The EMBO Journal Vol.1 No.10 pp.1149–1153, 1982

Visualization of Z sequences in form V of pBR322 by immuno-electron microscopy

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Communicated by C.Hélène
Received on 12 July 1982

Form V DNA has been prepared from pBR322 DNA by annealing covalently closed complementary single strands. Specific rabbit antibodies to Z-DNA were shown by radioimmunoassay and electron microscopy to react with form V DNA of pBR322. The bound antibodies were visualized either directly (on synthetic polynucleotides in Z-form), or after reaction with goat anti-rabbit immunoglobulin labeled with ferritin (on form V DNA).

Key words: antibodies to Z-DNA/form V DNA/immuno-electron microscopy

Introduction

Double-stranded DNA in a left-handed helix, named Z-DNA, was discovered from an X-ray study of oligo(dC-dG) crystals (Wang *et al.*, 1979; Crawford *et al.*, 1980; Drew *et al.*, 1980). Studies of poly(dG-dC).poly(dG-dC) in fibers (Arnott *et al.*, 1980; Behe *et al.*, 1981), films (Pilet and Leng, 1982), and in solution (Pohl and Jovin, 1972; Mitra *et al.*, 1981; Patel *et al.*, 1982; Thamann *et al.*, 1981; Wu *et al.*, 1981; Ramstein and Leng, 1980) have also led to the conclusion that this polymer can exist in Z-form. While there is no evidence for Z-form in naked calf thymus DNA (42% GC) or in *Micrococcus lysodeikticus* DNA (72% GC) (Nordheim *et al.*, 1981; Malfoy and Leng, 1981), DNA in the Z conformation has been visualized by indirect immunofluorescence in polytene chromosomes (Nordheim *et al.*, 1981; Rio *et al.*, 1982). Poly(dG-dC).poly(dG-dC) adopts the Z conformation in high salt concentration (Pohl and Jovin, 1972). The staining of polytene chromosomes was performed in low salt concentration. This raises numerous questions, one of which was whether the Z-form can be induced in some DNA sequences under conformational constraints.

Stettler *et al.* (1979) have reported that the annealing of covalently closed complementary single strands of PβG plasmid or PM2 phage DNA leads to the formation of a molecular species, form V, which they have characterized by several physical parameters. The comparison of c.d. spectra of form V DNA and poly(dG-dC).poly(dG-dC) in high salt concentration strongly suggested that some sequences in form V DNA could have Z-like conformation.

We have studied form V DNA prepared from plasmid pBR322. The presence of Z sequences was revealed with the use of antibodies to Z-DNA. These antibodies were elicited in rabbits immunized with a poly(dG-dC).poly(dG-dC) chemically modified by chlorodiethylenetriaminoplatinum (II) chloride. The antibodies are specific for the Z-form and do not cross-react with B- or A-DNA (Malfoy *et al.*, 1982; Malfoy and Leng, 1981). By competitive inhibition experiments, we show that the antibodies strongly interact with form V DNA. In addition we have visualized the binding of the antibodies to form V DNA by electron microscopy.

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Results

Radioimmunoassays

Antiserum to Z-DNA was elicited in rabbits immunized with poly(dG-dC).poly(dG-dC) modified by chlorodiethylenetriaminoplatinum (II) chloride (poly(dG-dC) dien-Pt). This antiserum (and the purified antibodies) reacts with poly(dG-dC) dien-Pt and several polynucleotides in the Z-form (Malfoy and Leng, 1981; Malfoy *et al.*, 1982).

The affinity of the Z-DNA antiserum towards form V DNA prepared from the plasmid pBR322 was first examined by radioimmunoassay. These experiments were performed under 0.1 M NaCl, 1 mM $MgCl_2$ or 3 M NaCl conditions in which the tracer, [^{3}H]poly(dG-dC) dien-Pt, was in Z-form (Malfoy *et al.*, 1981). Unlabeled poly(dG-dC) dien-Pt was used as a reference in our competitive inhibition experiments. As shown in Figure 1, the inhibition is independent of the ionic strength. In 0.1 M NaCl, 1 mM $MgCl_2$ and in 3 M NaCl, the concentration of poly(dG-dC) dien-Pt necessary to obtain 50% inhibition of the tracer binding is 1.5×10^{-8} M.

