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Searching for Potential *Z*-DNA in Genomic *Escherichia coli* DNA

Jörg D. Hoheisel and Fritz M. Pohl

Fakultät für Biologie
Universität Konstanz
Postfach 5560, D-7750 Konstanz, F.R.G.

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The Clarke–Carbon library with *Escherichia coli* DNA cloned into plasmid ColE1 was partially screened for *Z*-DNA with the monoclonal antibody Z-D11 using the retardation of the covalently closed circular DNA–protein complex by nitrocellulose filters. About 85% of the plasmids tested at "natural" supercoil density bound to the filter. Together with binding studies of the iodinated antibody, one *Z*-DNA segment per about 18,000 base-pairs of *E. coli* DNA is observed. One clone containing the region around the lactose operon, pLC20-30, was studied in detail. Subcloning a partial *Sau*3A digest and selection with antibodies gave three different *Z*-forming sites. They were mapped to within about ±20 base-pairs by preparing unidirectional deletion clones, selection of protein binding plasmids on nitrocellulose filters and subsequent sizing on agarose gels. The size of the *Z*-DNA-forming segments was estimated from two-dimensional gels of topoisomer mixtures. Together with results from sequencing of the plasmid DNA using exonuclease III to create single-stranded templates, stretches of alternating purine-pyrimidine tracts of 12 to 15 base-pairs were found to be responsible for *Z*-DNA formation. One of the sites was found in the middle of the *lacZ* gene, where it might be an obstacle for RNA polymerase. The methods used here should also be helpful for studying other DNA–protein sites, especially if they exist only in supercoiled DNA.

1. Introduction

Since the observation of a potential left-handed conformation of DNA (Pohl & Jovin, 1972) and the determination of its structure at atomic resolution (Wang *et al.*, 1979; Drew *et al.*, 1980), it has been shown that, for example, base modification, protein-binding or negative supercoiling of the DNA stabilize the left-handed helix at quasi-physiological conditions *in vitro* (for a review, see Rich *et al.*, 1984). *Z*-DNA tracts were identified in DNA of synthetic base sequence like alternating $(dC\text{-}dG)_n$ (Klysik *et al.*, 1981; Peck *et al.*, 1982; Singleton *et al.*, 1982), and in DNA from natural sources (e.g. Pohl *et al.*, 1982*a*; Nordheim *et al.*, 1982*a*; Nordheim & Rich, 1983*a*; DiCapua *et al.*, 1983).

On this basis, several studies were made to search for the presence of *Z*-DNA in the genomic material of living organisms, in an effort to learn about its possible biological role, but a major difficulty is the lack of suitable probes for labeling *in vivo*. Strong evidence supporting a physiological role for *Z*-DNA is the existence of *Z*-DNA binding proteins isolated from several organisms, such as *Drosophila* (Nordheim *et al.*, 1982*b*), wheat germ (Lafer *et al.*, 1985) and the simian virus (SV40) mini-chromosomes (Azorin & Rich, 1985), but no function has been revealed for any of these proteins.

Using a different approach, Kmiec *et al.* (1985) found that the rec1 protein, essential for the catalysis of genetic recombination in *Ustilago*, binds to *Z*-DNA 75 times more tightly than to *B*-DNA.

Results obtained by indirect immunofluorescence staining of polytene chromosomes of *Drosophila* and *Chironomus* species (Nordheim *et al.*, 1981; Lemeunier *et al.*, 1982; Arndt-Jovin *et al.*, 1983; Hill & Stollar, 1983; Robert-Nicoud *et al.*, 1984), mammalian metaphase chromosomes (Viegas-Pequignot *et al.*, 1983) and interphase nuclei (Morgenegg *et al.*, 1983) with antibodies against *Z*-DNA are controversial. They are in agreement with the widespread presence of $(dT\text{-}dG)_n \cdot (dC\text{-}dA)_n$ sequences in eukaryotic DNA (Hamada *et al.*, 1982), which can adopt the *Z*-conformation (Nordheim & Rich, 1983*b*; Haniford & Pulleyblank, 1983; Singleton *et al.*, 1984), but are contradictory to other experiments (Gross *et al.*, 1985). The most reproducible results for the detection of potential, natural *Z*-DNA tracts on a sequence-based

