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Transcription is associated with Z-DNA formation in metabolically active permeabilized mammalian cell nuclei

(negative supercoiling/DNA replication/Z-DNA-specific antibody/biotin labeling)

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ABSTRACT **Mammalian cells have been encapsulated in agarose microbeads, and from these cells metabolically active permeabilized nuclei were prepared. Previously, we showed that biotin-labeled monoclonal antibodies against Z-DNA can be diffused into the nuclei and, over a specific concentration range, they will bind to Z-DNA within the nucleus in a concentration-independent manner. By using radiolabeled streptavidin, we showed that the amount of Z-DNA antibody bound is related to the torsional strain of the DNA in the nucleus. Relaxation of the DNA results in a decrease of Z-DNA formation, whereas increasing torsional strain through inhibiting topoisomerase I results in increased Z-DNA formation. Here we measure the influence of RNA transcription and DNA replication. Transcription is associated with a substantial increase in the binding of anti-Z-DNA antibodies, paralleling the increased level of RNA synthesized as the level of ribonucleoside triphosphate in the medium is increased. DNA replication yields smaller increases in the binding of Z-DNA antibodies. Stopping RNA transcription with inhibitors results in a large loss of Z-DNA antibody binding, whereas only a small decrease is associated with inhibition of DNA replication.**

Z-DNA exists in biological systems in a dynamic rather than a static state. B-DNA is the lower-energy conformation and to put DNA into the left-handed Z conformation, energy has to be expended. In the cell nucleus, DNA is found at various levels of unwinding or negative supercoiling. Negative supercoiling is a major factor in stabilizing left-handed Z-DNA (1–5). Through the action of topoisomerases, DNA loses negative supercoiling and the left-handed Z state flips back to the lower-energy right-handed B-DNA state. The experiments reported herein must be viewed in the context of an equilibrium between B-DNA and Z-DNA, which in the face of topoisomerases requires the input of a steady process to maintain the Z conformation.

A system has been developed for measuring the level of Z-DNA in metabolically active eukaryotic nuclei (6). Nuclear preparations developed by Jackson (7) and Jackson and Cook (8–10) are used in which living mammalian cells trapped in agarose microbeads maintain normal metabolism. Treating the cells with 0.5% Triton X-100 results in lysis of the cytoplasmic membrane and permeabilization of the nuclear membrane. The nucleus remains morphologically intact and these nuclei are metabolically active (7–10). They carry out DNA replication at a rate 85% that seen in the intact cell and maintain transcription. The nuclei are permeable to macromolecules and we have used (6) this system to measure the binding of monoclonal antibodies that bind to Z-DNA independent of the nucleotide sequence. A single biotin residue is conjugated to the monoclonal antibody so that the binding can be measured quantitatively by adding radiolabeled streptavidin. At low and intermediate antibody concentration, a plateau of constant antibody binding was found covering a hundredfold range of antibody concentration. At much higher antibody concentrations, significantly greater binding was observed. Experiments demonstrated that the high antibody concentrations were responsible for inducing the higher level of Z-DNA stabilization. The plateau of binding found at the lower antibody concentrations was called preexisting Z-DNA since the level of binding was independent of antibody concentration over a hundredfold range. The height of the binding plateau (or preexisting Z-DNA) was strongly influenced by torsional strain. Nicking DNA with nucleases resulted in a rapid lowering of the level of the binding plateau but had very little effect on the induced Z-DNA binding at higher antibody concentrations. However, stopping the action of the DNA-relaxing enzyme topoisomerase I with camptothecin (11) resulted in raising the level of the binding plateau (6). The question that naturally arises from these experiments is what physiological processes act *in vivo* to generate the negative supercoiling that is continuously being removed by the activity of topoisomerase I (12)? Here we have added either ribonucleoside triphosphates (rNTPs) or deoxynucleoside triphosphates (dNTPs) to measure the effect of transcription or replication on the binding of antibodies against Z-DNA. We find that transcription is associated with a marked increase in the level of Z-DNA antibodies bound in the plateau region. This may be one of the major physiological processes that generates negative supercoiling and thus stabilizes Z-DNA formation in the intact cell. Liu and Wang (13) have suggested that RNA polymerase movement generates negative supercoiling upstream from the transcription site. The increase in preexisting Z-DNA that we detect associated with transcription may be a consequence of that mechanism.

