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Isolation of Z-DNA-containing plasmids

(monoclonal antibody/recombinant *Escherichia coli* DNA/affinity chromatography)

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ABSTRACT **A purified, monoclonal antibody, specific for the left-handed Z-form of poly(dG-dC), was coupled covalently to Sephacryl S-1000 beads. Such an antibody column provides a convenient method to isolate and purify those plasmid DNAs that contain Z-DNA from a large excess of other DNAs, RNA, etc. From a library of *Escherichia coli* DNA, cloned into the vector plasmid pUC-8, several recombinant plasmids were isolated, which bind to this antibody. Thus, *E. coli* contains sequences, which in "natural" negatively supercoiled DNA, adopt a left-handed Z-DNA-like conformation.**

Physicochemical studies of poly- and oligo(dC-dG)·(dC-dG) in solution (e.g., refs. 1–4) and in the solid state (5–8) have established that under particular conditions such a DNA adopts a left-handed double-helical conformation, the so-called Z-DNA. [A recent symposium gives an excellent overview of the present state (9).] By inserting oligo(dC-dG) into plasmid DNA it has been shown that the topological stress in negatively supercoiled covalently closed circular DNA (ccc DNA) promotes the transition from right- to left-handed double-helical conformations under conditions—e.g., of low salt—in which such a transition would not occur in the corresponding linear molecules (10–13). By increasing the torsional stress, as exemplified most drastically in form V-DNA with a linking number of zero, such left-handed structures are also induced in DNA of natural sequence (refs. 13–16; unpublished data).

The discovery that Z-DNA is a strong immunogen (17, 18) has paved the way to look for the occurrence of such DNA structures in more complicated systems by taking advantage of the high specificity of immunochemical methods. For example, polytene chromosomes of *Drosophila* bind antibodies specific for Z-DNA (19), suggesting that such left-handed DNA conformations occur as structural elements *in vivo*.

Because we are interested in isolating DNA sequences from genomic DNAs that have a high probability for adopting such left-handed conformations, we developed a relatively simple and convenient method for that purpose. It allows the selection from recombinant plasmid libraries of those molecules that at natural supercoil density bind to an antibody column specific for Z-DNA. A monoclonal antibody, designated Z-D11 (14), was immobilized on a solid support and used to select Z-DNA-containing plasmids, which were constructed by recombinant DNA techniques.

MATERIALS AND METHODS

Enzymes and Strains. Restriction endonucleases and the Messing strains (20), including JM83, JM103, and pUC-8, were from Bethesda Research Laboratories (Neu Isenburg); T4 DNA ligase was isolated (21) or obtained, like calf intestine alkaline phosphatase, from Boehringer Mannheim. Chemicals were analytical-grade commercial products.

Monoclonal Antibodies to Z-DNA. Spleen cells of a BALB/c mouse, immunized with brominated poly(dG-dC) according to Lafer *et al.* (17), were used for cell fusion (22, 23). Cell supernatants were screened for precipitating ^{32}P-labeled poly(dG-dC) in 4 M NaCl. Seven monoclonal cell lines producing antibodies to Z-DNA were established (unpublished data). Antibodies (κ/γ2b) from ascites fluids, by using cell line Z-D11, were purified by protein A-Sepharose (Pharmacia) chromatography (24, 25). Antibody concentration was determined, by using A_{280} = 1.4 for a solution at 1 mg/ml. ^{125}I-Labeled antibodies with ≈10,000 cpm/ng were prepared by the Chloramin-T method (26).

Antibody Column. Sephacryl S-1000 (superfine; Pharmacia) was activated with cyanogen bromide in sodium carbonate buffer (27); 0.3 mg of antibody Z-D11 per g of wet Sephacryl S-1000 was quantitatively coupled in 0.1 sodium carbonate (pH 8.5) for 8 hr, 1 M ethanolamine (pH 8.6) was added for 4 hr, and several washing cycles were included. The binding capacity of a small aliquot was determined with ccc DNA of pLP32 and pUCZ-8. Such antibody columns have been used repeatedly and over months.