Form V DNA interacts with the antibodies to Z-DNA (Figure 1). It displaces the tracer, but at a higher concentrtion than poly(dG-dC) dien-Pt. The affinity also depends on the ionic strength. The concentrations necessary to produce 50% inhibition of the tracer-antibody binding are 8×10^{-8} M and 8×10^{-7} M in 0.1 M NaCl, 1 mM $MgCl_2$ and 3 M NaCl, respectively. Under the same experimental conditions, relaxed (form II) pBR322 do not compete.

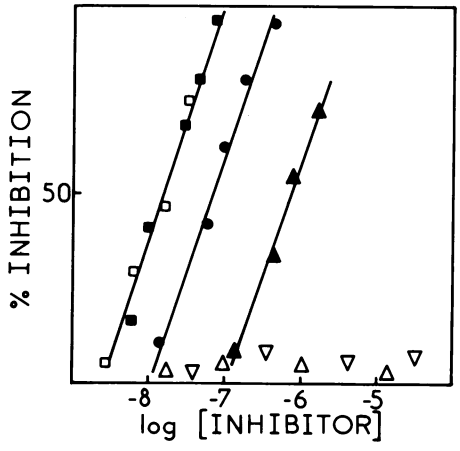
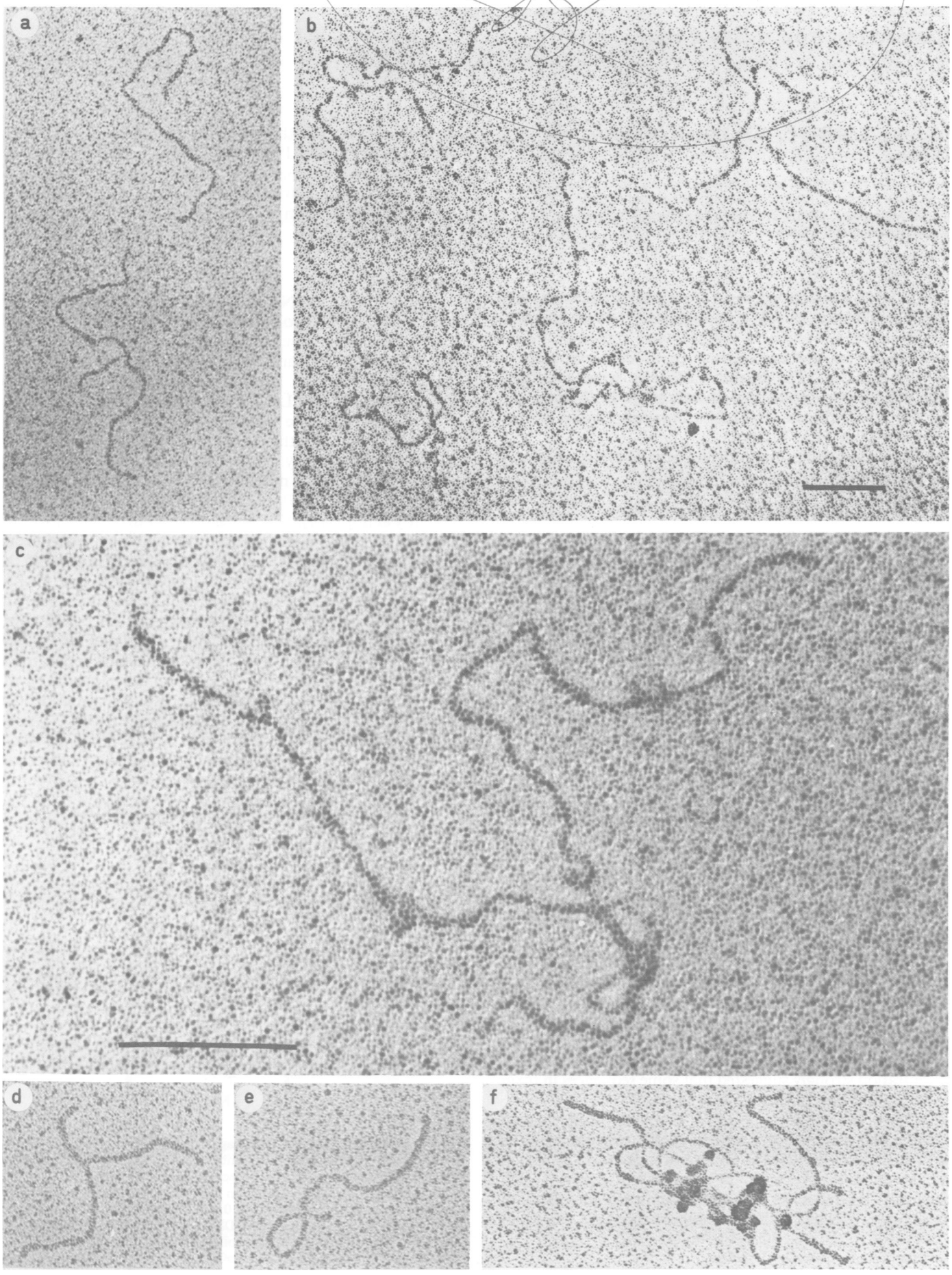


