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Antibodies to unusual DNA structures*

RATNA NANDI, NICOLAS VOGT, ABDERRAHIM RAHMOUNI, BERNARD MALFOY AND MARC LENG

Centre de Biophysique Moléculaire, 1 A, Avenue de la Recherche Scientifique, 45071 Orléans Cedex 2, France.

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Abstract

To illustrate the potential use of the antibodies to nucleic acids as biochemical reagents, two examples are given. The first one deals with the characterization, at the nucleotide level, of the conformation of the repeat unit of a satellite DNA which, *in situ*, is strongly recognized by the antibodies to Z-DNA. It is shown by means of the chemical probe diethylpyrocarbonate, in the presence/absence of the antibodies to Z-DNA that this repeat unit contains a (AC)-rich region which adopts the Z conformation under topological stress. It is also shown that the repeat unit contains two inverted sequences which form a cruciform structure under topological stress. The second example deals with the antibodies to poly (dG-dC) modified by the antitumor drug *cis*-diamminedichloroplatinum (II). These antibodies cross-react with the platinated $(dC\text{-}dG)_5$ with an interstrand adduct. The conclusion is that the platinated oligonucleotide is a good model for the interstrand adduct formed in platinated natural DNA.

Key words: Z-DNA, cruciform structures, chemical probes, *cis*-platinum.

1. Introduction

The antigenic functions of nucleic acids have been recognized later than those of proteins or polysaccharides but it is now well established that the synthesis of antibodies specifically recognizing bases, nucleotides, single stranded and multi-stranded polynucleotides can be induced in animals immunized with the right antigens[1-6]. However, it is not yet possible to induce experimentally the synthesis of antibodies to B-DNA. Despite this last point, antibodies to nucleic acids appear as promising biochemical reagents. We here report two examples which illustrate the potential of antibodies to nucleic acids. The first example deals with the antibodies to Z-DNA and their interactions with a (AC)-rich sequence from the monkey *Cebus*. The second example deals with the antibodies to poly(dG-dC).poly(dG-dC) modified by an antitumor drug the *cis*-diamminedichloro-platinum (II) and their interactions with a natural DNA modified by the drug.

*This paper is dedicated to Prof. T. M. Jacob.

2. Results and discussion

2.1. *Antibodies to Z-DNA*

Z-DNA is a strong immunogen in rabbits and mice. The antibodies to Z-DNA are specific for Z-DNA and do not cross-react with B-DNA or single-stranded nucleic acids[7–10]. *In situ* immunofluorescence techniques with these antibodies have shown that sequences which are able to adopt the Z conformation exist in the genome[11–16]. In the genus *Cebus*, the antibodies to Z-DNA bind to euchromatic and heterochromatic segments of fixed metaphase chromosomes. A very heavy staining is detected in large segments which correspond to R-band positive heterochromatin whereas euchromatin shows a weak heterogeneous staining which consistently reproduces the R- and T-banding pattern[14]. However, questions were raised about the possible role of fixatives (acetic acid, alcohol) used for cytological preparations in the induction of these stainings since acetic acid may have dramatic effects at the molecular level. The removal of the proteins of the chromatin can change DNA accessibility and topological stress. Moreover, the B-form-Z form transition is facilitated by protonation of the bases[17–20]. In order to characterize better the DNA segments recognized by the antibodies to Z-DNA and to give a molecular explanation for the cytological staining, these segments were isolated and sequenced[21]. They correspond to a satellite DNA with a repeat unit of about 1500 bp. Two regions of the repeat unit seem to be of particular interest (fig. 1). Around base 240, a (AC)-rich region is present. Around base 780, there are two long direct repeats preceded by one inverted repeat.

These two regions have been inserted in circular DNAs (named pCP1 and pCP5, respectively) and the conformational changes of these regions as a function of the superhelical density of the plasmids have been studied by means of the chemical probe diethylpyrocarbonate (DEPC). DEPC carbethoxylates purines at the N7 position and is hyperreactive with denatured DNA or Z-DNA as compared to B-DNA[22–24].

