The Level of Z-DNA in Metabolically Active, Permeabilized Mammalian Cell Nuclei Is Regulated by Torsional Strain

Burghardt Wittig,* Tomislav Dorbic,* and Alexander Rich‡

*Institut fur Molekularbiologie und Biochemie, Freie Universitat Berlin, D-1000 Berlin 33, West Germany; and
‡Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. Permeabilized nuclei from mammalian cells encapsulated within agarose microbeads in an isotonic buffer are active in transcription and replication (Jackson, D. A., and P. R. Cook. 1985. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:913–918). Their DNA is intact and the nuclei are accessible to macromolecules. Myeloma nuclei prepared in this way were used to probe the extent of DNA negative supercoiling and the effects of altering torsional strain by binding radioactively labeled monoclonal antibodies to Z-DNA. Control experiments used monoclonal antibodies against a nonhistone chromosomal protein, HMG-17. On increasing the amount of anti-HMG-17 added, a binding plateau was reached encompassing a 200-fold range of antibody concentration. On binding anti-Z-DNA antibody, a similar broad plateau of constant binding was found encompassing a 100-fold range of antibody concentration. The latter result was taken as a measure of preexisting Z-DNA in the nuclei. Additional anti-Z-DNA antibody binding can be "induced" in the presence of much higher concentration of antibody, apparently by perturbing the B-DNA/Z-DNA equilibrium. On inhibiting topoisomerase I with camptothecin, an elevated antibody binding plateau was found, suggesting that elastic torsional strain in the DNA is responsible for stabilizing the preexisting Z-DNA. This interpretation is supported by the fact that addition of small, nicking amounts of DNase I leads to a complete loss of antibody binding in the Z-DNA plateau region but not in the region of "induced" Z-DNA.

DNA can assume both right-handed and left-handed conformations. The left-handed Z-DNA conformation is in equilibrium with the right-handed conformation but under physiological solvent conditions it is at a higher energy state (see references 14 and 24 for reviews). Z-DNA can be stabilized in vivo either by negative supercoiling (13, 23, 26) or by Z-DNA binding proteins (1, 17). An unsolved problem is the extent to which DNA is negatively supercoiled in vivo, and the extent to which the Z-DNA conformation exists within the nucleus under physiologically relevant conditions. Several studies addressing this problem have been carried out using antibodies to Z-DNA and cells fixed for morphological investigation (15, 20, 22). However, chromatin in these studies was far from a physiological environment. Fixing cells generally involves the addition of substances such as acetic acid or ethanol that remove DNA bound proteins from the nucleus (4, 28). DNA in the nucleus is wound around proteins and removal of even a portion of those proteins releases negatively supercoiled, elastically strained DNA which may adopt the Z conformation. Likewise, microdissecting a *Drosophila* polytene chromosome and placing it in a buffer solution for staining with antibodies is far from the physiological environment of the intact nucleus (8).

We have addressed this problem using the novel technique developed by Jackson and Cook (3, 9–12). In this procedure, living cells are encapsulated within agarose microbeads which have large pores allowing free movement of macromolecules into and out of the beads (3, 10). When the cells are treated with a detergent (0.5% Triton X-100) in an isotonic salt solution, the plasma membrane and the soluble contents of cytoplasm are removed but the nucleus remains morphologically intact, and permeable to macromolecules. The DNA in the agarose embedded nucleus is unbroken and the nucleus remains active in both transcription and replication (9, 11, 12). Jackson and Cook have found that the intact chromatin template replicates DNA at 85% of the rate found in vivo (12). This represents a useful preparation for probing the torsional state of the DNA. Will antibodies to Z-DNA bind to these unfixed, metabolically active nuclei?

In the present work, we have used monoclonal antibodies that have been shown to bind to Z-DNA independent of its nucleotide sequence (19, 21, 25; reviewed in reference 27). The antibodies have been biotinylated. Subsequently, either radioactive or fluorescently labeled streptavidin has been added. Control experiments have used a monoclonal antibody against the nonhistone chromosomal protein HMG-17.

On adding increasing amounts of anti-HMG-17, an antibody concentration-independent binding plateau is found. At that plateau, there is approximately one antibody combining site per HMG-17 molecule. Anti-Z-DNA antibodies have been shown to "induce" Z-DNA formation by shifting

the B-Z equilibrium, especially at high antibody concentrations (16, 17). On raising the amount of anti-Z-DNA antibody added, a binding plateau was found here as well, and the amount of binding remained constant over a 100-fold increase of antibody concentration. This was taken as a measure of preexisting Z-DNA. However, at even higher antibody concentrations, the binding was observed to increase. We call this "inducible" Z-DNA.

It is likely that the plateau binding level of preexisting Z-DNA is maintained by torsional strain since addition of a topoisomerase I inhibitor results in an elevated level of the binding plateau. This interpretation is further supported by experiments with DNase I. Addition of small (nicking) amounts of DNase I results in the loss of all antibody binding in the preexisting Z-DNA binding plateau. However, it does not greatly affect the Z-DNA induced by much higher antibody concentrations.

Materials and Methods

Encapsulation of Cells

Mouse myeloma cells (X63-AG 8.6.5.3) were used in all of the experiments. The methods of cell preparation are adaptations of procedures published by Jackson and Cook (3, 9–12). Cells were grown in DME medium containing 10% FCS supplemented with L-glutamine (2 mM), pyruvate (1 mM), glucose (3.5 g/liter), and penicillin-streptomycin solution (100 U/ml). 5×10^8 cells were washed twice at room temperature with 50 ml each of PBS (150 mM NaCl, 8.4 mM Na_2PO_4, 1.5 mM KH_2PO_4, pH 7.2), suspended with 20 ml of PBS to yield 2.5×10^7 cells/ml, and kept at 39°C. In separate flasks, 5 ml of 2.5% (wt/wt) low melting agarose (type VII, No. A-4018; Sigma Chemical Co., St. Louis, MO) were liquified and subsequently cooled to 39°C, and 50 ml of liquid paraffin (model No. 7174; Merck, Bracco S. P. A., Milan) were warmed to 39°C. These three components were pooled in a 300-ml Erlenmeyer flask, prewarmed to 39°C, in the order paraffin–cells–agarose, emulsified by shaking at 400 rpm in a rotary shaker (model G-76; New Brunswick Scientific Co., Inc., Edison, NJ) at 20°C for 30 s, and equilibrated in an ice-water bath to 0°C for 5 min. Then 100 ml of ice-cold PBS were added and immediately mixed by manually rotating the flask. After quickly distributing the solution into glass centrifuge tubes (80 ml), the emulsion was centrifuged at 3,500 *g* and 0°C for 5 min. The supernatant phase was totally removed by aspiration. The microbead pellet was washed three times with 60 ml of ice-cold PBS for each tube, centrifuging at 4,800 *g*. The pellets were united resulting in 10–12 ml of microbead encapsulated living cells. Cell lysis and permeabilization of nuclei: Microbeads were suspended in 3 vol of ice-cold cell lysis buffer (0.5% [wt/vol] Triton X-100, 100 mM KCl, 25 mM Na_2 EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 7.6) and kept in an ice-water bath with gentle stirring for 20 min. After centrifugational sedimentation (4,800 *g*, 0°C, 5 min) and washing three times each with 5 vol (relative to the original pellet volume) of isotonic buffer (100 mM KCl, 25 mM $[NH_4]_2SO_4$, 1 mM EDTA, 1 mM DTT, 0.2% [wt/vol] BSA, 5% [vol/vol] glycerol, 20 mM Tris-HCl, pH 7.6), the microbeads were suspended with an equal volume of isotonic buffer.

