Enzyme-linked immunosorbent assays for Z-DNA

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Dot blot and transblot enzyme-linked immunosorbent assays (e.l.i.s.a.) are described which provide sensitive non-radioactive methods for screening Z-DNA-specific antisera and for detecting Z-DNA in polydeoxyribonucleotides and supercoiled plasmids. In the alkaline phosphatase dot blot e.l.i.s.a., Z-DNA, Br-poly(dG-dC)·poly(dG-dC), or B-DNA, poly(dG-dC)·poly(dG-dC), poly(dA-dT)·poly(dA-dT), Br-poly(dI-dC)·poly(dI-dC), or salmon sperm DNA were spotted onto nitrocellulose discs and baked. The e.l.i.s.a. was conducted in 48-well culture dishes at 37 °C using a rabbit polyclonal antiserum developed against Br-poly(dG-dC)·poly(dG-dC), an alkaline phosphatase-conjugated second antibody, and *p*-nitrophenol as the substrate. Under conditions where antibody concentrations were not limiting, alkaline phosphatase activity was linear for 2 h. Dot blot e.l.i.s.a. conditions are described which allow quantification of Z-DNA [Br-poly(dG-dC)·poly(dG-dC)] within the range 5–250 ng. Dot blot and transblot horseradish peroxidase e.l.i.s.a. are described that detect Z-DNA within supercoiled plasmid DNAs immobilized on diazophenylthioether (DPT) paper. In the transblot e.l.i.s.a., plasmid pUC8 derivatives containing 16, 24, or 32 residues of Z-DNA were electrophoresed in agarose gels and electrophoretically transferred to DPT paper. Z-DNA-antibody complexes were detected by the horseradish peroxidase-catalysed conversion of 4-chloro-1-naphthol to a coloured product that was covalently bound to the DPT paper. Z-DNA antibody reactivity was specific for supercoiled Z-DNA containing plasmids after removal of the antibodies cross-reactive with B-DNA by absorption onto native DNA-cellulose. The transblot e.l.i.s.a. was sensitive enough to detect 16 base pairs of alternating G-C residues in 100 ng of pUC8 DNA.

INTRODUCTION

Z-DNA is a left-handed form of the DNA helix that exists in dynamic equilibrium with right-handed helical conformations, usually B-DNA (reviewed by Rich *et al.*, 1984). Experiments with synthetic DNA polymers have shown that Z-DNA forms preferentially in regions of alternating purine-pyrimidine sequences, with the order of transition from B to Z occurring most readily in (dG-dC)·(dG-dC) > (dC-dA)·(dT-dG) > (dA-dT)·(dA-dT). Methylation of the C-5 position of cytosine and bromination of G and C residues (primarily at the C-8 position of guanine and the C-5 position of cytosine) stabilize poly(dG-dC)·poly(dG-dC) in the Z-conformation at physiological ionic strength (Behe & Felsenfeld, 1981; Moller *et al.*, 1984). The Z-DNA helix stabilized by bromination is a strong immunogen and has been used by several investigators to elicit antibodies that recognize and bind specifically to Z-DNA (Lafer *et al.*, 1981; Moller *et al.*, 1982).

The presence of widely dispersed alternating purine-pyrimidine sequences throughout eukaryotic and prokaryotic DNA and the ability of DNA in both eukaryotic and prokaryotic genomes to undergo negative supercoiling *in vivo* suggests that Z-DNA may have some biological significance (Hamada *et al.*, 1982, 1984; Lilley, 1984; Hoheisel & Pohl, 1987). Native plasmid negative superhelical density stabilizes the Z-conformation of alternating purine-pyrimidine DNA sequences (Nordheim *et al.*, 1982) and a variety of techniques have been

used to assay Z-DNA formation in nucleotide sequences which have been cloned into supercoiled plasmids. These include radioactive antibody binding techniques (Lafer *et al.*, 1981; Moller *et al.*, 1982; Nordheim *et al.*, 1981), two-dimensional gel electrophoresis of plasmid topoisomers (Peck & Wang, 1983), chemical reactivity with diethylpyrocarbonate (Herr, 1985) or tris(4,7-phenyl-1,10-phenanthroline)cobalt (III) (Barton & Raphael, 1985), chemical footprinting of antibody binding sites (Runkel & Nordheim, 1986), 4,5',8-trimethylpsoralen cross-linking kinetics (Sinden & Kochel, 1987), and the topoisomer gel retardation assay (Nordheim & Meese, 1988). Two e.l.i.s.a. approaches to Z-DNA detection are described in this report. These simple and sensitive non-radioactive methods discriminate between the B- and Z-DNA conformation, and will facilitate rapid screening of anti-DNA sera and DNA sequences for Z-DNA formation.