2.2. *(AC)-rich region*

As shown in fig. 1, between bases 253 and 282 there is a stretch of 15(GT/CA) (written (GT)15). To the left of this stretch up to base 180, the sequence is rich in (GT/CA) (19 over 33 dinucleotides) which are in phase with those of the (GT)15 stretch. In addition, there are 1 dinucleotide (CG/GC) (out of phase), 5 dinucleotides (GG/CC) and the sequences (ATCT/TAGA) and (GGGATTTT/CCCTAAAA). On the right of (GT)15 stretch, the sequence does not show any apparent particularity.

DEPC was reacted with pCP1 at various superhelical densities (σ), in the presence/absence of the antibodies to Z-DNA (fig. 2).

Let us first consider the (GT) strand. No reaction was detected between DEPC and relaxed pCP1. When pCP1 is negatively supercoiled ($\sigma = -0.10$), not only the G residues within the (GT)15 stretch but also most of the G and a few of the A residues outside the stretch become hyperreactive. The reactive sequence lies approximatively between bases

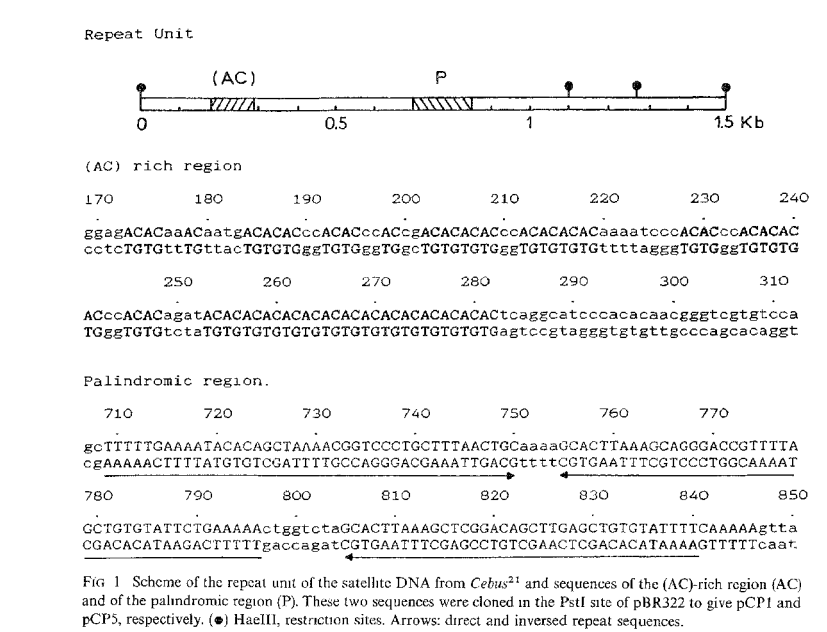

FIG 1 Scheme of the repeat unit of the satellite DNA from *Cebus*[21] and sequences of the (AC)-rich region (AC) and of the palindromic region (P). These two sequences were cloned in the PstI site of pBR322 to give pCP1 and pCP5, respectively. (●) HaeIII, restriction sites. Arrows: direct and inversed repeat sequences.

180 and 290. As judged by DEPC, the sequence (180–290) adopts the Z conformation. It seems quite unlikely that the hyperreactivity is due to unpaired bases which would be present in loops resulting from the slippage of the two strands of the double helix or from the formation of cruciform structures. The sequence does not contain large inverted repeats and one does not expect all the purines to be reactive after slippage of the two strands. The DEPC reactivity pattern observed in the presence of the antibodies to Z-DNA strengthens the suggestion that the sequence (180–290) adopts the Z conformation. At $\sigma = -0.10$, the antibodies are bound to this sequence since the hyperreactivity of the G residues is largely decreased. The antibodies to Z-DNA stabilize the Z conformation which is illustrated by the behavior of pCP1 at $\sigma = -0.07$. In the absence of the antibodies, the sequence (220–290) is in the Z conformation as judged by DEPC. In the presence of the antibodies, again the hyperreactivity of the G residues within this sequence is decreased, but some more G residues become slightly hyperreactive (up to base 200). These results confirm that the antibodies to Z-DNA shift the B-DNA-Z-DNA equilibrium towards the right[8,25–27].

