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Isolation of *Drosophila* proteins that bind selectively to left-handed Z-DNA

[affinity chromatography/filter binding assays/poly(dG-dC)/negatively supercoiled plasmids/DNA binding proteins]

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ABSTRACT **An affinity column for isolating Z-DNA binding proteins was made by attaching brominated poly(dG-dC) to Sephadex. Proteins from *Drosophila* nuclei were prepared and those that could bind to *Escherichia coli* B-DNA were removed from the solution. The remaining proteins were passed over the Z-DNA affinity column and then eluted with NaCl. Using both direct and competitive filter binding assays, we found that the eluted proteins bind to brominated poly(dG-dC) (Z-DNA) and poly(dG-m^{5}dC) but not to poly(dG-dC) (B-DNA), native or denatured *E. coli* or calf thymus DNA, or brominated oligonucleotides. The proteins also bind to negatively supercoiled plasmids carrying Z-DNA sequences but not to relaxed or linearized plasmids in which the Z-DNA conformation is no longer present. Gel analysis reveals a mixture of several large proteins up to approximately 150,000 daltons.**

Z-DNA is a left-handed conformation of the double helix which is favored by segments having alternations in purine and pyrimidine base sequences (1). Z-DNA is stabilized in poly(dG-dC) by high concentrations of NaCl and somewhat lower concentrations of $MgCl_2$ (2, 3). It is also stabilized by chemical modifications including methylation of cytosine at C5 (4) or bromination of poly(dG-dC) at guanine C8 and cytosine C5 (5). Brominated poly(dG-dC) is found as Z-DNA in a low-salt medium because the bromine atom stabilizes the guanine residues in the *syn* conformation which is found in alternate residues in Z-DNA, in contrast to the *anti* conformation which is found throughout in right-handed B-DNA. The brominated polymer has been used to induce the production of antibodies which are specific for left-handed Z-DNA (5). These antibodies have been used to identify Z-DNA in various organisms, including the polytene chromosome of *Drosophila* (6), the macronucleus of the ciliated protozoan *Stylonichia* (7), and certain plant nuclei (unpublished data). The left-handed Z-DNA conformation is also stabilized by negative supercoiling (8–10). The specificity of the antibodies for Z-DNA has also been demonstrated recently by their ability to combine with identified Z-DNA segments of negatively supercoiled plasmids but not with relaxed plasmids (10).

If Z-DNA plays a role in biological systems, it is likely to do so through the use of proteins, some of which should bind specifically to Z-DNA and not to B-DNA. Such proteins could have various roles in biological systems, including the stabilization of Z-DNA conformation. Because of our earlier demonstration of Z-DNA in *Drosophila* polytene chromosomes (6), we chose to undertake the isolation of Z-DNA binding proteins from that organism. We have used this method of affinity chromatography (11). Here we report the isolation of a group of proteins from *Drosophila* which have the property of binding selectively to Z-DNA. The proteins are large—up to 150,000 daltons. They bind to poly(dG-dC) when it is in the Z-DNA form but not when it is in the B-DNA form. Furthermore, they bind to negatively supercoiled plasmids under conditions such that segments of these plasmids exist as Z-DNA but not when these plasmids are in the relaxed form.

MATERIALS AND METHODS

Nucleic Acids and Z-DNA Affinity Matrix. Poly(dG-dC) (P-L Biochemicals) was converted to the left-handed Z-DNA conformation with 3.7 M NaCl (2) and stabilized in this form by bromination [Br-poly(dG-dC)] (5). Radioactive poly(dG-dC) polymer was synthesized by nick-translation according to Rigby *et al.* (12) with [^{3}H]XTPs as precursors. The methylated polymer poly[^{3}H](dG-m^{5}dC) was synthesized according to Behe and Felsenfeld (4) by incorporating [^{3}H]dGTP and was purified with DNase I (13). Plasmid DNAs were prepared as described (10); the constuction of plasmid pANO 64, carrying a 64-base-pair $(C\text{-}A/T\text{-}G)_n$ sequence will be outlined elsewhere. For preparation of the Z-DNA affinity matrix, 200 A_{260} units of Br-poly(dG-dC) (Z-form) was covalently attached to 5.0 g of Sephadex G-25 (Sigma; 40–120 μm) by using 1-cyclohexyl-3(2-mopholinoethyl)carbodiimide metho-*p*-toluenesulfonate (Sigma) as the coupling reagent (14).