MATERIALS AND METHODS

Encapsulation of Cells and Permeabilization of Nuclei. The human cell line HL60 (a gift of K. Langmach, UKRVC Berlin) was grown in RPMI 1640 medium (GIBCO/BRL) supplemented with folic acid (40 mg/liter), $NaHCO_2$ (2 g/liter), nonessential amino acids (100-fold concentrate, 10 ml/liter), L-glutamine (4 mM), streptomycin/penicillin solution (10 units/ml), and 10% (vol/vol) horse serum; pH was adjusted to 7.2 with 1 M HCl. Mouse myeloma cells (X63-AG 8.6.5.3) used to relate our previous results (6) to the experimental conditions herein were grown in Dulbecco's modified Eagle's medium supplemented with $NaHCO_2$ (3.7 g/liter), L-glutamine (2 mM), pyruvate (1 mM), glucose (3.5 g/liter), streptomycin/penicillin solution (10 units/ml), and 10% (vol/vol) fetal calf serum. Encapsulation of cells was identical to published procedures (6–10). Permeabilization of nuclei was performed in the "new" buffer developed by Jackson *et al.* (14). After encapsulation and washing, the agarose microbeads were suspended in 3 vol of ice-cold new buffer (130 mM

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KCl/10 mM Na_2HPO_4/1 mM $MgCl_2$/1 mM Na_2ATP/1 mM dithithreitol, pH adjusted to 7.4 by adding 100 mM $KHPO_4$). Free Ca^{2+} and varying acidity of ATP batches were treated as in ref. 14. The microbead suspension was pelleted (3500 × *g*, 0°C, 5 min) and resuspended in new buffer containing 0.5% Triton X-100. The suspension was kept in an ice-water bath with gentle stirring for 20 min. After centrifugation (4800 × *g*, 0°C, 5 min), the microbead suspension was washed three times with 5 vol of new buffer without Triton X-100. New buffer was supplemented with 0.2% bovine serum albumin in the last wash.

Transcription and Replication in Encapsulated and Permeabilized Nuclei. Transcription and replication were assayed as described (7, 9). To maintain transcription or replication over a long period of time (to allow for quantitative binding of Z-DNA specific antibody), much higher concentrations of all four rNTPs and all four dNTPs were used. For transcription, the microbead suspension was washed once in 5 vol of transcription buffer [50 mM KCl/2 mM EDTA/10 mM spermidine/200 mM $(NH_4)_2SO_4$/1 mM dithithreitol/10% (vol/vol) glycerol/100 mM Tris·HCl, pH 7.6]. The microbead preparation was pelleted (4800 × *g*, 0°C, 5 min), resuspended in 10 ml of transcription buffer, and distributed into 500-μl volumes. For inhibitor assays, the inhibitor was added at this step. The suspension was kept on ice for 10 min. An ice-cold solution of 2 mM $MgCl_2$, 50 μM *S*-adenosylmethionine, ^{32}P-labeled UTP (74 kBq, 111 TBq/mmol, DuPont), and nonlabeled rNTPs in transcription buffer at concentrations indicated were added, and the suspension was kept on ice for another 10 min. $MgCl_2$ (10 mM) was added to compensate for chelation by the NTPs. Samples were incubated at 37°C for 90 min. For replication, the microbead suspension was washed once in 5 vol of replication buffer (new buffer supplemented with 4 mM $MgCl_2$) and kept on ice for 10 min. A solution of 0.1 mM GTP, 0.1 mM CTP, 0.1 mM UTP (1.0 mM ATP is already present in new buffer), ^{32}P-labeled dATP (74 kBq, 185 TBq/mmol, DuPont), and nonlabeled dNTPs at concentrations indicated was added. The suspension was kept on ice for another 10 min. $MgCl_2$ (10 mM) was added to compensate for chelation. Samples were incubated at 37°C for 90 min.

