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Isolation and Characterization of Z-DNA Binding Proteins from Wheat Germ†

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ABSTRACT: The preparation of a heterogeneous non-histone protein extract from wheat germ utilizing Br–poly(dG-dC)·poly(dG-dC) (Z-DNA) affinity chromatography is described. The binding characteristics of antibodies against Z-DNA are used as a model system to define important criteria that the DNA binding behavior of a Z-DNA binding protein should display. We show that the wheat germ extract contains DNA binding proteins specific for left-handed Z-DNA by these criteria. The affinity of the proteins measured by competition experiments was approximately 10^5 greater for Br–poly(dG-dC)·poly(dG-dC) (Z-DNA) than for poly(dG-dC)·poly(dG-dC) (B-DNA). The affinity of the proteins for plasmid DNA increases with increasing negative superhelicity which is known to stabilize Z-DNA. The proteins are shown to compete with Z-DNA antibodies for binding to supercoiled plasmids. Finally, the affinity for two plasmids at a given superhelical density is greater for the plasmid containing an insert known to form Z-DNA than for a plasmid without the insert. The proteins exhibit a 2–3-fold greater affinity for stretches of $(dC\text{-}dA)_n\cdot(dT\text{-}dG)_n$ over stretches of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ when both sequences are induced to form Z-DNA by supercoiling.

Right-handed B-DNA and left-handed Z-DNA [reviewed in Rich et al. (1984)] are two equilibrium structures of double-stranded DNA. Z-DNA is the higher energy form, but it can be stabilized at physiological ionic strengths in a closed circular molecule by negative supercoiling (Singleton et al., 1982; Peck et al., 1982; Nordheim et al., 1982b; Haniford & Pulleyblank, 1983a,b) or by antibody binding (Lafer et al., 1985). Linear polymers can be stabilized as Z-DNA by chemical modifications such as bromination (Moller et al., 1984) or methylation (Behe & Felsenfeld, 1981). Early work on Z-DNA implied a strict requirement for alternating purine–pyrimidine sequences (Wang et al., 1979), but recently nonalternating sequences have been shown in X-ray crystallographic and solution studies to be capable of forming Z-DNA (Wang et al., 1985; Feigon et al., 1985). To assist in the elucidation of the biological role(s) of Z-DNA, we have isolated and characterized naturally occurring Z-DNA specific binding proteins from wheat germ. Wheat germ is a useful tissue to use for protein purification because it is both readily available and low in protease activity. Much work has been done defining the interactions between sequence-specific B-DNA binding proteins and their substrates [reviewed in Pabo & Sauer (1984)]. These studies have only limited usefulness as model systems for Z-DNA binding proteins. Unlike a specific sequence contained within a much longer B-DNA sequence, Z-DNA is a dynamic feature of the double-stranded DNA molecule which can be affected by superhelicity, ionic strength, and protein binding. An example of specific Z-DNA binding proteins is found in antibodies that we have prepared with both low and high affinities (Lafer et al., 1981, 1983, 1985). We consider these antibodies as a model system for naturally occurring Z-DNA binding activity.

By using the binding characteristics of the Z-DNA antibodies, we can define the DNA binding behavior that a Z-DNA binding protein should display. Bromination of poly(dG-dC)·poly(dG-dC) in high salt stabilizes Z-DNA, so a

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natural Z-DNA binding protein should show a greater affinity for Br–poly(dG-dC)·poly(dG-dC) than for poly(dG-dC)·poly(dG-dC) or native B-DNA. Z-DNA is also stabilized by negative supercoiling in a closed circular molecule so a Z-DNA binding protein should show a greater relative affinity for negatively supercoiled plasmid DNA than for relaxed plasmid DNA. It is important, however, to distinguish proteins that bind preferentially to supercoiled DNA alone from proteins that bind to Z-DNA stabilized by supercoiling. For example, histone H1 has been shown to bind preferentially to supercoiled SV40 DNA (Vogel & Singer, 1975), and it also displays a higher affinity for Br–poly(dG-dC)·poly(dG-dC) than for poly(dG-dC)·poly(dG-dC) (Mura & Stollar, 1984). However, histone H1 is not a Z-DNA binding protein because its binding to SV40 DNA is enhanced by either positive or negative supercoiling (Vogel & Singer, 1975), and positive supercoiling destabilizes Z-DNA. Therefore, satisfying the two criteria cited above is necessary but not sufficient in order to demonstrate Z-DNA specific binding activity.

Although the requirement for alternating purine–pyrimidine sequences for forming a Z-DNA structure has been shown not to be absolute, Z-DNA formation is still favored in such sequences. Two-dimensional gel electrophoretic analysis has shown that Z-DNA forms at lower negative superhelical densities in plasmids that contain stretches of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ or $(dC\text{-}dA)_n\cdot(dT\text{-}dG)_n$ than in plasmids that lack such sequences (Peck & Wang, 1983; Haniford & Pulleyblank, 1983a,b). Z-DNA antibodies have been shown to have a higher affinity for a plasmid containing an insert of alternating purine–pyrimidine sequence than for a plasmid lacking this insert, when both plasmids are sufficiently supercoiled to stabilize Z-DNA in the insert containing plasmid but not in the plasmid lacking the insert (Nordheim et al., 1982b). This defines a further important criterion to be met.

