*Nucleic Acids Research, 1993, Vol. 21, No. 11* **2669–2672**

# A method to identify and characterize Z-DNA binding proteins using a linear oligodeoxynucleotide

Alan G.Herbert and Alexander Rich
Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

Received January 20, 1993; Revised and Accepted April 27, 1993

## ABSTRACT

**An oligodeoxynucleotide that readily flips to the Z-DNA conformation in 10mM $MgCl_2$ was produced by using Klenow enzyme to incorporate 5-bromodeoxycytosine and deoxyguanosine into a $(dC\text{-}dG)_{22}$ template. During synthesis the oligomer can be labeled with $^{32}P$ to high specific activity. The labeled oligodeoxynucleotide can be used in bandshift experiment to detect proteins that bind Z-DNA. This allows the binding specificity of such proteins to be determined with high reliability using unlabeled linear and supercoiled DNA competitors. In addition, because the radioactive oligodeoxynucleotide contains bromine atoms, DNA-protein complexes can be readily crosslinked using UV light. This allows an estimate to be made of the molecular weight of the proteins that bind to the radioactive probe. Both techniques are demonstrated using a goat polyclonal anti-Z-DNA antiserum.**

## INTRODUCTION

The left handed conformer of DNA, Z-DNA, is a high energy DNA structure that is stabilized by negative supercoiling(1). In closed topological domains such as those found *in vivo*, the formation of Z-DNA alters the conformation of all other sequences in that domain by redistributing the tortional strain. This may affect the accessibility of particular sequences to proteins involved in transcription, replication and recombination. Thus the manner in which the formation and stabilization of Z-DNA is regulated *in vivo* has important implications for cell biology. While it is clear that topoisomerases represent one level of regulation of domain energy (2), the role in this process of proteins that specifically recognize the Z-DNA conformer is currently unknown.

Previous attempts at demonstrating the existence of Z-DNA binding proteins in nuclear extracts have been hampered by methodological problems inherent in studying any energetically unstable structure (3). There exists a need to establish a robust, yet sensitive, method to be used in the purification of proteins that bind Z-DNA. In this paper two methods are described that facilitate progress towards this goal. The first method relies on the observation that alternating stretches of deoxycytosine and deoxyguanosine can be stabilized in the Z-DNA conformation under physiological salt conditions by bromination or methylation of the 5 position of the deoxycytosine ring (4, 5). We show that an oligomer of alternating dC-dG can be used as a substrate to produce a radiolabeled partially brominated Z-DNA probe of high specific activity, which is suitable for use in a band shift assay. Because it contains bromine atoms, the radioactive probe also can be crosslinked to proteins that bind it by ultra-violet light. Following removal of non-covalently bound probe by digestion with nucleases, the molecular weight of the radioactively tagged protein can be estimated by SDS-PAGE.

In this paper these methods are demonstrated using a Z-DNA specific goat antiserum. In another paper, the use of these methods to identify a Z-DNA binding protein in nuclear extracts from chicken red blood nuclei is described (6).