Binding of Monoclonal Antibodies. Biotinylated monoclonal Z-DNA-specific antibody (Z-22) was used in all experiments (15, 16). Antibody binding was measured as described (6), and 20 μl (740 kBq) of ^{125}I-labeled streptavidin (Amersham; 740–1480 kBq/μg brought to 74–148 kBq/μg with nonlabeled streptavidin) was used for the quantitation of antibody binding. The dependence of Z-DNA specific antibody binding on concentration of Z-22 was determined in new buffer supplemented with 0.2% bovine serum albumin. The influence of transcription or replication on Z-DNA-specific antibody binding was determined in transcription buffer or replication buffer, respectively. The influence of transcription and replication was assayed in transcription buffer supplemented with all four dNTPs, each at a concentration of 2.5 mM (hereafter when equimolar amounts of all four dNTPs or rNTPs are added, that molarity of dNTPs or rNTPs will be used, i.e., 2.5 mM dNTPs). Z-22 antibody [2.8 μg (4 μl)] was added to 500 μl of the microbead suspension (corresponding to 250 μl of the packed preparation containing about 2.5×10^6 permeabilized nuclei) after the above described preincubation on ice. $MgCl_2$ (10 mM) was then added and samples were incubated at 37°C for 120 min. Streptavidin was labeled with ^{125}I as described (6).

RESULTS

If an isotonic buffer is used to prepare naked nuclei, the nuclear material aggregates and cannot be used for metabolic experiments. Agarose encapsulation prevents aggregation. Our earlier experiments were carried out using an isotonic buffer ["old" buffer; 100 mM KCl/25 mM $(NH_4)_2SO_4$/1 mM EDTA/20 mM Tris·HCl, pH 7.6] (6). However, Jackson *et al.* (14) have found that the architecture of the nucleus is better preserved by slightly modifying the buffer. Phosphate buffer was used instead of Tris buffer and EDTA was eliminated. In its place 1 mM $MgCl_2$ and 1 mM ATP was added. In the old buffer, EDTA was used to chelate Mg^{2+} that might activate nucleases. In the new buffer, Mg^{2+} is present but its effective concentration is somewhat reduced by the addition of ATP, a physiological chelating agent. At this concentration, there is sufficient free Mg^{2+} to preserve heterochromatin structure but insufficient to activate nucleases because it is nearly all complexed to ATP. This formulation also enables most ATP-utilizing enzymes to function (14).

Our first experiments were carried out to measure the binding of antibodies against Z-DNA to these nuclear preparations in both the old (6) and the new buffers. Fig. 1*A* shows the result of binding the Z-DNA-specific monoclonal antibody to permeabilized myeloma nuclei in the old buffer. There was a binding plateau covering almost a hundredfold change of antibody concentration. At higher concentrations of antibody, increased Z-DNA antibody was found associated with the formation of induced Z-DNA (6). In earlier control experiments it was shown that monoclonal antibodies directed against a nonmammalian protein did not bind to the nuclei, even at high antibody concentrations (6, 17). By using the new buffer (Fig. 1*B*), the binding plateau was present at lower antibody concentrations and higher antibody concentrations still induced the formation of Z-DNA. This is similar to the results in Fig. 1*A* except that the binding plateau was somewhat narrower, covering a 50-fold range of antibody concentrations compared to the hundredfold range in Fig. 1*A*.