Here we describe the isolation of naturally occurring Z-DNA binding proteins that fulfill these criteria. An earlier publication from this laboratory developed a methodology for the isolation of Z-DNA-specific binding proteins. The DNA binding properties of a *Drosophila* nuclear extract prepared by this methodology (Nordheim et al., 1982a) were characterized. The extract displayed a very high differential affinity for Br–poly(dG-dC)·poly(dG-dC) over B-DNA. However, the data for binding to negatively supercoiled plasmid DNA were inconclusive but not inconsistent with properties anticipated for Z-DNA binding proteins. The affinity of the extract correlated positively with negative supercoiling, but the binding was not identified as due to Z-DNA. More recently, some proteins that are not Z-DNA binding proteins have been shown to bind better to Br–poly(dG-dC)·poly(dG-dC) than to B-DNA, including histones H1 and H5 (Mura & Stollar, 1984). The differential affinities of these proteins for Br–poly(dG-dC)·poly(dG-dC) vs. B-DNA are on the order of 5–10-fold, which is much less than the differential affinity seen for the proteins isolated from *Drosophila* nuclei. Here we use Z-DNA affinity chromatography to isolate a group of proteins from wheat germ that shows a high differential affinity for Br–poly(dG-dC)·poly(dG-dC) compared to B-DNA. These proteins also have the additional property of binding to a negatively supercoiled plasmid DNA with an affinity that correlates positively with the presence of Z-DNA in the plasmid.

Materials and Methods

Protein Purification. A total of 500 g of raw unprocessed wheat germ (kindly provided by General Mills) was suspended in 1 L of 10 mM tris(hydroxymethyl)aminomethane (Tris),[1] 2.5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF, pH 8.0 (buffer A) at 4 °C. The thick slurry was incubated for 30 min with periodic stirring at 4 °C and then homogenized by blending at high speed for 4 min in a Waring blender. The total volume was brought to 2 L with cold buffer A, and then the homogenate was filtered successively through a double layer of cheesecloth, a 250-μm nylon filter, and a 100-μm nylon filter. The filtered homogenate was brought to 2 L with cold buffer A and then centrifuged in a Beckman J-6B at 4000 rpm for 60 min. The supernatant was discarded, and the upper yellowish phase of the pellet containing the chromatin fraction was resuspended with a test tube brush in 2 L of cold buffer A; the lower hard-packed white layer of the pellet is mostly starch and was discarded. This step was repeated twice, and the crude chromatin pellet was then resuspended in 200 mL of cold buffer A. Then 4.5 mL of 250 mM EDTA (pH 8.0) was added to the resuspended chromatin to give a final EDTA concentration of 5 mM, and 13 mL of 5 M NaCl was added to give a final NaCl concentration of 0.3 M. At this point the preparation of chromatin became very viscous. The chromatin was extracted in 0.3 M NaCl at 4 °C for 60 min with periodic stirring. This preparation was centrifuged at 20000 rpm in an SS-34 Sorvall rotor for 60 min, and the pellet was discarded. The volume at this point was approximately 150 mL. A total of 50 mL of sonicated (average size 1–2 kb) 2 mg/mL calf thymus DNA in 10 mM Tris (pH 8.0) and 1 mM EDTA was added to the supernatant. The preparation was then dialyzed against 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 140 mM NaCl (buffer B) at 4 °C overnight. This resulted in the formation of a B-DNA precipitate that was removed by a 60-min centrifugation in an SS-34 at 20000 rpm. The supernatant was then filtered successively through 0.45 and 0.2-μm filters to remove protein–protein or protein–DNA aggregates that did not sediment. This preparation was loaded onto a 10-mL column of 5 mg of Br–poly(dG-dC)·poly(dG-dC) coupled to Sephadex G-25 [prepared as in Nordheim et al. (1982a)] and extensively washed with cold buffer B. The bound material was eluted with 10 mM Tris (pH 8.0), 1 mM EDTA, 1 M NaCl, 1 mM DTT, and 1 mM PMSF. The eluate usually came off in approximately 20 mL and was concentrated 10-fold with a YM-30 Amicon membrane and then dialyzed into 20% glycerol, 0.1 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM DTT, and 1 mM PMSF at 4 °C. The concentrated, dialyzed proteins were stored at −70 °C in 20-μL aliquots. Typical yields were approximately 1 mg of protein/100 g of wheat germ.