Fluorescence Microscopy

For fluorescence microscopic studies, FITC-labeled streptavidin was used. In the case of the FITC label, to each 500 μl aliquot, 5 μl of FITC-streptavidin (RPN.1232; Amersham Corp., Arlington Heights, IL) was added. The suspension was then incubated at 20°C for 60 min, washed as described above, and mounted for fluorescence microscopy. As a control, total DNA staining was used with the dye DAPI (4,6-diamine-2 phenyl indole). 0.1 μg/ml DAPI was added to the last wash. In these experiments DAPI- and FITC-labeling were never used simultaneously.

Binding of Monoclonal Antibodies

500 μl of the suspended microbeads (corresponding to 250 μl of the packed preparation containing 2.5×10^6 permeabilized nuclei) were transferred to 1.5-ml Eppendorf reaction vials (model No. 3810; Brinkman Instruments, Inc., Westbury, NY) using blue Eppendorf pipette tips with enlarged openings. The monoclonal anti-Z-DNA antibody Z22 (19) as well as the anti-HMG-17 monoclonal antibody (5, 6) were biotinylated as prescribed in the Enzotin biotinylation kit (Enzo Biochem, Inc., New York). A fixed amount of the respective antibody was added to the microbead suspension. Incubation was for 2 h at room temperature, briefly vortexing the suspension at intervals of 20 min. To dispose of unbound antibody the vials were centrifuged at 4,800 *g* at 0°C for 5 min. The supernatant was aspirated using a specially mounted yellow Eppendorf pipette tip that guaranteed rapid and reproducible removal. The pellets were each washed three times with 1 ml of isotonic buffer, allowing 20 min for sufficient diffusion of unbound antibody each time, and resuspended with 500 μl of isotonic buffer.

Radioactive Labeling of Antibodies

For radioactive labeling, the Z22 antibody suspension was incubated with 11.1 kBq of ^{125}I-labeled streptavidin (Amersham Corp.; IM.236, 740–1480 kBq/μg, 3.7 MBq/ml, brought to 74–148 kBq/μg with nonlabeled streptavidin; Bethesda Research Laboratories, Gaithersburg, MD; Gibco Laboratories, Grand Island, NY). Labeling of bound HMG-17-specific monoclonal antibodies was with 11.1 kBq ^{125}I-labeled streptavidin brought to 7–15 kBq/μg with nonlabeled streptavidin. Cpm values were standardized to 74–148 kBq/μg. The incubation was the same as the fluorescence labeling with the exception of the final wash. The supernatant and upper parts of the pellet were removed by aspiration to leave exactly 100 μl. 800 μl of Bray's solution were then added, the mixture was homogenized on a vortex, and radioactivity determined in a liquid scintillation counter by directly inserting the vial into a scintillation vial.

Binding of Antibodies in the Presence of Camptothecin

Encapsulation of cells and permeabilization of nuclei were performed as described above. After sedimentation of microbeads, subsequent washes in isotonic buffer as well as incubation with the respective antibody were in the presence of 60 μM camptothecin. Camptothecin was diluted from a 20-mM stock solution in dimethylsulfoxide (29). The respective control experiments with no antibody added contained 0.5% (vol/vol) dimethylsulfoxide. Washes to remove nonbound antibody and labeling with ^{125}I-labeled streptavidin were in the presence of 60 μM camptothecin.

Preincubation with Polyclonal Anti-Z-DNA Antibodies

A nonbiotinylated, polyclonal Z-DNA-specific antibody preparation from goats was used of which ~3% (wt/wt) represent the Z-DNA specific fraction (17); the varying amounts of antibody shown in Fig. 7 were corrected accordingly. Incubation with the polyclonal antibody preparation was for 2 h at room temperature as described above for the monoclonal antibodies but in the presence of 60 μM camptothecin. Most of the unbound polyclonal antibodies were removed by washing the suspension once in isotonic buffer, followed by incubation with either 0.28 μg of Z-DNA-specific monoclonal antibody or 2.8 μg HMG-17-specific monoclonal antibody for 2 h still in the presence of 60 μM camptothecin. Labeling with ^{125}I-labeled streptadivin was also performed in the presence of 60 μM camptothecin.

Results

An overview of agarose microbeads containing live mouse myeloma cells is shown in Fig. 1 *a*. The focal plane cuts across the sphere yielding a sharp circular boundary at the periphery. The well-preserved morphology of the cells is demonstrated at a higher magnification of individual cell groups within the beads (Fig. 1 *b*). In these pictures, drops containing the encapsulating microbeads were visualized using Nomarski optics. The apparent three-dimensionality of the cells is due to differences in refractive index, and nucleoli are especially prominent. The cells maintain their positions in the agarose gel, and cells located in the focal plane produce a sharp image. The cytoplasm is clearly distinguished from the nucleus, which shows nucleoli if the cell is situated