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molecular level were obtained with extracted, closed circular DNAs by means of several simple assays, in particular with polyclonal or monoclonal antibodies as detector molecules (Lafer *et al.*, 1981; Malfoy *et al.*, 1982; Pohl *et al.*, 1982*a*; DiCapua *et al.*, 1983). The torsional stress in negatively supercoiled DNA induces *Z*-DNA (Singleton *et al.*, 1982; Peck *et al.*, 1982) and stabilizes the left-handed conformation in recombinant plasmids, and in the circular genomes of PM2, SV40 and ϕX174 (Azorin *et al.*, 1983; DiCapua *et al.*, 1983; Thomae *et al.*, 1983; Stockton *et al.*, 1983; Miller *et al.*, 1983; Nordheim & Rich, 1983*a*; Zarling *et al.*, 1984). Correlation with biological functions mapping in such areas suggested a physiological role for *Z*-DNA; for example, enhancer activity (Nordheim & Rich, 1983*a*) or recombination (Kmiec & Holloman, 1984).

To obtain more information about the existence, the distribution and a functional role for *Z*-DNA sequences within a genomic DNA, we used the *Escherichia coli* genome, recombinant in a ColE1-library (Clarke & Carbon, 1975). The effective superhelical density of the DNA inside the living bacterium is considered to be only about $-0{\cdot}025$ (Lilley, 1986). This is less than that necessary for the *B* to *Z* transition but, due to the structural organization of the genomic DNA, the supercoiling of the DNA probably varies locally. This supports the proposal that the superhelicity is not an artefact of the preparation but is essential for various intracellular processes (Gellert, 1981).

An effective and convenient isolation method for circular DNA containing *Z*-DNA was developed and, based on this assay, a simple and rapid mapping protocol of high precision of protein binding-sites was established.

With the data obtained from the library, we were able to make a rough estimate of the frequency of potential *Z*-DNA tracts in the *E. coli* genome. Some particular, isolated stretches, located within or around the *lac* operon, were analyzed in more detail.

2. Materials and Methods

(a) *Strains, plasmids, monoclonal antibodies, restriction enzymes and chemicals*

All bacterial strains used are derivatives of *E. coli* K12. Strain HB101, the plasmid pBR322 (Bolivar *et al.*, 1977), the Messing strain JM83, pUC8 (Vieira & Messing, 1982) and phage M13mp18 DNA (Yanisch-Perron *et al.*, 1985) were obtained from Bethesda Research Laboratories. To get pUC18, the pUC8 polylinker was replaced by that of M13mp18. The same procedure was carried out with pUCZ8 (Thomae *et al.*, 1983) to obtain pUCZ18 as a positive *Z*-DNA standard molecule, which carries $(dC\text{-}dG)_{16}$ *in Z-form* 313 bp† *upstream from the PstI site* of the polylinker.

† Abbreviations used: bp, base-pair(s); ccc, covalently closed circular.

ColE1, vector of the *E. coli* library, in the bacterial strain CR34 (Appleyard, 1954) was a gift from Dr E. Bade.

As a standard for DNA size in gel electrophoresis, different recombinant derivatives of pUC18 and deletion clones of pUCZ18 were used, whose length is known from restriction analysis.

Z-D11 and Z-H3 are antibodies to *Z*-DNA from established monoclonal cell lines (Thomae *et al.*, 1983; Thomae, 1984).

Restriction endonucleases, dideoxy- and deoxy-ribonucleotide triphosphates were from Bethesda Research Laboratories. Boehringer-Mannheim, or New England Biolabs.

For selection of colicin E1, a crude extract was prepared (Spudich *et al.*, 1970): strain CR34 was grown at 37 °C in 200 ml of medium until the absorbance at 578 nm was 1. Mitomycin was added at a concentration of 1 μg/ml and bacterial growth was continued for about 3 h. The cells were pelleted and resuspended in 5 ml of 10 mM-potassium phosphate (pH 7·1), 1 M-NaCl. Lysis occurred upon addition of 0·5 ml of chloroform. A brief centrifugation removed debris and the chloroform: 200 μl of supernatant was used for each agar plate.

All chemicals were analytical grade commercial products.

(b) *Plasmid DNA preparation*

All plasmid-containing bacterial strains were grown overnight at 37 °C in a medium of 1·6% Difco tryptone, 1% Difco yeast extract, 0·5% NaCl or on plates with 1·5% Difco agar.

