specifically recognize both Z-RNA and Z-DNA. These results show that the bromine atoms are not required for recognition of the Z conformation by the antibodies. The affinity of these anti-Z-RNA IgGs for Z-RNA is about 10-fold higher than for Z-DNA. Rabbit polyclonal anti-Z-DNA IgGs also have different affinities for Z-RNA that depend on the degree of bromination of the Br-poly[d(C-G)] immunogens (Hardin et al., 1987). The relative intensities of circular dichroism bands at 260 and 295 (or 285) nm in the spectra of both Br-poly[r(C-G)] (295 nm) and Br-poly[d(C-G)] (285 nm) are shown to correlate with the degree of bromination of the polynucleotides. Anti-Br-poly[r(C-G)] and anti-Br-poly[d(C-G)] antibodies that are elicited against the more highly modified polynucleotides have an increased sensitivity to phosphate inhibition in binding to Br-poly[r(C-G)]. This suggests a greater dependence on recognition of Z-RNA phosphodiester backbone determinants. Quantitative immunoprecipitin assay results indicate that the FAB binding sites for recognition of Br-poly[r(C-G)] by both types of anti-Z-RNA antibodies contain about five base pairs. On the basis of these results and an energy-minimized structure for Z-RNA, likely features of the multivalent antigenic epitopes are considered. We conclude that likely determinants include (1) 2′-OH group(s), (2) the zigzagged phosphodiester backbone, and (3) exposed portions of the bases on the convex hydrophobic surface including the C5 and G8 bromine atoms.

Several studies have demonstrated the utility of antibodies directed against nucleosides and polynucleotides in elucidating structural features of both native and modified nucleic acids [Guigues & Leng, 1976; Johnston & Stollar, 1978, and references cited therein; Sage et al., 1979; Zarling et al., 1984a; Sundquist et al., 1986; Runkel & Nordheim, 1986; reviewed by Stollar (1986)]. Johnston and Stollar showed that recognition by antibodies directed against various double-stranded RNAs depends primarily on differences in phosphodiester and ribofuranose structure, thus providing excellent probes for helical conformational variants. The antibody binding sites span about 4 bp[1]/FAB, indicating the recognition of an extended region of conformational features in the polynucleotide [see also Guigues and Leng (1976) and Runkel and Nordheim (1986)].

The left-handed Z-form of DNA, originally inferred from spectroscopic studies of poly[d(C-G)] at NaCl concentrations above 2.25 M (Pohl & Jovin, 1972), was described at atomic resolution for the hexanucleotide pentaphosphate $[d(C\text{-}G)_3]_2$ on the basis of X-ray crystallography studies (Wang et al., 1979, 1981; Drew & Dickerson, 1981). Distinctive features include syn guanine residues, stacking of guanine residues on the adjacent deoxyribose O1′, stacking of cytosine residues from opposite strands on each other, and a zigzag phosphodiester backbone conformation resulting in a dinucleotide repeat unit.

It was subsequently found that poly[d(C-G)] that is stabilized in the Z conformation under physiological conditions by chemical bromination at C8 of guanine residues and C5 of cytosine residues is highly immunogenic in both rabbits and

† This investigation was sponsored by USPHS–NIH Grant GM 10840 (I.T.), DOE Grant FG03-86-ER60406 (I.T.), ONR Contract N00014-18-C-0570 (D.Z.), and USPHS–NIH Grants GM 37114 and 38424 (D.Z.). C.C.H. was supported in part by NIH–NRSA Postdoctoral Fellowship GM 11638.

‡ University of California.

§ SRI International.

[1] Abbreviations: bp, base pairs; BSA, bovine serum albumin; CD, circular dichroism; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FAB, fragment antigen binding; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; IgG, immunoglobulin G; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; RIA, radioimmunoassay; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride.

0006-2960/88/0427-4169$01.50/0

mice (Lafer et al., 1981; Möller et al., 1982; Malfoy et al., 1982; Zarling et al., 1984a,b). Purified antibodies were found to be specific for the Z-form, and it was shown that recognition of the polynucleotide does not depend on the presence of the modification. Some of these antibodies specifically recognize Z-DNA tracts in a variety of synthetic polynucleotides, negatively supercoiled plasmids, and viral, bacterial, plant and animal genomes [reviewed by Rich et al. (1984) and Zarling et al. (1984a,b)]. It has been demonstrated that anti-Z-DNA antibodies can shift the B–Z equilibrium to the Z-form (Zarling et al., 1984b; Lafer et al. 1986). In addition, these antibodies have been shown to be useful as structural and mechanistic probes in the study of homologous recombination catalyzed by *Ustilago* rec 1 protein (Kmiec et al., 1984) and as kinetic probes in studying the B to Z transition in plasmid DNA (Peck et al., 1986).

The right-handed (A-form) to left-handed (Z-form) transition in double-stranded RNA was first described by Hall et al. (1984a) for the synthetic alternating copolymer poly[r(C-G)] in 6 M $NaClO_4$ buffer at 45 °C [see Cruz et al. (1986) and Tinoco et al. (1986) for reviews]. We recently showed (Hardin et al., 1987) that chemical bromination of poly[r(C-G)]results in stabilization of Z-RNA under physiological conditions. In addition, it was demonstrated that rabbit polyclonal anti-Br-poly[d(C-G)]antibodies T4 and Z6 (Zarling et al., 1984a,b) specifically recognize the Z-form of RNA under these conditions, indicating the presence of a Z-DNA-like structural element in Z-RNA (Hardin et al., 1987). Results also indicate that the Z-forms of RNA and DNA share a common phosphodiester backbone determinant.

In this paper we describe properties of rabbit polyclonal antibodies elicited against the Z-form of Br-poly[r(C-G)]. We also demonstrate specific recognition of both modified and unmodified Z-DNA by anti-Z-RNA antibodies and show that the affinities for Z-RNA and Z-DNA differ depending on the degree of bromination of the antigen used to produce the antibody. The IgG binding sites span 10 ± 4 bp (5 ± 2 bp/FAB), and features of the phosphodiester backbone, bases, and 2′-OH groups appear to be recognized.

Experimental Procedures

Materials

RNA polymerase was kindly provided by Professor Michael Chamberlin (Department of Biochemistry, University of California, Berkeley). Deoxyribonuclease I, protein A–Sepharose CL-4B, DEAE-Sephadex A-50, and polynucleotide kinase were from Pharmacia Biochemicals. Poly[d(A)·d(T)], 16S/23S rRNA, poly[d(C-G)], poly[d(br^5C-G)], poly[d-(m^5C-G)], poly[r(I-C)], and single-stranded polyribonucleotides were from Sigma or Pharmacia Biochemicals; synthetic polynucleotides were prepared enzymatically (Zarling et al., 1984a; Hardin et al., 1987). All polynucleotides were phenol extracted and dialyzed prior to use. Polynucleotides were ^{32}P 5′-end-labeled and purified for RIA experiments as described previously (Hardin et al., 1987). Affinity-purified goat anti-rabbit IgG (2.4 mg/mL) was from Jackson Immunoresearch, and goat anti-rabbit IgG–alkaline phosphatase conjugate (26 units/μL) was from Tago, Inc. Poly(L-lysine) (M_r 5000) and poly(L-glutamate) (M_r 26 500) were from Sigma. Millipore Millititer-STHA09610 96-well nitrocellulose plates and Dynatech Immulon I 96-well polystyrene microtiter plates were used for nitrocellulose filter binding assay and ELISA, respectively. Bradford reagent was from Bio-Rad.