Fig. 1. Radioimmunoassays. Percentage of inhibition of tracer precipitation as a function of the logarithm of inhibitor concentration. Tracer [^{3}H]poly-(dG-dC) dien-Pt, 1.5×10^{-8} M; antiserum dilution, 1:10^5; buffer, 5 mM Tris-HCl (pH 7.5). The concentration of inhibitors are expressed in M nucleotide. Poly(dG-dC) dien-Pt: □ 0.1 M NaCl/1 mM $MgCl_2$; poly(dG-dC) dien-Pt: ■ 3 M NaCl; form V DNA: ● 0.1 M NaCl/1 mM $MgCl_2$; form V DNA: ▲ 3 M NaCl; supercoiled pBR322: ▽ 0.1 M NaCl/1 mM $MgCl_2$; relaxed pBR322: △ 0.1 M NaCl/1 mM $MgCl_2$.


a
b
c
d
e
f

Electron microscopy

The results presented in Figure 1 show that the antibodies to Z-DNA react with poly(dG-dC) dien-Pt and with form V DNA. We then investigated whether there were several short sequences distributed throughout the entire molecule of DNA or one long sequence reacting with the antibodies to Z-DNA. We therefore studied the binding of the antibodies to poly(dG-dC).poly(dG-dC), poly(dG-dC) dien-Pt, and form V DNA by electron microscopy.

Figures 2a, d, and e are electron micrographs of poly(dG-dC) dien-Pt spread according to Dubochet *et al.* (1971) and to the modified procedure of Kleinschmidt (Davis *et al.*, 1971), respectively. Most of the molecules are branched. Figure 2b is a micrograph of the purified complexes between the antibodies to Z-DNA and poly(dG-dC) dien-Pt spread according to the procedure of Dubochet. Figure 2c is an enlargement of one such molecule. The antibodies are distributed along the poly(dG-dC) dien-Pt as dark dots. Under our experimental conditions, the IgG molecules look circular with an average diameter of 90 Å (de Murcia *et al.*, 1979). Using the modified method of Kleinschmidt, we were unable to distinguish the bound IgG molecules. However, their presence is revealed by binding of ferritin-labeled goat antibodies to the rabbit IgG as shown in Figure 2f.

In Figure 2b there are only a few bound IgG molecules per molecule of poly(dG-dC) dien-Pt. All attempts to increase the amount of binding were unsuccessful. When greater concentrations of antibodies were mixed with the polynucleotide, precipitation occurred, and the complexes could not be recovered from the Sepharose 4B column.