EXPERIMENTAL

Production and purification of antisera

Poly(dG-dC)·poly(dG-dC) ($s_{20,w}$ 8.9; Pharmacia, Piscataway, NJ, U.S.A.) was brominated to stabilize the Z-conformation by using the method of Moller *et al.* (1984). The B-to-Z transition was monitored spectrophotometrically by calculating the A_{295}/A_{260} ratio in aliquots of the reaction mixture. The B and Z conformations of poly(dG-dC)·poly(dG-dC) exhibited an $A_{295}/$

Abbreviations used: e.l.i.s.a., enzyme-linked immunosorbent assay(s); DPT, diazophenylthioether; BSA, bovine serum albumin; PBS, phosphate-buffered saline (0.14 M-NaCl, 2.6 mM-KCl, 4.2 mM-NaH₂PO₄, 1 mM-KH₂PO₄, pH 7.4); TBS, Tris-buffered saline (20 mM-Tris/HCl, pH 7.5, containing 0.5 M-NaCl).

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A_{260} ratio of approx. 0.12 and 0.36, respectively. The Z-DNA was divided into portions and stored at -70°C .

Two New Zealand White rabbits (Green Meadows, Murrysville, PA, U.S.A.) were inoculated with $50\text{ }\mu\text{g}$ of brominated (Br)-poly(dG-dC)·poly(dG-dC) complexed with $75\text{ }\mu\text{g}$ of methylated BSA (bovine serum albumin) (Sigma) in Freund's complete adjuvant (Cappel, West Chester, PA, U.S.A.) at multiple intradermal injection sites. Booster shots of Br-poly(dG-dC)·poly(dG-dC) were administered in Freund's incomplete adjuvant on weeks 2, 3, 5, 7 and 9. Serum samples were taken on alternate weeks to monitor the development of antibodies to Br-poly(dG-dC)·poly(dG-dC). Rabbits were killed on week 14 and blood collected by cardiac puncture. Blood samples were centrifuged at 1700 g for 30 min at 4°C ; the resulting serum was heated at 56°C for 10 min and then stored at -20°C .

The crude antiserum was pre-absorbed using native calf thymus DNA bound to cellulose (Pharmacia; 1.14 mg of DNA/ml of DNA-cellulose slurry). Antiserum ($100\text{ }\mu\text{l}$) was mixed with the pellet from $100\text{ }\mu\text{l}$ of the DNA-cellulose slurry for 2 h at room temperature. The mixture was centrifuged for 5 min (11000 g) and the supernatant (antiserum) transferred to a tube containing fresh DNA-cellulose. This procedure was repeated three times for each batch of antiserum.

Alkaline phosphatase dot blot e.l.i.s.a.

Synthetic DNA polymers or native salmon sperm DNA were spotted onto dry nitrocellulose discs ($0.2\text{ }\mu\text{m}$ pore size, $\frac{5}{16}$ inch diameter; Schleicher and Schuell, Keene, NH, U.S.A.), air dried, and baked *in vacuo* at 80°C for 90 min. Discs could be stored for 2–3 days at -20°C in the dark before proceeding with the assay with no loss in sensitivity. Blank discs treated in an identical manner were used to determine non-specific background reactivity in the e.l.i.s.a. Discs were distributed into the wells of a flat-bottom 48-well cell culture plate; then the following steps were carried out at room temperature with gentle agitation. Blocking buffer [1% (w/v) BSA (Sigma; 77–99% albumin) in PBS] was added to each well, and incubated for 1 h. The blocking buffer was removed and discs were incubated for 90 min with 0.25 ml of antisera diluted in PBS. The antiserum was removed and the wells rinsed three times with 0.2% BSA in PBS. Next, 0.5 ml /well of alkaline-phosphatase-conjugated anti-(rabbit IgG) (Miles Yeda, Israel) diluted $1\text{ }\mu\text{l}$: 5 ml in diethanolamine buffer (48 mg of MgCl_2 and 91 ml of diethanolamine/l, pH 9.8) was added and incubated for 1 h. The conjugated antibody solution was removed and the discs rinsed four times with 0.2% BSA in PBS, and twice with PBS.

The alkaline phosphatase reactions were initiated by the addition of 0.5 ml /well of substrate (disodium *p*-nitrophenyl phosphate, $5\text{ mg}/5\text{ ml}$ of diethanolamine buffer) and allowed to proceed at 37°C for up to 18 h. Alkaline phosphatase enzymically converts *p*-nitrophenyl phosphate to *p*-nitrophenol, a coloured product with an absorbance maximum at 405 nm . Absorbance readings were taken to assess enzyme activity using a Titerak Multiscan or Varian DMS 80 spectrophotometer. Control absorbance readings of DEA buffer alone, alkaline phosphatase substrate alone, rabbit antiserum alone, and conjugated antibody alone were low. Results were expressed directly as A_{405} or on a graded numerical scale from 0 to 9 generated by the Titerak Multiscan. In the

latter case, the absorbance of the diethanolamine buffer blank was assigned a value of 0 and the maximum absorbance achieved in the assay a value of 9. Comparisons of data obtained in different experiments were more readily performed using the numerical scale.