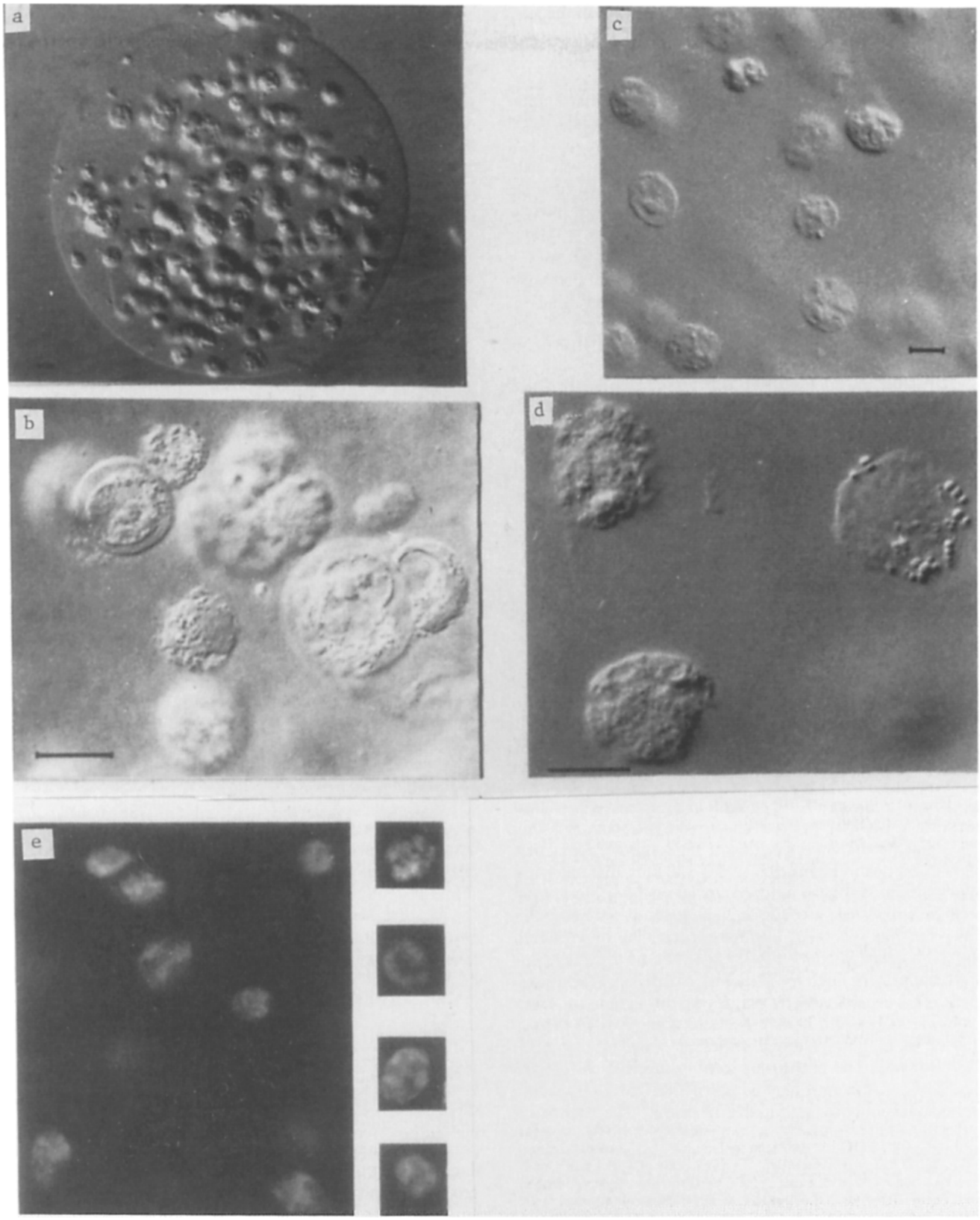

Figure 1. Microscopy of mouse myeloma cells and permeabilized nuclei encapsulated in agarose microbeads. (*a*) A single agarose bead containing cells (10 μm marker). (*b*) Higher magnification (10 μm marker). Nomarski optics is used, which is based on differences in refractive index, and nucleoli are especially prominent. (*c*) Microscopy of permeabilized nuclei encapsulated in agarose microbeads (10 μm marker). (*d*) Nuclei at higher magnification (10 μm marker). (*e*) Fluorescence of isotonic encapsulated permeabilized nuclei exposed to a low concentration of anti-Z-DNA antibody (0.28 μg/500 μl). This was followed by addition of FITC-labeled streptavadin. Four single nuclei on the right were selected for optically favorable conditions when they were found in the focal plane.

in an optically favorable position. The permeabilized nuclei are shown in Figs. 1, *c* and *d* after the cytoplasm has been lysed. The nuclei are morphologically intact and nucleoli remain prominent.

To obtain information about the content and distribution of Z-DNA in the permeabilized, microbead-encapsulated nuclei, they were incubated with biotinylated monoclonal anti-Z-DNA antibody (Z22). This anti-Z-DNA antibody reacts with Z-DNA irrespective of its nucleotide sequence (19, 21, 25). For microscopic observation a second incubation with FITC-streptavidin was carried out to produce fluorescent-labeled antibody-Z-DNA complexes. Micrographs of such fluorescent nuclei are shown in Fig. 1 *e*. Low concentrations of antibody were used, as discussed below. For purposes of comparison, encapsulated permeabilized nuclei were also stained with DAPI, which labels total nuclear DNA (results not shown). Fig. 1 *e* shows the formation of Z-DNA antibody complexes in unfixed, metabolically active nuclei in a native state under isotonic conditions. Isotonic conditions are taken as 150 mM in monovalent cations (10, 11). The distribution of these fluorescent complexes within the nuclear substructure is similar to the pattern of total DNA staining with DAPI. Control experiments were carried out under identical conditions, using a biotinylated monoclonal antibody against bacterial chloramphenicol acetyltransferase (CAT),[1] an enzyme that does not occur in myeloma cell nuclei. A weak, diffuse fluorescence staining was obtained without any substructure (results not shown).

Binding of Radioactively Labeled Monoclonal Antibodies to Permeabilized Nuclei

Quantitative binding experiments were carried out by adding radioactive streptavidin after the Z22 monoclonal antibodies had been incubated with the permeabilized nuclei in the same isotonic medium. The rate at which antibody molecules become bound to the nucleus is shown in Fig. 2. On incubation with a low concentration of anti-Z-DNA antibody, an initial lag period (20–40 min) is seen, possibly reflecting the diffusion kinetics of the antibody molecules into the permeabilized nuclei. By 80–120 min, maximum antibody attachment has developed. In the antibody binding experiments reported here, 120-min incubations were used unless otherwise specified. Control experiments were carried out using a monoclonal antibody against a nonhistone chromosomal protein, high mobility group protein 17 (HMG-17) (5, 6). This protein is found distributed throughout the chromatin and is believed to be associated with ~5% of the nucleosomes. HMG-17 is found at a higher concentration in active chromatin and at a lower level in inactive chromatin. Fig. 2 shows the rate of binding of the HMG-17-specific monoclonal antibody over a 2-h time period. The binding kinetics are similar to that found with the Z22 antibody, except that binding is somewhat faster at early times. By 2 h the binding is beginning to plateau in a manner similar to that seen with the Z-DNA-specific monoclonal antibody.

Fig. 3 *a* shows the binding of the monoclonal antibody against the nonhistone chromatin protein HMG-17 as a function of increasing antibody concentration. An increase in antibody binding begins to occur at 0.15 μg and it reaches a plateau of maximal binding near 0.7 μg. Thereafter there is no significant increase in binding even though the antibody concentration increases 200-fold. HMG-17 is a chromatin protein that has been well-characterized both in terms of its distribution and the amounts present. Each binding experiment contains 2.5×10^6 nuclei, and it is known that each nucleus contains ~2×10^7 nucleosomes. It has been estimated that ~5% of the nucleosomes contain HMG-17 (5); thus, using the measured specific activity of the antibody-streptavidin complex we can estimate that there are about twice as many HMG-17 molecules in the nuclei as there are HMG antibody molecules bound in the plateau (Fig. 3 *a*). This control experiment suggests that the antibody is diffusing through the entire nucleus rather than simply penetrating into its peripheral segment. That there are twice as many HMG-17 molecules as antibody molecules may be related to the fact that there are two antibody combining sites per antibody molecule. There is no rise in the binding of the HMG-17-specific monoclonal antibody at very high antibody concentrations.

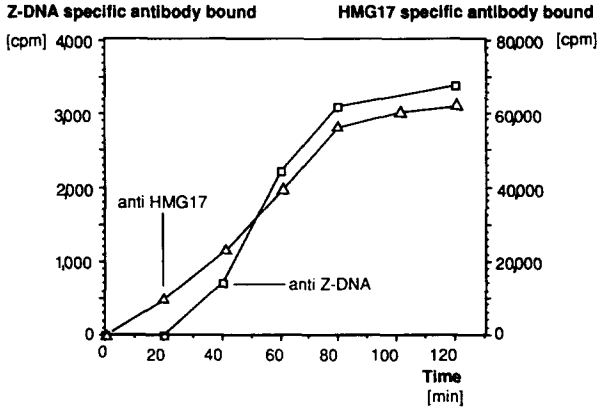

Figure 2. Kinetics of the binding of radioactively labeled anti-Z-DNA or anti-HMG-17 antibody to permeabilized nuclei under isotonic conditions. 0.28 μg of anti-Z-DNA antibody Z22 or 2.8 μg of anti-HMG-17 was added per 500 μl of agarose microbead suspension. The final incubation time with the antibody was varied to obtain a time course for binding. All points are the average of three independent experiments.