Preparation of Nuclear Proteins. Cells of *Drosophila melanogaster* Schneider line 2L (gift of M. L. Pardue) were grown at 25°C in 2 liters of a mammalian cell medium to a density of 6–8 × 10^6 cells per ml (15, 16). The cells were harvested by centrifugation, washed twice in washing buffer [20 mM Tris, pH 7.5/100 mM NaCl/3 mM $MgCl_2$/0.1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$)], and resuspended in 50 ml of washing buffer containing 0.3% Nonidet P-40. The cells were homogenized with a Dounce homogenizer (B pestle; 25 strokes) and the nuclei were sedimented at 1,000 × *g* for 10 min. The pelleted nuclei were rehomogenized twice (B pestle; 6 strokes) and finally resuspended in 12 ml of extraction buffer (20 mM Tris, pH 7.5/400 mM NaCl/1 mM dithiothreitol/0.1 mM $PhMeSO_2F$). Nuclei were disrupted by sonication (two 20-sec bursts; Ultrasonics). The NaCl concentration was then brought to 1.5 M, and the mixture was centrifuged at 16,000 × *g* for 10 min. The supernatant was mixed with polyethyleneimine (Sigma) at pH 7.9 to a final concentration of 0.4%. The resulting precipitate was removed by centrifugation at 16,000 × *g* for 15 min. Ammonium sulfate was added to the supernatant to 70% saturation, the mixture was centrifuged at 27,000 × *g* for 15 min, and the pellet was resuspended in 10 ml of buffer A [5 mM Tris·HCl, pH 8.0/150 mM NaCl/0.1 mM EDTA/0.1 mM dithiothreitol/0.1 mM $PhMeSO_2F$/5% (wt/vol) glycerol]. Material that did not redissolve was removed by centrifugation at 27,000 × *g* for 15 min. To the extract (A_{280} = 2.0), 3 mg of sonicated *Escherichia coli* DNA (Sigma) was added and this mixture was dialyzed for several hours against buffer A. A precipitate

Abbreviation: $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

formed and was removed by centrifugation (27,000 × *g* for 15 min).

The extract was then passed through the Br-poly(dG-dC)-Sephadex G-25 affinity column which had been equilibrated with buffer A. After extensive washing of the column with buffer A, the proteins were eluted with a linear 0.15–1.50 M NaCl gradient in buffer A. The fractionated eluate was assayed for binding to Br-poly[^{3}H](dG-dC) (Z-form) and poly[^{3}H](dG-dC) (B-form) in 0.15 M NaCl. A second column purification was then carried out: fractions (11–21 in Fig. 1*A*) with high Z-DNA binding ability were pooled, supplemented with 2 mg of native *E. coli* DNA and 1 mg of denatured *E. coli* DNA, and dialyzed against buffer A. This was passed over the Z-DNA affinity column. After the column was washed, the material was eluted in a single step with 50 ml of 1.0 M NaCl (in buffer A). Those 2-ml fractions of the salt eluate that showed preferential binding to Br-poly[^{3}H](dG-dC) (Z-DNA) over poly[^{3}H](dG-dC) (B-DNA) were pooled and dialyzed against buffer A. The glycerol concentration was raised to 50% and the proteins were stored at −20°C. For polyacrylamide gel analysis, the proteins were dialyzed exhaustively against 10 mM ammonium bicarbonate, concentrated by lyophilization, and electrophoresed as described by Laemmli *et al.* (17).

Nitrocellulose Filter Binding Assays. Millipore type HA 0.45-μm nitrocellulose filters (prewashed in 0.3 M NaOH) were used to retain DNA–protein complexes (18). A typical binding reaction was performed in 1.0 ml of buffer A with 0.1 μg of [^{3}H]DNA incubated with the protein sample for 20 min at room temperature. Filters were rinsed with buffer A and the reaction mixture was filtered through at low speed (1 ml/30 sec) on a multiple filtration apparatus (Hoeffer Scientific, San Francisco). The filters were washed with 1 ml of buffer A and dried, and the amount of radioactivity retained on the filter was measured by liquid scintillation counting. In competitive filter binding assays, retention of Br-poly[^{3}H](dG-dC) (Z-form) was measured after preincubation of the proteins with increasing amounts of nonradioactive competitor nucleic acid material. Preincubations were for 20 min at room temperature before Z-[^{3}H]DNA was added; the reaction mixture then was incubated for another 20 min.