Plasmid DNA Preparation. Plasmid pLP32, a pBR322 derivative with $(dC\text{-}dG)_{16}$ inserted into the filled in *Bam*HI site, was kindly provided by L. Peck and J. Wang (12). ^{3}H-Labeled ccc DNA (5–20 cpm/ng) was obtained by growing the cells in minimal medium (28) to A_{600} = 0.2 and adding [^{3}H]dThd (New England Nuclear). ccc DNAs were prepared by the alkaline lysis method (29), as described (30).

Construction of Plasmid pUCZ-8. For obtaining a small, Z-DNA-containing, vector plasmid, pLP32 was completely digested with endonuclease *Hae* II and the $(dC\text{-}dG)_{16}$-containing fragment was isolated from a low-melting agarose gel. pUC-8 DNA was linearized by limited digestion with *Hae* II, phosphatase treated, ligated with the fragment, and transformed into competent JM83 cells. A clone with the fragment inserted before the *lac* promoter was characterized by restriction and antibody mapping. The ccc DNA, as isolated from *Escherichia coli* JM83, had Z-DNA about 280 base pairs upstream of the *Bam*HI site in the polylinker.

***E. coli* Plasmid Library.** High molecular weight DNA from *E. coli* strain JM103 was prepared by lysozyme, proteinase K, and RNase digestion and by phenol extraction (31). In a particular experiment the *E. coli* DNA was partially digested with endonuclease *Sau*3A and 12 μg was ligated with 5 μg of *Bam*HI-cleaved, phosphatase-treated, pUC-8 DNA in 500 μl of ligase buffer for 2 hr at room temperature. Twenty microliters of this mixture was transformed into competent, Ca^{2+}-treated JM83 cells and plated on 2× concentrated YT-agar plates with am-

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Abbreivation: ccc DNA, covalently closed circular DNA.

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picillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside as indicator dye (32). About 10,000 colorless and 2,500 blue colonies were obtained and washed off of the plates, and plasmid DNA was prepared, yielding 140 μg of ccc DNA as an *E. coli* library.

Selection of Z-DNA-Containing Plasmids. Fourteen micrograms of this ccc DNA in 0.5 M NaCl/10 mM Tris·HCl/1 mM EDTA, pH 8.0, was loaded on a new antibody column (1 ml) and washed with 40 ml of the same buffer. The bound DNA was eluted with 100 μM ethidium bromide in the same buffer and ethanol precipitated, and an aliquot was transformed into JM83, yielding 1,500 colorless and 19 blue colonies. To select for strong binding plasmids with high yield this procedure was repeated twice; then single colonies were picked and amplified and their plasmid DNA characterized.

Antibody Binding to Plasmid DNA. ^{3}H-Labeled ccc DNA was incubated with antibody in 0.2 M NaCl/60 mM Na phosphate/30 mM EDTA, pH 8.0, for 1 hr at room temperature and carefully filtered through a nitrocellulose filter (Schleicher & Schüll) and the bound DNA was determined by scintillation spectroscopy (13).

In a semiquantitative assay the mobility change of ccc DNA, due to the binding of the antibody, in 1% agarose gel electrophoresis was employed (14). The gel contained as buffer 36 mM Tris/30 mM NaH_2PO_4/1 mM EDTA and was run at 5 V/cm, and the DNA was visualized by UV illumination after staining with 0.5 μg of ethidium bromide per ml.

The bound antibody was quantitated by using ^{125}I-labeled Z-D11 in the gel assay, the corresponding DNA band was excised, and the radioactivity was determined.

Direct Blotting. For the autoradiography of ^{125}I-labeled antibody bound to plasmid DNA, after electrophoresis a "direct blotting" electrophoresis apparatus was used (ref. 33; unpublished data). At the end of a short 1% agarose gel a conveyor belt with DE-81 ion exchange paper (Whatman) moved with constant speed and collected the molecules as they left the gel. The paper was covered with x-ray film and exposed overnight. The DNA on the paper was visualized by ethidium bromide staining and photographed by UV illumination.