## METHODS

### Synthesis of $^{Br}CG$ probe

The synthesis of the probe involves the extension by the DNA-polymerase Klenow fragment of a self-complementary, self-primed template consisting of $(dC\text{-}dG)_{22}$. This oligomer was synthesized on an ABI-380B DNA synthesizer by the phosphite triester method using the manufacturer's protocol and is used without purification. One $\mu$l of a stock solution of this oligomer ($0.2\mu g/\mu l$) stored at 4°C is added to 20$\mu$l of $H_2O$, 2$\mu$l of stock buffer (1M KOAc, 100 mM $MgCl_2$, 250 mM Tris.HCl, pH 7.6), 1$\mu$l of 0.2 mM 5-bromodeoxycytosine triphosphate (Pharmacia, Piscataway, N.J.), 10$\mu$l of $\alpha^{32}$P-dGTP (6000 Ci/mM, NEN, Boston, MA.) and 20 units of Klenow enzyme (New England Biolabs, Beverly, MA.). The mixture is incubated at 25°C for a minimum of 30 minutes. A further 20 units of Klenow enzyme is then added along with 2$\mu$l of 0.2mM deoxyguanosine and incubation continued at least 15 minutes. The reaction is stopped by adding EDTA to a final concentration of 20mM. The conditions for this reaction are critical, especially the temperature and the nucleotide concentration. The labeled DNA is separated from unincorporated nucleotide by non-denaturing PAGE in a 7.5% gel run in TBE (Tris-Borate-EDTA buffer (7)). Typically two labeled bands are identified by autoradiography using a 10–30s exposure of Kodak X-OMAT AR film (figure 1). The lower band has a mobility similar to an oligodeoxynucleotide of $(dC\text{-}dG)_{33}$ and probably arises from extension of a single duplex DNA molecule. The upper band probably arises from intermolecular priming of synthesis. Both

bands are duplex and both are suitable for use in assays.The bands are excised and crushed in a Spin-X microfuge cartridge (Costar, Cambridge, MA.), to which 400μl of TE solution (10mM Tris.HCl, 1mM EDTA, pH 7.6) is added. Following incubation at room temperature for a period longer than one hour, probe can be recovered by microfuging the Spin-X for 5 minutes. The yield is about $2\times10^5$ cpm/μl. The labeled deoxyguanosine is incorporated along with 5-bromodeoxycytosine into the probe, resulting in the selective labeling of Z-DNA forming sequences. The specific activity of the probe when determined by purification using an elutip-D column (Schleicher and Schuell, Keene, NH.) is approximately $5-10\times10^8$cpm/μg, meaning that the recovery of total radioactivity from the two bands in the gel is approximately 50%. The probe can be stored at room temperature and can be used for more than one month following manufacture.

### Band shift assay

Typically protein fractions are incubated with 40,000 cpm probe, which represents 100−200pg of DNA, in the presence of 10mM $MgCl_2$, 50mM NaCl, 10mM Tris.HCl (pH 7.5) for 10 minutes at room temperature in a final volume of 20μl. Complexes are resolved from free probe by electrophoresis of these samples on a 6% non-denaturing gel run at room temperature in TB buffer (Tris-Borate buffer(7)) at 300V (8). Electrophoresis is continued until a bromophenol blue marker band is at the bottom of the gel.

All bandshift experiments shown here contain 1μg of unlabeled sheared salmon sperm DNA in each reaction mixture. In competition experiments, plasmids were either used at bacterial superhelical density or after linearization with the restriction enzyme Hind III.

### UV crosslink experiments

For all crosslink experiments, incubation mixtures containing protein and probe were prepared as described for the bandshift experiments. The brominated radioactive probe was crosslinked to protein by irradiating an incubation mixture in microfuge tubes for 30 minutes with unfiltered UV light from an inverted Model C-51 transilluminator (Ultra-Violet Products, San Gabriel, CA.). The transilluminator was suspended 5cm above the base of the tubes and the time of illumination was determined in trial experiments in which time points ranging from 5 to 45 minutes were tested. Additional competition experiments were carried out by adding to the incubation mixture poly(dC-dG) (Pharmacia, Piscataway, N.J.) in the B-DNA form or stabilized in the Z-DNA form by chemical bromination (4). Following irradiation with UV light, free probe and non-crosslinked parts of the bound probe were removed by using DNase-I and micrococcal nuclease(9). To each 25μl sample, 25μl of a solution containing 0.4% sarkosyl and 2mM PMSF (phenylmethylsulfonyl fluoride) and 50μl of a solution containing 25mM Tris.HCl(pH7.4), 2mM $CaCl_2$, 7.5mM $MgCl_2$,and 1% of the detergent NP40 (nonidet P-40) were added. Nuclease digestion was carried out with 5U of DNase-I (Boehringer Mannheim, Indianapolis, IN.) and 7.5U of micrococcal nuclease (Worthington Biochemical Corporation, Freehold, NJ.) at 37°C for 60 minutes. Samples were then precipitated in 15% trichloroacetic acid, washed with 80% acetone before being resuspended in SDS-PAGE sample buffer. Samples may be stored frozen at −20°C if necessary, then resolved on a 10% gel using SDS-PAGE and $^{14}$C-labeled molecular weight markers. Gels were dried and autoradiographed using Kodak X-OMAT AR film. Alternatively, gels can be silver stained (10) without loss of signal. This facilitates the visualization of radioactively tagged proteins in non-crosslinked protein samples.