The HL60 cell type was adopted to explore the binding of Z-DNA antibodies in a human nucleus that can undergo a differentiation sequence. The pattern of binding of Z-DNA-specific antibodies to HL60 nuclei (Fig. 1 *C* and *D*) was very similar to that found for the mouse myeloma nuclei in the same buffer. The binding plateau was somewhat narrower; however, there was a clear distinction between the plateau of binding of preexisting Z-DNA and the induced binding at higher antibody concentrations. Antibody binding experiments were also carried out in the presence of 60 μM camptothecin to inhibit topoisomerase I. As reported earlier (6), by using the old buffer, a plateau 80–85% higher was found for preexisting DNA, in both myeloma and HL60 cells (Fig. 1 *A* and *C*). However, in the buffer containing Mg^{2+} and ATP, a much smaller (9–10%) increase is seen (Fig. 1 *B* and *D*). It is possible that the difference may be related to release of mRNA from DNA, due to the presence of ATP and Mg^{2+} (18).

We measured the effect of transcription on the binding of Z-DNA-specific antibodies in the plateau region. Permeabilized nuclei were incubated with various concentrations of rNTPs and both RNA synthesis and antibody binding was measured (Fig. 2*A*). The rNTPs complex Mg^{2+} and so additional Mg^{2+} was added. For these experiments, 2.8 μg of antibodies was added, an amount in the middle of the plateau of preexisting antibody binding (Fig. 1*D*). The cells were encapsulated and lysed in the new buffer but were then resuspended in the lysis buffer with 13 mM $MgCl_2$ and various concentrations of rNTPs. The nuclei were incubated for 120 min in the presence of antibodies against Z-DNA. In parallel experiments, the level of incorporation of radioactive UTP in newly synthesized RNA was measured. Control experiments showed the antibody had no effect on the synthesis of RNA (results not shown). Both RNA synthesis and binding of antibodies in the plateau region showed a strong dependence on rNTP concentration. At 10 mM rNTPs, the antibody binding had reached a level almost four times that found at 0.1