To determine the efficiency of DNA binding to the nitrocellulose discs, radiolabelled poly(dG-dC)·poly(dG-dC), poly(dA-dT)·poly(dA-dT), and native salmon sperm DNA were prepared by nick-translation using, as appropriate [^3H]dCTP or [^3H]dATP (Amersham International; $50\text{ Ci}/\text{mmol}$) (Rigby *et al.*, 1977). Radiolabelled poly(dG-dC)·poly(dG-dC) was brominated in 4 M-NaCl to form [^3H]Z-DNA. The specific activity of these polymers ranged from 2×10^7 to $5 \times 10^7\text{ c.p.m.}/\mu\text{g}$ of DNA. Aliquots containing $2\text{ }\mu\text{g}$ of the radiolabelled polymers were spotted onto nitrocellulose discs in triplicate and DNA binding was determined by scintillation counting. The percentage retention of all [^3H]DNAs by the nitrocellulose filters was $\geq 95\%$, using total trichloroacetic-acid-precipitable ^3H counts in $2\text{ }\mu\text{g}$ aliquots of DNA as 100% . The binding of ^3H -labelled-poly(dG-dC)·poly(dG-dC) to nitrocellulose discs was quantitative throughout the range of concentrations used in these studies.

Plasmids containing Z-DNA

Plasmids pUC8 and pBR322 contained less than 14 bp of alternating purine–pyrimidine sequence, which do not readily form Z-DNA (Nordheim *et al.*, 1982). Derivatives of pUC8 and pBR322 containing cloned inserts of alternating purine–pyrimidine sequence form Z-DNA at native negative superhelical density (Thomae *et al.*, 1983; Pohl, 1986). Plasmids pFP316, pFP324, and pFP332 contain 16, 24, and 32 bp, respectively, of alternating G-C residues inserted within the *Bam*H1 site of pUC8. Plasmid pFP124 contains the 24 bp DNA sequence $-(\text{G}(\text{CGCATGCG})_3\text{C}-)$ within the *Bam*H1 site in pUC8. Plasmid pJWTG42 contains 42 bp of alternating T-G residues in the *Eco*RI-*Bam*H1 region of pBR322 (M. Becker, personal communication).

Horseradish peroxidase dot blot and transblot e.l.i.s.a.

For the dot blot e.l.i.s.a., $5\text{ }\mu\text{g}$ of each DNA were spotted onto fresh DPT paper discs and incubated for 30 min on ice. [^3H]DNA binding after spotting onto freshly prepared DPT paper discs or slow filtration through a sampling manifold containing fresh DPT paper ranged from 50 to 100% .

In the transblot e.l.i.s.a., $5\text{ }\mu\text{g}$ of each DNA were electrophoresed in a 0.7% agarose gel in $89\text{ mM-Tris}/89\text{ mM-boric acid}/2\text{ mM-EDTA}$, pH 8.2, buffer at 100 V for 6 h to resolve relaxed and supercoiled plasmids. The gel was stained briefly in ethidium bromide ($1\text{ }\mu\text{g}/\text{l}$) to allow visualization of the DNA, and then equilibrated for 3 h at 4°C in electroblotting buffer ($20\text{ mM-sodium acetate}$, pH 4.0). DNA was transblotted onto DPT paper (Alwine *et al.*, 1979) using a Hoefer Scientific Instruments Apparatus (San Francisco, CA, U.S.A.) cooled to $\leq 4^{\circ}\text{C}$ with circulating ice water. Prolonged soaking of the gel in the electroblot equilibration buffer or heating during the transfer can result in DNA band fuzziness. Electroblotting was conducted for 1 h at 15 V (0.22 A) and 4 h at 40 V (0.68 A). Under these conditions, 10% of the ^3H -labelled poly(dG-dC)·poly(dG-dC) and Br-poly(dG-dC)·poly(dG-dC) applied to the gel was transferred to the DPT paper as determined by cutting the

Fig. 1. Alkaline phosphatase dot blot e.l.i.s.a. timecourse

Br-poly(dG-dC)·poly(dG-dC), (2 µg) bound to nitrocellulose discs (□) or blank NC discs (■) were treated with rabbit Z-DNA antiserum and alkaline phosphatase-conjugated anti-(rabbit IgG) as described in the Experimental section. Alkaline phosphatase reactions were initiated at time zero by the addition of diethanolamine buffer + substrate pre-warmed to 37 °C. Reactions were conducted at 37 °C for 15, 30, 60 min and 2, 6 and 18 h, then terminated by chilling in ice. Alkaline phosphatase activity was measured at each time point by the absorbance at 405 nm (A_{405}). The mean of triplicate determinations in a single experiment which was duplicated with similar results is presented. The S.D. (not shown) was < 10 % at every time point.