Anti-Z-DNA antibodies influence the equilibrium between right-handed B-DNA and left-handed Z-DNA (16, 17). Our interest is not directed toward Z-DNA that accumulates due to the presence of the anti-Z-DNA antibody but rather toward the presence, if any, of preexisting Z-DNA. To this end, we explored the effect of increasing antibody concentration on the binding of anti-Z-DNA antibodies to permeabilized nuclei. As above, the standard experiment used 500 μl of suspended agarose beads containing 2.5×10^6 nuclei with ~5 μg of DNA. The anti-Z-DNA antibody (Z22) was added to permeabilized nuclei in an isotonic medium with amounts ranging from 0.0056 to 280 μg. After considerable washing, an excess of radioactively labeled streptavidin was added. Fig. 3 *b* shows that the binding of biotinylated Z22-antibody to the permeabilized nuclei is constant from 0.28 to >28 μg, a 100-fold change in concentration. The controls in Fig. 3 *b* show low and constant values for either anti-

1. *Abbreviations used in this paper*: CAT, bacterial chloramphenicol acetyltransferase; HMG-17, high mobility group protein 17; LiDS, lithium dodecyl sulfate.

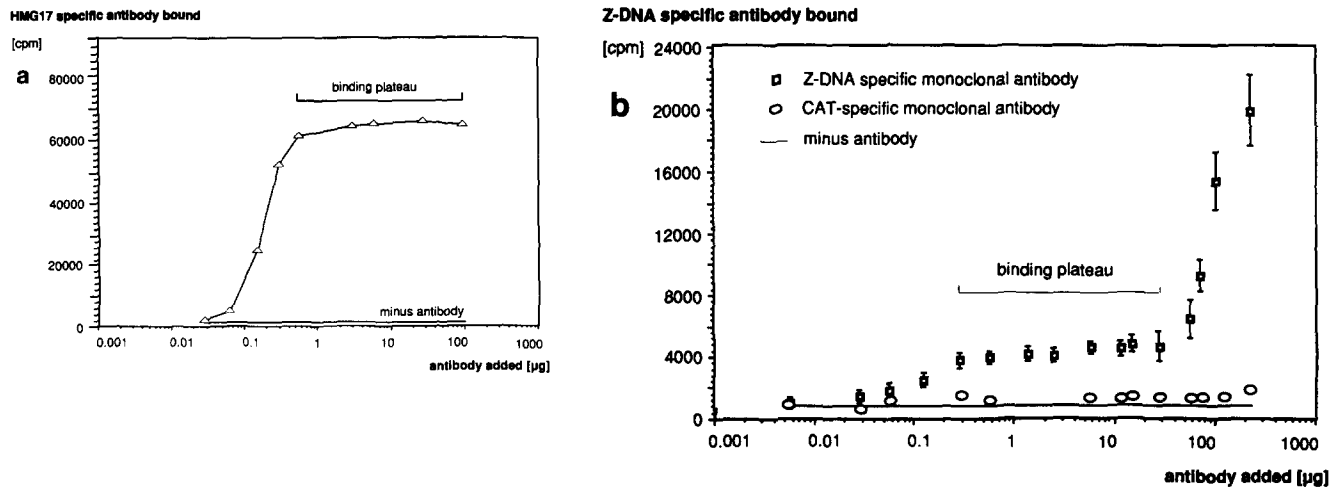

Figure 3. Quantitation of the binding of antibodies to permeabilized nuclei under isotonic conditions. Complex formation is measured as a function of antibody concentration. (*a*) Binding of HMG-17-specific monoclonal antibody: Permeabilized nuclei were incubated with varying amounts of anti-HMG-17 antibody in the same isotonic medium. Labeling was carried out as described in Fig. 2. The amount of complex remaining after washing is shown. All points are the average of three independent experiments. The control with no antibody added is the solid line. A plateau of binding is seen but without a sharp rise at high antibody concentrations. (*b*) Binding of Z-DNA-specific monoclonal antibody: Two nonspecific background binding experiments are shown here. In one, the antibody was omitted from the reaction mixture (*solid line*). In the second, nonspecific biotinylated monoclonal anti-CAT antibody was added. Standard deviation of the mean values is indicated as calculated from 5 to 20 single determinations. It can be seen that the antibody to Z-DNA reaches a plateau of binding over a 100-fold increase in antibody concentration. More binding is seen at even higher antibody concentration. The radioactive labeling was as described in Materials and Methods. The suspension was incubated with 11.1 kBq of ^{125}I-labeled streptavidin or 111 kBq in assays containing <2.8 µg of Z22 antibody.

body to CAT or for the buffer alone. Because the binding of Z22 antibody is insensitive to antibody concentration in the plateau region, we suggest that it is due to preexisting Z-DNA.

At much higher concentrations of antibody the amount of antibody binding increases dramatically, in striking contrast to the binding of HMG-17-specific antibodies (Fig. 3 *a*). We know that anti-Z-DNA antibodies can perturb the B-Z equilibrium by trapping Z-DNA, especially at high concentrations (16, 17). Thus, it seems likely that this rise in binding at high antibody concentration represents the induction of Z-DNA either by antibody molecules directly or by displacement of Z-DNA binding proteins followed by antibody attachment.

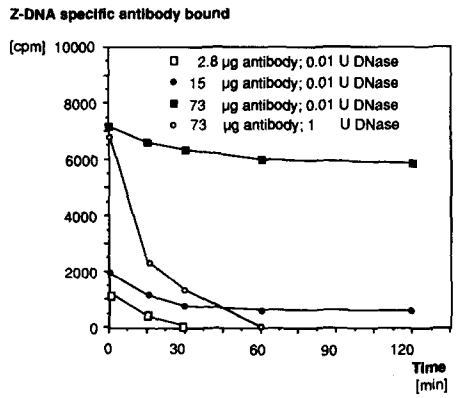

Figure 4. The effect of preincubation with DNase I on the binding of anti-Z-DNA antibodies to permeabilized nuclei. The preincubation was carried out at room temperature and nicking amounts of *E. coli* DNase I (0.01 U) as well as larger degrading amounts (1.0 U) were used. The lowest antibody concentration (2.8 µg) is from the concentration independent plateau region of Fig. 3. The higher concentrations are associated with antibody "induced" Z-DNA formation.

Torsional Strain in DNA Stabilizes Antibody-Z-DNA Complexes

The DNA in these nuclei has been shown to be intact (3). We can ask whether the Z-DNA antibody attachment is due to torsional strain in the DNA. This was addressed by experiments in which the nuclei were first preincubated with low, nicking amounts of *Escherichia coli* DNase I (Fig. 4).

Using an amount of antibody found in the binding plateau (2.8 µg as in Fig. 3 *b*), preincubating with 0.01 U DNase leads to a complete loss of binding within 30 min. The antibody binding at this concentration was interpreted as due to preexisting Z-DNA because it was independent of antibody concentration. This binding is entirely eliminated by small, nicking amounts of DNase I.