RESULTS

We used the method of affinity chromatography in searching for Z-DNA binding proteins. The elution pattern is shown in Fig. 1*A*. The protein came off the column in a large peak near fraction 20 and a smaller, broader peak near fraction 30. The fractions were assayed in filter binding experiments at 150 mM NaCl with radioactive Z-DNA in the form of Br-poly[^{3}H](dG-dC) or radioactive B-DNA in the form of poly[^{3}H](dG-dC). The percentage retention of the two radioactive polymers is shown in Fig. 1*B*. More Z-DNA than B-DNA was retained by the material, and the retention was in two major peaks, one near fraction 17 and another (smaller) peak near fraction 38.

The first passage over the column produced protein fractions that had enhanced binding for Z-DNA relative to B-DNA but, because some B-DNA binding activity was still present, a second purification was run on the Z-DNA column. Fractions 11–21 were pooled and an excess of double-stranded *E. coli* DNA and single-stranded, denatured, *E. coli* DNA were also added to the mixture which was then dialyzed against buffer A. No precipitate was observed at this step, and the material was then loaded on the column in 150 mM NaCl and eluted in a single step with 1 M NaCl. The eluate was reassayed for B-DNA binding and Z-DNA binding (Fig. 1*C*). The background of B-DNA binding was considerably reduced relative to that in Fig. 1*A*; a sharp peak of Z-DNA binding activity was eluted.

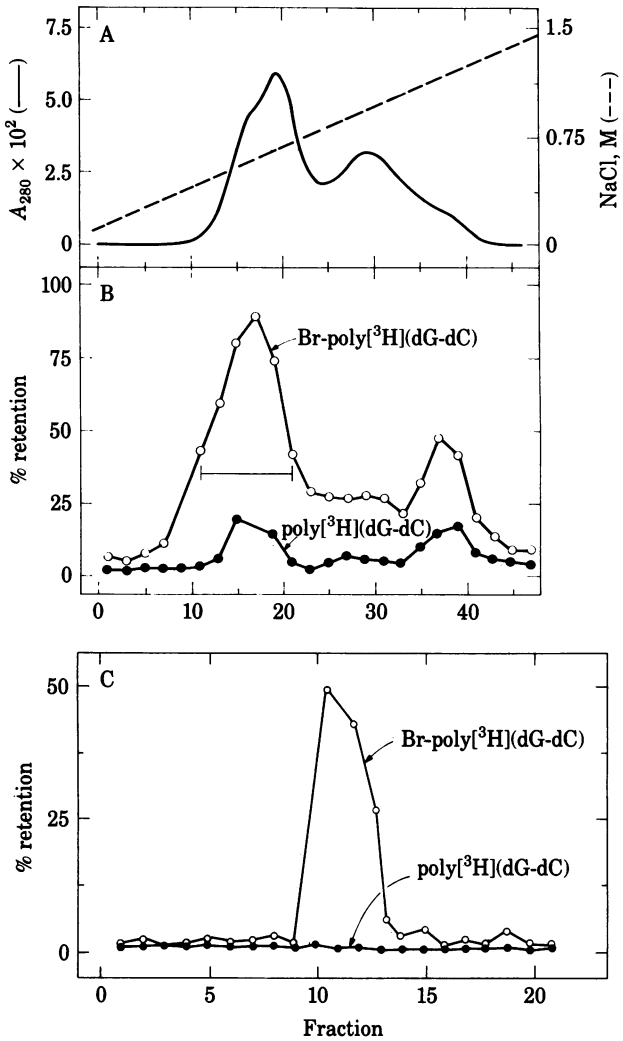

FIG. 1. Column chromatography of Z-DNA binding proteins from *Drosophila* nuclei. (*A*) Protein elution profile (A_{280}) as a function of fraction number; the eluting concentration of NaCl is also given. (*B*) Results of filter binding assays of the various fractions with labeled Z-DNA [Br-poly[^{3}H](dG-dC)] and B-DNA [poly[^{3}H](dG-dC)]. The fractions indicated by the horizontal bar in the main peak of Z-DNA activity were pooled and repurified in a second affinity column chromatography run. (*C*) Binding activity of the material eluted from the second affinity column for both Z-DNA and B-DNA.