Methods

Preparation of Br-poly[r(C-G)]. Poly[r(C-G)] was prepared by transcription from a poly[d(I-C)] template as described by Hall et al. (1984b). Chemical bromination of poly[r(C-G)] was carried out in 6 M NaBr buffer as described by Hardin et al. (1987). Reactions were quenched by dilution into ice-cold 10 mM sodium phosphate (pH 7) and 1 mM EDTA; excess bromine was removed by bubbling N_2(g) through the diluted reaction mixtures as described previously (Hardin et al., 1987).

CD and ^{1}H NMR Spectroscopy. CD spectra were recorded on a Jasco J500C spectropolarimeter in 1-cm path length quartz cuvettes. Proton NMR spectra were obtained at 500 MHz on a Bruker AM 500 spectrometer in 10 mM sodium phosphate (pH 7), 1 mM EDTA, 100 mM NaCl, and 99.98% D_2O buffer. Spectral impurities were removed by several ethanol precipitations followed by extensive dialysis. The HDO signal was reduced by three to four lyophilizations from 99.98% D_2O (Aldrich). Samples were prepared in a glovebag under N_2; chemical shifts are reported relative to internal standard TSP.

Pure phase NOESY data sets (128 × 2K) were accumulated by using a 50-ms mixing time in the standard pulse sequence (Bodenhausen et al., 1984). Data sets were transferred via magnetic tape to a VAX 11/785 minicomputer and processed on a MicroVAX computer using software kindly provided by Dr. Dennis Hare. The data were apodized by applying an exponential line broadening of 10 Hz before Fourier transforming in the t_2 dimension. The data were then apodized in the t_1 dimension by using a phase-shifted skewed sine bell of length 128 points, zero-filled to 1024 points, and Fourier transformed; the final matrix size was 1K × 1K. Two-dimensional contour plots were obtained by using a Hewlett-Packard 7475A plotter interfaced to the computer.

Preparation of Anti-Z-RNA Antibodies. Chemically brominated poly[r(C-G)] containing ca. either 50% Z-RNA or >80% Z-RNA was prepared by using stoichiometries of 3 and 6 Br_2/nucleotide in 55 °C reactions as described above. Extinction coefficients for the polynucleotides were reported previously (Gray et al., 1981; Hardin et al. 1987). Polynucleotides (60 μg) were resuspended into 400 μL of 40 mM Tris-HCl (pH 7.5) buffer containing 4 mM EDTA, 220 mM NaCl, and 90 μg of methylated BSA (Sigma). Solutions were emulsified in 1 mL of Freund's complete adjuvant, and female New Zealand white rabbits were immunized by multiple intradermal and subcutaneous injections. Sera were collected at approximately 1-month intervals over the course of 8–12 months. Positive anti-Z-RNA titers were obtained in 4/4 rabbits designated A–D; rabbits A and B were injected with the less modified antigens (32% br^8G, 26% br^5C; 50% Z-RNA) while rabbits C and D were injected with polynucleotides containing 49% br^8G and 43% br^5C (>80% Z-RNA). IgG preparations are referred to by animal and bleed number; e.g., B6 IgG was prepared from rabbit B serum, sixth bleed. Balb/C mice were immunized as described above except that the injection volume was reduced 5-fold and 40 μg of polynucleotide was used as the immunogen. Sera were heat inactivated by incubating at 55 °C for 15 min; sodium azide was added, and the solutions were stored frozen at −70 °C.

Immunoglobulin G fractions were prepared as follows: sera were thawed, 10 mM sodium acetate (pH 5.5) was added (1:5 v/v), and protein was precipitated at 4 °C by addition of solid $(NH_4)_2SO_4$ to 26.5% (w/v). Precipitates were collected by centrifuging at 15K rpm for 20 min (4 °C), and pellets were resuspended into 10 mM sodium acetate (pH 5.5) buffer at 20% of the original volume. Insoluble material was removed by dialyzing extensively against this buffer at 4 °C and centrifuging as described above. Negative polar impurities were batch extracted by adding $^1/_5$ packed volume buffer-pre-

equilibrated DEAE-Sephadex A-50 and rocking the slurry at 22 °C for 1 h. Following centrifugation at 2K rpm and removal of the supernate, the resin was washed with $^1/_{10}$ volume fresh buffer. The combined supernates were stored at −70 °C. Thawed solutions were adjusted to 0.2 M sodium phosphate (pH 8) and adsorbed to a protein A–Sepharose CL-4B column preequilibrated in 0.2 M sodium phosphate buffer, and after extensive washing, 0.5-mL aliquots were eluted with 100 mM glycine hydrochloride (pH 3) buffer into sterile tubes containing 60 μL of 1 M HEPES (pH 8). Peak fractions (determined by A_{280}) were pooled, extensively dialyzed versus 10 mM Tris-HCl (pH 7) and 1 mM EDTA at 4 °C, and stored at −70 °C.

Immunochemical Assays. Immunoprecipitation and nitrocellulose filter binding assays were performed by using immune sera or purified IgG fractions and radiolabeled polynucleotides prepared with polynucleotide kinase as described previously (Hardin et al., 1987). Purified normal rabbit IgG, nonimmune serum, or buffer alone was used as negative control samples.

ELISAs were carried out by using 150-μL reaction volumes at 22 °C as follows: Poly(L-lysine)(50 mg/mL in H_2O) was incubated for 30 min in polystyrene microtiter plate wells, the coated wells were washed twice with TES buffer (40 mM Tris-HCl, pH 7.5, 4 mM EDTA, 220 mM NaCl), and plates were allowed to dry briefly. Polynucleotides (0.05 or 2.5 μg/mL in TES) were then incubated in the wells for 2 h followed by three washes with TES. Wells were blocked to prevent nonspecific adsorption by incubation with poly(L-glutamate) (50 μg/mL in TES) for 1 h and then with 1% BSA (in TES) for 30 min. The wells were washed with TES containing 0.1% Tween-20 after each blocking step. Antigen-coated wells were incubated with antibody in TES for 1 h followed by washes with 200 μL of TES and TES/Tween-20. Goat anti-rabbit IgG conjugated to alkaline phosphatase (2000-fold diluted in TES) was then incubated in the wells for 90 min followed by two washes with TES and TES/Tween-20. *p*-Nitrophenyl phosphate (150 μL, 1 mg/mL) in 50 mM sodium bicarbonate (pH 9.5) and 2 mM $MgCl_2$ was then incubated in the wells for 1 h. Finally, 100 μL of 4 N NaOH was added to stop each reaction. The absorbance at 405 nm was read either in a 5-mm cuvette or directly in the wells by using a Dynatech MR580 ELISA plate reader.

Quantitative immunoprecipitin assays were performed by adding increasing amounts of Br-poly[r(C-G)] (49% br^{8}G, 43% br^{5}C) to 200 μL of purified IgG in a total volume of 250 μL of TES buffer. Reactions were incubated at 37 °C for 2 h and then for 72 h at 4 °C. Precipitates were obtained by centrifuging for 30 min at 13K rpm, washed twice with 100 μL of TES, and assayed for protein by the method of Bradford (1976). The amount of protein depleted from the supernate was also determined in each case; these results agreed with the precipitate data. Molar protein concentrations were determined by assuming a molecular weight of 150 000/bivalent IgG molecule. The amount of polynucleotide (in base pairs) precipitated per IgG was corrected relative to the total input polynucleotide concentration for the 60% binding levels measured by filter-binding assay (Figure 1B) or by RIA with ^{32}P-labeled Br-poly[r(C-G)]. Similar subquantitative binding has also been noted with anti-Br-poly[d(C-G)] antibodies (Lafer et al., 1981; Möller et al., 1982; Zarling et al., 1984a).