Plasmid DNA was prepared by the alkaline lysis method (Birnboim & Doly, 1979), modified as described by Pohl *et al.* (1982*b*). The DNA was purified by extraction with phenol and chloroform/isoamyl alcohol (24 : 1, v/v), followed by precipitation with ethanol. The pelleted DNA was taken up in 10 mM-Tris·HCl (pH 8·0), 0·1 mM-EDTA and stored frozen at -20 °C.

DNA concentration was determined in a pH 12 buffer (20 mM-K_3PO_4, 0·5 mM-EDTA, 1 μg ethidium bromide/ml) by measuring the increased fluorescence of the DNA-intercalated ethidium bromide (Morgan *et al.*, 1979; Pohl *et al.*, 1982*b*). The average yield of a preparation from liquid medium was 4 μg DNA/ml, in the case of pUC18-derivatives, with more than 95% being ccc DNA, or about 0·6 μg of ColE1 DNA.

All digestions with restriction nucleases were done in 33 mM-Tris-acetate (pH 7·9), 65 mM-potassium acetate, 10 mM-magnesium acetate, 0·5 mM-dithiothreitol at the appropriate temperature with an excess of 4 to 5 units of enzyme/μg DNA. After digestion, the DNA was extracted with phenol and chloroform to remove the enzyme and precipitated with ethanol.

(c) *Unidirectional deletion clones*

Unidirectional deletion with exonuclease III and S_1 nuclease (Henikoff, 1984) was carried out as described (Hoheisel & Pohl, 1986). DNA was double-digested with 2 restriction nucleases generating a 3′ protrusion, which protects the DNA from digestion by exonuclease III (Yang & Wu, cited by Guo & Wu, 1982). The DNA was then deproteinized with organic solvent and precipitated as described above. Linear DNA (150 μg/ml) was incubated with 60 units of exonuclease III (Boehringer-Mannheim) per pmol of susceptible 3′ ends. Every minute, a portion was removed from the sample and the