Experiments were also carried out to confirm that the retention of Z-DNA on filter binding was due to protein rather than to other materials. In these experiments, proteinase K was added to the DNA protein binding mixture in the absence of $PhMeSO_2F$, and a rapid decay of DNA binding activity was noted (data not shown).

Polyacrylamide Gel Analysis. The material eluted from the affinity column was characterized by polyacrylamide gel electrophoresis. Fig. 2 shows a typical electrophoretic pattern. After the *Drosophila* DNA was removed by polyethyleneimine precipitation, the total proteins were precipitated with ammonium sulfate at 70% saturation. The pellet was then resuspended and analyzed on a 7–20% polyacrylamide/$NaDodSO_4$ gel (18) (Fig. 2*A*). After the first affinity chromatography run, a large number of the smaller proteins were removed, including virtually all of the histones (Fig. 2*B*). However, some larger proteins remained in the solution. A number of proteins were detected in lanes C and D and most of them had molecular weights up to 150,000. At least four different proteins (P) were seen as major peaks and

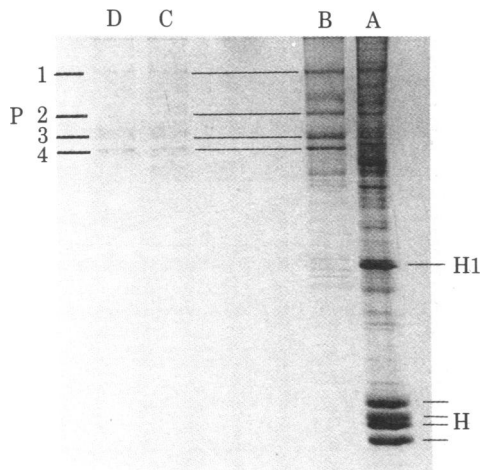

FIG. 2. Gel electrophoretic analysis (7–20% acrylamide) of *Drosophila* nuclear proteins before and after affinity chromatography. Lanes: A, Total proteins of *Drosophila* nuclei before affinity chromatography [histone H1 (H1) and core histones (H) are indicated]; B, pooled fractions 11–21 of Fig. 1*A*; C and D, two fractions of the material eluted from the second affinity column as shown in Fig. 1*C*. Prominent peaks are labeled P. It can be seen that the bands P1 and P2 are also seen as bands in the original unfractionated mixture in lane A.

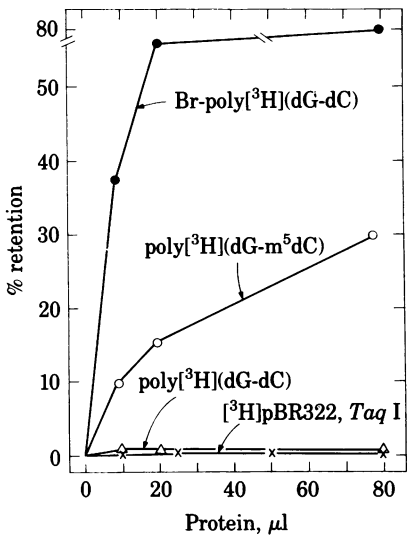

FIG. 3. Filter binding assays for four different radioactive DNA samples as a function of increasing amounts of Z-DNA binding proteins. The Br-poly[^{3}H](dG-dC) is in the Z-DNA form; poly[^{3}H](dG-dC) and the *Taq* I digest of [^{3}H]pBR322 are in the B-DNA form. The methylated polymer poly[^{3}H](dG-m^{5}dC) was partially retained in this assay. All binding experiments were carried out in 150 mM NaCl.

there were several additional minor peaks. Two of the proteins (P1 and P2) could be identified as bands in the original unfractionated extract. This suggests that these proteins are not minor components in the nucleus.

Acrylamide gel analyses were also carried out on the material in Fig. 1*A* that eluted at 1 M NaCl. The results were similar to those seen in lane B of Fig. 2. Most of the bands were present but there was some change in their relative intensities, and other minor bands appeared.