A control experiment was performed in 0.1 M NaCl with poly(dG-dC).poly(dG-dC). Under this salt condition, poly(dG-dC).poly(dG-dC) is in the B-form. Poly(dG-dC).poly(dG-dC) was mixed with the antibodies as described in Materials and methods. The presence of antibodies bound to poly(dG-dC).poly(dG-dC) was not detected by electron microscopy. The same experiment was repeated in 4 M NaCl, where poly(dG-dC).poly(dG-dC) is in Z-form. Poly(dG-dC).poly(dG-dC) was mixed with the antibodies. The complexes were purified in the cold on the Sepharose 4B column equilibrated in 200 mM NaCl, and spread on the grid no later than 2 h after the beginning of the experiment. The presence of antibodies bound to poly(dG-dC).poly(dG-dC) was detected and the results looked like those of Figure 2b (data not shown).

Spreading of form V DNA was achieved by the modified method of Kleinschmidt. We could not obtain good results for spreading of form V DNA with the procedure of Dubochet. Figures 3a, and b show the form V DNA molecules, which resemble supercoiled DNA molecules. Nevertheless, we observed a few single-stranded regions (Figure 3b, arrow). Figures 3d, and e show the ferritin-labeled goat antibodies bound to form V DNA having been reacted with Z-DNA rabbit IgG. In these micrographs one can see the ferritin-labeled antibodies bound to molecules of DNA. In Figure 3e, several DNA molecules are cross-linked by the antibodies. The IgGs are distributed all along the DNA molecules. A control experiment was performed by incubating form V DNA alone with the ferritin-labeled immunoglobulins. No bound antibody was detected.

Discussion

The presence of DNA sequences in the Z-form has been shown in polytene chromosomes by indirect immunofluorescence, but until now not in naked linear DNA or RNA even at high ionic strength (Nordheim *et al.*, 1981; Rio *et al.*, 1982). In polytene chromosomes the presence of Z-DNA may be the result of long stretches of G-C sequences. These regions could be stabilized by proteins preferentially bound to the Z-form, or by topological constraints due to the organization of the chromosomes. Because of this latter possibility, we felt that Z-DNA might be present as a stabilizing factor in form V DNA. We have shown here by radioimmunoassay and electron microscopy that antibodies to Z-DNA do indeed bind to form V DNA. These antibodies, elicited in rabbits immunized with poly(dG-dC) dien-Pt, are specific for the Z-form: the platinum residues are not involved in the antigenic determinant (Malfoy and Leng, 1981) and the antibodies do not cross-react with A-form, B-form, or denatured DNA (Malfoy *et al.*, 1982; Malfoy and Leng, 1981). In the competition experiment reported here, ~5-fold more form V DNA than poly(dG-dC) dien-Pt are necessary to inhibit the tracer-antibody binding in 0.1 M NaCl, 1 mM $MgCl_2$, and ~50-fold more is required in 3 M NaCl.

Malfoy *et al.* (1982) reported that the affinity of the antibodies toward several Z-form polynucleotides decreases as the ionic strength increases. The conclusion was that phosphate groups of the polynucleotide interact electrostatically with the positively charged amino acid residues of the antibody-binding sites. The same decrease in the affinity of Z-DNA antibodies toward form V DNA is observed going from 0.1 M to 3 M NaCl. This effect of ionic strength is not observed with poly(dG-dC) dien-Pt because this polynucleotide is less negatively charged since each modified guanine bears two positive charges. In the present study we found no increase in the amount of Z-form in form V DNA as the ionic strength is increased and thus factors other than salt concentration are of major importance in the stabilization of the Z-form.

To discuss the relative affinity of the antibodies toward form V DNA and poly(dG-dC) dien-Pt, we have to consider K_1, K_2 the association constants and n_1, n_2 the percentage of antigenic sites of poly(dG-dC) dien-Pt and form V DNA, respectively. We know (Malfoy *et al.*, 1982) that each Fab fragment binding site covers about four nucleotide residues in the Z-form of poly(dG-dC).poly(dG-dC) and we assume the same values for form V DNA. From our data in 3 M NaCl we obtain a K_1n_1/K_2n_2 ratio of ~50. We should make a comparison using association constants under standard conditions (i.e., measured in 1 M NaCl, Record *et al.*, 1976, 1981), but for an initial approximation we will use the results obtained in 3 M NaCl.