Nucleic Acids. Calf thymus DNA was obtained from Sigma. Poly(dG-dm^{5}C)·poly(dG-dm^{5}C) was prepared as described by Behe & Felsenfeld (1981). Br–poly(dG-dC)·poly(dG-dC) was prepared as described (Moller et al., 1984). The Z-DNA conformation of the polynucleotide was verified by UV absorption and CD spectroscopy. All other polymers were purchased from either P-L Biochemicals, Collaborative Research, or Boehringer-Mannheim. [^{3}H]Thymidine-labeled *Escherichia coli* DNA was prepared from a mutant strain B3, as described previously (Papalian et al., 1980) but with a pulse of 0.5 mCi of [^{3}H]thymidine (New England Nuclear). ^{3}H-Labeled poly(dG-dm^{5}C)·poly(dG-dm^{5}C) was synthesized according to Behe & Felsenfeld (1981) except that 0.065 mCi of [^{3}H]dGTP (Amersham) was included in the synthesis re-

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase.

action mixture. The other polymers were radiolabeled by nick translation (Rigby et al., 1977). Radiolabeled plasmid DNA was prepared by growing transformed DH1 TL3 (Thy$^-$) in 500-mL cultures of minimal media supplemented with 2 μg/mL cold thymidine and 1 μCi/mL [^{3}H]thymidine. Typical yields of plasmid were 0.5 μg/mL labeled to 2×10^4 cpm/μg. Plasmids with different degrees of supercoiling were prepared by treating the plasmid with topoisomerase I (purchased from Bethesda Research Labs) in the presence of varying concentrations of ethidium bromide: relaxed plasmid, 0 μg/mL ethidium bromide; superhelical density of −0.03, 0.5 μg/mL ethidium bromide; −0.05, 0.8 μg/mL ethidium bromide; −0.08, 1.5 μg/mL ethidium bromide. Two units of topoisomerase I was used per microgram of DNA in a reaction mixture containing 10 μg/mL DNA, 10 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM EDTA. The reaction was carried out overnight at room temperature. Superhelical densities were determined by the band counting method of Keller (1975).

Filter Binding Assays. Binding assays were done by incubating 0.1 μg of labeled polymer or *E. coli* DNA with varying amounts of protein in 1 mL of 10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF for 30 min at room temperature and then measuring retention of the label on a nitrocellulose filter. Assays with plasmid DNA were done in exactly the same fashion but with 0.5 μg of DNA. Competitive assays were done with a 30-min preincubation of cold competitor DNA with protein followed by addition of the labeled DNA.

Competitive Radioimmunoassay. Varying concentrations of Z-DNA binding proteins were incubated with 0.1 μg of supercoiled plasmid in 350 μL of 10 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 0.5 mM PMSF, for 30 min at room temperature followed by the addition of 100 μL of a DEAE-purified goat anti-Z-DNA antibody preparation which contained 0.3 μg of specific IgG. The incubation was continued for an additional 30 min followed by the addition of 50 μL of a rabbit anti-goat Ig. After a third 30-min incubation period the resulting precipitate was centrifuged in a Beckman microcentrifuge. The pellet was washed twice and resuspended in 0.2 mL of 100 mM NaOH. Three milliliters of Aquasol-2 scintillation cocktail was added and the radioactivity measured in a Beckman scintillation counter. The anti-Z-DNA antibodies were prepared as described in Lafer et al. (1981). Rabbit anti-goat IgG was purchased from Antibodies Incorporated (Davis, CA).

Results

The strategy behind our protein purification centered on purifying a Z-DNA binding activity away from any B-DNA binding activity. This was achieved by salt-extracting wheat germ chromatin at an ionic strength known to enrich for non-histone chromatin proteins. These extracted proteins were precipitated with B-DNA to further deplete B-DNA binding proteins. The remaining material was then bound to Sephadex-coupled Br–poly(dG-dC)·poly(dG-dC) (Z-DNA affinity column) in the presence of excess competitor B-DNA. The results of each of these steps in the purification are seen in Figure 1. In lane 8 we see the initial total chromosomal protein showing a large number of bands spanning a wide range of molecular weights. Lane 7 shows the proteins that come off of the chromatin in 0.3 M NaCl. Lanes 5 and 6 show the proteins remaining after B-DNA precipitation, prior to chromatography on Br–poly(dG-dC)·poly(dG-dC)–Sephadex G-25. Lanes 1–3 show three different concentrations of the purified proteins that were eluted from the Z-DNA affinity column. Lane 4 shows the run-through material; however, it

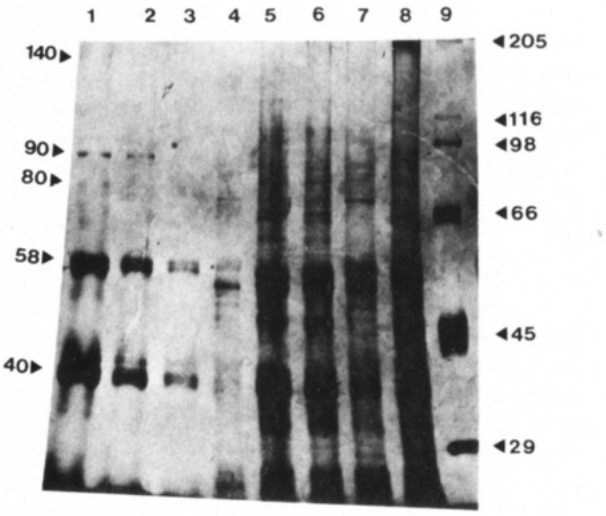

FIGURE 1: SDS–PAGE (10% acrylamide) analysis of purification of naturally occurring Z-DNA binding proteins from wheat germ. The gel was silver stained by the procedure of Morrissey (1981). Lanes 1–3 are purified proteins in three different concentrations. Lane 4 is the Br–poly(dG-dC)·poly(dG-dC) affinity column flow through. Lanes 5 and 6 are the affinity column input. Lane 7 is a 0.3 M salt wash of chromatin while lane 8 is the initial total chromosomal protein. Lane 9 contains calibrating marker proteins with their molecular weights ($\times 10^3$) listed at the right.