Binding Characteristics. The material that eluted from the Z-DNA affinity column (Fig. 1*C*) was pooled and assayed for its ability to retain various radioactive DNA polymers on nitrocellulose filters. Fig. 3 shows the effect of adding increasing amounts of protein to solutions of various radioactive polymers. In 150 mM NaCl, the Br-poly(dG-dC) is in the Z-DNA form and poly(dG-dC) and a *Taq* I restriction digest of *E. coli* plasmid pBR322 are in the B-DNA form. *Taq* I cleavage of pBR322 generates DNA fragments ranging from approximately 150 to 1,500 base pairs (19) which resembles the size distribution of the poly(dG-dC) preparation used in this study. Addition of up to 80 μl of the protein solution resulted in the retention of >80% of the Br-poly(dG-dC). However, none of the *Taq* I-cleaved pBR322 was retained on the filter. Similarly, poly(dG-dC) also was not retained in the filter binding assay.

Methylation of poly(dG-dC) at cytosine C5 shifted the B-DNA-to-Z-DNA equilibrium position closer to Z-DNA. In 150 mM NaCl this polymer is still in the form of B-DNA (4). However, 30% of the methylated polymer, poly(dG-m^{5}dC), was retained by 80 μl of protein. This suggested that the proteins may have the ability to stabilize the Z conformation in addition to binding to it. This was tested by adding antibodies specific for Z-DNA to the methylated polymer in the presence of Z-binding proteins in order to determine whether an immunoprecipitate could be formed. The results of this experiment (data not shown) indicated that about 10% of the methylated polymer was recognized by the Z-DNA-specific antibody whereas in the absence of Z-DNA binding proteins, none of the methylated polymer was incorporated into the immunoprecipitate. Thus the Z-DNA binding proteins appear to have the capacity to stabilize to a certain extent the Z conformation of the methylated polymer.

Competitive Binding Experiments. To test the specificity of the Z-DNA binding proteins, competition experiments were carried out to determine whether other kinds of DNA could bind to the proteins (Fig. 4). Radiolabeled Z-DNA [Br-poly[^{3}H](dG-dC)] was bound by the proteins. Addition of nonradioactive competitor in the form of Br-poly(dG-dC) decreased the amount of radioactive polymer bound by the protein; when the nonradioactive polymer exceeded the radioactive polymer by 5-fold, there was an ≈50% decrease in radioactive material bound. A 50-fold excess of nonradioactive brominated polymer decreased the retention of radioactive polymer to <5%. A number of other nonradioactive materials were tested for competition in a similar way. Even at 50-fold excess, double-stranded and single-stranded *E. coli* DNA decreased the binding <15%.

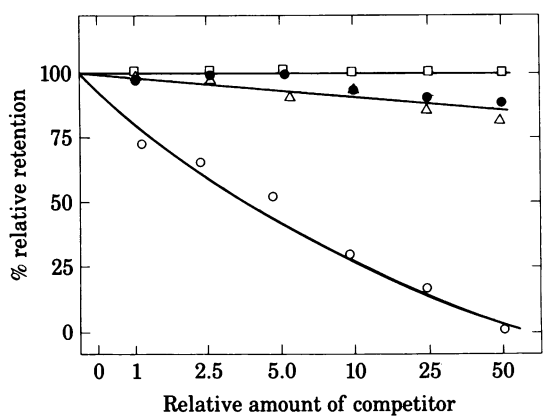

FIG. 4. Binding of radioactive Z-DNA [Br-poly[^{3}H](dG-dC)] after preincubation of proteins with varying amounts of competing nonradioactive material. The retention of radioactive Z-DNA on the filter relative to its retention in the absence of the competitor is plotted as a function of excess of competitor over Z-[^{3}H]DNA. Competitor DNAs used: ○, nonradioactive Br-poly(dG-dC) (Z-DNA); □, brominated nucleotides as well as brominated deoxynucleotide octamers; ●, double-stranded *E. coli* DNA; △, denatured single-stranded *E. coli* DNA.

Brominated nucleotides and nucleosides, native and denatured calf thymus DNA, poly(dG-dC), and brominated octanucleotides of DNA that did not have alternating purine-pyrimidine sequences, had no appreciable ability to bind to these proteins. These preparations of Z-DNA binding proteins appeared to be fairly pure, showing selectivity in binding toward Z-DNA.