Fig. 2 a–d. Electron micrographs of poly(dG-dC) dien-Pt. **a**: Unbound polynucleotide spread according to the Dubochet procedure in 100 mM NaCl/1 mM $MgCl_2$/5 mM Tris-HCl, pH 7.5. **b, c**: Electron micrographs (Dubochet procedure) of poly(dG-dC) dien-Pt incubated with the antibodies to Z-DNA at an antibody-to-antigen molar ratio of 10^{-3} and then purified on a Sepharose 4B column. Complexation, purification, and spreading were performed in 100 mM NaCl/1 mM $MgCl_2$/5 mM Tris-HCl, pH 7.5. **d, e**: Unbound polynucleotide spread according to the modified Kleinschmidt procedure (final conditions: 0.5 μg/ml polynucleotide with 25% formamide in 100 mM NaCl/20 mM EDTA/10 mM Tris-HCl, pH 7.5). **f**: Electron micrographs of ferritin-labeled goat antibodies bound to the poly(dG-dC) dien-Pt-rabbit IgG complex. The DNA was incubated with antibodies to Z-DNA, purified as in **b, c**, followed by incubation with ferritin-labeled goat antibodies, and then purified by filtration on a Sepharose 4B column. Spreading was performed as in **d**. The bars represent 1000 Å. The micrographs are at the same magnification, except **c**, which is an enlargement.