## RESULTS AND DISCUSSION

### Bandshift assay

Oligonucleotides of $(dC\text{-}dG)_n$ exist in equilibrium between the B-DNA and Z-DNA conformations. This is demonstrated by the observation that Z-DNA was initially crystallized from a solution of a $(dC\text{-}dG)_3$ which by circular dichroism criteria contains only B-DNA (11). It has been found that by increasing the salt concentration or by adding organic solvents such as ethanol the equilibrium can be shifted towards the formation of Z-DNA (12). This shift is potentiated by bromination of the 5 position of the deoxycytosine ring (4). Preliminary experiments using circular dichroism measurements indicated that a 22-base pair oligomer consisting of alternating $^{5Br}$dC-dG flips to the Z-DNA conformation at 50mM NaCl in the presence of either 10mM $MgCl_2$ or 50μM $Co(NH_3)_6Cl_3$. Such an oligomer was a poor substrate for either polynucleotide kinase or polymerase enzymes and was not useful for generating a high specific activity probe needed in assays of biological material. However, it was found that a non-brominated 44 base-pair dC-dG oligomer could be used as a substrate by Klenow polymerase to incorporate both $^{32}$P dGTP and $^{5Br}$dCTP. This yielded a duplex 3′ partially brominated probe of high specific activity that formed Z-DNA in 50 mM NaCl at 10−20 mM $MgCl_2$. Circular dichroism studies of a chemically synthesized 3′ brominated oligodeoxynucleotide consisting of $(dC\text{-}dG)_{22}(d^{5Br}C\text{-}dG)_{11}$ show that at least 60% of the probe is in the Z-DNA conformation (Jon Madison and A.H., unpublished results) under these conditions.

This probe is suitable for use in testing the specificity of proteins for Z-DNA in a bandshift assay (8). Protein is incubated with the radioactively labeled probe and protein-probe complexes are resolved from free probe by nondenaturing PAGE. Results obtained using a goat anti Z-DNA antiserum (13) are shown in

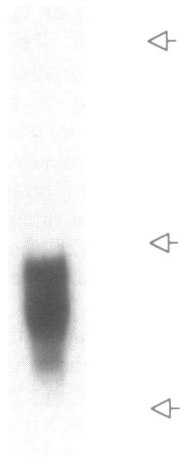

**Figure 1.** An autoradiograph of a gel used to prepare the oligonucleotide probe described in the text. Exposure time was 25 seconds. The upper arrow indicates the origin of electrophoresis, the middle arrow the migration of xylene cyanol and the lower arrow the migration of bromophenol blue.

figure 2. Significant retardation of probe mobility occurs in the presence of the Z-DNA specific antiserum. Three separate bandshifts can be identified in these gels. The most intense band (marked with an open arrowhead) is likely to be the faster moving monomer band. The fainter, slower moving bands probably have two and three antibody molecules per probe. Assuming this stoichemistry, a plot of log(molecular weight of antibody bound) versus distance moved from the origin of electrophoresis is linear.