Binding to Supercoiled Plasmids. Recently it has been established that negative supercoiling of DNA is one of the features that strongly stabilize Z-DNA (8–10). Upon negative supercoiling of DNA plasmids, it has been possible to detect the conversion of B-DNA segments to Z-DNA by changes in sedimentation behavior, mobility in agarose gels, and induction of binding to antibodies specific for Z-DNA. This has been demonstrated for the plasmid pBR322 (10) as well as for pBR322 derivatives with inserts of $(C\text{-}G/G\text{-}C)_n$ (10) or $(C\text{-}A/G\text{-}T)_n$ (unpublished data). Crosslinking the antibody to the plasmid has made it possible to detect the segments that form Z-DNA (10). Experiments were carried out to determine whether these isolated Z-DNA binding proteins would have the ability to retain, on filters, negatively supercoiled plasmids carrying Z-DNA segments. In addition, we wanted to learn whether relaxed or linearized plasmids that have their Z-DNA segment converted back to B-DNA would pass through the filter.

Results of this type of experiment are shown in Fig. 5*A* for pBR322 at three different levels of negative superhelicity as well as pBR322 linearized by *Hin*dIII digestion. The plasmid with the highest number of negative superhelical turns was retained on the filter at the highest level. Increased negative superhelicity induces a higher concentration of Z-DNA as judged independently by the binding of Z-DNA-specific antibodies (10). There was a significant level of binding to plasmids having 14–22 negative superhelical turns, which are found in plasmids isolated from bacterial cells. Recent experiments have demonstrated that in 150 mM NaCl these plasmids have significant levels of Z-DNA as judged by their ability to bind anti-Z-DNA antibodies (unpublished data). It is interesting that antibodies do not bind to plasmids containing 0–4 negative superhelical turns (10) but nonetheless, there was a decreased but still significant retention of plasmids at this level of negative supercoiling. This experiment suggests that the Z-DNA binding proteins may have the ability to stabilize the Z-DNA conformation at low levels of negative supercoiling. The absence of binding to linearized plasmids demonstrates the complete removal of general B-DNA binding by the protein purification strategy used.

Results of similar experiments are shown for plasmid pANO 64 which is pBR322 with a 250-base-pair insert containing a segment of 64 alternating base pairs $(C\text{-}A/G\text{-}T)_{32}$, except that one C is replaced by T (20). This plasmid has been shown to bind antibodies to Z-DNA when it is negatively supercoiled. The retention of pANO 64 on nitrocellulose filters by Z-DNA binding proteins was slight at the lowest level of negative supercoiling and increased to >40% at the highest level of supercoiling (Fig. 5*B*). The number of negative superhelical turns in these plasmids is measured in the absence of Z-DNA formation (10); the formation of Z-DNA decreases the number of negatively superhelical turns. The top curve of Fig. 5*B* is shown as having an average of 35 negative superhelical turns in the absence of Z-DNA; the number decreases to 23 if all its 67 base pairs of alternating purine and pyrimidine are in the Z-DNA form. Thus, it may be compared with plasmid pBR322 of Fig. 5*A* having 18 negative superhelical turns. More than 40% of pANO 64 was retained by the Z-DNA binding proteins compared to an estimated 25% retained in pBR322 itself at 18 negative superhelical turns; this may suggest that the $(C\text{-}A/G\text{-}T)_{32}$ segment has contributed to an enhanced binding of the Z-DNA