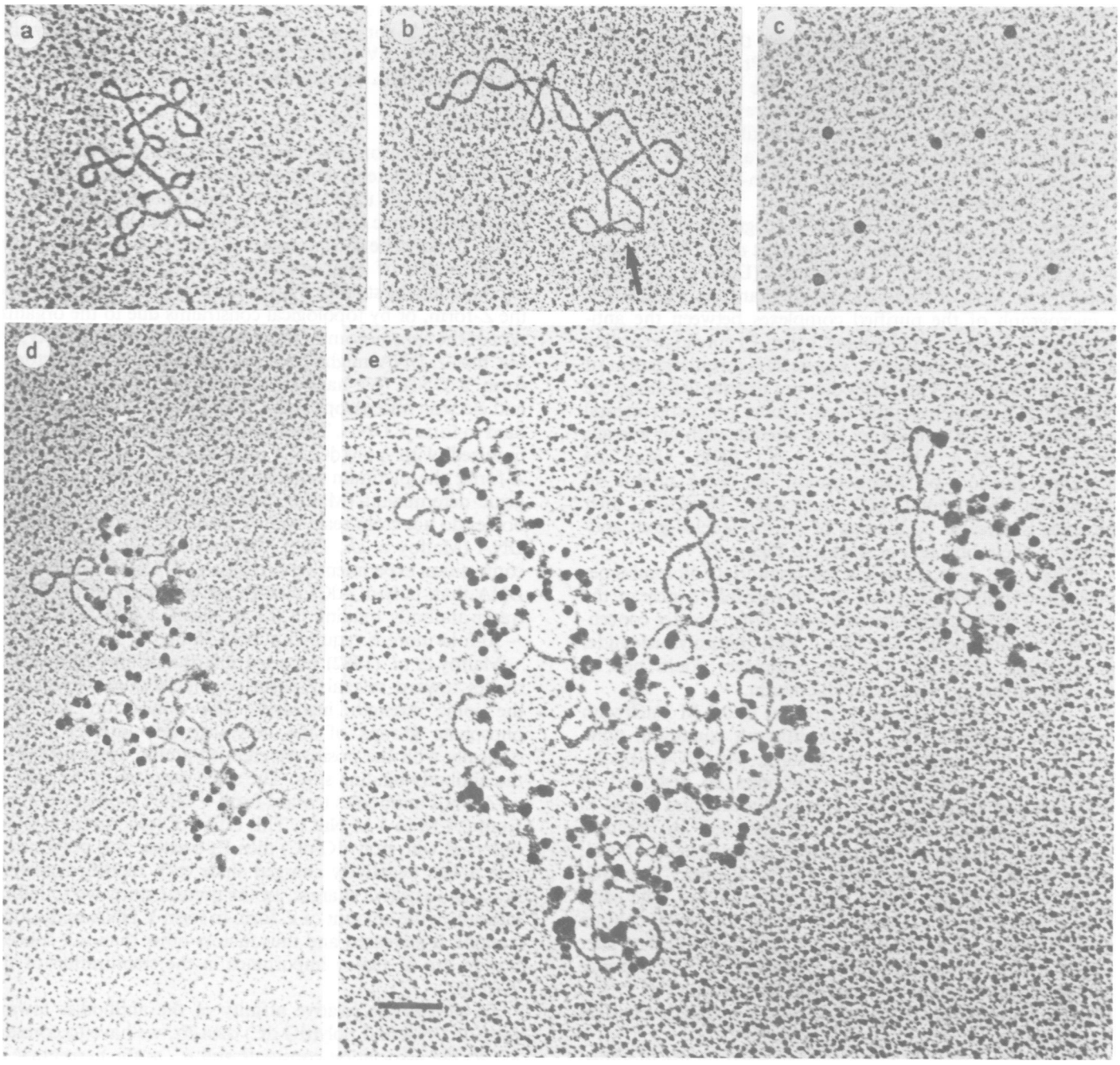


Fig. 3 a,b: Electron micrographs of form V DNA. Spreading according to the modified Kleinschmidt method (Davis *et al.*, 1971). Spreading buffer: 25% formamide in 100 mM NaCl/20 mM EDTA/10 mM Tris, pH 7.5. **c:** Electron micrograph of the ferritin-labeled goat antibodies to rabbit IgG; spreading conditions of **a**. **d, e:** electron micrographs of ferritin-labeled goat antibodies bound to the form V DNA-rabbit IgG complexes. Samples of form V DNA (10 μg/ml) were mixed with the anti-Z rabbit IgG at an antigen-to-antibody molar ratio of 10^3 in 100 mM NaCl/1 mM $MgCl_2$/10 mM Tris-HCl, pH 7.5. After purification on a Sepharose 4B column, this complex was incubated with the ferritin-labeled goat antibodies and then filtered on a Sepharose 4B column. Spreading conditions as in **a**. In a control experiment, the sample was treated similarly except that the incubation with the anti-Z antibodies was omitted. In that case, similar pictures to **a** were obtained. The bar represents 1000 Å. All the micrographs are at the same magnification.

If we assume $K_1 = K_2$, the number n_2 of Z binding sites in form V DNA would be only $\sim 0.02\ n_1$. From inspection of the base sequence of pBR322 (4362 bp), only one GCGCGC sequence and 21 GCGC sequences are found, which may account for the observed 2% Z-form, on the basis of 100% Z-form in poly(dG-dC) dien-Pt. Nevertheless, this apparent agreement is not compatible with the results of c.d. studies (Stettler *et al.*, 1979 and our unpublished data). Assuming that form V DNA is a mixture of B-form DNA and Z-form, and using the $\Delta\epsilon$ value of poly(dG-dC).poly(dG-dC) in Z-form (Pohl, 1976), we calculate that ~40% of the base residues are in Z-form. This value has to be considered with caution. From the work of Stettler *et al.* (1979) and our results, one can conclude that most of the molecules have an ordered structure that excludes large single-stranded regions. Even if form V DNA is a mixture of right-handed and left-handed helices, the $\Delta\epsilon$ of these helices and long linear helices can be slightly different. There is no experimental evidence for the presence of right-handed B sequences in form V DNA. Up to this point, an accurate estimate of the percen-

tage of Z-form by c.d. cannot be given but it seems reasonable to assume that much more than 2% of the base pairs are in Z-form in form V DNA. The sites must include sequences other than stretches of alternating GC, since even the number of GC doublets could account for at best 10% of the total number of base pairs. Therefore, we conclude that sequences recognized by the antibodies contain both AT and GC pairs. At variance with some results on crystal structures of minihelices (Dickerson and Drew, 1981; Conner *et al.*, 1982) we conclude that A and T residues do not prevent some sequences from adopting in solution a Z conformation under strong topological constraints.

It should be noted that if 40% form V DNA has the Z conformation, K_1/K_2 is equal to 20. This can be explained by the polymorphism of Z-DNA, which has been observed in crystals (Wang *et al.*, 1979; Dickerson and Drew, 1981) and in solution by means of the antibodies to Z-DNA (Malfoy *et al.*, 1982).