DPT paper into 0.5 cm fractions and scintillation counting. The percentage transfer of high (> 23 kbp) and low (< 0.5 kbp) molecular mass ³H-labelled polymer DNA to the DPT paper was equivalent.

All remaining reactions were performed at room temperature with gentle agitation. The DPT paper discs were transferred into individual wells of a 48-well cell culture plate and the DPT paper transblot (5 cm × 7 cm) was placed in a 150 mm² petri dish. The DPT paper transblot was placed in 100 ml of 1 % BSA/1 % (w/v) glycine in TBS for 1 h then transferred to 25 ml of fresh TBS containing antiserum diluted 1:600 for 90 min. After rinsing three times with 100 ml of TBS to remove non-specifically bound antiserum, the DPT paper was

incubated with 25 ml of TBS plus a 1:500 dilution of horseradish peroxidase-conjugated anti-(rabbit IgG) (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) for 1 h. The DPT paper was washed six times with 100 ml of TBS. Antibody binding was evident after a 30 min reaction with 0.05 % 4-chloro-1-naphthol (Sigma) and 0.015 % H₂O₂ in water/methanol (5:1, v/v) which yielded a coloured reaction product covalently bound to the paper at the antibody binding sites (Limberger & Charon, 1986).

Identical reaction conditions, with a 100-fold reduction in volume, were used for the horseradish peroxidase dot blot e.l.i.s.a. was quantified in arbitrary densitometric step, the DPT paper discs were transferred in triplicate to 100 mm² petri dishes and washed six times with 10 ml of TBS.

Antibody reactivity in the horseradish peroxidase dot blot e.l.i.s.a. was quantified in arbitrary densitometric units. The dots were arranged in an array on white paper and photographed with Type 55 Polaroid film and a Wratten No. 15 yellow filter (Kodak). The photographic negatives were developed in 18 % sodium sulphite, washed thoroughly in tap water, and analysed with a Joyce-Loebl Magiscan Image Analyzer (Nikon, Garden City, NJ, U.S.A.). The integrated density of each dot in the photographic negative is inversely related to the extent of antibody reactivity.

RESULTS

Characterization of the alkaline phosphatase dot blot e.l.i.s.a. using rabbit antisera to Br-poly(dG-dC)·poly(dG-dC)

The production of antibodies to Br-poly(dG-dC)·poly(dG-dC) by two rabbits was monitored using the alkaline phosphatase dot blot e.l.i.s.a. with the immunogen, Br-poly(dG-dC)·poly(dG-dC) affixed to nitrocellulose paper discs. Pre-immune sera did not react with the immunogen. IgG antibodies to Br-poly(dG-dC)·poly(dG-dC) developed within 4 weeks in both rabbits; the titre of rabbit A antiserum was higher than that of rabbit B at all times, and was maximal at week 12. The immunoreactivity of week 12 rabbit A antiserum diminished proportionally upon dilution greater than 1:800. All subsequent experiments were performed with week 12 rabbit A antiserum diluted 1:600.

Fig. 1 shows the time course of the enzymic activity of the alkaline phosphatase-conjugated antibody retained

Table 1. Antisera specificity for polydeoxyribonucleotides immobilized on nitrocellulose in an alkaline phosphatase dot blot e.l.i.s.a.

Abbreviations used: polydeoxyribonucleotides are abbreviated to, e.g., poly(dA-dT) for poly(dA-dT)·poly(dA-dT); SS DNA, native salmon sperm DNA.

Antiserum and dilution	Polydeoxyribonucleotide (5 µg)					
	Br-poly(dG-dC)	poly(dG-dC)	poly(dA-dT)	Br-poly(dI-dC)	SS DNA	None
Rabbit*	1:300	7.3 ± 0.6	1.3 ± 0.6	1.3 ± 0.6	1.0 ± 0.4	1.3 ± 0.6
	1:600	7.3 ± 1.2	1.0 ± 0.0	1.0 ± 0.0	1.5 ± 0.5	1.0 ± 0.0
Goat†	1:300	5.0	1.0	0.0	0.0	0.0
	1:600	3.0	0.0	0.0	0.0	0.0

* Results are the mean ± S.E. of three independent assays.

† Results are the mean of triplicate determinations in a single assay.