Molecular Mechanics Calculations. Atomic coordinates for Z_I- and Z_{II}-DNA were taken from published X-ray crystallographic analyses (Wang et al., 1981). Data files for 12 bp of alternating r(br^{5}C-br^{8}G) were prepared from these initial coordinates by using the energy-minimization program AMBER (Weiner & Kollman, 1981; Weiner et al., 1984) on a VAX 11/785 computer. These structures were then transferred to a MicroVAX computer and energy minimized with a distance-dependent dielectric constant ($\epsilon = R_{ij}$) by using the "all atom" AMBER force field (Weiner et al., 1984). Energy minimizations were also carried out in the presence of counterions and H_2O by using the Cray XMP version of AMBER (Singh et al., 1985). Final structures were viewed on an Evans and Sutherland PS300 graphics terminal supported by INSIGHT software. A detailed description of the calculations may be obtained from the authors.

RESULTS

We obtained 500-MHz ^{1}H NMR spectra from Br-poly[r(C-G)] samples containing either (i) 32% br^{8}G and 26% br^{5}C or (ii) 49% br^{8}G and 43% br^{5}C, respectively (Figure S1A, and B, supplementary materials). The peaks in these spectra were previously assigned on the basis of one-dimensional nuclear Overhauser effect (NOE) studies (Hardin et al., 1987; Hall et al., 1984a). The NOE is a result of a distance-dependent dipolar coupling between proximal nuclei and has been successfully used in the determination of many nucleic acid structures in solution [e.g., Patel et al. (1987) and Suzuki et al. (1986)]. The most informative NOE in the spectrum of Br-poly[r(C-G)] occurs between the GH8 and GH1′ peaks since the distance between these atoms decreases from >4 Å in the A-form nucleotide to ~2.3 Å in the Z-form nucleotide. Thus, the GH8 ↔ GH1′ NOE (~30%) in the Z-form spectrum is of approximately the same magnitude as the NOE between the fixed-distance nuclei CH5 and CH6 [see Hardin et al. (1987)]. The two-dimensional NOESY contour spectrum for the more extensively brominated poly[r(C-G)] sample was obtained under conditions where spin-diffusion effects (higher order NOEs) are minimized (Figure S1C). Cross-peaks corresponding to GH8 ↔ GH1′ (7.79 ppm/5.83 ppm) and CH5 ↔ CH6 (4.98 ppm/7.13 ppm) NOEs are present. In contrast to the result obtained with the less modified Br-poly[r(C-G)] sample (Hardin et al., 1987), no A-form CH5 ↔ CH6 NOEs are present. The A-form GH8 and CH6 resonances were previously assigned to 7.42 and 7.59 ppm, respectively. The integral of the peak at 7.21 ppm correlates well with the degree of bromination at C5 and was thus assigned to br^{5}CH6 (Hardin et al., 1987). Integration of the peaks in the spectrum obtained from the more highly modified (Figure S1B) sample demonstrates that >80% of the polynucleotide is in the Z conformation. These data show that *unmodified* residues located adjacent to the site(s) of modification are stabilized in the Z-form. The same is true for the less modified polynucleotide (Hardin et al., 1987); however, in that case only ca. 50% of the unmodified residues are in the Z-form (Figure S1A).

Rabbits were inoculated with the two Br-poly[r(C-G)] samples described above in order to assess their immunogenicities. Rabbits A and B were injected with the less modified polynucleotide, while the more highly modified sample was used with rabbits C and D. Figure 1A shows the recognition of Br-poly[r(C-G)](Z-RNA) and poly[d(br^{5}C-G)](Z-DNA) by rabbit serum B2 antibodies as measured by RIA. The amount of polynucleotide that was precipitated was dependent on the amount of immune serum (Figure 1A). Antibodies in preimmune serum from rabbit B or other normal rabbit sera did not recognize these polynucleotides. Less than 3% of the polynucleotide was precipitated in the absence of primary immune serum or purified IgG. Poly[d(C-G)] (B-DNA), right-handed poly[d(A)·d(T)], A-RNAs including poly[r(C-G)] and poly[r(I-C)], all four types of homopolyribo-