was underloaded on the gel in Figure 1. When we load more of this material on the gel, we see all the bands present in lanes 5 and 6 although the bands present in the eluate are weakened. The column eluate contains major bands at 58K and 40K, a major band at 90K, and minor bands at 140K and 80K. All of these bands are substantially enriched relative to their representation in the crude chromatin preparation. The band at 40K is the only band that is a major band in the crude extract. The three major bands at 90K, 58K, and 40K are reproducibly the major polypeptide species visible on a silver-stained gel from purification to purification. Some variability is observed in the relative amounts of each of these bands: in some preparations there is proportionally more of the 90K band present. The protein stain running below 29K is, in fact, running with the dye front and is presumably composed of protein degradation products.

We characterized the DNA binding properties of our protein preparation by using direct binding as well as competitive filter binding assays. These assays rely on the ability of a nitrocellulose filter to retain protein and any ligand bound to the protein, but these filters do not retain uncomplexed double-stranded nucleic acids. Using a radiolabelled DNA, we were able to separate bound from free DNA by retention on a nitrocellulose filter. The fraction of input DNA bound by the filter was measured by scintillation counting. Nonspecific retention of free DNA to the filter was controlled by subtracting the binding of the DNA to the filter in the absence of protein from that measured in the presence of protein. In the direct binding assay we measured the retention of a constant concentration of DNA by a variable concentration of protein. The results from direct binding studies done with synthetic DNA polymers and native DNA from *E. coli* are shown plotted in Figure 2. In the range of protein concentrations shown in Figure 2 no appreciable binding to any of the polymers except Br–poly(dG-dC)·poly(dG-dC) is seen.

Two methods were used to measure the relative affinity of the extract for these polymers. By doing the same kind of experiment described in Figure 2 but using higher concentrations of protein, we were able to determine the concentration of protein that would retain 50% of the input DNA. We

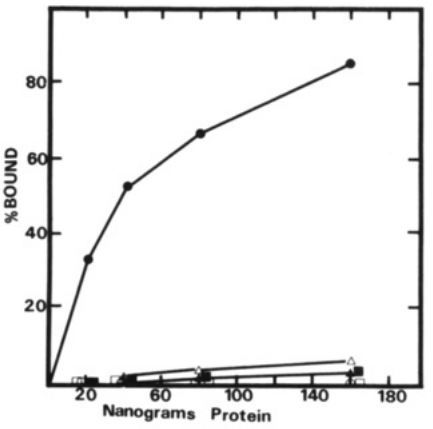

FIGURE 2: Binding of naturally occurring Z-DNA binding proteins from wheat germ to Br–poly(dG-dC)·poly(dG-dC) (●), poly(dG-dC)·poly(dG-dC) (○), native *E. coli* DNA (▲), poly(dG-dm^{5}C)·poly(dG-dm^{5}C) (■), poly(dA-dT)·poly(dA-dT) (□), and poly(dC-dA)·poly(dG-dT) (△). Nitrocellulose filter binding experiment in 10 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 1.0 mM PMSF.

Table I: Relative Affinity of Wheat Germ Z-DNA Binding Proteins for Polynucleotides of Approximately Equal Lengths[a]

polymer	direct binding	competition
Br–poly(dG-dC)·poly(dG-dC)	1	1
poly(dC-dA)·poly(dG-dT)	1×10^{-2}	ND
poly(dG-dm^{5}C)·poly(dG-dm^{5}C)	1×10^{-2}	ND
double-stranded *E. coli* DNA	5×10^{-3}	1×10^{-4}
poly(dA-dT)·poly(dA-dT)	5×10^{-3}	ND
poly(dG-dC)·poly(dG-dC)	1×10^{-3}	1×10^{-5}
single-stranded *E. coli* DNA	ND	2×10^{-4}

[a] Filter binding experiments were carried out in 10 mM Tris (pH 8.0), 1 mM EDTA, and 150 mM NaCl. ND = not determined.

assigned an affinity of 1.0 to the protein concentration that bound 50% of the Br–poly(dG-dC)·poly(dG-dC) and calculated the affinity of the proteins for the other polymers relative to this (Table I, direct binding column). Three orders of magnitude more protein was required to bind 50% of the poly(dG-dC)·poly(dG-dC) than to bind 50% of the Br–poly(dG-dC)·poly(dG-dC). The direct binding assay gives us information on the binding properties of the protein mixture that, although enriched significantly for Z-DNA binding proteins, may still contain some contaminating B-DNA binding proteins.