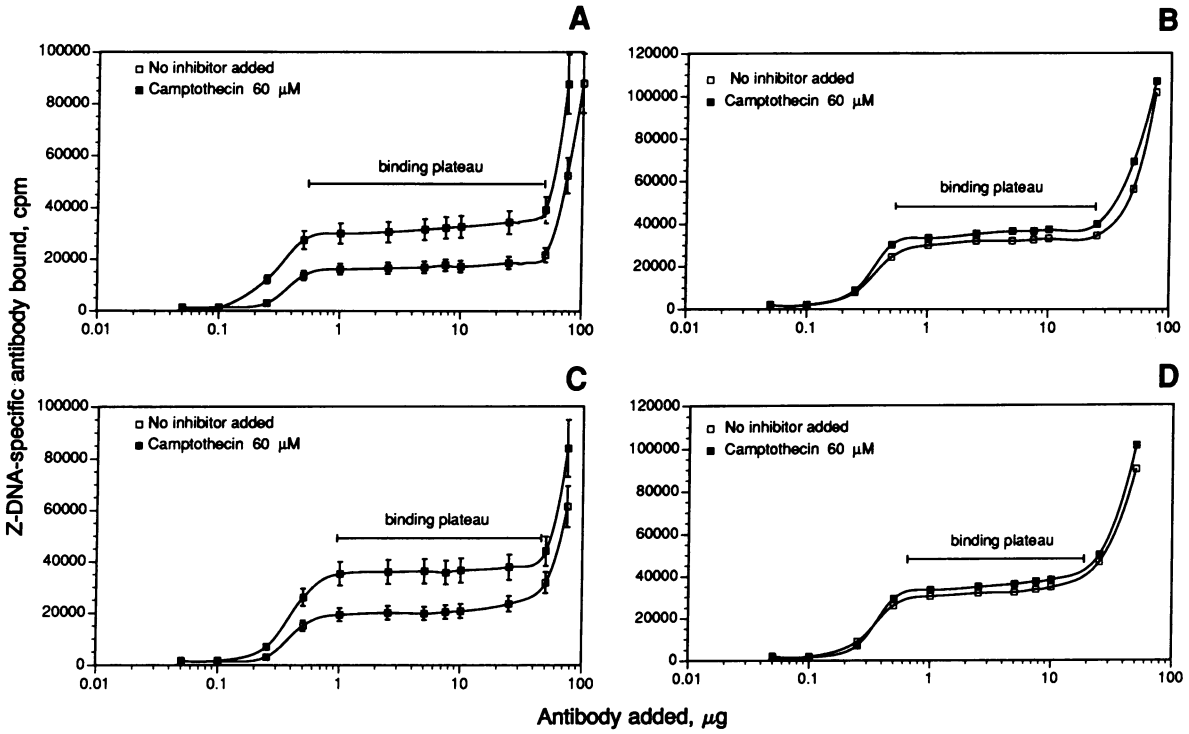

FIG. 1. Binding of Z-DNA-specific antibodies to agarose-encapsulated permeabilized mammalian nuclei as a function of increasing antibody concentration. The effects of adding the topoisomerase I inhibitor camptothecin are shown as well as the effects of changing the buffer system. (*A*) Mouse myeloma nuclei using the old buffer show a broad binding plateau and increased binding due to topoisomerase I inhibition. (*B*) Mouse myeloma nuclei in the presence of new buffer containing ATP and Mg^{2+}. Topoisomerase inhibition has only a small effect on antibody binding. (*C*) Human HL60 nuclei in old buffer. (*D*) Human HL60 cells in new buffer, where topoisomerase inhibition has only a negligible effect as in *B*. Three measurements were made for each point in the experiments in *A*, nine measurements were made for experiments in *B* and *C*, and 12 were made for experiments in *D*. Standard deviation error bars were plotted for *A* and *C* and were of similar size in *B* and *D* but were omitted for clarity.

mM rNTPs. The RNA synthesis was carried out in 13 mM Mg^{2+} because rNTPs complex Mg^{2+}; thus, less Mg^{2+} was available at higher rNTP concentrations. Experiments similar to those shown in Fig. 2 *A–C* were carried out with only 5 mM Mg^{2+} in the incubating buffer. Those gave similar antibody binding curves up to nearly 5 mM NTPs, but higher levels resulted in decreased antibody binding (results not shown). These experiments reinforced our interpretation of the critical balance of NTP and Mg^{2+}.

The next experiments determined the effect of DNA replication on the binding of Z-DNA antibodies in the plateau region (Fig. 2*B*). Increased dNTP concentration resulted in a steady increase in the amount of DNA synthesized during the 30-min incubation period. The binding of antibodies in the plateau region showed a slight increase with a maximum at 10 mM. At that point, there was about twice as much Z-DNA antibody bound to the nuclei compared to 0.1 mM dNTPs. At higher concentrations of dNTPs, the amount of antibody bound dropped as well as the amount of DNA synthesized, probably due to Mg^{2+} complexation. DNA synthesis is more sensitive to Mg^{2+} concentration than is RNA synthesis (10).

The binding of Z-DNA antibodies was measured in a nucleus carrying out both replication and transcription. The nuclei were given 2.5 mM dNTPs and various concentrations of rNTPs. There was a steady increase in the level of RNA synthesized as the concentration of rNTPs was increased (Fig. 2*C*) up to a concentration of 10 mM. At 25 mM rNTP, there was a drop of incorporation and Z-DNA antibody binding, probably associated with Mg^{2+} complexation.