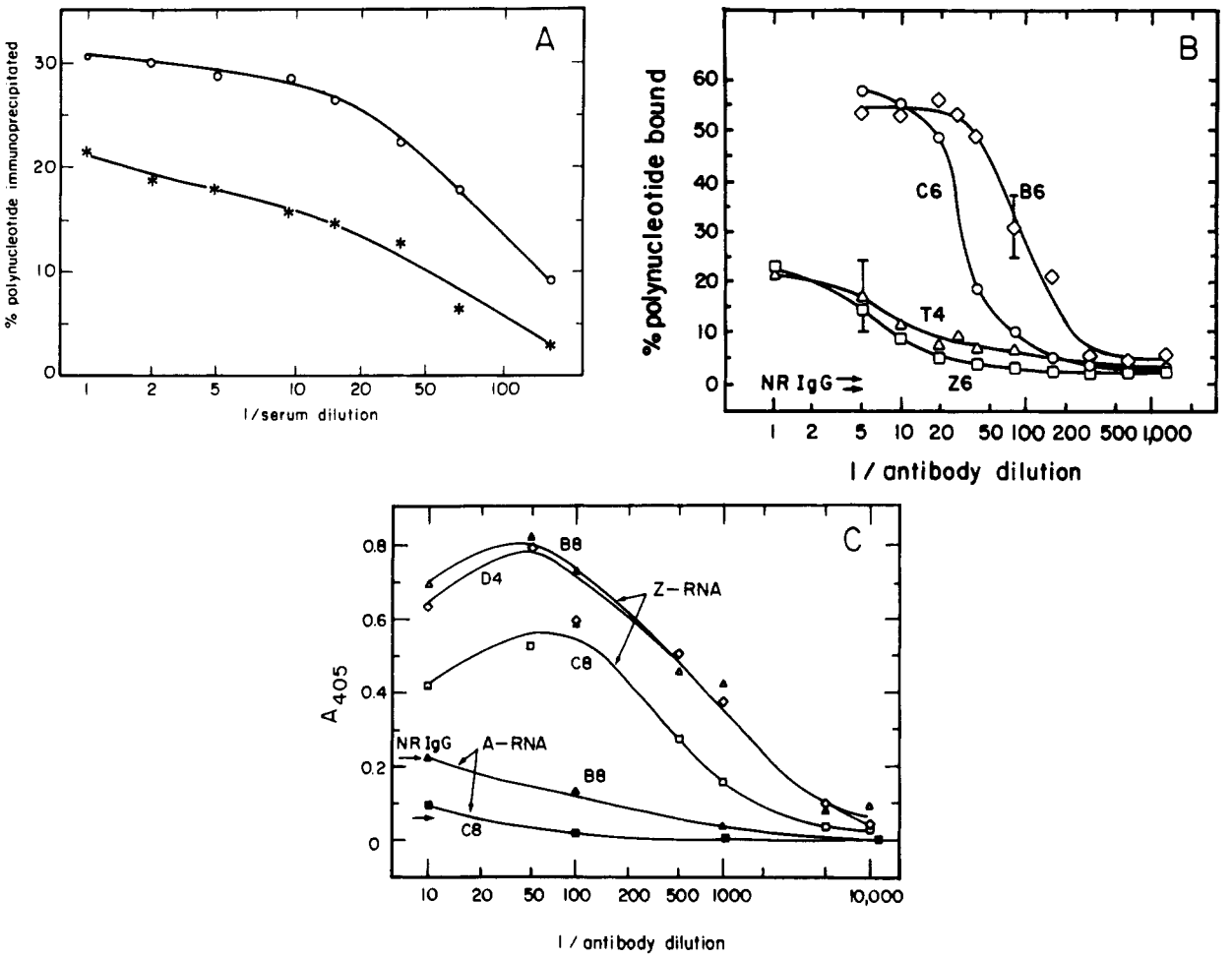

FIGURE 1: Binding of anti-Br-poly[r(C-G)] antibodies to ^{32}P-labeled Br-poly[r(C-G)] (O) and poly[d(br^{5}C-G)] (*). (A) Dilutions of serum B2 were tested by RIA in TES buffer using goat anti-rabbit IgG as second antibody (Hardin et al., 1987). Recognition of Br-poly[r(C-G)] (32% br^{8}G, 26% br^{5}C) was tested by using 5 μL of B2 serum and 2.4 μg of second antibody in a 25-μL reaction. Binding levels for A-RNAs, B-DNAs, and all four homopolyribonucleotides were <3% [see Hardin et al. (1987)]. (B) Second antibody independent nitrocellulose filter binding assay results showing the binding of purified rabbit anti-Br-poly[r(C-G)] (anti-Z-RNA) IgGs B6 (◇) and C6 (O), and anti-Br-poly[d(C-G)] (anti-Z-DNA) IgGs T4 (△) and Z6 (□) (Zarling et al., 1984a,b; Hardin et al., 1987) to [^{32}P]Br-poly[r(C-G)] (49% br^{8}G, 43% br^{5}C). Antibody concentration were adjusted to 2.5 mg of protein mL^{-1} = IgG/1 (assuming 1.4 A_{280} units = 1 mg of protein mL^{-1}) and normalized for specific anti-Z-RNA IgG content (see Figure 4). Assays contained 20 ng of polynucleotide (2.5 ng of ^{32}P-labeled and 17.5 ng of unlabeled polynucleotide). Background values [buffer →; normal rabbit (NR) IgG →] and the levels of recognition of radiolabeled A-RNA (poly[r(C-G)]) and B-DNA (poly[d(C-G)]) by B6 and C6 IgGs were <3%, as seen with anti-Br-poly[d(C-G)] IgGs T4 and Z6 in previous filter-binding experiments (Hardin et al., 1987). Error bars represent the average standard deviation in four determinations; the range was 0.7–4.1%. (C) ELISA results comparing the recognition of Br-poly[r(C-G)] (Z-RNA) and poly[r(C-G)] (A-RNA) by purified B8, C8, and D4 IgGs. Background levels were measured by incubating the wells with either antibody or nucleic acid and then adding normal rabbit (NR) IgG → or buffer (→) in the ELISA procedure.

nucleotides, and an *Escherichia coli* 16S/23S rRNA mixture were not recognized by B2 serum antibodies or the purified IgGs discussed below [also see Zarling et al. (1987)].

IgG fractions prepared from later immune sera were characterized more extensively by nitrocellulose filter binding assay and ELISA (Figure 1B,C). Figure 1B shows the relative degree of recognition of Br-poly[r(C-G)] by anti-Br-poly[r-(C-G)] IgGs B6 and C6 and the anti-Br-poly[d(C-G)] IgGs T4 and Z6. The anti-Z-RNA IgGs were purified from sera obtained from the peak regions of the titer versus time curves (Zarling et al., 1987). Results shown represent the averages of four determinations; standard deviations ranged from 0.7 to 4.1%. The data have been normalized for the amounts of specific anti-Z-RNA IgG in the preparations determined as discussed below. Comparing the relative inflection points in the binding curves shows that the anti-Z-RNA IgG that was elicited against the more highly modified polynucleotides (C6) binds with a lower affinity to Z-RNA than the IgG that was elicited against the less modified polynucleotides (B6). These results show that the anti-Z-RNA antibodies bind about 10-fold tighter to Br-poly[r(C-G)] than the anti-Z-DNA antibodies. Thus, the major covalent structural difference between Z-RNA and Z-DNA, the 2′-hydroxyl group, appears to be part of the anti-Z-RNA IgG determinant and partially inhibits anti-Z-DNA IgG recognition.

Figure 1C shows the recognition of Br-poly[r(C-G)] by anti-Z-RNA IgGs as determined by solid-phase ELISA. Relative to background values there was no significant binding of any of these antibodies to A-RNA. Thus, by these criteria (Figure 1A–C) the Z-form of Br-poly[r(C-G)] is immunogenic and produces specific anti-Z-RNA antibodies in rabbits. In addition, rabbits do not normally have significant amounts of endogenous circulating anti-Z-RNA antibodies. Similar results were also obtained with Balb/C mice [see also Zarling et al. (1987)].

The degree of recognition of several different forms of Z-DNA by the anti-Z-RNA IgGs B6 and C6 is shown in Figure 2. B6 IgG recognizes the Z-forms of poly[d(br^{5}C-G)] in 220 mM NaCl, poly[d(C-G)] in 4 M NaCl, and poly[d-(m^{5}C-G)] in 1.5 M NaCl. However, C6 IgG does not rec-

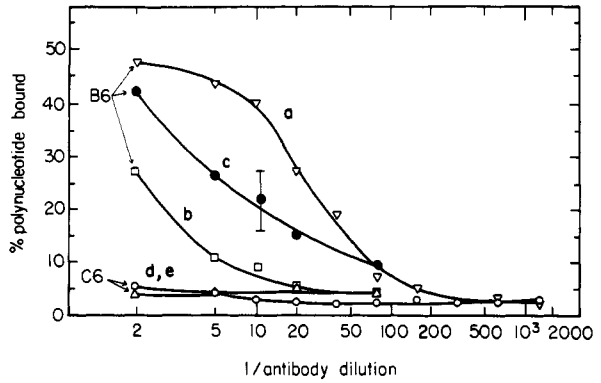

FIGURE 2: Binding of B6 and C6 IgGs to Z-DNA determined by nitrocellulose filter binding assay. Binding curves are labeled as follows: B6 IgG binding to (a) poly[d(br^{5}C-G)] (220 mM NaCl), (b) poly[d(m^{5}C-G)] (1.5 M NaCl), and (c) poly[d(C-G)] (4 M NaCl); C6 IgG binding to (d) poly[d(br^{5}C-G)] (220 mM NaCl), (e) poly[d(m^{5}C-G)] (1.5 M NaCl), and (f) poly[d(C-G)] (4 M NaCl) with *C6* IgG. Dilution curves are scaled to 0.5 mg of protein mL^{-1} = IgG/5. Assays contained 20 ng of polynucleotide (2.5 ng of ^{32}P-labeled polynucleotide and 17.5 ng of unlabeled polynucleotide). Error bars represent the average standard deviation in four determinations. The range was similar to that stated in Figure 1B.

ognize any of these forms of Z-DNA. A similar lack of recognition was also obtained with D4 IgG. In addition, C6 and D6 and D4 IgGs do not recognize Z-DNA stabilized by negative superhelical stress in M13 DNA, while B6 IgG does (Zarling et al., 1987). Thus, the degree of cross-reactivity with Z-DNA is correlated with the extent of modification of the Z-RNA. More extensive bromination of the poly[r(C-G)] immunogens used in rabbits C and D produced IgGs that are specific for Z-RNA, yet do not recognize Z-DNA. Similar results were also obtained with mouse polyclonal anti-Br-poly[r(C-G)] antibodies. The different extents of recognition of the different forms of Z-DNA by B6 IgG are correlated with the C5 substituent as follows: Br > H > CH_3. This order was also obtained with these polynucleotides in 4 M NaCl buffer, indicating that this is not due to different ion conditions. The higher affinity for the brominated polynucleotide is not surprising since the RNA antigen was partially brominated at C5. These results imply that the C5 bromine atom is part of the epitope. Similar increased affinities of proteins for brominated polynucleotides have also been observed with the *lac* repressor (Lin & Riggs, 1972) and autoimmune monoclonal FABs (Lee et al., 1982).