Fig. 2. Alkaline phosphatase dot blot e.l.i.s.a. concentration-response relationship

Br-poly(dG-dC) · poly(dG-dC) was bound to nitrocellulose discs in amounts ranging from 5 ng to 2 μ g. Discs were treated with rabbit Z-DNA antiserum and alkaline phosphatase-conjugated anti-(rabbit IgG) as described in the Experimental section. Alkaline phosphatase reactions were initiated at time zero by the addition of diethanolamine buffer + substrate pre-warmed to 37 °C. Reactions were conducted at 37 °C for 1 h or 18 h then terminated by chilling on ice. Alkaline phosphatase activity was measured by the A_{405} and is reported after subtracting the absorbance of nitrocellulose paper blanks. The mean $\pm$ S.D. of triplicate determinations in single experiments which were repeated three times is reported. The S.D. was < 10% of the uncorrected absorbances at every data point. For clarity, data are presented only over the linear range of the concentration-response relationship.

by the rabbit Z-DNA antibodies complexed with 2 μ g of Br-poly(dG-dC) · poly(dG-dC) on nitrocellulose paper. Product formation was linear for 2 h but continued to accumulate throughout the 18 h time course. Background reactivity with blank nitrocellulose discs was consistently observed. This could be reduced slightly by the use of nitrocellulose discs spotted with 5 μ g of salmon sperm DNA (Table 1).

To determine the sensitivity of the alkaline phosphatase dot blot e.l.i.s.a., various concentrations ranging between 1 ng and 2 μ g of Br-poly(dG-dC) · poly(dG-dC) were spotted onto nitrocellulose in triplicate. Alkaline phosphatase activity in 1 h or 18 h reactions is depicted

in Fig. 2 as absorbance (A_{405}) after correction for the nitrocellulose paper blanks. In an 18 h reaction, specific Z-DNA antibody reactivity with 5 ng of Br-poly(dG-dC) · poly(dG-dC) was routinely detectable using both blank nitrocellulose discs and poly(dG-dC) · poly(dG-dC) spotted nitrocellulose discs as controls (results not shown). Alkaline phosphatase activity (A_{405}) was linear between 5 and 25 ng in an 18 h reaction. In a 1 h reaction, the detection limit of Br-poly(dG-dC) · poly(dG-dC) was between 25 and 50 ng; alkaline phosphatase activity (A_{405}) was linear between 10 and 250 ng. Thus, the sensitivity of the 18 h alkaline phosphatase e.l.i.s.a. reaction is greater. In addition to being highly sensitive, the data in Fig. 2 show that the alkaline phosphatase dot blot e.l.i.s.a. is also suitable for quantitative analyses.

The specificity of rabbit A antiserum in the dot blot e.l.i.s.a. and that of a goat Z-DNA antiserum provided by Dr. Alexander Rich (M.I.T.) is demonstrated in Table 1. Absorbance readings reflecting alkaline phosphatase activity are reported on a numerical scale ranging from 0 to 9. Alkaline phosphatase activity in wells containing the Z-DNA polymer ranged from 3 to 5 for the goat antiserum to 7 for the rabbit antiserum. Alkaline phosphatase activity in reactions with blank nitrocellulose discs or nitrocellulose discs containing the right-handed polydeoxyribonucleotides [poly(dG-dC) · poly(dG-dC), poly(dA-dT) · poly(dA-dT), and native salmon sperm DNA] ranged from 0 to 1.5, indicating that the cross-reactivity of these antisera with B-form DNA was low. Little antibody reactivity occurred with the brominated B-DNA polymer, Br-poly(dI-dC) · poly(dI-dC), indicating that the bromination is not an important epitope for these antisera.

The alkaline phosphatase dot blot e.l.i.s.a. was also tested for its ability to detect Z-DNA sequences in supercoiled plasmid DNA. In contrast with the synthetic polymers, Z-DNA forming sequences within plasmid DNA occur in short alternating purine-pyrimidine stretches comprising less than 1% of the total DNA. In these experiments, the positive control Br-poly(dG-dC) · poly(dG-dC) was immunoreactive. However, there was no detectable immunoreactivity with the plasmids containing Z-DNA (pFP124, pFP316, pFP324, pFP332 and pJWTG42) or the control plasmids, pUC8 and pBR322 (results not shown). Increasing the amount of plasmid DNA up to 15 μ g, increasing the amount of antiserum, or affixing plasmid DNA onto DPT paper, did not increase the sensitivity of this alkaline phosphatase assay. We suggest that baking the nitrocellulose discs to affix the DNA masks or alters the short Z-DNA regions within the plasmids, leaving only the predominant B-DNA accessible to antibody interactions. DNA binding to DPT paper occurs covalently at room temperature and therefore another explanation for the lack of sensitivity of the plasmid/DPT paper dot blot e.l.i.s.a. is needed. Presently, an observed increase in the background binding of antibodies to the DPT paper discs in the alkaline phosphatase dot blot e.l.i.s.a. is the only explanation we can offer. The problem is overcome in the horseradish peroxidase dot blot and transblot e.l.i.s.a. techniques described below.