To measure the relative affinity of only the population of proteins that binds to Br–poly(dG-dC)·poly(dG-dC), we used a competitive filter binding assay. This assay also has the advantage that it can be used with single-stranded polynucleotides which could not be used in the direct binding assay because single-stranded DNA is retained on nitrocellulose. In this assay the amount of a nonradioactive competitor DNA was measured, which inhibits by 50% binding of the proteins to radiolabelled Br–poly(dG-dC)·poly(dG-dC). An affinity of 1 was assigned to Br–poly(dG-dC)·poly(dG-dC), and the relative affinities of the Br–poly(dG-dC)·poly(dG-dC) binding proteins for the other polymers were calculated relative to this (Table I, competition column). When relative affinities were determined by competition assays, the proteins showed a 10^5 stronger affinity for Br–poly(dG-dC)·poly(dG-dC) than for poly(dG-dC)·poly(dG-dC). The difference between the relative affinities as determined by direct binding vs. competition implies that there is some contaminating B-DNA binding activity still present in the preparation. In other experiments it was found that radioactive B-DNA binding was competed for more effectively by nonradioactive Br–poly(dG-dC)·poly(dG-dC) than by B-DNA (data not shown). However, some B-DNA binding proteins show enhanced binding to Br–poly(dG-dC)·poly(dG-dC) (Mura & Stollar, 1984), and such proteins might well be present as contaminants in the preparation. A Z-DNA binding protein should show a high relative affinity for Z-DNA over B-DNA. This relative affinity is reflected in the numbers derived from the competition assay while the direct binding assay is sensitive to small amounts of contaminating B-DNA binding activity.

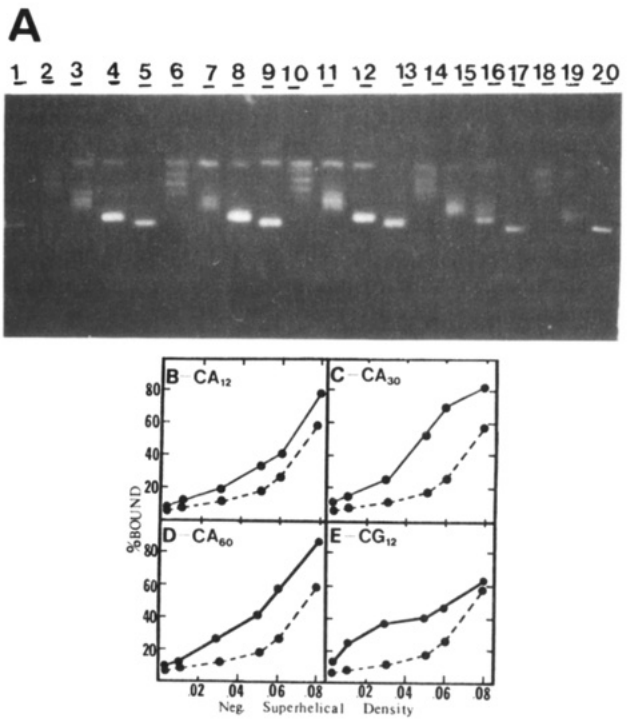

FIGURE 3: Binding of naturally occurring Z-DNA binding proteins from wheat germ to plasmids as a function of negative superhelicity. (A) 1% agarose gel showing the topoisomers of different superhelical densities used in these binding assays: lane 1, linearized pDPL6; lanes 2–5, pDPL6 relaxed, −0.03, −0.05, and −0.08 (numbers refer to negative superhelical density); lanes 6–9, pDPL6 with $(CA)_{12}$ insert relaxed, −0.03, −0.05, and −0.08; lanes 10–13, pDPL6 with $(CA)_{30}$ insert relaxed, −0.03, −0.05, and −0.08; lanes 14–17, pDPL6 with $(CA)_{60}$ insert relaxed, −0.03, −0.05, and −0.08; lanes 18–20, pDPL6 with $(CG)_{12}$ insert relaxed, −0.05 and −0.08. In panels B–E the dashed line represents binding to the parent vector, pDPL6, and the solid line shows binding to a pDPL6 construct containing the indicated insert of alternating purine–pyrimidine sequence. B, binding to pDPL6 with a $(CA)_{12}$ insert. C, binding to pDPL6 with a $(CA)_{30}$ insert. D, binding to pDPL6 with a $(CA)_{60}$ insert. E, binding to pDPL6 with a $(CG)_{12}$ insert. The filter binding was in 10 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 1.0 mM PMSF with 0.5 μg/mL protein.

Bromination of poly(dG-dC)·poly(dG-dC) in 4 M NaCl yields a molecule that is irreversibly in the Z-DNA conformation even as the ionic strength is lowered to within the physiological range (Moller et al., 1984). A Br–poly(dG-dC)·poly(dG-dC) chromatography purification is expected to enrich for Br–poly(dG-dC)·poly(dG-dC) binding proteins. To determine if this Br–poly(dG-dC)·poly(dG-dC) binding represents binding to Z-DNA, we asked whether the proteins could recognize another form of Z-DNA that did not contain bromine atoms as shown in Figure 3. Segments of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ [abbreviated $(CG)_n$] and $(dC\text{-}dA)_n\cdot(dG\text{-}dT)_n$ [abbreviated (CA)n], when contained in a closed circular DNA molecule, can be stabilized in the Z form by the free energy of supercoiling. We measured the binding of the proteins to a plasmid containing an insert of $(CA)_{30}$ as a function of negative superhelical density (Figure 3, panel C, solid line). As the negative superhelical density of the plasmid