Figure 3 shows competition radioimmunoassay results that verify the specific recognition of Z-DNA by the anti-Z-RNA IgG B6. Unlabeled Br-poly[r(C-G)] competes effectively with [^{32}P]poly[d(br^{5}C-G)] binding to B6 IgG (Figure 3A). These results are compared with the homologous competition reactions containing Z-DNA and Z-RNA, respectively. Note that poly[d(br^{5}C-G)] does not effectively compete with Z-RNA in the competitor concentration range tested here (≤2 μg of total polynucleotide). This is expected since the results in Figure 1B show that the affinity of B6 IgG for Z-DNA is more than 10-fold lower than for Z-RNA. Similar results were obtained in the inverse situation with the anti-Z-DNA [anti-Br-poly[d(C-G)]] IgG T4 and Br-poly[r(C-G)] (Hardin et al., 1987). Figure 3B shows that Br-poly[r(C-G)] competes effectively with unmodified Z-form [^{32}P]poly[d(C-G)] in 4 M NaCl buffer. Since Z-RNA competes with unmodified Z-DNA, the same populations in the polyclonal IgG preparation bind to both types of polynucleotide. This demonstrates that Br-poly[r(C-G)] has Z-DNA-like antigenic epitopes.

The binding stoichiometries in the complexes formed between Br-poly[r(C-G)] and anti-Z-RNA IgGs were determined by quantitative immunoprecipitin assay (Figure 4). Stoichiometries at the equivalence point (i.e., where the slopes in the binding curves change from positive to negative) correspond to 10 ± 4 bp/bivalent IgG molecule. Thus, the maximum epitope size, per FAB unit, is 5 ± 2 bp. Similar results were obtained with B8, C8 and D4 IgGs (Figure 4).

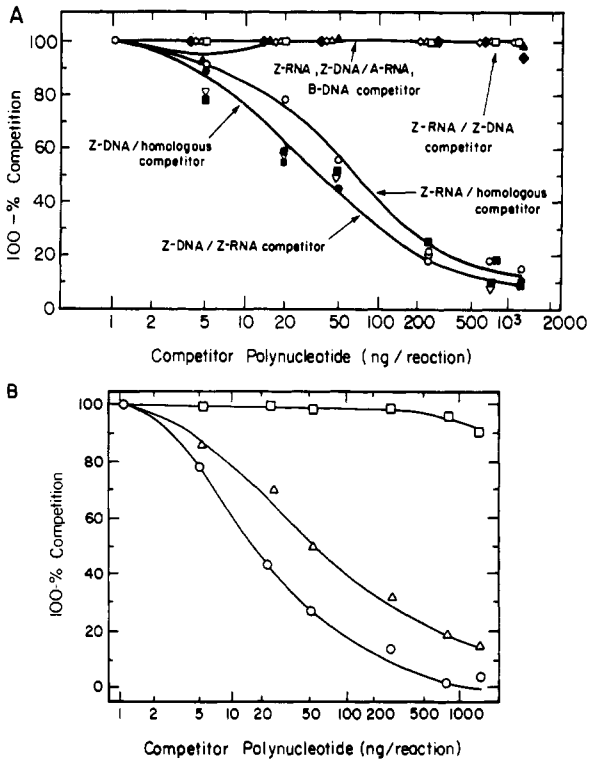

FIGURE 3: Specificity in the binding of anti-Br-poly[r(C-G)] IgG B6 to Z-DNA. (A) Competition filter-binding assay results obtained by preincubating 2.5 ng ^{32}P of ^{32}P-labeled polynucleotide and 17.5 ng of unlabeled polynucleotide with the specified amounts of unlabeled competitor in TES buffer at 37 °C prior to adding B6 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. (B) Results obtained by preincubating labeled poly[d(C-G) with unlabeled, unmodified poly[d(C-G)] (Z-DNA) competitor (Δ), Br-poly[r(C-G)] (Z-RNA) competitor (○), and poly[r(C-G)] (A-RNA) (□) in 4 M NaCl TES buffer prior to assay by RIA.

Figure 5A shows the CD spectra for the Br-poly[r(C-G)] samples used to elicit anti-Br-poly[r(C-G)] IgGs in rabbits B and C (D), respectively. The spectrum obtained with the less modified antigen (B6) has nearly symmetrical positive and negative CD intensities at 260 and 295 nm, respectively. In contrast, the Br-poly[r(C-G)] antigen used to elicit the Z-RNA-specific IgGs in rabbits C and D (C6, D4) has a lower intensity at 260 nm and a well-resolved shoulder at 282 nm. This shoulder is seen in the spectrum of Z_R-RNA [see Hardin et al. (1987)] but not in that of Z-DNA (Figure 5B). The structural origin of the spectral differences is not understood; however, Raman spectroscopy provides complementary evidence for different structures in Z-RNA and Z-DNA and suggests that differences in glycosidic torsion angles may be involved (Tinoco et al., 1986; Hardin et al., 1987). This result suggests that the specificity of C6 (C8) and D4 IgGs in binding to Z-RNA relative to Z-DNA may be in part due to the spectroscopically detected structural differences between the antigens.

Symmetric CD bands at 260 and 285 nm are also seen in the spectrum of the Br-poly[d(C-G)] sample used to elicit the anti-Z-DNA IgG T4 (Figure 5B). Note that the 282-nm shoulder is not present in either of these spectra. As seen with

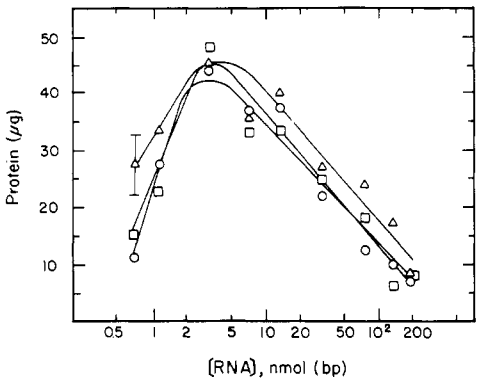

FIGURE 4: Binding stoichiometries in the complexes formed between Br-poly[r(C-G)] and B8 (○), C8 (□), and D4 (△) IgGs determined by quantitative immunoprecipitin assay. Protein concentrations were determined by Bradford assay of the precipitates and diluted IgG-depleted supernates. Stoichiometries at the equivalence point (45 μg of IgG, 3 nmol of bp) correspond to 10 ± 4 bp/bivalent IgG. These results show that ca. 9% of the total protein in the polyclonal preparation is specific for Br-poly[r(C-G)].