Confirmation that the binding of the antiserum to the probe is Z-DNA specific is provided by the competition experiments shown in figure 2. pDHg16 (14) is a 2.3kb plasmid containing a $(dC\text{-}dG)_{13}$ insert which is in the Z-DNA conformation at bacterial superhelical density This plasmid efficiently competes for the band shift (panel C) while the supercoiled parental plasmid pDPL6 without such an insert does not (panel A). As the concentration of pDHg16 is increased the slowest migrating bandshift is lost first, as expected, because of the higher antibody-probe stoichiometry. At the point where competition is complete (Fig. 2, C-3), the weight ratio between plasmid Z-DNA, which is approximately 1% of the plasmid weight, and probe, which is approximately 100pg per lane, is 20:1. If pDHg16 is linearized prior to assay, (panel B) competition for the binding of the antiserum is also lost. This confirms that binding of the antibody to the plasmid is supercoil dependent and thus Z-DNA specific. It is important to note that this competition behaviour eliminates the possibility that the antibody is solely specific for some feature of the brominated deoxycytosine residue, or that it binds to single strand ends.

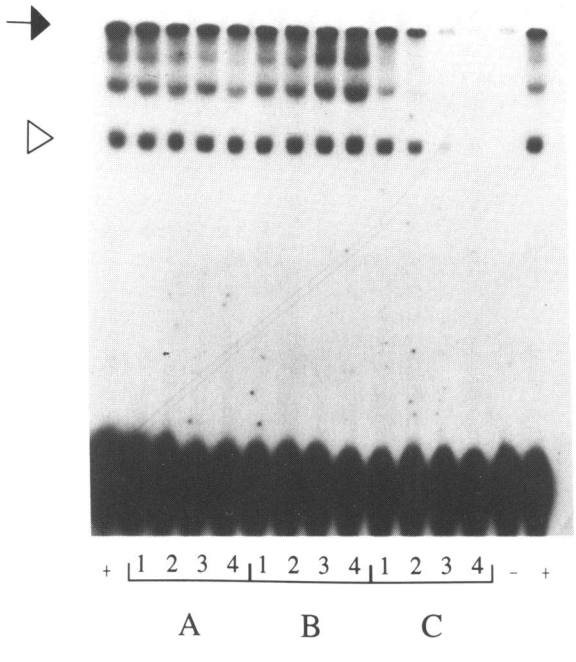


**Figure 2.** Band Shift Assay to demonstrate the specificity of a Goat anti Z-DNA antiserum: A $10^{-6}$ dilution of serum, obtained from a goat immunized with Z-form brominated poly(dC-dG), was incubated as described in the text with 100pg of radiolabeled probe in the presence of 1$\mu$g of sheared salmon sperm DNA, 10mM $MgCl_2$, 10mM Tris.HCl (pH 7.5) and 50 mM NaCl in a final volume of 20$\mu$l. The following amounts of unlabeled plasmid were added to each mixture; Lane 1 (0.008 $\mu$g), lane 2 (0.04 $\mu$g), lane 3 (0.2 $\mu$g) and lane 4 (1.0 $\mu$g). Lanes containing antibody and probe without added plasmid (+) and probe alone (−) are as indicated. Panel A contains lanes with the supercoiled plasmid pDPL6, panel B contains lanes with the plasmid pDHg16 linearized with Hind III, while panel C contains supercoiled pDHg16. pDHg16 is derived from pDPL6 and contains a $(dC\text{-}dG)_{13}$ insert which forms Z-DNA at bacterial superhelical density (14). The origin of electrophoresis is indicated by an arrow. The open arrowhead shows the monomeric complex of antibody and probe; the weaker bands above contain two and three antibody molecules per probe respectively. A plot (not shown) of log(molecular weight of antibody bound) versus distance moved from the origin of electrophoresis is linear.