Experiments were next carried out using inhibitors of transcription and replication. The nuclei were given both 7.5 mM rNTPs and 2.5 mM dNTPs so that both replication and transcription were going on. These concentration levels were chosen since both gave significant stimuli for nucleic acid synthesis as well as antibody binding. Replicative DNA synthesis was inhibited through the use of aphidicolin (19). Aphidicolin resulted in very little change in the binding of antibodies specific for Z-DNA in the plateau region (Fig. 3*A*) or on RNA synthesis (Fig. 3*B*) but had a large effect in decreasing the synthesis of DNA (Fig. 3*C*). These results are compatible with results shown in Fig. 2 where the synthesis of DNA was not associated with significant increases in Z-DNA antibody binding. α-Amanitin is widely used as an inhibitor of transcription (20). At low concentrations, it inhibits polymerase II and at higher concentrations it inhibits polymerase III as well. There was a significant decrease in the binding of antibody to Z-DNA at three concentrations of α-amanitin (Fig. 3*A*). It is possible that the major contribution to antibody binding was due to the polymerase II activity rather than polymerase III. There was a marked inhibition of RNA synthesis but α-amanitin had only a small effect inhibiting the incorporation of ^{32}P-labeled dATP in DNA synthesis (Fig. 3*C*). The synthesis of the Okazaki lagging strand during DNA replication is initiated by synthesis of an oligoribonucleotide. It is possible that the small decrease in DNA replication observed is associated with the inhibition of RNA primer synthesis used in DNA replication. Actinomycin inhibits both replication and transcription (21). Unlike the previous inhibitors that bind mostly to the polymerizing enzymes, actinomycin binds to DNA. In the presence of actinomycin there was a significant decrease of antibody binding and a decrease of both RNA and DNA synthesis. The experiments in Fig. 3 are consistent with the observation that inhibiting transcription results in a significant loss of Z-DNA antibody binding in the concentration range of the plateau region whereas inhibiting DNA synthesis does not have as large an effect.

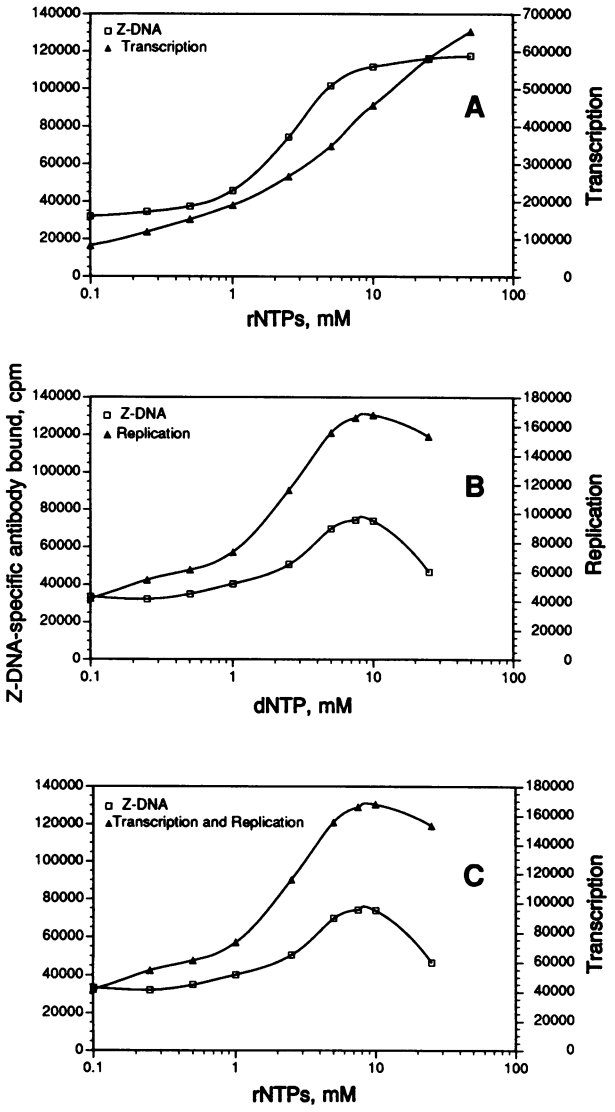

FIG. 2. Human HL60 agarose-encapsulated permeabilized nuclei were incubated with various concentrations of rNTPs or dNTPs. Transcription or replication was measured by incorporation of radioactive nucleotides into RNA or DNA. At the same time, Z-DNA-specific antibodies were added to the nuclei and their binding was measured. Transcription is measured as the incorporation of [^{32}P]UTP into RNA and expressed as cpm. Replication is measured as the incorporation of [^{32}P]dATP into DNA and expressed as cpm. (*A*) The binding of antibodies increases with transcriptional rate over a wide range of rNTPs. (*B*) Antibody binding is much smaller during replication. The drop in both curves at the right is probably due to Mg^{2+} deprivation. (*C*) Transcription is measured as a function of rNTP in the presence of 2.5 mM dNTPs. Each experiment was repeated three times.