When larger amounts of antibody are added (15 or 73 µg), higher initial levels of radioactive binding are observed in agreement with Fig. 3 *b*. However, preincubation with nicking amounts of DNase I removes only part of the binding of anti-Z-DNA antibodies. Nicking the DNA to release torsional strain does not prevent the antibody from inducing Z-DNA formation (or trapping the formed Z-DNA) when the antibody is added later at high concentrations. However, preincubation with lytic amounts of DNase I (1 U) results in rapid loss of DNA and an inability to induce Z-DNA formation after 60 min, even with high concentrations of antibody (Fig. 4).

The experiments with nicking amounts of DNase I suggest that torsional strain in the DNA may be important in maintaining the apparently preexisting Z-DNA that is detected in the concentration-independent range of antibody binding in

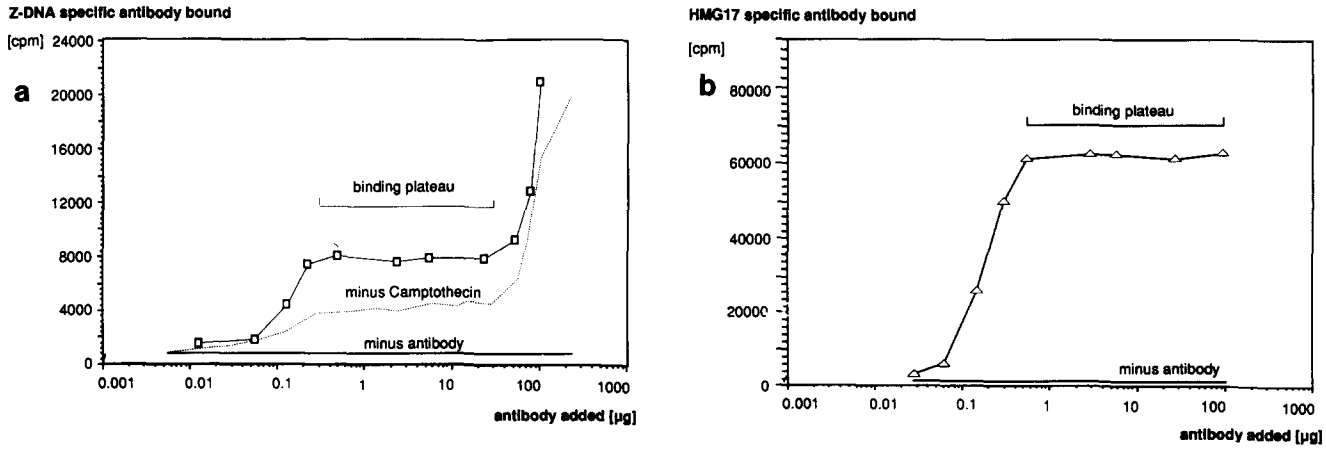

Figure 5. The binding of Z-DNA-specific and HMG-17-specific monoclonal antibodies to encapsulated permeabilized nuclei in the presence of the topoisomerase I inhibitor camptothecin (60 μM) as a function of antibody concentration. All points are the average of three independent experiments. The solid line is the minus antibody control. (*a*) The binding of Z-DNA-specific monoclonal antibody (Z22). The level of binding in the absence of camptothecin (Fig. 3 *b*) is represented by the dashed line. A plateau of binding is found in the presence of camptothecin but at a higher level than in Fig. 3 *b*. At high antibody concentrations where increased binding is seen in Fig. 3 *b*, a similar increase in binding is seen in the presence of camptothecin. (*b*) The binding of HMG-17 specific monoclonal antibody.

the plateau region. DNA torsional strain is believed to be maintained by the cellular DNA topoisomerases. Eukaryotic cells contain topoisomerases I and II, and the latter is believed to be important in disentangling daughter DNA duplexes after replication. Topoisomerase I relaxes DNA and it is believed to have an important role in maintaining the level of torsional strain. We have investigated this directly by using the topoisomerase I inhibitor camptothecin (7). Experiments were carried out in which the nuclei were incubated with 60 μM camptothecin. Monoclonal antibodies were then added at varying concentrations against Z-DNA and against HMG-17, respectively. The results of the anti-Z-DNA experiments are shown in Fig. 5 *a*. The dotted line shows the level of antibody bound in the absence of camptothecin, as presented in Fig. 3. It can be seen that with camptothecin treatment the plateau is maintained but at slightly more than a twofold increase in the level of anti-Z-DNA binding. As in previous experiments, very high levels of antibody give rise to the much larger binding that we have termed induced Z-DNA. In contrast, the anti-HMG-17 control experiments in the presence of camptothecin (Fig. 5 *b*) are essentially unchanged from results without the inhibitor (Fig. 3 *a*). These findings are consistent with the results of experiments involving nicking amounts of DNase I. Inhibition of topoisomerase I prevents the gradual loss of DNA torsional strain. The higher level of torsional strain stabilizes more Z-DNA; this is reflected in a higher level of bound antibody but it still remains independent of antibody concentration in the plateau region. This reinforces our interpretation of the plateau as a measure of preexisting Z-DNA. In contrast, the binding of HMG-17-specific monoclonal antibody, which is simply a measure of the amount of that protein in the nuclei, is unaffected by the change in DNA torsional strain.

We have carried out experiments to ask what is the effect of preincubation on the binding of anti-Z-DNA antibodies. Fig. 6 *a* shows the effect of preincubation at room temperature on the subsequent binding of anti-Z-DNA antibodies. Three different antibody concentrations are used, the low level in the plateau region (2.8 μg) and higher levels associated with induced Z-DNA formation (15 and 73 μg). Preincubation results in a steady decrease in the amount of bound antibody. The values drop slightly after 30 min preincubation and by 120 min most of the complex formation is lost for both the 2.8 and 15 μg additions of antibody. The higher value of 73 μg antibody still retains a significant component of the induced Z-DNA complex.

The decay in antibody binding shown in Fig. 6 *a* could be due to the disintegration of nuclei and the release of nucleases or, alternatively, to the continued action of topoisomerase I leading to a relaxation of DNA and the loss of Z-DNA formation. To distinguish between these interpretations, experiments were carried out using nuclei that were incubated in the presence of 60 μM camptothecin to inhibit topoisomerase I. As shown in Fig. 6 *b*, the binding of the anti-Z-DNA antibody did not change as a function of time when the cells were incubated in the presence of the topoisomerase I inhibitor in contrast to the gradual loss of binding seen in Fig. 6 *a*. To test for the breakdown of nuclei, similar experiments were done to measure the binding of the antibody against HMG-17 as a function of time (Fig. 6 *b*). Its binding remains constant over the time course of the experiment, suggesting that nuclei are not disintegrating. Thus, simple preincubation results in relaxation of DNA with time due to the action of topoisomerase I, with no nuclear disintegration. Topoisomerase I inhibition leaves the anti-Z-DNA antibody binding at a constant level in the plateau region throughout the 2-h incubation period.