At high molar ratios of poly(dG-dC) dien-Pt over antibody, the antibody molecules are located along the polynucleotide molecule as shown by electron microscopy. The two binding sites of the antibody are probably bound to the antigen (Nahon-Merlin *et al.*, 1980) and each IgG covers ~24 bp. At lower ratios, each Fab fragment binding site binds to two different polynucleotide molecules and precipitation occurs. Similar results were obtained with Z-poly(dG-dC).poly-(dG-dC)-antibody complexes.

The antibodies to Z-DNA bind to form V DNA as shown in Figure 3. A major point is that the antibodies seem to be distributed all over the form V DNA molecules, which shows the presence of several sequences in Z-form. As in the case of poly(dG-dC).poly(dG-dC), the experiments were performed at a high molar ratio of form V DNA to antibodies. At low ratios the complexes precipitate.

In conclusion, this study shows that a Z or Z-like conformation can be induced in a DNA of natural sequence under topological stress.

Materials and methods

Poly(dG-dC).poly(dG-dC) was purchased from P.L. Biochemicals (St Goar, FRG). [^{3}H]Poly(dG-dC).poly(dG-dC) (4 x 10^6 c.p.m./μg) was synthesized with the *Escherichia coli* DNA polymerase large fragment (Boehringer, Mannheim) in the presence of [^{3}H]dGTP (New England Nuclear) and unlabeled dCTP (P.L. Biochemicals, St Goar, FRG). The chemical modification of poly(dG-dC).poly(dG-dC) by chlorodiethylenetriaminoplatinum (II) chloride (poly(dG-dC) dien-Pt) has been described previously (Malfoy *et al.*, 1981); 12% of the total bases were modified. Form V DNA was prepared from plasmid pBR322 according to Stettler *et al.* (1979). C.d. and electrophoretic behaviour of form V DNA of pBR322 and PβG were similar (data not shown). Immunization of rabbits and characterization of the antibodies to Z-DNA has been previously described (Malfoy and Leng, 1981; Malfoy *et al.*, 1982). The IgG fraction was isolated by gel filtration on an ultragel ACA 34 column. Radioimmunoassays and c.d. were performed as previously described (Malfoy *et al.*, 1982).

Electron microscopy

The IgG fraction in 5 mM Tris-HCl, pH 7.5, containing various concentrations of salt was allowed to react with either synthetic polynucleotides or form V DNA at room temperature for 60 min. The DNA concentration was ~100 μg/ml or 10 μg/ml for synthetic polynucleotides and form V DNA, respectively. The antibody/nucleotide concentration ratio used was 10^{-3}. After incubation, the unbound IgG was removed by gel filtration on a Sepharose 4B column (0.8 x 15 cm) equilibrated in the corresponding buffer. Ferritin-labeled goat immunoglobulin IgG against rabbit IgG (Miles-Yeda, Rehovot) was purified on a 5–28% sucrose gradient. The goat immunoglobulin was then incubated at room temperature for 2 h with the anti-Z antibody-polynucleotide complexes. Unbound goat immunoglobulin was removed by gel filtration on a Sepharose 4B column. As previously described (de Murcia *et al.*, 1979), synthetic polynucleotides (0.5 μg/ml) or the same bound to Z-DNA antibodies were spread on positively charged, carbon coated grids (400 mesh) according to the procedure of Dubochet *et al.* (1971). Synthetic polynucleotides or form V DNA alone or the corresponding complexes with Z-DNA antibodies and ferritin-labeled Ig were spread using cytochrome *c* according to the Kleinschmidt method as modified by Davis *et al.* (1971). The grids were examined on a Siemens 101 Elmiskop electron microscope.

Acknowledgements

This work was supported in part by INSERM (Contrat no. 120019) and by DGRST (Contrat no. 81E 1213). The authors wish to thank Drs.Daney de Marcillac and G.de Murcia for helpful discussions. S.Schmid is gratefully acknowledged for his critical reading of the manuscript.