RESULTS

Binding of Monoclonal Antibody Z-D11 to a Plasmid DNA. Monoclonal antibodies are of considerable advantage in quantitative binding studies, because they can be reproducibly obtained in large amounts. From our collection of such antibodies to Z-DNA we have used antibody Z-D11 exclusively in the experiments reported here, because it shows the strongest binding to the Z form of poly(dG-dC)·poly(dG-dC). Fig. 1 *Upper* gives the titration of the ccc DNA of plasmid pLP32 at natural supercoil density with the purified antibody, as revealed by the nitrocellulose binding assay. No interaction of the antibody—e.g., with the linear form of this DNA or with pBR322, lacking the $(dC\text{-}dG)_{16}$ sequence—at natural supercoil density is observed (13, 14, 34). This indicates the importance of the negative superhelical density for sustaining left-handed Z-DNA at low salt concentration.

By the intercalation of, for example, ethidium bromide into ccc DNA the negative supercoil density can be changed in a simple and reversible way (35). Fig. 1 *Lower* shows that such a decrease of the negative supercoil density abolishes the binding of the antibody to the plasmid DNA above a certain ethidium bromide concentration (34), due to the loss of left-handed double-helical structure.

Testing the Antibody Column. Different materials have been tried for covalently coupling the antibody to a solid support. The best results for the present purpose have been obtained with highly porous Sephacryl S-1000 beads. As expected, a considerable dependence of the binding capacity for Z-DNA-containing plasmids of different molecular weight has been observed. For the small plasmid pUCZ-8, which was constructed for testing the approach, about 10 μg of ccc DNA was bound per ml of bed volume. Fig. 2 illustrates the selectivity of the binding of plasmid DNA to the Z-D11 column. Only such ccc DNA, which contains Z-DNA, as known from other experiments, is bound to the column and eluted by ethidium bromide, whereas the other DNAs simply pass through the column. This property provides an alternative method to purify such ccc DNA.

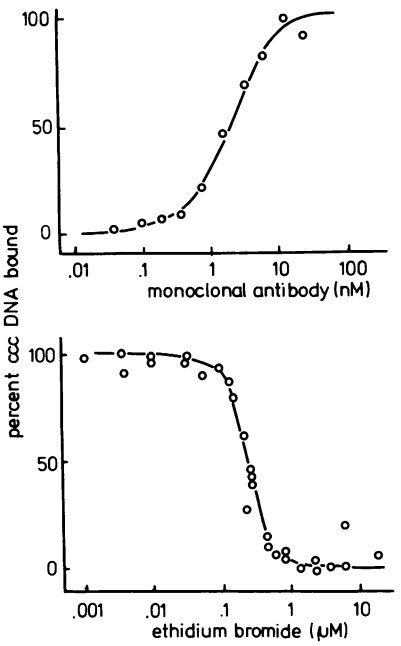

FIG. 1. Binding of monoclonal antibody Z-D11 to ^{3}H-labeled ccc DNA of plasmid pLP32 (12), as measured by the nitrocellulose filter binding assay. (*Upper*) One hundred seventy nanograms (1,000 cpm) of ccc DNA was incubated with varying amounts of antibody in 100 μl of 0.2 M NaCl/60 mM Na phosphate/30 mM EDTA, pH 8.0, for 1 hr at room temperature and slowly filtered, and the bound DNA was determined by scintillation spectroscopy. (*Lower*) pLP32-DNA was reacted with saturating amounts of antibody as above, ethidium bromide was added to the solution for 1 hr at the indicated concentration, and the bound complex was determined.