Competition between Monoclonal and Polyclonal Antibodies against Z-DNA

Polyclonal antibodies are available that have been raised against poly(dG-dC) stabilized in the Z conformation (16, 17). The monoclonal antibody Z22 raised against the same antigen was selected for these experiments because it is known to bind to Z-DNA independently of nucleotide sequence (19). It has been studied extensively and footprints

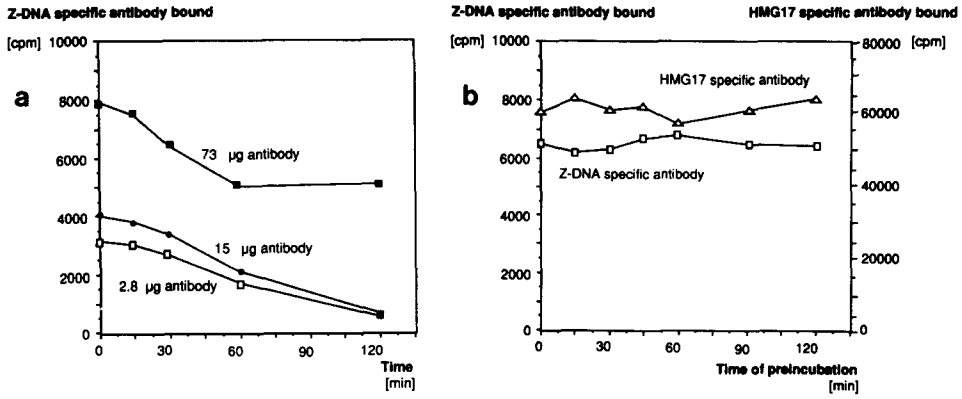

Figure 6. The effect of preincubation at room temperature of encapsulated permeabilized nuclei on the binding of the monoclonal antibodies. All points are the average of three independent experiments. (*a*) The binding of anti-Z-DNA antibody (Z-22) at three different concentrations. The lowest concentration is in the plateau region. It can be seen that there is a steady drop with time, although the binding finally is stabilized at the highest antibody concentration. (*b*) The effect of the topoisomerase I inhibitor camptothecin (60 μM) on the binding of anti-Z-DNA antibody Z22 (0.28 μg) and anti-HMG-17 (2.8 μg). The binding is independent of time for this preincubation period for both antibodies.

have been obtained of it against Z-DNA (25). The epitope for its binding is probably found in the sugar-phosphate backbone of the Z-DNA conformation. In contrast, the polyclonal antibodies are known to contain antibodies directed against a large number of epitopes. Some immunogenic epitopes may be associated with the purine and pyrimidine rings. It could be argued that the binding of the monoclonal antibody Z22 is nonspecific, despite its sensitivity to torsional strain. If it were specific, there should be competition between it and the nonbiotinylated polyclonal antibody. In such a competition experiment, longer incubation periods are used. To prevent the loss of the Z-DNA due to continued topoisomerase activity as in Fig. 6 *a*, the nuclei were maintained in 60 μM camptothecin to inhibit topoisomerase I. The nuclei were first incubated for 2 h with varying amounts of IgG fractions containing unlabeled polyclonal antibody against Z-DNA. After this the nonbiotinylated antibody was washed out and the nuclei were then incubated for 2 h with 0.28 μg of labeled Z22 monoclonal antibody. Fig. 7 shows the binding of the monoclonal antibody as a function of increasing concentration of polyclonal antibody. Little effect is seen up to a concentration of ~0.3 μg of added polyclonal antibody. At higher concentrations, there is a gradual loss of the binding of the labeled monoclonal antibody. There is competition between the binding of unlabeled polyclonal antibody and the labeled monoclonal antibody. Included in Fig. 7 are also control experiments in which the binding of monoclonal antibodies against the chromatin protein HMG-17 was measured. This binding is unaffected by the addition of increasing amounts of polyclonal antibody against Z-DNA.

The Effect of Deproteinization and Cytological Fixation

It is known that changes in solvent conditions can cause conformational changes in chromatin, and these changes may be crucial for the transition between B- and Z-DNA. We have investigated the effect of varying the treatment of the encapsulated nuclei on anti-Z-DNA antibody binding. Fig. 8 *a* shows experiments in which binding was measured after the nuclei had been exposed to different salt concentrations during permeabilization. A "noninducing" antibody concentration was used in these experiments. Only slight changes are observed on varying salt from 0.025 to 0.17 molarity of monovalent cations. In the experiments shown in Fig. 8, controls were also carried out by adding biotinylated anti-CAT antibodies followed by radioactively labeled streptavidin. Those results (not shown) were 10–15% greater than the "no antibody" control that is plotted in the figure. Anti-Z-DNA antibody binding is not very sensitive to changes in this range of salt concentration. The effect of treating cells with higher concentrations of salt will be presented elsewhere.

What is the effect of protein removal? Lithium dodecyl sulfate (LiDS) treatment is known to remove most proteins. Cook (3) has shown that this treatment yields DNA that is unbroken, and it has maximum negative supercoiling, which would stabilize Z-DNA. Under this treatment, fourfold increase of complex formation with the anti-Z-DNA antibody is observed (Fig. 8 *b*). Z-DNA has been frequently studied in tissues by fixation, followed by addition of anti-Z-DNA antibodies for fluorescence staining (15, 20, 22). The effect of two such treatments is shown quantitatively in Fig. 8 *b*. These lead to an approximately threefold elevated signal compared to the preexisting Z-DNA found in the isotonic permeabilization. The level of the signal is not as high as with LiDS, presumably because the fixation treatment strips away only part of the proteins from the chromosomal DNA (2, 4).

Discussion

Much work has been carried out in an attempt to measure Z-DNA content of nuclei using anti-Z-DNA antibodies. These experiments all have to be analyzed in the light of two significant artifacts that can perturb the measurement. The first is the state of the chromatin in the nucleus. To what extent is it close to in vivo or have experimental conditions significantly altered the chromatin? The second question is the extent to which the use of anti-Z-DNA antibody binding to the DNA significantly disturbs the B-DNA/Z-DNA equilibrium? In the experiments described above we have considered both of these issues.

The quality of the encapsulated permeabilized physiological active nuclei has been addressed by using the novel approach of Jackson and Cook. They have shown that HeLa cells trapped in agarose beads as well as their permeabilized nuclei are morphologically intact (9). The myeloma cells used in these experiments are also intact and the permeabilized nuclei prepared after treatment with 0.5% Triton X-100

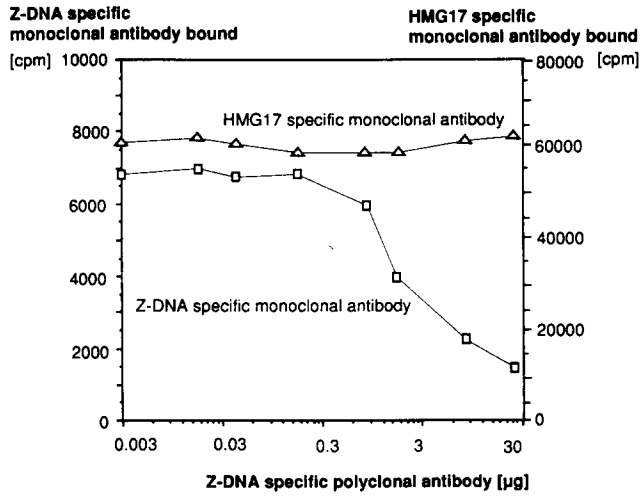

Figure 7. Effect of adding polyclonal anti-Z-DNA antibodies on the binding of monoclonal antibodies against Z-DNA and HMG-17 nonhistone chromatin proteins. The encapsulated permeabilized nuclei were first incubated with unlabeled polyclonal anti-Z-DNA antibodies at varying concentrations. Subsequently, the binding of monoclonal antibodies against Z-DNA and HMG-17 was measured. All points are the average of three independent experiments. The binding of anti-HMG-17 is not affected by the preincubation. However, competitive binding is seen for the anti-Z-DNA antibodies.