References

Arnott,S., Chandrasekaran,R., Birdsall,D.L., Leslie,A.G.W., and Ratliff, R.L. (1980) *Nature,* **283**, 743-745.
Behe,M., Zimmerman,S., and Felsenfeld,G. (1981) *Nature,* **293**, 233-235.
Conner,B.N., Takano,T., Tanaka,S., Itakura,K., and Dickerson,R.E. (1982) *Nature,* **295**, 294-299.
Crawford,J.L., Kolpak,F.J., Wang,A.H.J., Quigley,G.J., Van Boom,J.H., Van der Marel,G., and Rich,A. (1980) *Proc. Natl. Acad. Sci. USA,* **77**, 4016-4020.
Davis,R.W., Simon,M., and Davidson,N. (1971) *Methods Enzymol.,* **21**, 413.
de Murcia,G., Lang,M.C.E., Freund,A.M., Fuchs,R.P.P., Daune,M.P., Sage,E., and Leng,M. (1979) *Proc. Natl. Acad. Sci. USA,* **76**, 6076-6080.
Dickerson,R.E., and Drew,H.T. (1981) *J. Mol. Biol.,* **149**, 761-786.
Dubochet,J., Ducommun,M., Zollinger,M., and Kellenberg,E. (1971) *J. Ultrastruct. Res.,* **35**, 147-167.
Drew,H., Takano,T., Tanaka,S., Itakura,K., and Dickerson,R.E. (1980) *Nature,* **286**, 567-573.
Malfoy,B., and Leng,M. (1981) *FEBS Lett.,* **132**, 45-48.
Malfoy,B., Hartmann,B., and Leng,M. (1981) *Nucleic Acids Res.,* **9**, 5659-5669.
Malfoy,B., Rousseau,N., and Leng,M. (1982), *Biochemistry (Wash.),* in press.
Mitra,C.K., Sarma,M.H. and Sarma,R.H. (1981) *Biochemistry (Wash.),* **20**, 2036-2041.
Nahon-Merlin,E., Delain,E., Coulaud,D., and Lacour,F. (1980) *Nucleic Acids Res.,* **8**, 1805-1822.
Nordheim,A., Pardue,M.L., Lafer,E.M., Möller,A., Stollar,B.D., and Rich, A. (1981) *Nature,* **294**, 417-422.
Patel,D.J., Kozlowski,S.A., Nordheim,A., and Rich,A. (1982) *Proc. Natl. Acad. Sci. USA,* **79**, 1413-1417.
Pilet,J., and Leng,M. (1982) *Proc. Natl. Acad. Sci. USA,* **79**, 26-30.
Pohl,F.M., and Jovin,J.M. (1972) *J. Mol. Biol.,* **67**, 375-396.
Pohl,F.M. (1976) *Nature,* **260**, 365-366.
Ramstein,J.,and Leng,M. (1980) *Nature,* **288**, 413-414.
Record,M.T., Lohman,T.M., and de Haseth,P. (1976) *J. Mol. Biol.,* **107**, 145-158.
Record,M.T., Mazur,S.J., Melancon,P., Roe,J.H., Shaner,S.L., and Unger, L. (1981) *Annu. Rev. Biochem.,* **50**, 997-1024.
Rio,P., Malfoy,B., Sage,E., and Leng,M. (1982) *Environ. Health Perspect.,* in press.
Stettler,U.H., Weber,H., Koller,Th., and Weissmann,C.H. (1979) *J. Mol. Biol.,* **131**, 21-40.
Thamann,T.J., Lord,R.C., Wang,A.H.J., and Rich,A. (1981) *Nucleic Acids Res.,* **9**, 5443-5457.
Wang,A.H.J., Quigley,G.J., Kolpak,F.J., Crawford,J.L., Van Boom,J.H., Van der Marel,G., and Rich,A. (1979) *Nature,* **282**, 680-686.
Wu,H.M., Dattagupta,N., and Crothers,D.M. (1981) *Proc. Natl. Acad. Sci. USA,* **78**, 6806-6811.

Note added in proof

After this work had been completed, we learned that Pohl *et al.* found a positive reaction between antibodies to Z-DNA and form V DNA by radioimmunoassay.