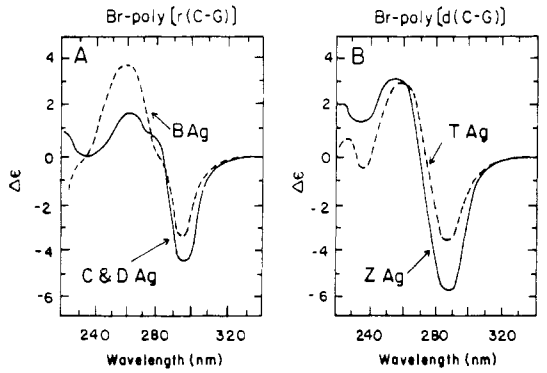

FIGURE 5: (A) Circular dichroism spectra of Br-poly[r(C-G)] antigens used to produce anti-Br-poly[r(C-G)] IgGs in rabbits B–D in 10 mM sodium phosphate (pH 7), 100 mM NaCl, and 1 mM EDTA. (B) CD spectra of the antigens used to elicit anti-Br-poly[d(C-G)] IgGs T4 and Z6 in 20 mM Tris-HCl (pH 7.2), 10 mM NaCl, and 0.1 mM EDTA (Zarling et al., 1984a).

the antigens used in rabbits C and D, more extensive modification produces CD spectra with deeper negative CD bands at 285 nm. This is seen in Figure 5B for the Br-poly[d(C-G)] antigen used to elicit Z6 IgG. In our previous study of the recognition of Br-poly[r(C-G)] by anti-Z-DNA IgGs (Hardin et al., 1987), we found a lower affinity for Z-RNA with Z6 IgG than with T4 IgG. In contrast to the present case, the decreased affinity for Z-RNA was not nearly as dramatic as that seen with C6 and D4 IgGs in binding to Z-DNA.

Figure 6 shows the inhibitory effects of sodium phosphate on the recognition of Br-poly[r(C-G)] by both anti-Z-RNA and anti-Z-DNA antibodies. For comparison, the results obtained with anti-Br-poly[d(C-G)] IgGs T4 and Z6 are reproduced from our previous study (Hardin et al., 1987). Similar results were obtained with anti-Z-RNA and anti-Z-DNA IgGs (Figure 6). Increasing the sodium phosphate concentration above 100 mM in solutions containing 220 mM NaCl reduces the binding levels to <70% of those obtained in the absence of the phosphate anion. The effects of NaCl on the binding levels were similar to those seen with T4 and Z6 IgGs (Hardin et al., 1987); at 1 M NaCl the binding levels were reduced to about 85% of those obtained in the 100 mM NaCl range (Figure 6). Direct comparison with the phosphate inhibition data at 1 M sodium phosphate (Figure 6) shows that the binding levels are reduced to about 50% of those obtained in the absence of the anion. As noted previously with anti-

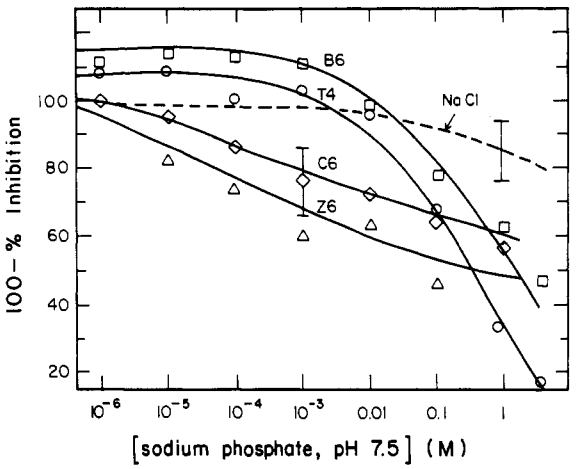

FIGURE 6: Effects of phosphate ions on the binding of anti-Br-poly[r(C-G)] IgGs B6 (□) and C6 (◇) and anti-Br-poly[d(C-G)] IgGs T4 (○) and Z6 (△) to Z-RNA (49% br^{8}G, 43% br^{5}C) in TES buffer. ^{32}P-labeled (2.5 ng) and (17.5 ng) unlabeled polynucleotide were incubated with 5 μL of antibody solution (0.1 mg of anti-Br-poly-[r(C-G)] IgG mL^{-1}; 0.5 mg of anti-Z-DNA IgG mL^{-1}) at the specified sodium phosphate concentrations. Filter-binding assays were performed as described under Methods. Error bars indicate the average standard deviation in four determinations. The range was as stated in Figure 2. All reactions contained 220 mM NaCl. Averaged NaCl inhibition results were corrected for the difference in ionic strength relative to sodium phosphate. A nonlinear least-squares fit of the NaCl inhibition results (---) and the average standard deviation (indicated by error bars) allow direct comparison of the effects of phosphate and chloride ions on the antibody-binding reactions.

Z-DNA IgGs (Hardin et al., 1987), the general dependence on ionic strength and the specific inhibition by phosphate anion strongly suggest the recognition of a phosphodiester backbone determinant common to the Z-forms of RNA and DNA. In addition, the antibodies that were elicited against the more highly modified polynucleotides, C6 and Z6, are sensitive to much lower phosphate concentration. These data suggest that the binding of C6 and Z6 IgGs to Br-poly[r(C-G)] is more dependent on the recognition of phosphodiester determinants than the binding of B6 and T4 IgG.

Discussion

In this paper we complement and extend our previous observations (Hardin et al., 1987) by demonstrating that Z-RNA and Z-DNA are structurally similar by the criterion of immunochemical cross-reactivity. Polyclonal anti-Z-DNA IgGs T4 and Z6 specifically recognize Z-RNA in the form of Br-poly[r(C-G)] (Hardin et al., 1987), while polyclonal anti-Z-RNA IgGs from rabbit B specifically recognize the Z-DNA conformation (Figures 2 and 3). In each case the affinity for the heterologous polynucleotide is reduced by at least 10-fold relative to that of the homologous (antigenic) polynucleotide. Thus, Z-DNA can be immunochemically differentiated from Z-RNA by its relative affinities to both anti-Z-RNA and anti-Z-DNA antibodies. This is demonstrated most dramatically with anti-Z-RNA IgGs from rabbits C and D. These antibodies do not recognize Z-DNA stabilized by C5 bromination, C5 methylation (in 1.5 M NaCl), 4 M NaCl (Figure 2), or negative superhelical stress (Zarling et al., 1987). These antibodies are thus useful probes for Z-RNA under conditions where Z-DNA is also present. This property has been used to specifically detect Z-RNA in the cytoplasm of fixed protozoan cells (Zarling et al., 1987).

Several properties of the epitope(s) recognized by these antibodies were delineated by these studies. The attenuated