Searching for Z-DNA-Forming Sequences. Fig. 3 outlines the procedure for constructing recombinant plasmid libraries and the selection of those plasmids that have a Z-DNA at nat-

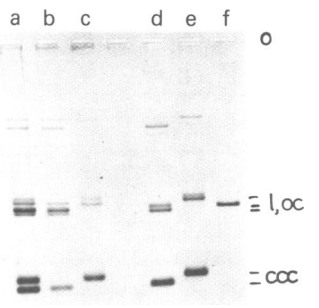

FIG. 2. Separation of a mixture of plasmid DNAs by affinity chromatography, by using a column with monoclonal antibody Z-D11 coupled to cyanogen bromide-activated Sephacryl S-1000 beads, as revealed by agarose gel electrophoresis. Lanes: a, mixture of DNAs, as shown in lanes d and e, before loading on the column; b, DNA passing through the column; c, eluted with 100 μM ethidium bromide; d, ccc DNA of plasmid pUC-8; e, ccc DNA of plasmid pUCZ-8; f, linear DNA of plasmid pUCZ-8, digested with *Eco*RI.

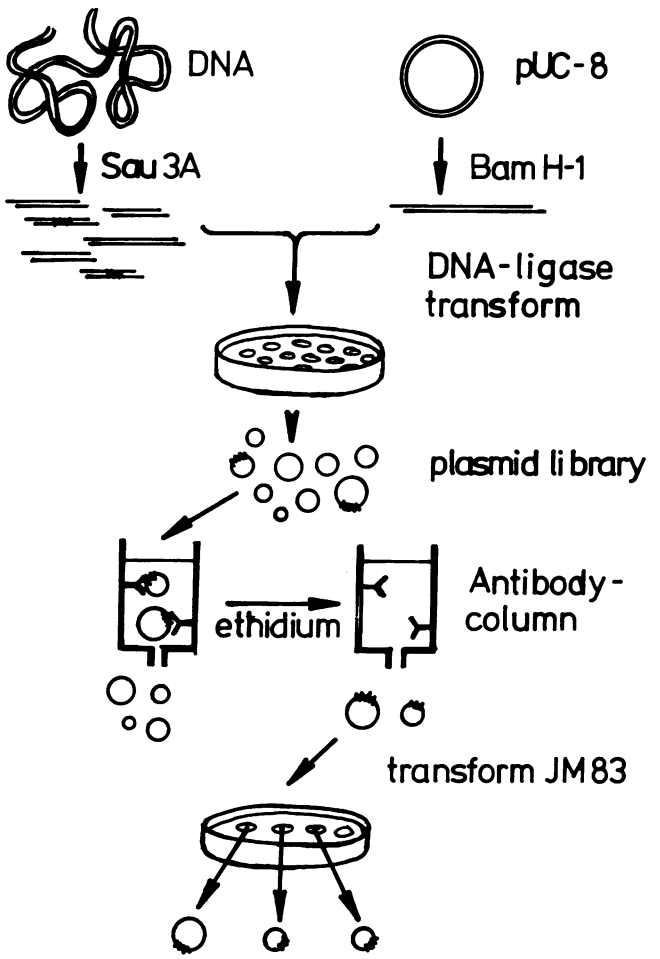

FIG. 3. Scheme for the selection of Z-DNA-containing plasmids from a genomic library by affinity chromatography.

ural supercoil density by using for the affinity chromatography a monoclonal antibody column. The vector pUC-8 DNA is cleaved at the single *Bam*HI site and treated with phosphatase to reduce self-ligation. High molecular weight DNA is partially digested with endonuclease *Sau*3A, creating tetranucleotide single-stranded ends, which can hybridize with those produced by *Bam*HI and thus with high efficiency cloned into the vector DNA. This recombinant DNA is transformed into *E. coli* strain JM83 and spread onto plates containing ampicillin as antibiotic and an indicator dye. From the resulting colonies with recombinant plasmids, ccc DNA is prepared and loaded onto the antibody column. After extensive washing, the bound DNA is eluted with ethidium bromide and transformed again into JM83. This protocol gives a rather general way for isolating those DNA sequences from genomic DNA, which—e.g., at "natural" negative supercoil density—form left-handed Z-DNA.