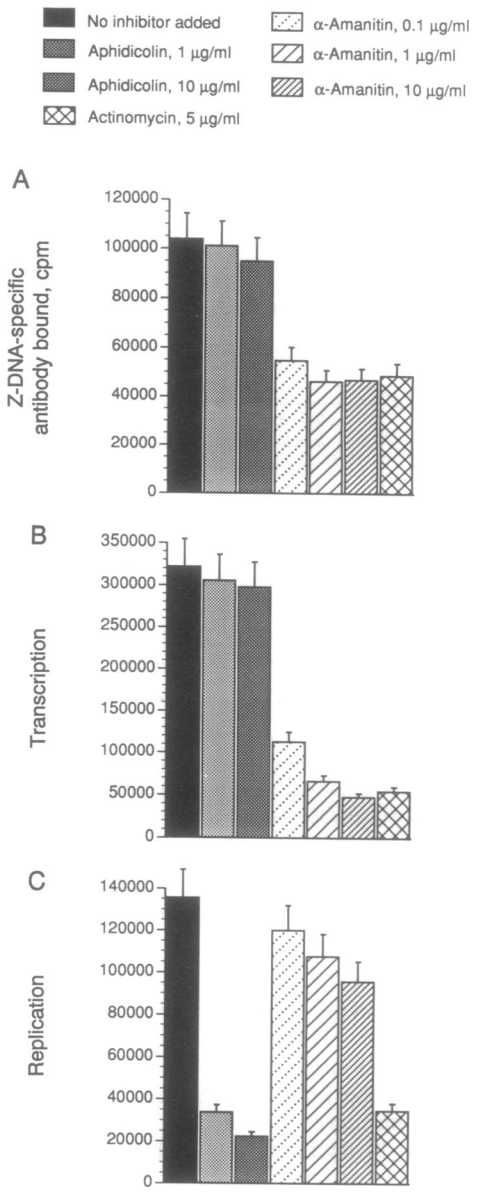

FIG. 3. HL60 permeabilized nuclei in agarose beads were incubated to determine the effects of inhibitors of transcription or replication on the binding of Z-DNA-specific antibody. Transcription and replication were measured and are expressed as in Fig. 2. (*A*) The incubation medium contained 7.5 mM rNTPs, 2.5 mM dNTPs, and 2.8 μg of Z-DNA-specific antibodies. Incubation time was 120 min. (*B*) The incubation medium contained 7.5 mM rNTPs, 2.5 mM dNTPs, 74 kBq [^{32}P]UTP, and the incubation time was 90 min. (*C*) The incubation medium contained 2.5 mM dNTPs, 0.1 mM rNTPs, and 74 kBq [^{32}P]dATP and the incubation time was 90 min. The concentration of inhibitors is indicated at the top. The experiment was repeated six times and the error bars indicate standard deviation.

DISCUSSION

The nuclear preparations of Jackson (7) and Jackson and Cook (8–10) have unique properties. The DNA is unbroken and even though the nuclear membrane is permeabilized, the nuclei maintain a complete complement of nuclear proteins that allow them to carry out both replication and transcription activities. In our earlier work (6, 17), control experiments were carried out using a monoclonal antibody against the nonhistone chromosomal protein HMG-17 (22). The antibody penetrated the entire nucleus, binding the same amount of HMG-17 protein as is found in the nucleus *in vivo*. These nuclei have the property of being able to carry out transcription or replication depending upon whether the substrates for the relevant polymerizing enzymes are added. Previous experiments demonstrated that the level of preexisting Z-DNA is determined by the torsional strain in the DNA. Consequently, the changes seen in antibody binding in the present experiment are most directly interpreted as being due to changes in torsional strain. However, an important caveat must be mentioned. If any of the physiological processes that we are carrying out results in a significant release of Z-DNA binding proteins, this might register as an increased binding of antibody even though it is not associated with a net increase in Z-DNA content.