Horseradish peroxidase dot blot and transblot e.l.i.s.a. of plasmid DNA

A transblot e.l.i.s.a. was developed that detects short stretches of Z-DNA in supercoiled plasmids and distin-

Fig. 3. Transblot e.l.i.s.a. of Z-DNA

DNA ($2.5 \mu\text{g}/\text{lane}$) was electrophoresed at 100 V in a 0.7% agarose gel for 3 h at 4°C in Tris/borate/EDTA buffer: lane 1, pUC8; lane 2, pFP124; lane 3, pFP316; lane 4, pFP332; lane 5, Br-poly(dG-dC)·poly(dG-dC); lane 6, poly(dG-dC)·poly(dG-dC); lane 7, pFP332; lane 8, Br-poly(dG-dC)·poly(dG-dC); lane 9, poly(dG-dC)·poly(dG-dC). The DNA was transblotted to DPT paper as described in the Experimental section and an e.l.i.s.a. was performed using crude rabbit A 12 week antiserum (1:600) (lanes 1–6) or pre-immune antiserum (1:600) (lanes 7–9).

guishes between antibody binding to supercoiled and non-supercoiled plasmid forms. Fig. 3 shows the reactivity of rabbit A pre-immune and 12 week crude antiserum with various DNAs after transblotting from an agarose gel onto DPT paper. Lane 1 contains the control plasmid pUC8 that does not form Z-DNA under negatively supercoiled conditions, and lanes 2, 3 and 4, three pUC8 derivatives, pFP124, pFP316, and pFP332, that contain 16–32 bp of Z-DNA under negatively supercoiled conditions. Only the plasmids in lanes 2, 3, and 4 containing potential Z-DNA forming regions reacted with the Z-DNA antiserum; however, antibody reactivity occurred with the linear (form III) and nicked circular (form II) states of these plasmids, which were not expected to form Z-DNA as well as with the supercoiled plasmids (form I) containing Z-DNA. Lane 5 shows that rabbit A crude antiserum reacted strongly with the Z-DNA polymer Br-poly(dG-dC)·poly(dG-dC) and also reacted to a much lesser degree with the B-form of this polymer, unbrominated poly(dG-dC)·poly(dG-dC) (lane 6). Pre-immune rabbit A antiserum did not react with either the brominated and unbrominated forms of poly(dG-dC)·poly(dG-dC) or with the Z-DNA containing plasmid, pFP332 (lanes 7, 8, 9).

The polyclonal antiserum used in these studies may contain antibodies directed against B-form poly(dG-dC)·poly(dG-dC) which reacted with the G-C inserts in

Fig. 4. Transblot e.l.i.s.a. of Z-DNA using pre-absorbed antiserum

DNA ($5 \mu\text{g}/\text{lane}$) was electrophoresed at 100 V in a 0.7% agarose gel (left panel) for 6 h at 4°C in Tris/borate/EDTA buffer: lane 1, pFP332; lane 2, pFP124; lane 3, pUC8; lane 4, poly(dG-dC)·poly(dG-dC); and lane 5, Br-poly(dG-dC)·poly(dG-dC). DNA was transblotted onto DPT paper and an e.l.i.s.a. performed as described in the Experimental section using rabbit A antisera (12 week, 1:600, pre-absorbed with DNA-cellulose). Immunoreactive DNA bound to DPT paper is shown in the right panel (1*–5*) corresponding to the ethidium bromide stained DNA in lanes 1–5 at left. The migration of Form I, II, III, and dimeric (d) supercoiled plasmid DNA is indicated.

the linearized and nicked-circular forms of pFP332 and pFP316. This explanation is less likely to account for the antibody reactions with nicked and linearized forms of pFP124. Alternatively, consistent with the observations of Lafer *et al.*, (1985), the antiserum may contain antibodies that stabilize Z-DNA within linear and relaxed plasmid DNA.

The crude antiserum was pre-absorbed to native DNA-cellulose and the specificity of the pre-absorbed antiserum tested in a transblot e.l.i.s.a. containing Br-poly(dG-dC)·poly(dG-dC) and poly(dG-dC)·poly(dG-dC). The transblot e.l.i.s.a. in Fig. 4 shows the conformation specificity of the pre-absorbed Z-DNA antiserum: $5 \mu\text{g}$ of the control plasmid, pUC8, and two Z-DNA-containing pUC8 derivatives pFP124 and pFP332, were electrophoresed in a 0.7% agarose gel, then transferred onto DPT paper. Poly(dG-dC)·poly(dG-dC) and Br-poly(dG-dC)·poly(dG-dC) were run in adjacent lanes of the gel.