Potential Z-DNA in the *E. coli* Genome. A plasmid library with *E. coli* DNA was constructed as described above and Z-DNA-containing plasmids were selected by their ability to bind to the antibody column. Fig. 4 *Upper* gives the size distribution of recombinant plasmid libraries, as revealed by gel electrophoresis of the ccc DNA. Before applying to the antibody column a very smooth distribution of the 10^4 recombinant plasmids is observed.

But already after the first passage through the column discrete bands can be seen, and we estimate very roughly that there are about 100 plasmids of different size released by ethidium bromide from the column. With further selection cycles, the discrete nature becomes more pronounced. Fig. 4 *Lower* gives individual plasmid clones picked randomly from single colonies

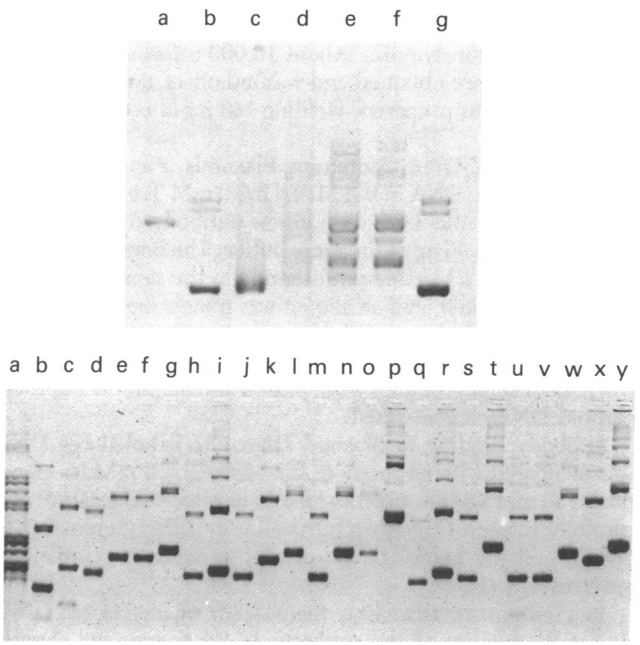

FIG. 4. Isolation of recombinant plasmids with Z-DNA from an *E. coli* plasmid library. (*Upper*) Agarose gel electrophoresis of recombinant plasmid libraries. Lanes: a, ccc DNA from pLP32; b and g, pUCZ-8 as standard; c, ccc DNA prepared from about 10^4 single colonies with recombinant plasmids containing *E. coli* DNA, before loading onto the antibody column; d, DNA after elution from the antibody column by ethidium bromide and transformation; e, after a second selection cycle; f, after a third selection cycle. (*Lower*) Lanes: a, mixture of ccc DNA from the third selection cycle; b, pUC-8; c–y, plasmids DNA from individual clones, picked randomly from the third selection cycle and prepared according to ref. 36.

after three selection cycles. From the size distribution for the individual clones we estimate that this library, designated ZE3, consists of about 20 plasmids with inserts of different size. (For some of these plasmids an unusually high percentage of multimeric ccc DNA molecules up to pentamers is observed!) Thus this multiple selection procedure has resulted in a manageable number of plasmids with inserted *E. coli* DNA sequences, which when isolated as ccc DNA bind to the monoclonal antibody Z-D11. Further experiments will allow a more quantitative estimate of the possible number of such sequences in the *E. coli* chromosome.

Binding of the Antibody Z-D11 to Selected Plasmids. Some of the plasmids of different size shown in Fig. 4 *Lower* were used for an independent test for antibody binding. The ccc DNA was incubated with ^{125}I-labeled antibody and subjected to agarose gel electrophoresis. Because antibody that is not bound stays at the origin, the radioactivity moving with the DNA represents the bound molecules. As can be judged qualitatively from the autoradiogram in Fig. 5 *Upper*, all of the *E. coli* plasmids still have antibody bound after moving through the gel, although bound apparently to different degrees.