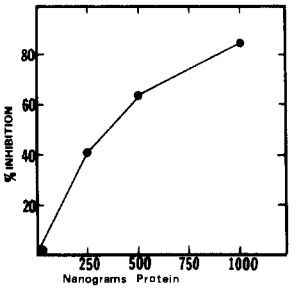

FIGURE 4: Naturally occurring wheat germ Z-DNA binding proteins are used to inhibit anti-Z-DNA antibody binding to plasmids. Plasmid pDPL6 was used containing an insert of $(CA)_{30}$ at a superhelical density of −0.08. Experiments were carried out in 10 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 1.0 mM PMSF. The 450-μL reaction mixture contained 0.3 μg of specific anti-Z-DNA antibody and 0.1 μg of supercoiled DNA.

increases from 0 to −0.08, a 25-fold increase in the affinity of the proteins for the plasmid is observed. If the proteins are binding to the $(CA)_{30}$ insert which has been previously shown to form Z-DNA in this range of superhelicities (Haniford & Pulleyblank, 1983a,b; Nordheim & Rich, 1983), they should compete for Z-DNA antibody binding to the plasmid. We measured the inhibition of antibody binding to plasmid DNA by varying the concentrations of protein. Low concentrations of protein compete effectively for Z-DNA antibody binding (Figure 4).

However, an alternative explanation to the data presented in Figure 3 (panel C) and Figure 4 is that these proteins are "supercoil binding" proteins: either proteins like *lac* repressor that unwind DNA when bound (Wang et al., 1974) or proteins like histone H1 that interact directly with supercoils (Vogel & Singer, 1975). Such proteins would show enhanced binding to supercoiled DNA and could also "compete" for Z-DNA antibody binding by removing torsional strain from the plasmid upon binding and thus destabilizing Z-DNA.

That this is not the case is shown by the other data presented in Figure 3. We examined the binding of the proteins to a series of plasmids containing inserts known to form Z-DNA as a function of negative superhelical density. An agarose gel of the topoisomers used in these experiments is shown in Figure 3A. The binding of the proteins to plasmid pDPL6, a 2.2-kb pBR322 derivative is shown in panels B–E (dashed line). The binding of the proteins to pDPL6 containing inserts of $(CA)_{12}$, $(CA)_{30}$, $(CA)_{60}$, and $(CG)_{12}$ is shown in panels B–E, respectively. As the negative superhelical density of each of the plasmid molecules increases from 0 to −0.08, an increase in the affinity of the proteins for the plasmid is observed. Qualitatively, it can be seen that there is more retention of the plasmids with the Z-forming insert than with the parent plasmid. The superhelical density at which the proteins bind 50% of the plasmid is lowest for the plasmid containing the $(CA)_{30}$ insert (−0.047) followed by plasmids containing $(CA)_{60}$, $(CA)_{12}$, or $(CG)_{12}$ inserts at superhelical densities of −0.055, −0.064, and −0.064, respectively. The superhelical density at which the proteins bind 50% of the plasmid is highest for the parent vector pDPL6, which contains no insert (−0.074).

The proteins show enhanced binding to plasmids that contain inserts of sequences known to form Z-DNA readily upon supercoiling. The differential affinities of the proteins for these plasmids at a superhelical density of −0.06 are summarized in Table II. In order to determine the percentage of each of these plasmids which contains Z-DNA at this superhelical density, we determined the percentage of each plasmid DNA retained in a filter binding assay by a 6.4 nM concentration of a low-affinity Z-DNA antibody preparation. Previous work has shown that, at this concentration, these antibodies are relatively nonperturbing to the B–Z equilibrium in a supercoiled plasmid (Lafer et al., 1985). The amounts of Z-DNA detected by antibody binding are consistent with the ability of each insert to form Z-DNA as measured by two-dimensional gel electrophoresis (Peck & Wang, 1983; Haniford & Pulleyblank, 1983a,b). The percent of plasmids bound by the antibody is listed together with the relative affinities determined for these proteins in Table II. For the $(CA)_n$ family of inserts the affinity increases with the amount of Z-DNA present as detected by antibody binding. At superhelical density −0.06 the $(CA)_n$ plasmids contain less Z-DNA than the $(CG)_n$ plasmid as shown by antibody binding, but they display equal $[(CA)_{12}]$ or enhanced $[(CA)_{30},(CA)_{60}]$ affinities for the proteins. The proteins are thus showing a sequence specificity in preferring $(CA)_n$ in the Z conformation to $(CG)_n$ in the same conformation by 2–3-fold as judged by filter retention.