## UV crosslink

The gel-mobility shift assay provides information about the presence of Z-DNA binding proteins. In order to estimate the size of proteins bound to this probe, a modification of previously published protocols (9) can be used. The presence of the bromine group attached to the cytosine group allows the probe to be crosslinked to protein by irradiation with low intensity UV light. The complex is then digested with DNase-I and micrococcal nuclease to remove free probe and those parts of the probe not crosslinked to the protein. This allows the molecular weight of the radioactively tagged protein to be determined by autoradiography of a SDS-PAGE gel. Results for the goat antiserum are shown in figure 3. On a reducing SDS-PAGE gel radiolabeled bands are observed. As judged by comparision with siver stained gels of goat antibody, these correspond to light and heavy chains of IgG (lane +). The diffuse nature of the bands arises in part from variation in size of the DNA tag attached to the protein due to the formation of multiple crosslinks between the partially brominated probe and the DNA binding site of the antibody. The most accurate estimate of molecular weight is obtained by using the fastest migrating edge of the band which contains antibody with the smallest size DNA tag. The molecular weight of the heavy chain is approximately 55,000 while that of the light chain is 28,000. It is interesting that considerably more crosslinking to the heavy chain is observed. Competition with non-radioactively labeled B-form and Z-form poly(dC-dG) is also shown. The loss of signal in the presence of Z-form poly(dC-dG) confirms the specificity of the anti-serum for Z-DNA.

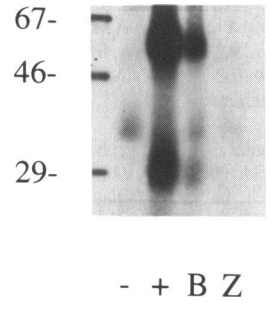


**Figure 3.** SDS-PAGE gel of proteins from a goat anti Z-DNA antiserum that were radiolabeled by UV-induced crosslinks to the Z-DNA probe. A $10^{-4}$ dilution of antiserum was incubated with radiolabeled probe in the presence of 1$\mu$g of salmon sperm DNA and irradiated with UV light as described in the text. Non-covalently bound DNA and free probe was removed by digestion with DNase-I and micrococcal nuclease. Following precipitation with trichloroacetic acid, proteins were analyzed by SDS-PAGE and the result is shown in the lane marked (+). To test the specificity of binding, 100ng of unlabeled B-form (B) or Z-form (Z) linear DNA competitor was added to the incubation mixture as indicated. The molecular weight ($\times 10^{-3}$) of $^{14}C$ labeled protein markers is as shown. Probe exposed to UV light in the absence of protein is in the lane marked (−).

The methods described here for detecting Z-DNA binding proteins have many useful features. The probe exists in the Z-DNA conformer at physiological salt concentrations allowing buffers to be optimized for protein binding. The probe is short with a restricted number of binding sites, allowing resolution of different DNA-binding activities. B-Z junctions and other potential regions of single-stranded DNA are unlikely to exist under the assay conditions used (15). The high specific-activity radiolabeling of the probe allows high sensitivity in detection and label-transfer to proteins. The ease with which competition studies can be conducted using unlabeled substrates allow assignment of binding-specificity with a high degree of certainty.

The methods described here are complementary to those involving supercoiled plasmids (16) or supercoiled minicircles (17). These supercoil-dependent assays are potentially very useful in verifying the binding specificity of proteins once they have been purified. The supercoil dependent assays are less useful in assaying crude protein mixtures and are susceptible to interference by nucleases, topoisomerases and other non-Z-DNA, supercoil dependent DNA-binding proteins present in nuclear extracts.

The methods described here may also be extended to those proteins that have both B-DNA and Z-DNA binding sites on separate domains. By using partial proteolysis it may be possible to separate the two domains. The Z-DNA binding fragment or the B-DNA binding fragment could then be identified in the bandshift assay or the UV crosslink assay by appropriate use of unlabeled competitor DNA.

## ACKNOWLEDGMENTS

This work is supported by grants by the Office for Naval Research, the National Institutes of Health, the American Cancer Society, the National Aeronautics and Space Administration and the National Science Foundation. We thank Dr Richard Fishel and Dr Ky Lowenhaupt for helpful discussions.

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