The cleavage pattern of the (AC) strand after reaction with DEPC confirms that the

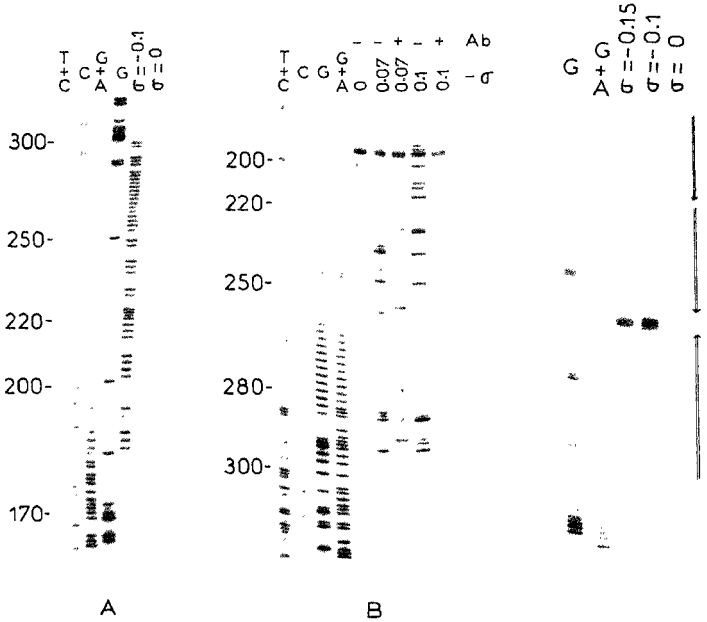

FIG. 2

FIG. 3

FIG. 2. A. Supercoil-induced DEPC hyperreactivity of the (AC)-rich region of pCP1. (AC) strand. pCP1 was reacted with 50 mM DEPC for 15 minutes at 20°C in 50 mM sodium cacodylate, 1 mM EDTA pH 7[22]. After ethanol precipitation, the plasmid was digested by StyI and labelled at 5′ ends (the insert from *Cebus* contains a StyI site near one of its ends). Cleavage by HpaII allowed to isolate a unique end-labelled fragment which contains the (AC)-rich region. Eluted, uniquely labelled fragments were precipited with ethanol. Piperidine treatment, preparation of the samples were identical as for sequencing reaction[39]. Equivalent amounts of radioactivity for each sample were loaded on a 6% buffer gradient sequencing gel[40]. Chemical degradation sequencing reactions were as described[39].

T + C, C, G + A, G: Maxam- and Gilbert-specific reactions. DNAs were reacted at a superhelical density of $\sigma = 0$ and $\sigma = -0.1$, respectively.

B. DEPC footprint of the anti Z-DNA antibodies on the (AC)-rich region of pCP1.(GT) strand. Preincubation for one hour at 20°C of 2 μg of plasmid with 20 μl of antibodies to Z-DNA[8] were carried out in 150 μL of 60 mM NaCl, 5 mM Tris-HCl pH 7. Tubes were transferred in ice, following adjustment of the medium at 50 mM NaCl, 25 mM sodium cacodylate, 0.25 mM EDTA, pH, 7. Samples were further processed as in A, with a phenol extraction step to remove antibodies prior to precipitation with ethanol.

T + C, C, G, G + A; Maxam- and Gilbert-specific reactions. DNAs at indicated superhelical density ($-\sigma$) were reacted with DEPC in the presence (+) or absence (−) of anti Z-DNA antibodies. Numeration refers to fig. 1.

(*continued on p. 7*)

sequence (180–290) is in the Z conformation. Again, not only the A residues within the (GT)15 stretch but also most of the A residues outside of the stretch are hyperreactive. A striking result is that the cleavage pattern is unchanged when the DEPC reaction is performed in the presence of the antibodies to Z-DNA (results not shown).

In conclusion, these results show that under topological stress, the (GT)15 stretch adopts the Z conformation in agreement with other data[28–30]. In addition, a long sequence rich in (AC) doublets but in which several bases are out of alternation is also in Z conformation. All these bases represent about 8% of the repeat unit which can explain the heavy staining of the fixed metaphase chromosomes from *Cebus* by the antibodies to Z-DNA[14].

By *in situ* hybridization, the correspondence between the (AC)-rich region and the heterochromatic regions of the metaphase chromosomes has been verified. The (AC)-rich region was inserted in M13mp18 and the single-stranded form of the phage modified by acetylaminofluorene residues was used as a non-radioactive probe. This probe hybridized specifically on the heterochromatic R-positive regions which are also reactive with the antibodies to Z-DNA[21–31].