Table II: Relative Affinities of Wheat Germ Z-DNA Binding Proteins for Plasmids Containing a Z-DNA Insert at Superhelical Density −0.06

plasmid	relative affinity	% antibody bound
pDPL6	1	0
pDPL6 + $(CA)_{12}$·$(GT)_{12}$	2–3	10
pDPL6 + $(CA)_{60}$·$(GT)_{60}$	4	30
pDPL6 + $(CA)_{30}$·$(GT)_{30}$	5	40
pDPL6 + $(CG)_{12}$·$(GC)_{12}$	2–3	80

The $(CG)_n$ inserts form Z-DNA at a lower negative superhelical density than do the $(CA)_n$ inserts. This accounts for the rapid rise in retention of the plasmid with $(CG)_{12}$ at lower levels of negative supercoiling (Figure 3E). However, at higher negative superhelical density, formation of Z-DNA occurs in the (CA)n inserts, and this produces their enhanced retention (Figure 3B–D).

Discussion

Here we describe a protein mixture in which the DNA binding characteristics fulfill the three criteria outlined above which provide necessary and sufficient evidence to substantiate a claim for a naturally occurring Z-DNA binding activity. The use of wheat germ made it possible to isolate proteins in sufficient quantities to do extensive testing. The relative affinities of the proteins for plasmids with Z-forming inserts compared to their parent vector are much smaller than the relative affinities determined for the Z polymer Br–poly(dG-dC)·poly(dG-dC) vs. B-DNA. This would be expected given the mass action principles that govern the filter binding assay. At a superhelical density of −0.06, for instance, the $(CG)_{12}$ plasmid contains approximately 1% Z-DNA and 99% B-DNA [$(CG)_{12}$ on a 2.2-kb plasmid] while its parent vector contains no Z-DNA as measured by antibody retention. The relative affinity of the proteins as assayed by direct binding is approximately 2-fold greater for the $(CG)_{12}$ plasmid than for its parent vector, but the relative affinity for Br–poly(dG-dC)·poly(dG-dC) vs. linear B-DNA is 100–1000-fold greater.

A simple analytical treatment is useful here. We can define K_Z as the affinity of an extract protein P for Z-DNA and K_B as the affinity of the same extract protein for B-DNA. We define $[B_0]$, $[Z_0]$, and $[P_0]$ as the initial concentrations of B-DNA, Z-DNA, and the protein, respectively. At equilibrium, we have P_Z of the protein complexed to Z-DNA and P_B of the protein complexed to B-DNA. The equilibrium equations for association of the protein to the two forms of DNA are

$$[P{\cdot}B]/([B_0{-}B][P_0{-}P_Z{-}P_B]) = K_B$$
$$[P{\cdot}Z]/([Z_0{-}Z][P_0{-}P_Z{-}P_B]) = K_Z \quad (1)$$

where [P·Z] and [P·B] are the concentrations of proteins bound to Z-DNA or to B-DNA, respectively. In this analysis, let us suppose that the binding constant of the protein to Z-DNA is 100 times greater than its binding to B-DNA, or $K_Z = 100K_B$. However, in our plasmid there is 1% Z-DNA and 99% B-DNA, or approximately $[B_0] = 100[Z_0]$ for the $(CG)_{12}$ insert at a superhelical density of −0.06. We are interested in the ratio of the Z complex to the B complex. Thus

$$[P{\cdot}Z]/[P{\cdot}B] = (K_Z[Z_0{-}Z][P_0{-}P_Z{-}P_B])/(K_B[B_0{-}B][P_0{-}P_Z{-}P_B]) \quad (2)$$

In general, we have less than 100% retention of the plasmid on the filter. The relative amount of B-DNA involved in interaction with protein is small since only one protein molecule is needed to retain the entire plasmid on the filter. Thus, we can approximate $[B_0{-}B] = [B_0]$. If we now use the relations $K_Z = 100K_B$, and $[B_0] = 100[Z_0]$, eq 1 reduces to

$$[P{\cdot}Z]/[P{\cdot}B] = [Z_0{-}Z]/[Z_0] \quad (3)$$

The concentration of protein bound to B-DNA is always greater than the concentration of protein bound to Z-DNA for the situation defined by the parameters given above.

In this simple analysis, the affinity of the protein for the B-DNA segment (99% of the plasmid) is equal to the affinity of the protein for the Z-DNA segment (1% of the plasmid) because of the 100-fold greater binding constant to Z-DNA. If we now compare the filter binding of a plasmid with the Z-forming insert to the binding of the same plasmid without the insert, it is clear that the plasmid with the insert will be retained to a greater extent, since it has retention from both its B and Z components. If we find that $1/n$ of the plasmids without the insert are retained on the filter, we can express this in terms of the concentration of protein bound to B-DNA, i.e., [P·B]. Each of the $1/n$ of the retained plasmids has one protein bound to it; $1/n$ of those have two proteins bound, $1/n$ of those have three proteins bound, etc. Thus, the total of bound protein is $[P{\cdot}B] = [\text{plasmid}](1/n + 1/n^2 + 1/n^3 + \ldots)$ or $[P{\cdot}B] = [\text{plasmid}](1/[n-1])$. Hence, the fraction of retained plasmids is $1/n = [P{\cdot}B]/([\text{plasmid}] + [P{\cdot}B])$. If n is large, the concentration of complexed protein [P·B] is small, and $1/n$ reduces to [P·B]/[plasmid]. However, if n is small, [P·B] is large and $1/n$ approaches 1.