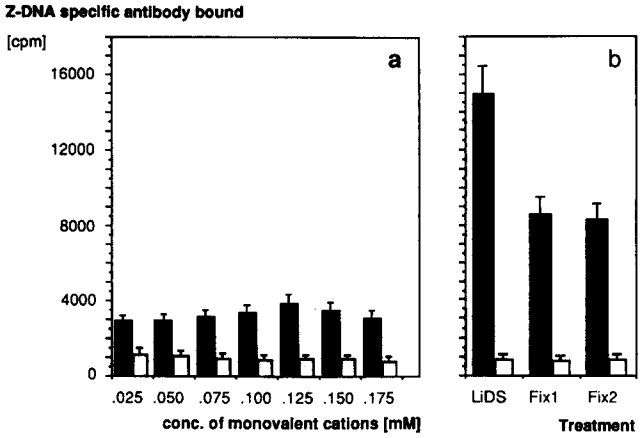

Figure 8. The influence of deproteinization and fixation conditions on complex formation at a noninducing antibody concentration (2.8 μg). (*a*) Dependence on the concentration of monovalent cations below and near the isotonic range. The cation concentrations indicated on the horizontal axis were used during the permeabilization treatment. (*b*) The effect of LiDS (cell lysis in 1% [wt/vol] LiDS, 100 mM EDTA, 10 mM Tris-HCl, pH 8.0, see Cook [reference 3]). Two different cytological fixation conditions are also shown. These were carried out on encapsulated permeabilized nuclei. (Fix 1: 45% acetic acid, 10 min; fix 2: ethanol/acetic acid 3:1, 10 min, followed by 45% acetic acid, 10 min [15, 22].) 2.8 μg of Z22 antibody were used per assay. The standard deviations of the mean refer to six determinations each. In these experiments, an anti-CAT antibody control was also carried out. The results (not shown) were 10–15% higher than the minus antibody control.

in an isotonic buffer look similar to those in the intact cell (Fig. 1). The nuclei are transcriptionally active (11) and in quantitative experiments Jackson and Cook have shown that the rate of DNA synthesis in these nuclei is 85% of the rate in vivo (12). In these preparations the DNA has been shown to be unbroken and under torsional strain (3, 9, 10, 11). The buffer medium is isoltonic but it should be noted that it contains 1 mM EDTA and no divalent cations to keep nucleases inactive. This is the only preparative method that allows the DNA of chromatin to remain intact so it can be studied in an isotonic salt solution. Individual nuclei generally aggregate under these conditions, but the agarose matrix keeps them separate. These permeabilized nuclei are good preparations for accessing DNA torsional strain and measuring the Z-DNA content, since they are metabolically active.

The control experiments using anti-HMG-17 antibody address the question of how well antibodies measure the content of nuclear components. On increasing the antibody concentration, a binding plateau is found near one antibody molecule per two HMG-17 molecules (Fig. 3 *a*). This could be due to the fact that only half of them are accessible; alternatively, each antibody binding site may find an HMG-17. Further, this binding plateau does not change in other experiments in which torsional strain in DNA is altered.

It has been shown that anti-Z-DNA antibodies can induce Z-DNA formation, especially if the antibodies bind with high affinity (16, 17). The monoclonal antibody used in this experiment is not a high affinity antibody but it has the property of recognizing Z-DNA independent of nucleotide sequence (19, 21). A "footprint" has revealed how the antibody binds to Z-DNA (25). The binding of anti-Z-DNA antibodies to the permeabilized nuclei is a multiphasic process as a function of antibody concentration: no binding is found at very low concentrations but once a threshold concentration is reached, the binding remains constant. The amount of radioactive antibody bound to the nuclei remains constant over a 100-fold change in antibody concentration (Fig. 3 *b*). Only upon going to much higher antibody concentrations does the amount of bound antibody increase. This is probably due to the antibody trapping Z-DNA in the B-Z equilibrium as implied in calling it induced Z-DNA. Addition of nicking amounts of DNase I cause a rapid loss in the binding of antibody present at a concentration in the plateau range. However, the induced Z-DNA-antibody complex is not greatly affected by DNA nicking. Likewise, inhibition of topoisomerase I leads to a higher plateau level of Z-DNA binding. We have interpreted these experiments as providing a basis for separating the Z-DNA found into preexisting and induced components. The lower antibody concentrations in the plateau region measure the preexisting component. That is taken as a measure of the amount of Z-DNA found in the nucleus under conditions in which the DNA is unbroken, the salt solution is isotonic, and the nucleus is active in both transcription and replication.

Is it likely that the plateau binding of anti-Z-DNA antibodies (Fig. 3 *b*) is not due to preexisting Z-DNA? For example, could a low concentration of the antibody "trigger" the induction of Z-DNA to produce the plateau? There are many reasons that such an interpretation is improbable. The constancy of binding over a 100-fold increase in concentration is most simply interpreted as preexisting Z-DNA. The variability of the plateau binding level in response to changes in DNA tor-

sional strain (increasing with topoisomerase I inhibition, lost entirely with DNA nicking) is fully interpretable in terms of our current understanding of the behavior and stabilization of Z-DNA.

In addition to trapping Z-DNA in the B-Z equilibrium, other mechanisms can be considered in the production of induced Z-DNA associated with high concentrations of antibody. Z-DNA binding proteins may be displaced by the antibody. Alternatively, chromosomal or nucleosomal proteins may be displaced leading to regions of unrestrained torsional stress in the nuclear DNA. Finally, there may be direct induction of the Z conformation in protein-free stretches of DNA by cooperative binding of antibody molecules. To get a correct assessment of preexisting Z-DNA, it is important to use conditions that prevent all of these from occurring.

The experiments shown in Fig. 8 *b* reveal the extent of fixation artifacts. Removal of virtually all chromosomal proteins by LiDS without nicking the DNA results in a fourfold increase in the binding of anti-Z-DNA antibodies even at the low concentration of antibodies found in the plateau region. This Z-DNA stabilization is undoubtedly due to the release of negative supercoiling in the DNA even in the presence of noninducing antibody concentrations. Classical fixation procedures produce approximately a threefold increase in antibody binding. Proteins are removed by fixation, but not as many as with the LiDS treatment. It is likely that even higher levels of antibody binding would be found if concentrations of antibody were used that induced Z-DNA formation.

Our conclusions are different from those of Hill and Stollar who failed to observe antibody binding in microdissected *Drosophila* polytene chromosomes (8). The different results could be due to two effects. First, the microdissected chromosome is put into a buffer environment that is quite different from that of the intact nucleus which could lead to a loss of Z-DNA. In addition, it is likely that during the microdissection and further processing of polytene chromosomes, endogeneous topoisomerases were active and resulted in a loss of preexisting Z-DNA as we have observed during preincubation (Fig. 4 *b*). Hill and Stollar found that it required a removal of chromosomal proteins by a fixation process to produce antibody binding. Recent work by Lancillotti et al. have demonstrated binding of anti-Z-DNA antibodies to unfixed *Drosophila* polytene chromosomes (18). The difference between their results and Hill and Stollar's suggests that specific manipulations used in isolating individual polytene chromosomes may result in the loss of Z-DNA.