Ethidium bromide staining of the agarose gel revealed that the plasmids electrophoretically separated into supercoiled (form I), supercoiled dimers (d), relaxed (form II), and linear forms (form III). Unbrominated and brominated poly(dG-dC)·poly(dG-dC) are heterogeneous in length. Consistent with the Z-DNA conformation the Br-poly(dG-dC)·poly(dG-dC) in lane 5 showed little staining with ethidium bromide (Walker *et al.*, 1985).

The transblot e.l.i.s.a. shown on the right side of Fig. 4 is a mirror image of the gel following transfer of the DNA onto the DPT paper. Peroxidase-conjugated antibody binding to the DNA immobilized on the DPT paper is evident by the dark staining. The rabbit

Fig. 5. Sensitivity of the transblot e.l.i.s.a. for Z-DNA using pre-absorbed antiserum

Various amounts of DNA were electrophoresed at 100 V in a 0.7% agarose gel for 6 h at 4°C in Tris/borate/EDTA buffer: lane 1, Br-poly(dG-dC)·poly(dG-dC) (5.0 µg); lane 2, poly(dG-dC)·poly(dG-dC) (5.0 µg); lane 3, pUC8 (2.5 µg); lane 4, pFP332 (1.0 µg); lane 5, pFP332 (0.5 µg); lane 6, pFP332 (0.1 µg); lane 7, pFP316 (1.0 µg); lane 8, pFP316 (0.5 µg); lane 9, pFP316 (0.1 µg); lane 10, pFP124 (1.0 µg); lane 11, pFP124 (0.5 µg); lane 12, pFP124 (0.1 µg). DNA was transblotted onto DPT paper and an e.l.i.s.a. performed as described in the Experimental section using rabbit A antiserum (12 week, 1:600, pre-absorbed with DNA-cellulose) to detect immunoreactivity. Dimeric and monomeric forms of the supercoiled plasmids are indicated by the bands labelled 'd' and 'm', respectively. The structures of the dimeric and monomeric plasmids were confirmed by the generation of discrete ladders of topoisomers in topoisomerase I relaxation assays.

antiserum bound strongly to the immunogen, Br-poly(dG-dC)·poly(dG-dC) (lane 5*) and the supercoiled forms of pFP124, and pFP332 (lanes 1* and 2*, respectively) which contain 24 and 32 bp of Z-DNA, respectively, at native plasmid negative superhelical density. The failure of the dimeric supercoiled plasmids containing Z-DNA to react with the antibody in this assay is unusual. The relaxed and linear forms of these plasmids did not react with the pre-absorbed antibody. pUC8, which lacks sequences capable of Z-DNA formation, did not react with the antiserum (lane 3*) nor did the unbrominated poly(dG-dC)·poly(dG-dC). Thus, using pre-absorbed antiserum, the transblot e.l.i.s.a. is Z-DNA conformation-specific and sensitive enough to detect short Z-DNA regions in supercoiled plasmids. The results also suggest that pre-absorption of polyclonal antisera to Z-DNA with native DNA bound to cellulose may be a useful technique to remove antibodies capable of Z-DNA stabilization as well as those cross-reactive with B-form DNA.

The sensitivity of the transblot e.l.i.s.a. for Z-DNA in plasmids was tested using 1.0, 0.5, and 0.1 µg of pFP332, pFP316, and pFP124 containing respectively, 32, 16, and 24 bp of Z-DNA (Fig. 5). Immunoreactivity with the pre-absorbed Z-DNA antiserum was specific for the monomeric and dimeric supercoiled forms of the Z-DNA-containing plasmids; 2.5 µg of the control plasmid, pUC8, did not cross-react with the antibody. The sensitivity limit of this transblot e.l.i.s.a. is approx. 0.1 µg

Table 2. Detection of Z-DNA in supercoiled plasmids and Br-poly(dG-dC) in a horseradish peroxidase dot blot e.l.i.s.a

Crude rabbit A antiserum or Rabbit A antiserum pre-absorbed with DNA-cellulose as described in the Experimental section were used at a 1:600 dilution. Antiserum reactivity was quantified densitometrically using photographic negatives as described in the Experimental section. Increasing antiserum reactivity results in decreasing densitometric units. Data represent the mean ± s.d. of triplicate determinations. Abbreviations used are defined in Table 1.