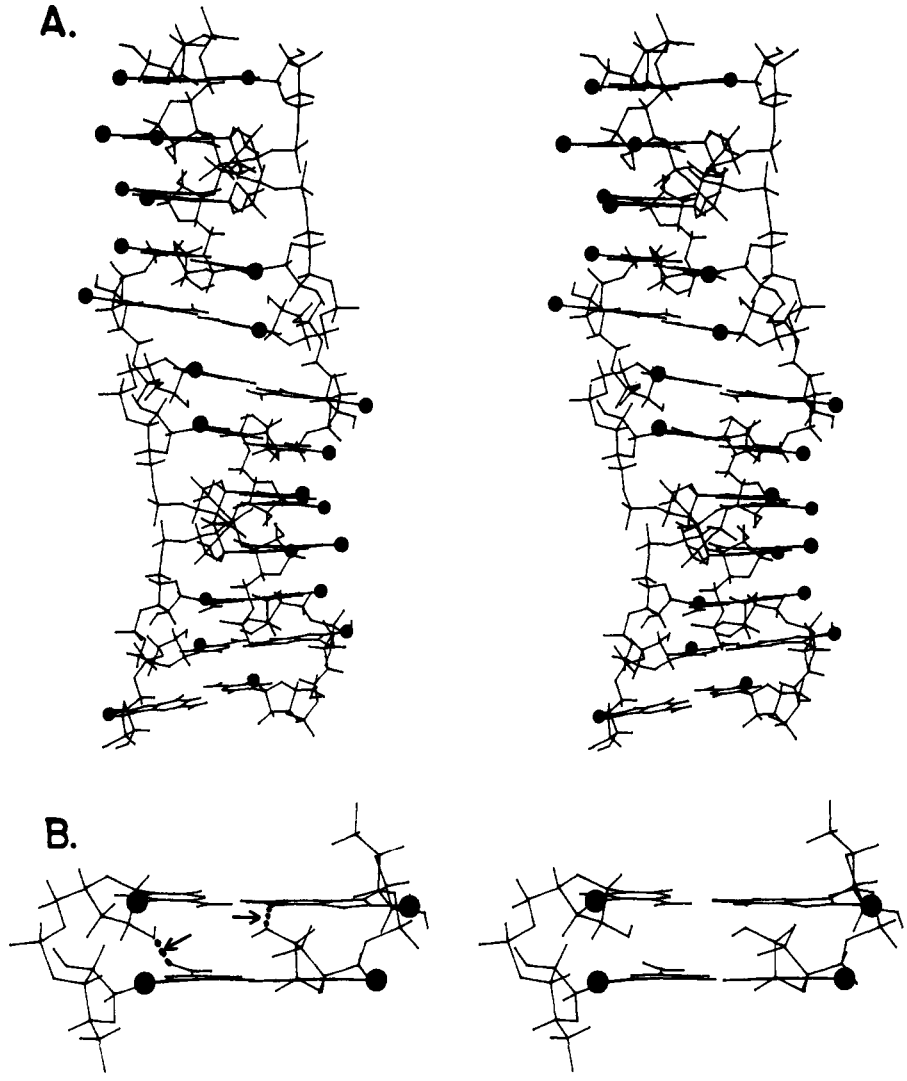

FIGURE 7: (A) Structure of Z_{II}-form poly[r(br^{5}C-br^{8}G)] generated by energy minimization using the molecular mechanics program AMBER (Weiner et al., 1984; Weiner & Kollman, 1981) starting with the X-ray coordinates of Z_{II}-DNA. Bromine atoms are represented as blackened spheres; structures are presented as stereo pairs. Panel B shows a dinucleotide section from the structure shown in (A) in order to emphasize the guanine NH_2 hydrogen–cytidine OH oxygen hydrogen-bonding interaction (indicated by the arrows).

cross-reactivities with Z-DNA found with B6 and C6(D4) IgGs strongly imply that the 2′-hydroxyl group is a determinant in the recognition by both types of antibody. Phosphate-inhibition studies (Figure 6) show that the anion specifically interferes with the recognition of Z-RNA by anti-Z-RNA and anti-Z-DNA antibodies. These results imply that the phosphate groups on the phosphodiester backbone are recognized determinants and are in qualitatively similar conformations in both Z-RNA and Z-DNA. Raman spectroscopy and ^{31}P NMR results support the presence of a Z-form phosphodiester conformation with two distinct phosphate environments in Br-poly[r(C-G)] in low-salt buffer (Hardin et al., 1987) and in poly[r(C-G)] in 6 M $NaClO_4$ or NaBr buffers (Cruz et al., 1986).

B6 IgG specifically recognizes unmodified Z-form poly[d(C-G)] (Figures 2 and 3), demonstrating that bromine is not required for recognition. However, it was also shown that the affinity for the Z-forms of poly[d(x^{5}C-G)] (x = Br > methyl > H) correlates with the electronegativity of the cytosine C5 substituent (Figure 2). This result was also obtained with all three polynucleotides in 4 M NaCl buffer, confirming that it is not due to different ionic conditions. Thus, the cytosine C5 substituents are involved in anti-Z-RNA IgG recognition. Since the G8 bromine atom is probably exposed in the antigen (see below), it is likely that it is also recognized by the antibodies. The presence of these exposed antigens is probably responsible for the potent immunogenicity of the polynucleotides.

Quantitative immunoprecipitin assay results show that these antibodies bind 10 ± 4 bp or 5 ± 2 bp/FAB (Figure 5). These results agree with those of Runkel and Nordheim (1986) obtained with monoclonal anti-Z-DNA IgGs using a diethyl pyrocarbonate footprinting technique. Two different monoclonal anti-Br-poly[d(C-G)] IgGs were shown to cover about 6 bp/FAB and, on the basis of protection patterns, must have approximate binding sizes of 18 × 20 Å. Malfoy et al. (1982) found a binding-site size of 4 bp/FAB on the basis of quantitative immunoprecipitin studies with anti-Z-DNA [chloro(diethylenetriamine)platinum(II)-modified poly[d(C-G)]] polyclonal antibodies.

Figures 7 shows the structure of Br-poly[r(C-G)] in the Z_{II}-form calculated by energy minimizing a 12-bp segment of alternating (br^{5}C-br^{8}G) with the molecular mechanics program AMBER using the coordinates of Z_{II}-DNA obtained from the crystal structure (Wang et al., 1981) as the initial Z-RNA geometries. The structure resembles the parent DNA conformations; however, there are features that are unique to the RNA. For example, as noted by Rao and Kollman (1986) with r(CGCGCG)$_2$ and r(Cm^{8}GCm^{8}GCm^{8}G)$_2$, the br^{5}C(C) 2′-hydroxyl oxygen atoms are hydrogen bonded to the N2

hydrogen atoms of 5′-adjacent br^{8}G residues (Figure 7). The ribose and guanine moieties reorient to accommodate the hydrogen bond. This interaction is also evident in the crystal structure of r(Cbr^{8}GCbr^{8}G) (Nakamura et al., 1985). This altered guanosine conformation may be responsible for the different guanine imidazole ring-breathing mode frequencies in the Raman spectra of Z-RNA and Z-DNA (Tinoco et al., 1986; Hardin et al., 1987). A feature noted only in the energy-minimized Z_{II}-form poly[r(br^{5}C-br^{8}G)] structure involves a hydrogen bond between the br^{8}G 2′-hydroxyl hydrogen and 3′-adjacent phosphate oxygen atoms. This interaction was not seen in the crystal structure. Rao and Kollman (1986) advise caution in interpreting intra-RNA hydrogen bonding without the inclusion of explicit H_2O molecules. The hydrogen-bonding interactions between the C2′ OH and the GNH_2 and G2′ OH and the CpG phosphate were also present when the Z_{II}-form of poly[r(br^{5}C-br^{8}G)] was minimized in the presence of H_2O and monovalent cations. An interesting feature noted in the crystal structure of r(Cbr^{8}GCbr^{8}G)$_2$ was the presence of a Z_{II}-form GpC conformation in which the phosphate is rotated away from the narrow deep groove region. This is in contrast with the Z-DNA crystal sturctures where Z_I-form phosphodiester conformations predominate [see Drew and Dickerson (1981) and Westhof et al. (1986)]. Note that the bromine atoms in the Z-form Br-poly[r(C-G)] structures are clustered along the interface between the convex hydrophobic surface and the phosphodiester backbone (Figure 7).

A review of the evidence presented in this paper indicates that the antigenic epitope(s) recognized by anti-Z-RNA antibodies are multivalent and are likely to be comprised of one or more of the following components: (1) the 2′-hydroxyl(s); (2) the zigzagged phosphodiester backbone; (3) exposed bases in the vicinity of the C5 and G8 bromine atoms; and (4) similar extended Z-form conformational features in RNA and DNA with anti-Z-RNA IgG epitopes of approximately 5 bp in length.