The DNA in the nucleus is likely to be in a dynamic state, with some processes generating negative torsional strain and other processes reducing the strain. Topoisomerase I reduces negative torsional strain. We have made two measurements of Z-DNA antibody binding in the noninducing plateau region. One measurement after 120 min of incubation without camptothecin (Fig. 3 *b*) and a second in the presence of camptothecin (Fig. 5 *a*). Based on the experiments described in this article, we conclude that a level of preexisting Z-DNA is found in the myeloma nucleus when it is measured under conditions in which there is no induced Z-DNA formation. Using the quantitative data presented above, the known specific activity of streptavidin and the fact that there is one biotinylation site per antibody, we find one antibody molecule is bound per 100 kbp of DNA in the absence of camptothecin and one antibody per 50 kbp when topoisomerase I is inhibited. If we assume that topologically isolated domains of chromatin contain ~100 kb (~500 nucleosomes), we estimate that there is on the average about one or two preexisting Z-DNA binding sites for an antibody molecule per domain, or one or two per 500 nucleosomes. However, there is no reason to believe that these sites are randomly distributed, and they may in fact be concentrated in certain regions.

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References

1. Azorin, F., and A. Rich. 1985. Isolation of Z-DNA binding proteins from SV40 minichromosomes: evidence for binding to the viral control region. *Cell.* 41:365–374.
2. Cartwright, I. L., S. M. Abmayr, G. Fleischmann, K. Lowenhaupt, S. C. R. Elgin, M. A. Keene, and G. C. Howard. 1982. Chromatin structure and gene activity: the role of nonhistone chromosomal proteins. *Crit. Rev. Biochem.* 13:1–86.
3. Cook, P. R. 1984. A general method for preparing intact nuclear DNA. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1837–1842.
4. Dick, C., and E. W. Jones. 1968. The effect of acetic acid containing fixatives on the histone content of calf thymus deoxyribonucleoprotein and calf thymus tissue. *Exp. Cell Res.* 51:626–638.
5. Dorbic, T., and B. Wittig. 1986. Isolation of oligonucleosomes from active chromatin using HMG17-specific monoclonal antibodies. *Nucleic Acids Res.* 14:3363–3376.
6. Dorbic, T., and B. Wittig. 1987. Chromatin from transcribed genes contains HMG17 only downstream from the starting point of transcription. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2393–2399.
7. Drlica, K., and R. J. Franco. 1988. Inhibitors of DNA Topoisomerases. *Biochemistry.* 27:2253–2259.
8. Hill, R. J., and B. D. Stollar. 1983. Dependence of Z-DNA antibody binding to polytene chromosomes on acid fixation and DNA torsional strain. *Nature (Lond.).* 305:338–340.
9. Jackson, D. A. 1986. Replication occurs at a nucleoskeleton. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1403–1410.
10. Jackson, D.A., and P. R. Cook. 1985. A general method for preparing chromatin containing intact DNA. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:913–918.
11. Jackson, D. A., and P. R. Cook. 1985. Transcription occurs at a nucleoskeleton. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:919–925.
12. Jackson, D. A., and P. R. Cook. 1986. A cell cycle dependent DNA polymerase activity that replicates intact DNA in chromatin. *J. Mol. Biol.* 192:65–76.
13. Jaworski, A., W. T. Hsieh, J. A. Blaho, J. E. Larson, and R. D. Wells. 1987. Left-handed Z-DNA in vivo. *Science (Wash. DC).* 238:773–777.
14. Jovin, T. M., D. M. Soumpasis, and L. P. McIntosh. 1987. The transition between B-DNA and Z-DNA. *Annu. Rev. Phys. Chem.* 38:521–557.
15. Jovin, T. M., J. H. van de Sande, D. A. Zarling, D. J. Arndt-Jovin, F. Eckstein, H. H. Fuldner, C. Greider, I. Grieger, E. Hamori, B. Kalisch, L. P. McIntosh, and M. Robert-Nicoud. 1982. Generation of left-handed Z-DNA in solution and visualization in polytene chromosomes by immunofluorescence. *Cold Spring Harbor Symp. Quant. Biol. XLVII.* 1: 143–154.
16. Lafer, E. M., R. Sousa, A. Rashid, A. Rich, and B. D. Stollar. 1986. The effect of anti-Z-DNA antibodies on the B-DNA-Z-DNA equilibrium. *J. Biol. Chem.* 261:6439–6443.
17. Lafer, E., R. Sousa, and A. Rich. 1985. Anti-Z-DNA antibody binding can stabilize Z-DNA in relaxed and linear plasmids under physiological conditions. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3655–3660.
18. Lancillotti, F., M. C. Lopez, P. Arias, and C. Alonso. 1987. Z-DNA in transcriptionally active chromosomes. *Proc. Natl. Acad. Sci. USA.* 84: 1560–1564.
19. Moller, A., J. E. Gabriels, E. M. Lafer, A. Nordheim, A. Rich, and B. D. Stollar. 1982. Monoclonal antibodies recognize different parts of Z-DNA.

J. Biol. Chem. 257:12081–12085.
20. Morgenegg, G., M. R. Celio, B. Malfoy, M. Leng, and C. C. Kuenzle. 1983. Z-DNA immunoreactivity in rat tissues. *Nature (Lond.).* 303: 540–543.
21. Nordheim, A., M. L. Pardue, L. M. Weiner, K. Lowenhaupt, P. Scholten, A. Moller, A. Rich, and B. D. Stollar. 1986. Analysis of Z-DNA in fixed polytene chromosomes with monoclonal antibodies that show base sequence-dependent selectivity in reactions with supercoiled plasmids and polynucleotides. *J. Biol. Chem.* 261:468–476.
22. Pardue, M. L., A. Nordheim, E. M. Lafer, B. D. Stollar, and A. Rich. 1982. Z-DNA and the polytene chromosome. *Cold Spring Harbor Symp. Quant. Biol.* 47:171–176.
23. Peck, L. J., A. Nordheim, A. Rich, and J. C. Wang. 1982. Flipping of cloned $d(pCpG)_n$ $d(pCpG)_n$ DNA sequences from right to left-handed helical structures by salt. *Proc. Natl. Acad. Sci. USA.* 79:4560–4564.
24. Rich, A., A. Nordheim, and A. H.-J. Wang. 1984. The chemistry and biology of left-handed Z-DNA. *Annu. Rev. Biochem.* 53:791–846.
25. Runkel, L., and A. Nordheim. 1986. Chemical footprinting of the interaction between left-handed Z-DNA and anti-Z-DNA antibodies by diethylpyrocarbonate carbethoxylation. *J. Mol. Biol.* 189:487–501.
26. Singleton, C. K., J. Klysik, S. M. Stirdivant, and R. D. Wells. 1982. Left-handed Z-DNA is induced by supercoiling in physiological ionic conditions. *Nature (Lond.).* 299:312–316.
27. Stollar, B. D. 1986. Antibodies to DNA. *Crit. Rev. Biochem.* 20:1–36.
28. Zarling, D. A., D. J. Arndt-Jovin, M. Robert-Nicoud, L. P. McIntosh, R. Thomae, and T. M. Jovin. 1984. Immunoglobulin recognition of synthetic and natural left-handed Z-DNA conformations and sequences. *J. Mol. Biol.* 176:369–377.
29. Zhang, H., J. C. Wang, and L. F. Liu. 1988. Involvement of DNA topoisomerase I in transcription of human ribosomal RNA genes. *Proc. Natl. Acad. Sci. USA.* 85:1060–1064.