DNA (5 µg)	Antiserum reactivity (arbitrary densitometric units)	
	Crude	Pre-absorbed
None	61.2 ± 6.5	49.6 ± 11.0
pUC18	64.8 ± 9.8	49.9 ± 9.6
poly(dG-dC)	41.0 ± 4.3	41.7 ± 9.9
Br-poly(dG-dC)	4.5 ± 4.1	12.4 ± 4.0
pFP332	20.8 ± 3.0	15.9 ± 8.2
pFP124	36.4 ± 8.1	25.5 ± 7.9

of pUC8 containing 16 bp (0.037%) of alternating G-C sequence (lane 9).

The horseradish peroxidase e.l.i.s.a. can be also used to screen for the presence of Z-DNA in plasmids spotted onto DPT paper. Table 2 shows the reactivity of both crude and DNA-cellulose pre-absorbed rabbit A 12 week antiserum with 5 µg of control plasmid pUC18, the Z-DNA containing plasmids pFP332 and pFP124, poly(dG-dC)·poly(dG-dC), and Br-poly(dG-dC)·poly(dG-dC) in this dot blot e.l.i.s.a. Z-DNA-specific reactivity with pFP332, pFP124, and Br-poly(dG-dC)·poly(dG-dC) was observed with both crude and pre-absorbed antiserum. Reactivity with the control plasmid pUC18 was indistinguishable from blank DPT paper discs with either antiserum preparation. The pre-absorbed antiserum reacted less strongly than the crude antiserum with the B-DNA polymer, poly(dG-dC)·poly(dG-dC). The possibility that the crude antiserum stabilizes the Z-conformation in nicked circular and linearized plasmid forms does not detract from the broad utility of the horseradish peroxidase dot blot e.l.i.s.a. as a simple and rapid primary screen for Z-DNA in sequences cloned into pUC plasmids.

DISCUSSION

Z-DNA antibody techniques have been used extensively to measure Z-DNA formation in plasmid DNA. The Z-DNA dot blot and transblot e.l.i.s.a. described in this report offer several advantages over the existing techniques. The dot blot e.l.i.s.a. is very sensitive and simple to perform. In the present studies, the alkaline phosphatase dot blot e.l.i.s.a. was used to monitor the development of polyclonal antisera directed against Br-poly(dG-dC)·poly(dG-dC), antibody titre, and DNA specificity. The technique is well suited to the screening and characterization of monoclonal antibodies as well and, as with any e.l.i.s.a. procedure, the screening process may be automated. In addition, the dot blot e.l.i.s.a. is not necessarily limited to the screening of conformation-

specific antibodies. Coupled with the ability to produce synthetic DNAs of any desired deoxyribonucleotide sequence, the dot blot e.l.i.s.a. might be used in the identification of DNA sequence-specific antibodies.

A disadvantage of the alkaline phosphatase dot blot e.l.i.s.a. is its inability to detect Z-DNA within supercoiled plasmids. Based upon the results obtained with polymer DNA, this does not appear to be a simple problem of sensitivity: 2 μ g of pFP16 DNA are retained nearly quantitatively on nitrocellulose paper and contain 0.037% or 74 ng of alternating G-C sequence in the Z-conformation (in solution). Fig. 2 shows that as little as 5 ng of alternating G-C sequence in the Z-DNA polymer Br-poly(dG-dC)·poly(dG-dC) is detectable. We have proposed that physical changes within the plasmid DNA during the nitrocellulose fixation process preclude detection of Z-DNA within plasmids.

Utilization of the more sensitive horseradish peroxidase dot blot and transblot e.l.i.s.a. (Hawkes *et al.*, 1982) with the DNA bound to DPT paper permits detection of Z-DNA within plasmid DNA. The horseradish peroxidase e.l.i.s.a. provide a rapid means to identify Z-DNA within cloned DNA sequences and could prove useful in screening plasmids for mutations that either stabilize or destabilize Z-DNA formation as well.

The chief advantage of the horseradish peroxidase dot blot and transblot e.l.i.s.a. over existing antibody binding techniques is that these techniques do not rely upon the use of radiolabelled supercoiled plasmid DNA. The production of 3 H-labelled supercoiled plasmids via the growth of specialized (Thy⁻) *Escherichia coli* in [3 H]-thymidine-supplemented medium is expensive (Lafer *et al.*, 1985). Alternatively, more time-consuming biochemical approaches have been used to produce radioactive supercoiled plasmids from linearized plasmid DNA (Nordheim & Meese, 1988). In addition, both the transblot e.l.i.s.a. and the recently described topoisoimer gel retardation assays for Z-DNA can be used to distinguish antibody binding to supercoiled, relaxed, and linear plasmid forms. This becomes an important consideration when discriminating between antibody detection of Z-DNA and antibody-induced Z-DNA formation.

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