2.3. *Palindromic region*

In this region, there are two direct repeats, the length of each being 42 base pairs, and preceded by one inverted repeat. The formation of a cruciform structure within negatively supercoiled DNA has been suggested by the sensitivity of this region to S1 nuclease[21] and is confirmed here by the cleavage pattern obtained after reaction of pCP5 and DEPC (fig. 3). The four A residues between the inverted repeats are strongly reactive with DEPC. It has been verified that the hyperreactivity of the A residues is abolished when DEPC was reacted with pCP5 in the presence of antibodies to adenosine (results not shown).

The cruciform extrusion kinetics of pCP5 have been studied according to the protocol described by Lilley[32]. The extrusion kinetics belong to the S-type[33] as characterized by the salt dependence (the optimal salt concentration for cruciform extrusion is 100 mM NaCl or 150 mM $MgCl_2$, experiments not shown) and by the value of the activation energy. In fig. 4, are plotted the logarithm of the fractions of extruded molecules (measured by gel electrophoresis) as a function of time, at various temperatures. Arrhenius plot of the rate constants *vs* the reciprocal absolute temperatures (inset fig. 4) gives an activation energy equal to 67 kcal/mole.

In conclusion, in addition to the sequence which adopts the Z conformation, the repeat unit of the *Cebus* satellite DNA contains a sequence which can form a cruciform structure.

(*continued from p. 6*)

FIG. 3. Supercoil-induced DEPC hyperreactivity of the direct and inverted repeat sequences of pCP5. After 10 minutes at 60°C in order to induce the cruciform extrusion, pCP5 was reacted with 50 mM DEPC for 15 minutes at 20°C in 50 mM sodium cacodylate, 1 mM EDTA pH, 7.1. The HpaII-HpaII fragment containing the repeat sequences was purified, labelled at 5′ ends and digested by Sau3AI. Samples were further processed as in fig. 1.

G, G + A: Maxam- and Gilbert-specific reactions. DNAs were reacted with DEPC at a superhelical density of $\sigma = 0$, -0.1 and -0.15 respectively. Arrows: direct and inverted repeat sequences (see fig. 1).

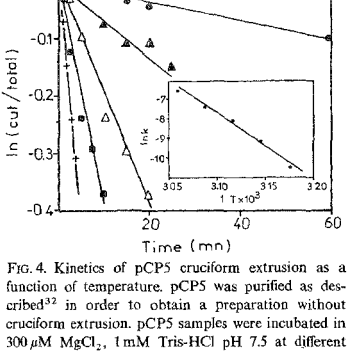

FIG. 4. Kinetics of pCP5 cruciform extrusion as a function of temperature. pCP5 was purified as described[32] in order to obtain a preparation without cruciform extrusion. pCP5 samples were incubated in 300 μM $MgCl_2$, 1 mM Tris-HCl pH 7.5 at different temperatures and aliquots withdrawn to ice at various time intervals. The medium was made of 50 mM sodium acetate and 3 mM $ZnSO_4$ pH 4.6. The DNAs were assayed for the presence of cruciform structure by S1 nuclease and EcoR1 cleavage. The fraction of extruded molecules was measured by gel electrophoresis and densitometry, and the natural logarithm plotted as a function of time of incubation. ●: 42°C; ▲ 45°C; △ 48°C; ■ 51°C; × 54°C.
Insert: Arrhenius plot of the rate consonants against the reciprocal absolute temperature.

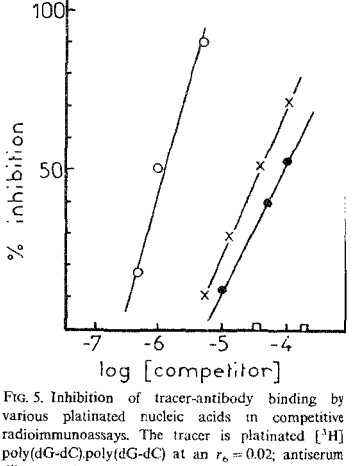

FIG. 5. Inhibition of tracer-antibody binding by various platinated nucleic acids in competitive radioimmunoassays. The tracer is platinated [^{3}H] poly(dG-dC).poly(dG-dC) at an $r_b = 0.02$; antiserum dilution 1/500. Competitors: (○) platinated poly (dG-dC).poly(dG-dC) at an $r_b = 0.02$; (×) [(dC-dG)$_5$]$_2$ Pt; (●) platinated pBR322 DNA at an $r_b = 0.1$; (□) (dC-dG)$_5$ Pt. Medium, 0, 15 M $NaClO_4$, 5 mM Tris-HCl buffer, pH 7.5 and 1 mM EDTA; temperature, 4°C.