Geysen et al. (1987) outlined the following characteristics for the most frequently recognized epitopes in protein antigens: (1) high local mobility; (2) convex surface shape; and (3) negative electrostatic potential. Similar factors are also likely to apply to Z-RNA and Z-DNA antigens. Although crystallographic temperature factor data are not available for Z-RNA, the relative mobilities are likely to be similar to those of Z-DNA (Drew & Dickerson, 1981; Westhof et al., 1986): GpC phosphate > deoxyriboses(riboses) > bases. The negative electrostatic potential in Z-DNA is strongest at the phosphates (especially CpG), while the base potentials are ordered as follows: N7(G) > O6(G) > O2(C) $\simeq$ N3(G) > C5(C) > N2(G) > N4(C) > C8(G); the accessibilities are N7(G) > O6(G) > C8(G) > O2(C) > C5(C), N4(C), N3(C) (Pullman & Pullman, 1981). The bromine substituents at G8 and C5 in the antigens will clearly increase the negative potentials at these positions. While numerically smaller than at the phosphates or bases, a reasonable negative potential also exists at the ribose oxygen atoms. Thus, criteria 1 and 3, above, implicate the phosphates, the bases in the vicinity of N7(G), O6(G), Br8(G), and Br5(C), and the riboses as likely antigenic determinants. The O2(C), N3(G), and N2(G) atoms, which are located in the deep minor groove of the Z-form polynucleotide, are probably relatively inaccessible to the antibodies. In contrast, the previously mentioned substituents are located at the interface between two convex surfaces (criterion 2, above).

Finally, we address a possible reason for the different specificities of antibodies obtained from rabbits B and C(D). Phosphate inhibition is much more pronounced with C6 and D4 IgGs compared to that with B6 IgG (Figure 6, data not shown for D4 IgG). Significantly, these IgGs are considerably less cross-reactive with Z-DNA than IgGs from rabbit B (Figure 1B). These results implicate phosphate and ribose moieties as being more important as determinants for recognition by C6 (D4) IgGs than by B6 IgG. Thus, although quantitative immunoprecipitin results show that the epitope sizes are similar for all three IgGs (Figure 4), the epitopes differ in that while C6 and D4 IgGs depend more on recognition of phosphodiester backbone determinants, B6 IgG recognition probably depends more on recognition of both the phosphodiester backbone and hydrophobic convex surface determinants. Thus, increased bromination of the RNA antigen apparently skews the antibody recognition toward phosphodiester (especially ribose) determinants. Increased bromination of Z-DNA also increases the antigenicity of phosphodiester backbone determinants (Möller et al., 1982; Zarling et al., 1984a,b), suggesting that this may be general phenomenon.

ACKNOWLEDGMENTS

We thank John Peitso and Barbara Dengler for assistance in synthesizing poly[r(C-G)], Connie Calhoun for technical assistance, and Drs. U. Chandra Singh, Shashidhar Rao, and Steve Holbrook for helpful advice regarding the use of AMBER and INSIGHT. Finally, we thank Professor Peter Kollman for the latest version of AMBER.

SUPPLEMENTARY MATERIAL AVAILABLE

^{1}H NMR spectra of Br-poly[r(C-G)] (2 pages). Ordering information is given on any current masthead page.

Registry No. Br-poly[r(C-G)], 114031-38-8; poly[r(C-G)], 49846-05-1.

REFERENCES

Bodenhausen, G., Kogler, H., & Ernst, R. R. (1984) *J. Magn. Reson. 58*, 370–388.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cruz, P., Hall, K., Puglisi, J., Davis, P., Hardin, C. C., Trulson, M. O., Mathies, R., Tinoco, I., Jr., Johnson, W. C., Jr., & Neilson, T. (1986) in *Proceedings of the Fourth Symposium in Biomolecular Stereodynamics III*, pp 179–200, Adenine, New York.

Drew, H. R., & Dickerson, R. E. (1981) *J. Mol. Biol. 152*, 723–736.

Geysen, H. M., Tainer, J. A., Rodda, S. J., Mason, T. J., Alexander, H., Getzoff, E. D., & Lerner, R. A. (1987) *Science (Washington, D.C.) 235*, 1184–1190.

Gray, D. M., Liu, J.-J., Ratliff, R. L., & Allen, F. S. (1981) *Biopolymers 20*, 1337–1382.

Guigues, M., & Leng, M. (1976) *Eur. J. Biochem. 69*, 615–624.

Hall, K., Cruz, P., Tinoco, I., Jr., Jovin, T. M., & van de Sande, J. H. (1984a) *Nature (London) 311*, 584–586.

Hall, K., Cruz, P., & Chamberlin, M. J. (1984b) *Arch. Biochem. Biophys. 236*, 47–51.

Hardin, C. C., Zarling, D. A., Puglisi, J. D., Trulson, M. O., Davis, P. W., & Tinoco, I., Jr. (1987) *Biochemistry 26*, 5191–5199.

Johnston, M. I., & Stollar, B. D. (1978) *Biochemistry 17*, 1959–1964.

Kmiec, E. B., Angelides, K. J., & Holloman, W. K. (1985)

Cell (Cambridge, Mass.) 40, 139–145.
Lafer, E. M., Möller, A., Nordheim, A., Stollar, B. D., & Rich, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3546–3550.
Lafer, E. M., Sousa, R., Ali, R., Rich, A., & Stollar, B. D. (1986) *J. Biol. Chem. 261*, 6438–6443.
Lee, J. S., Dombroski, D. F., & Mosmann, T. R. (1982) *Biochemistry 21*, 4940–4945.
Lin, S.-Y., & Riggs, A. D. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2574–2576.
Malfoy, B., Rousseau, N., & Leng, M. (1982) *Biochemistry 21*, 5463–5467.
Möller, A., Gabriels, J. E., Lafer, E. M., Nordheim, A., Rich, A., & Stollar, B. D. (1982) *J. Biol. Chem. 257*, 12081–12085.
Nakamura, Y., Fujii, S., Urata, H., Uesugi, S., Ikehara, M., & Tomita, K. (1985) *Nucleic Acids Symp. Ser. No. 16*, 29–32.
Patel, D. J., Shapiro, L., & Hare, D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 423–453.
Peck, L. J., Wang, J. C., Nordheim, A., & Rich, A. (1986) *J. Mol. Biol. 190*, 125–127.
Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396.
Pullman, A., & Pullman, B. (1981) *Q. Rev. Biophys. 14*, 289–380.
Rao, S. N., & Kollman, P. A. (1986) *J. Am. Chem. Soc. 108*, 3048–3053.
Rich, A., Nordheim, A., & Wang, A. H.-J. (1984) *Annu. Rev. Biochem. 53*, 791–846.
Runkel, L., & Nordheim, A. (1986) *J. Mol. Biol. 189*, 487–501.
Sage, E., Fuchs, R. P. P., & Leng, M. (1979) *Biochemistry 18*, 1328–1332.
Singh, U. C., Weiner, P. K., Caldwell, J. W., & Kollman, P. A. (1985) AMBER (UCSF), Version 3.0, Department of Pharmaceutical Chemistry, University of California, San Francisco.
Stollar, B. D. (1986) *CRC Crit. Rev. Biochem. 20*, 1–36.
Sundquist, W. I., Lippard, S. J., & Stollar, B. D. (1986) *Biochemistry 25*, 1520–1524.
Suzuki, E., Pattabiraman, N., Zon, G., & James, T. L. (1986) *Biochemistry 25*, 6854–6865.
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.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., von Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1981) *Science (Washington, D.C.) 211*, 171–176.
Weiner, P. K., & Kollman, P. A. (1981) *J. Comput. Chem. 2*, 287–202.
Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Ghio, C., Alagona, G., Profeta, S., Jr., & Weiner, P. (1984) *J. Am. Chem. Soc. 106*, 765–783.
Westhof, E., Chevrier, B., Gallion, S. L., Weiner, P. K., & Levy, R. M. (1986) *J. Mol. Biol. 190*, 699–712.
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.
Zarling, D. A., Calhoun, C. J., Hardin, C. C., & Zarling, A. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6117–6121.