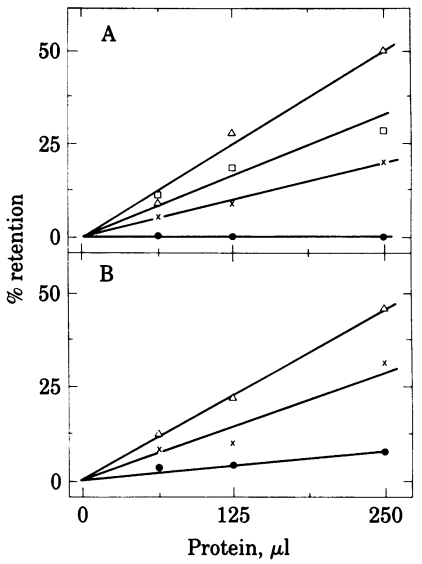

FIG. 5. Retention of radioactive plasmids on nitrocellulose filters as a function of increasing amounts of Z-DNA binding proteins. Results are shown for pBR322 (*A*) and pANO 64 (*B*). (*A*) Retention of pBR322 plasmids with three different levels of negative supercoiling; ×, 0–4 negative superhelical turns; □, 14–22 turns; △, 33–38 turns. ●, Binding of pBR322 linearized by *Hin*dIII cleavage. (*B*) Retention of pANO 64 [pBR322 with a 250-base-pair insert containing 64 base pairs of $(C\text{-}A/G\text{-}T)_n$] with three different levels of negative supercoiling: ●, 0–4 turns; ×, 29–35 turns; △, 33–38 turns.

binding proteins. Other experiments have been carried out with inserts of $(C\text{-}G/G\text{-}C)_n$. These plasmids also were retained by the Z-DNA binding proteins, but their incremental binding was not as great as for the plasmids containing the $(C\text{-}A/G\text{-}T)_n$ inserts.

DISCUSSION

The experiments described here were designed to search the nucleus of *Drosophila* cells for proteins that have the ability to bind Z-DNA but not B-DNA. To purify such Z-DNA binding proteins, DNA-affinity chromatography (11) on a Sephadex G-25 matrix carrying covalently attached left-handed Z-DNA was used. Before passage of the nuclear extract over this affinity column, B-DNA binding proteins were adsorbed to added *E. coli* DNA. This procedure may not capture all of the proteins that bind to Z-DNA because some of them might have the ability to bind to both Z-DNA and B-DNA. The experiments described here were designed to find those that bind selectively to Z-DNA rather than both forms. It should be pointed out that proteins in general do not have the ability to retain Br-poly(dG-dC) selectively in filter binding assays.

A number of proteins were tested for their ability to bind Br-poly(dG-dC) compared to their ability to bind B-DNA. These included cytochrome *c*, lysozyme, and bovine serum albumin. These proteins were able to bind poly(dG-dC) (B-DNA) equally as well as Br-poly(dG-dC) (Z-DNA), demonstrating absence of preferential binding for the brominated form of Z-DNA. The studies using competitive filter binding assays are central to interpreting the specificity of the Z-DNA binding proteins. Those experiments strongly suggest that the isolated proteins will only bind Z-DNA and not other types of DNA or, if they do bind other forms of DNA, the binding is significantly weaker than the Z-DNA binding. Those experiments also rule out the possibility that the bromine atom may play a special role in this Z-DNA binding. That interpretation is reinforced by the experiments with negative superhelical plasmids in which Z-DNA

is stabilized by supercoiling rather than by bromination.

The results in Fig. 5*B* showing binding to the plasmid with the insert of $(C\text{-}A/G\text{-}T)_{32}$ relative to pBR322 itself suggest that the binding is likely to be due to Z-DNA rather than just associated with supercoiling alone (21). In addition, more binding may be found with the $(C\text{-}A/G\text{-}T)_n$ insert than with the $(C\text{-}G/G\text{-}C)_n$ insert which is consistent with the recent results by Hamada *et al.* (22) showing that the *Drosophila* genome contains approximately 1,000 segments with at least 50 base pairs of the alternating $(C\text{-}A/G\text{-}T)_n$ sequence. However, they reported no detectable segments containing alternating C-G residues. This raises the distinct possibility that Z-DNA binding proteins from *Drosophila* also may have some sequence specificity as well.

The gel analysis indicates that four major species appear to have Z-DNA binding activity, as well as a number of minor species. Further work will be necessary to separate these individual species and to determine whether they have sequence preferences in binding and whether they have different biological activities. It becomes apparent from the present experiments that there exist in *Drosophila* classes of proteins that have the ability to recognize Z-DNA and bind to it. These proteins are likely to play a role in stabilizing Z-DNA and they may also function in the interconversions that may occur between B-DNA and Z-DNA in biological systems. It has been suggested that Z-DNA may be involved in the regulation of gene expression (6). If this is true, such regulatory functions may be performed by the Z-DNA binding proteins described here.

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