Work is in progress to characterize the conformational changes induced by torsional stress when both the palindromic region and the (AC)-rich region are within the same plasmid.

2.4. *Antibodies to platinated poly (dG-dC).poly (dG-dC)*

Numerous studies support the concept that DNA is the likely target for the antitumor drug *cis*-diamminedichloroplatinum (II). Most of the adducts formed in the reaction of *cis*-DDP and DNA have been identified. Two major adducts arise from an intrastrand crosslink between two adjacent guanine residues and between adjacent adenine and guanine residues. Minor adducts arise from intrastrand crosslinks between two guanine residues separated by one base and from interstrand crosslinks between two guanine residues. It is not yet known which adduct (s) is responsible for antitumor activity of *cis*-DDP[34,35]. The interstrand adducts represent only about 1% of the total adducts[36,37] and our aim was to find a model compound for these interstrand adducts.

In the reaction of poly(dG-dC).poly(dG-dC) and *cis*-DDP, at low level of platination, the main adduct is an interstrand crosslink between two guanine residues. This was deduced

from the analysis by gel electrophoresis in denaturing and non-denaturing conditions, respectively, of the fragments obtained by cleavage of the platinated polymer by the restriction enzyme HhaI[38]. The platinated polymer injected in rabbits induces the synthesis of antibodies. As shown in fig. 5, the antibodies crossreact with platinated natural DNA and with $[(dC\text{-}dG)_5]_2Pt$ but do not crossreact with $(dC\text{-}dG)_5Pt$ (intra and interstrand crosslinks are formed in the reaction of *cis*-DDP and $(dC\text{-}dG)_5.(dC\text{-}dG)_5$; the modified oligonucleotides with an interstrand adduct $[(dC\text{-}dG)_5]_2Pt$ or an intrastrand adduct $(dC\text{-}dG)_5Pt$ were purified by hplc, results not shown). The conclusion is that the interstrand adducts formed in the reaction of *cis*-DDP and natural DNA or $(dC\text{-}dG)_5.(dC\text{-}dG)_5$ have the same geometry and thus the physico-chemical study of $[(dC\text{-}dG)_5]_2Pt$ might help to the characterization of the interstrand adducts.

3. Conclusion

Four kinds of antibodies have been used in this work: antibodies against a nucleoside (adenosine), antibodies against a modified base (guanosine modified on the C(8) by acetylaminofluorene residues), antibodies against a double-stranded helix (Z-DNA) and antibodies against a distorted double helix (poly(dG-dC).poly(dG-dC) modified by *cis*-DDP). These antibodies appear to us as powerful tools to characterize the conformation of nucleic acids in complement to physico-chemical techniques.

References

1. STOLLAR, B. D. — The specificity and applications of antibodies to helical nucleic acid antigens, *CRC Crit. Rev. Biochem.*, 1975, **3**, 45–69.
2. MUMS, T. W. AND LISZEWSKI, M. K. — Antibodies specific for modified nucleosides: an immunochemical approach for the isolation and characterization of nucleic acids, *Prog. Nucleic Acid Res. Mol. Biol.*, 1980, **24**, 109–165.
3. STRICKLAND, P. T. AND BOYLE, J. M. — Immunoassay of carcinogen-modified DNA, *Prog. Nucleic Acid Mol. Biol.*, 1984, **31**, 1–58.
4. JACOB, T. M. AND SRIKUMAR, C. — Nucleic acid reactive antibodies—specificity and applications, *J. Biosci.*, 1985, **7**, 61–73.
5. LENG, M. — Immunological detection of lesions in DNA, *Biochimie*, 1985, **67**, 309–315.
6. STOLLAR, B. D. — Antibodies to DNA, *CRC Crit. Rev. Biochem.*, 1986, **20**, 1–36.
7. LAFER, E. M., MÖLLER, A., NORDHEIM, A., STOLLAR, B. D. AND RICH, A. — Antibodies specific for left-handed Z-DNA, *Proc. Natn. Acad. Sci. U.S.A.*, 1981, **78**, 3546–3550.
8. MALFOY, B. AND LENG, M. — Antiserum to Z-DNA, *FEBS Lett.*, 1981, **132**, 45–48.
9. HANAU, L. H., SANTELLA, R. M., GRUNBERGER, D. AND ERLANGER, B. F. — An immunochemical examination of acetylaminofluorene-modified poly(dG-dC).poly(dG-dC) in the Z conformation, *J. Biol. Chem.*, 1984, **259**, 173–178.