It is interesting that the binding plateau in the buffer containing 1 mM ATP and 1 mM $MgCl_2$ is somewhat narrower than the binding plateau observed in the buffer without

magnesium and ATP (Fig. 1 *A* and *B*). In the buffer containing no magnesium ion and EDTA, chromatin has a more compact appearance (23). It is conceivable that this change in morphology may be related to the width of the plateau. If in the Mg^{2+}-ATP buffer the chromatin is less compact, it may be freer to respond to the increased concentration of anti-Z-DNA antibodies and, therefore, may induce the formation of Z-DNA at a lower concentration than in the EDTA buffer. The inflection of increased antibody binding due to induced Z-DNA is lower in Fig. 1*B* than in Fig. 1*A*.

The permeabilized nucleus is a good model system that approximates the *in vivo* situation. At the lower concentrations of rNTPs, there seems to be a virtually parallel synthesis of RNA and increased formation of Z-DNA as measured by the binding of Z-DNA antibodies. It is not known precisely what the steady-state concentration of rNTPs is in the intact cell, but several experiments suggest that the actual concentration is much lower than 10 mM (9). The binding of Z-DNA antibody is measuring what is generally a balanced process, in that the rate at which negative supercoiling is generated by transcription is generally equal to the rate at which topoisomerase I diffuses into the DNA and relaxes it. However, transients are likely to occur. It is possible that, at the highest rate of RNA synthesis, the physical properties of the DNA have been modified so that the access of topoisomerase I is increased and the rate at which relaxation occurs is greater there than is occurring at a lower rate of RNA synthesis. These experiments largely measure the completion of RNA strand synthesis (9). Other experiments will be needed to study the initiation of RNA synthesis.

The experiments with DNA synthesis (Fig. 2*B*) show a smaller increase in Z-DNA antibody binding as the concentration of dNTPs increases. Above 10 mM dNTPs, there is a significant decrease. Jackson and Cook (9) found a maximal rate of DNA synthesis at 0.25 mM dNTPs. We are using much higher concentrations because we have to incubate for a longer time period to let the antibody diffuse into the nucleus. By continuing to supply more material, DNA synthesis continues on without being limited by substrate availability. The drop in Z-DNA antibody binding at higher dNTP concentrations might be due to a number of factors, such as greater access to topoisomerase I or II at higher rates of DNA synthesis. Or there might be some relaxing activity seen at the replication fork where single DNA strands are present. In interpreting the Z-DNA antibody binding during DNA replication, an important consideration limiting our interpretation is the question of cell cycle. DNA synthesis occurs only during the S phase, and at present we do not know the number of cells in the S phase. To resolve this issue, cells should be synchronized and the experiments in Fig. 2*B* should be repeated. Only then can the results be interpreted quantitatively.

The experiments dealing with inhibitors provide a strong reinforcement of the results seen previously (6). By using moderate concentrations of triphosphates in the nucleus where both DNA and RNA synthesis occurs, inhibition of DNA synthesis results in a small loss of antibody binding whereas inhibition of RNA synthesis results in a significant loss. These findings are consistent with the proposal by Liu and Wang (13) that transcription is associated with the generation of negative supercoiling in the upstream regions of the gene. Negative supercoiling may result in the formation of Z-DNA before relaxation occurs due to involvement of topoisomerases in the process. What emerges from this work and the previous study (6) is a picture of the generation and dissipation of Z-DNA as a dynamic process, fueled through the generation and dissipation of DNA negative supercoiling during normal metabolic processes. Transcription and to a lesser extent replication generate negative supercoiling and hence Z-DNA formation. The diffusion of topoisomerase I to these sites leads to a loss of negative supercoiling and loss of Z-DNA.

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