In a similar way, we can calculate the retention of the plasmid with the Z-DNA forming insert, as it will be equal to that retained by the B-DNA binding plus that due to Z-DNA binding. Since we assume the Z-DNA forming insert is small, the increased retention due to Z-DNA binding is $([P{\cdot}Z]/[\text{plasmid}])\,[1 - [[P{\cdot}B]/([\text{plasmid}] + [P{\cdot}B])]]$, and the total retention is $([P{\cdot}B] + [P{\cdot}Z])/([\text{plasmid}] + [P{\cdot}B])$. We can now compare the retention of a plasmid with the Z-DNA forming insert to that without the insert:

$$\text{retention with insert/retention without insert} = ([P{\cdot}B] + [P{\cdot}Z])/[P{\cdot}B] = 1 + ([P{\cdot}Z]/[P{\cdot}B]) = 1 + ([Z_0{-}Z]/[Z_0]) \quad (4)$$

using eq 3. For this example, the ratio ranges from 1 to 2. If, for example, 30% of the Z-DNA forms a protein complex, then $[Z_0{-}Z]/[Z_0]$ is 0.7 and the ratio for the two plasmids is 1.7. Adding more protein decreases the ratio as the amount of B-DNA binding goes up and the Z-DNA binding makes a smaller contribution.

We have simplifled the analysis by assuming that one protein could bind to both Z-DNA and B-DNA with a 100-fold difference in binding constant. However, it is likely that Z-DNA binding proteins would have much larger differences in binding constants, especially if they acted to effectively find and occupy the Z-DNA in vivo. The competition binding experiments showed a 10^5 greater affinity for Z-DNA than B-DNA while direct binding gave only a 100–1000-fold difference. The smaller differential affinity determined by the direct binding assay reflects the presence of contaminating B-DNA binding proteins in our heterogeneous extract. A more thorough analysis would take this into account. However, the results would be rather similar to those obtained above, especially under our experimental conditions where the proteins are in molar excess relative to the molar concentration of the plasmid. The above analysis predicts that the ratio of retention of a plasmid without an insert vs. the plasmid with an insert will be between 1 and 2; the observed differences are between 2- and 3-fold. This is consistent with the polymer binding results that give significantly more than a 100-fold difference in relative affinity of Z-DNA vs. B-DNA. As an individual Z-binding protein is freed of contaminating B-DNA binding proteins, the observed differences are likely to rise even further than 3-fold.

For the $(CA)_n$ family of plasmids these differences in relative affinity are even greater, and we interpret this as a sequence preference for $(CA)_n$ in Z over $(CG)_n$ in Z. It is possible that $(CA)_n$ is a sequence which these proteins bind to in vivo, but all we can conclude is that $(CA)_n$ resembles the natural sequence more than $(CG)_n$. In this regard, the sequence $(dC\text{-}dA)_n{\cdot}(dG\text{-}dT)_n$ with $n > 50$ is found as middle repetitive DNA widely distributed throughout eukaryotes (Hamada et al., 1982).

Some Z-DNA binding proteins, analogous to B-DNA binding proteins, probably display sequence specificity. The relative affinities of some B-DNA binding proteins for their sequences are very great: *lac* repressor binds with a 10^8-fold greater affinity for the operator than for random sequence DNA [reviewed in von Hippel (1979)]. However, this has been questioned recently as not representing a sufficiently large differential affinity to regulate a gene in a eukaryotic genome (Travers, 1984a,b; Ptashne, 1984). Eukaryotic genomes are much larger than prokaryotic genomes, and it has been suggested that a protein such as repressor would become "tied up" on the vast excess of DNA present in the eukaryotic genome because of its low affinity for random sequence DNA. A sequence-specific Z-DNA binding protein can take advantage of a level of recognition which a sequence-specific B-DNA binding protein does not have. If it also shows a 10^6–10^8 greater affinity for its specific Z-DNA sequence over random sequence Z-DNA, then this coupled with a 10^4–10^5 greater affinity for random sequence Z-DNA over random sequence B-DNA (as measured in our competition assay) would result in a sufficiently great differential affinity to regulate genes in the larger eukaryotic genome. It has been suggested that Z-DNA may be important in regulating gene expression [reviewed in Rich et al. (1984)].

A detailed study has been done of the stabilization of Z-DNA in polymers and plasmids by Z-DNA antibodies of low and high affinities (Lafer et al., 1985). In supercoiled plasmids stabilization by antibodies is characterized by a shifting of the midpoint of the B–Z transition, a broadening of the sharp transitional profile, and reduced discrimination between plasmids with inserts and their parent vectors. These features also characterize the behavior of the natural proteins with supercoiled plasmids as shown in Figure 3. However, without further experimental evidence it is not possible to decide whether these features are due to stabilization of Z-DNA by

a high-affinity protein or to contaminating B-DNA binding. Evidence to be presented elsewhere suggests that stabilization is, in fact, the correct interpretation.

It is clear that further characterization will require pure protein species. With them, it will be possible to search for the natural sequence(s) of the individual proteins and quantitatively characterize their stabilizing effect as well as search for possible biological functions.

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