10. ZARLING, D. A., ARNDT-JOVIN, D. J., ROBERT-NICOUD, M., MCINTOSH, L. P., THOMAE, R. AND JOVIN, T. M. — Immunoglobulin recognition of synthetic and natural left-handed Z-DNA conformations and sequences, *J. Mol. Biol.*, 1984, **176**, 369–415.

11. NORDHEIM, A., PARDUE, M. L., LAFER, E. M., MÖLLER, A., STOLLAR, B. D. AND RICH, A. — Antibodies to left-handed Z-DNA bind to interband regions of *Drosophila* polytene chromosomes, *Nature*, 1981, **294**, 417–422.

12. LIPPS, H. J., NORDHEIM, A., LAFER, E. M., AMMERMANN, D., STOLLAR, B. D. AND RICH, A. — Antibodies against Z DNA react with the macronucleus but not the micronucleus of the hypotrichous ciliate Stylonychia mytilus, *Cell*, 1983, **32**, 435–441.

13. MORGENEGG, G., CELIO, M. R., MALFOY, B., LENG, M. AND KUENZLE, C. C. — Z-DNA immunoreactivity in rat tissues, *Nature*, 1983, **303**, 540–543.

14. VIEGAS-PEQUIGNOT, E., DERBIN, C., MALFOY, B., TAILLANDIER, E., LENG, M. AND DUTRILLAUX, B. — Z-DNA immunoreactivity in fixed metaphse chromosomes of primates, *Proc. Natn. Acad. Sci. U.S.A.*, 1983, **80**, 5890–5894.

15. NORDHEIM, A., PARDUE, M. L., WEINER, L. M., LOWENHAUPT, K., SCHOLTEN, P., MÖLLER, A., RICH, A. AND STOLLAR, B. D. — 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.*, 1986, **261**, 468–476.

16. ARNDT-JOVIN, D. J., ROBERT-NICOUD, M., ZARLING, D. A., GREIDER, C., WEIMER, E. AND JOVIN, T. M. — Left-handed Z-DNA in bands of acid-fixed polytene chromosomes, *Proc. Natn. Acad. Sci., U.S.A.*, 1983, **80**, 4344–4348.

17. ROBERT-NICOUD, M., ARNDT-JOVIN, D. J., ZARLING, D. A. AND JOVIN, T. M. — Immunological detection of left-handed Z-DNA in isolated polytene chromosomes. Effect of ionic strength, pH, temperature and topological stress, *EMBO J.*, 1984, **3**, 721–731.

18. HILL, R. J. AND STOLLAR, B. D. — Dependence of Z-DNA antibody binding to polytene chromosomes on acid fixation and DNA torsional strain, *Nature*, 1983, **305**, 338–340.

19. HILL, R. J., WATT, F. AND STOLLAR, B. D. — Z-DNA immunoreactivity of *Drosophila* polytene chromosomes, *Expl Cell Res.*, 1984, **153**, 469–482.

20. CHEN, F. M. — Base protonation facilitates B-Z interconversions of poly(dG-dC). poly(dG-dC), *Biochemistry*, 1984, **23**, 6159–6165.

21. MALFOY, B., ROUSSEAU, N., VOGT, N., VIEGAS-PEQUIGNOT, E., DUTRILLAUX, B. AND LENG, M. — Nucleotide sequence of an heterochromatic segment recognized by the antibodies to Z-DNA in fixed metaphase chromosomes, *Nucleic Acids Res.*, 1986, **14**, 3197–3214.