DISCUSSION

The results show that the method of using antibodies against Z-DNA conformation on a solid support works for isolating sequences from genomic DNA, which have the propensity to form left-handed DNA at natural supercoil density. It provides a rather general and systematic approach for selecting such sequences from different genomes, and preliminary experiments, using mouse DNA, also gave rise to Z-DNA-containing recombinant plasmids. In a way, it supplements the method recently described for the isolation of proteins that bind selectively to Z-DNA (37).

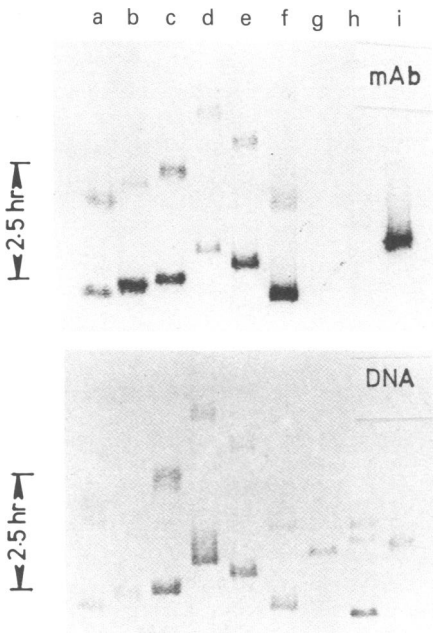

FIG. 5. Binding of 6 ng of ^{125}I-labeled monoclonal antibody Z-D11 to 50–100 ng of ccc DNA of different plasmids, after incubation for 1 hr in 0.2 M NaCl/60 mM Na phosphate/30 mM EDTA, pH 8.0, and electrophoresis on a short 1% agarose gel. The molecules leaving the gel were collected by direct blotting on DE-81 paper, which moved with constant speed across the end of the gel. (*Upper*) Autoradiography; (*Lower*) ethidium bromide staining. Lanes: a–e, individual recombinant *E. coli* clones, as shown in Fig. 4 *Lower* (lane b = lane g; c = i; d = g; e = e); f, ccc DNA of pUCZ-8; g, pUCZ-8, linearized with *Eco*RI; h, pUC-8; i, pLP32.

By all criteria employed, the antibody used here is highly specific for binding to a left-handed double-helical DNA conformation of the Z type. No crossreaction has been detected up to now for any substance that does not contain such a stereochemical arrangement of deoxyribonucleotides. It binds, for example, to acid-fixed polytene chromosomes of *Chironomus* (unpublished data) or the lampbrush chromosomes of *Pleurodeles* (unpublished data) in a characteristic pattern, as revealed by indirect immunofluorescence staining. This suggests that such chromosomes contain sequences that adopt Z-DNA-like conformation.

We have shown that a bacterium, *E. coli*, contains such sequences and estimate that about 100 clones (about 1% of the original plasmid library) contain Z-DNA-like structures in negatively supercoiled DNA, which are recognized by this specific, monoclonal antibody. Although a monoclonal hybridoma cell line is a convenient and rich source of antibody, it is not expected that the whole range of possible left-handed structures is recognized. But because one of the aims in searching for such sequences in genomic DNA is also to define the specificity of this particular antibody, it is not a pronounced disadvantage in this initial stage. By using other monoclonal or polyclonal antibodies for the selection procedure it should be possible to overcome this limitation. Also, if a less stringent selection pressure is applied—such as higher salt concentration or more negatively supercoiled DNA—a larger variety of sequences is expected to be obtained.

Further studies will show which sequences in the isolated plasmids adopt a Z-DNA structure, where they are located on the genome, and whether they serve some special biological function, as in the maintenance of topological stress, in the packing of DNA in the cell, in replication, or the regulation of gene expression.

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