22. HERR, W. — Diethyl pyrocarbonate: A chemical probe for secondary structure in negatively supercoiled DNA, *Proc. Natn. Acad. Sci., U.S.A.*, 1985, **82**, 8009–8013.

23. JOHNSTON, B. H. AND RICH, A. — Chemical probes of DNA conformation: detection of Z-DNA at nucleotide resolution, *Cell*, 1985, **42**, 713–724.

24. SCHOLTEN, P. M. AND NORDHEIM, A. — Diethyl pyrocarbonate: a chemical probe for DNA cruciforms, *Nucleic Acids Res.*, 1986, **14**, 3981–3993.

25. RUNKEL, L. AND NORDHEIM, A. — Chemical footprinting of the interaction between left-handed Z-DNA and anti-Z-DNA antibodies by diethylpyrocarbonate carbethoxylation, *J. Mol. Biol.*, 1986, **189**, 487–501.

26. JOVIN, T. M., MCINTOSH, L. P., ARNDT-JOVIN, D. J., ZARLING, D. A., ROBERT-NICOUD, M., VAN DE SANDE, J. H., JORGENSON, K. F. AND ECKSTEIN, F. — Left-handed DNA: from synthetic polymers to chromosomes, *J. Biomol. Struct. Dyn.*, 1984, **1**, 21–57.

27. LAFER, E. M., SOUSA, R., ALI, R., RICH, A. AND STOLLAR, B. D. — The effect of anti-Z-DNA antibodies on the B-DNA-Z-DNA equilibrium, *J. Biol. Chem.*, 1986, **261**, 6438–6443.

28. RICH, A., NORDHEIM, A. AND WANG, A. H. J. — The chemistry and biology of left-handed Z-DNA, *Annu. Rev. Biochem.*, 1984, **53**, 791–846.

29. LENG, M. — Left-handed Z-DNA, *Biochem. Biophys. Acta*, 1985, **825**, 339–344.

30. WELLS, R. D. — Unusual DNA structure, *J. Biol. Chem.*, 1988, **263**, 1095–1098.

31. VIEGAS-PEQUIGNOT, E., MALFOY, B., LENG, M., DUTRILLAUX, B. AND TCHEN, P. — *In situ* hybridization of an acetylamminofluorene-modified probe recognized by Z-DNA antibodies *in vitro*, *Cytogenet. Cell Genet.*, 1986, **42**, 105–109.

32. LILLEY, D. M. J. — The kinetic properties of cruciform extrusion are determined by DNA base-sequence, *Nucleic Acids Res.*, 1985, **13**, 1443–1465.

33. SULLIVAN, K. M. AND LILLEY, D. M. J. — A dominant influence of flanking sequences on a local structural transition in DNA, *Cell*, 1986, **47**, 817–827.

34. PINTO, A. L. AND LIPPARD, S. J. — Binding of the antitumor drug *cis*-diamminedichloroplatinum (II) (*cis*Platin) to DNA, *Biochem. Biophys. Acta*, 1985, **780**, 167–180.

35. REEDIJK, J. — The mechanism of action of platinum anti-tumor drugs, *Pure Appl. Chem.*, 1987, **59**, 181–192.

36. PLOOY, A. C. M., VAN DIJK, M. AND LOHMAN, P. H. M. — Induction and repair of DNA cross-links in Chinese hamster ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity and antitumor activity, *Cancer Res.*, 1984, **44**, 2043–2051.

37. RAWLINGS, C. J. AND ROBERTS, J. J. — Walker rat carcinoma cells are exceptionally sensitive to *cis*-diamminedichloroplatinum (II) (*cis*platin) and other difunctional agents but not defective in the removal of platinum-DNA adducts, *Mutation Res.*, 1986, **166**, 157–168.

38. RAHMOUNI, A. AND LENG, M. — Reaction of nucleic acids with *cis*-diamminedichloroplatinum (II): interstrand crosslinks, *Biochemistry*, 1987, **26**, 7229–7234.

39. MAXAM, A. M. AND GILBERT, W — Sequencing end-labelled DNA with base specific chemical cleavages, *Meth. Enzymol.*, 1980, **65**, 499–560.

40. BIGGIN, M. D., GIBSON, T. J. AND HONG, G. F. — Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination, *Proc. Natn. Acad. Sci. U.S.A.*, 1983, **80**, 3963–3965.