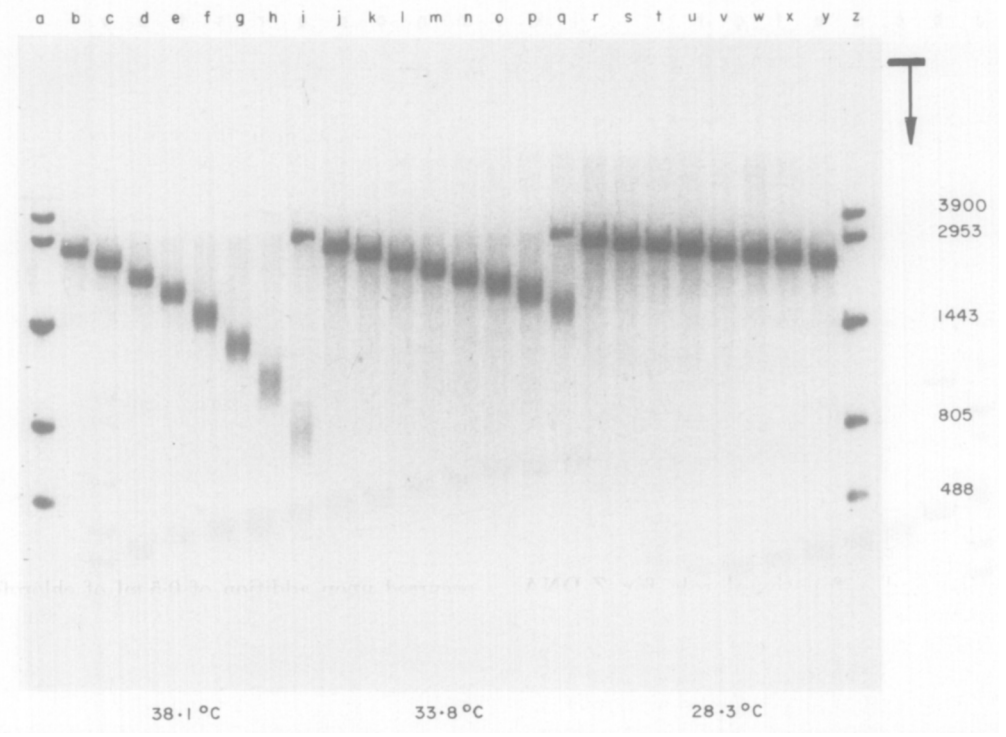

Figure 1. Unidirectional deletion in pUCZ18 linearized by digestion with *Pst*I and *Sma*I digestion. The plasmid DNA was digested with exonuclease III at the indicated temperatures. Samples were taken at 1 min intervals and the reaction was immediately stopped by heating, followed by digestion with S_1 nuclease at room temperature as described in the text. The rate of deletion in the linear DNA, 270 bp/min at 38·1 °C, 135 bp/min at 33·8 °C, and 80 bp/min at 28·3 °C, was determined by electrophoresis in a 1% agarose gel. The standards (lanes a and z) have a size of 3900 bp, 2953 bp, 1443 bp, 805 bp and 488 bp.

enzyme was inactivated by heat. S_1 buffer and S_1 nuclease (18 units, PL Biochemicals) were added to digest the remaining single strand. The addition of a 3rd buffer shifting the pH to 8·0 stopped this reaction. Klenow fragment, purified from recombinant bacteria (Joyce & Grindley, 1983), and nucleotide triphosphates were added to create blunt ends. The DNA was ligated with high-titer phage T4 DNA ligase (isolated as described by Tait *et al.*, 1980). Calcium-treated JM83 cells were transformed using 10 µl of the 45-µl ligation mix. This "1-tube" protocol involves the minimum of handling and avoids the loss of any DNA molecules. Changing the incubation temperature of the exonuclease III digestion is an easy way to manipulate the rate of deletion (Fig. 1), which is doubled by a temperature increase of about 6 deg.C (e.g. about 200 bp/min at 37 °C, 30 bp/min at 22 °C). Figure 2 shows some sets of unidirectional deleted ccc plasmid DNA, each set prepared from 1 agar plate. The deletion rate was synchronous; the background of none or bidirectional-digested DNA was very small and is directly dependent upon the quality of the cut by the restriction nucleases. To check the distribution of length within a set, more than 100 plasmids from single, randomly selected colonies were screened in 4 different experiments. At least 75% were within a range of 10% of the removed number of nucleotides around the average size.

(d) *Isolation of Z-DNA-containing plasmids*

DNA (0·1 to 0·5 µg) was mixed with 2 µg of Z-D11 10 mM-Tris·HCl (pH 8·0), 0·1 mM-EDTA, 50 mM-NaCl in a final volume of 10 to 30 µl and incubated for about 40 min at room temperature. After incubation, 60 µl of buffer was added. The sample was passed through a sterile Millipore Millititer-HA nitrocellulose filter (in a Millititer vacuum filtration holder; pore size 0·45 µm) followed by 100 µl of buffer and collected in a well of a Microtiter plate. Then the filter was washed with about 1·5 ml of buffer to remove all DNA not completed with the antibody. The plasmids, retained on the membrane by the antibody, were eluted twice, after incubation for about 5 min at room temperature, in 2 × 75 µl of buffer containing 50 µM-ethidium bromide. To recover also the DNA in the drop hanging beneath the filter, another 20 µl of buffer was passed through. To the filtrate and filter eluate, respectively, NaCl was added to a final concn of 0·3 M. The DNA was concentrated by precipitation with ethanol. Occasionally, 10 µg of tRNA was added to enhance the precipitation.

(e) *Antibody binding*

Antibody binding to plasmid DNA was determined semi-quantitatively by the electrophoretic retention of the DNA–antibody complex in agarose gels (Pohl *et al.*, 1982*a*).

Quantitative results were obtained with ^{125}I-labeled antibodies with a specific activity of about 10,000 cts/min per ng, prepared by the Chloramine T method (Hunter & Greenwood, 1962). A 500 µl column of Sepharose CL-4B (Pharmacia) was equilibrated in 10 mM-Tris·HCl (pH 8·0), 30 mM-EDTA, 50 mM-NaCl, 10 µg bovine serum albumin/ml (Serva, Heidelberg). This buffer was used in all steps. DNA (1·8 nM) and ^{125}I-labeled antibodies (14 nM) were incubated for about 40 min at room temperature. Glutaraldehyde was added (final concn 0·1%) to crosslink bound antibodies to DNA (37 °C for 15 min: Nordheim *et al.*, 1982*a*). The sample was loaded

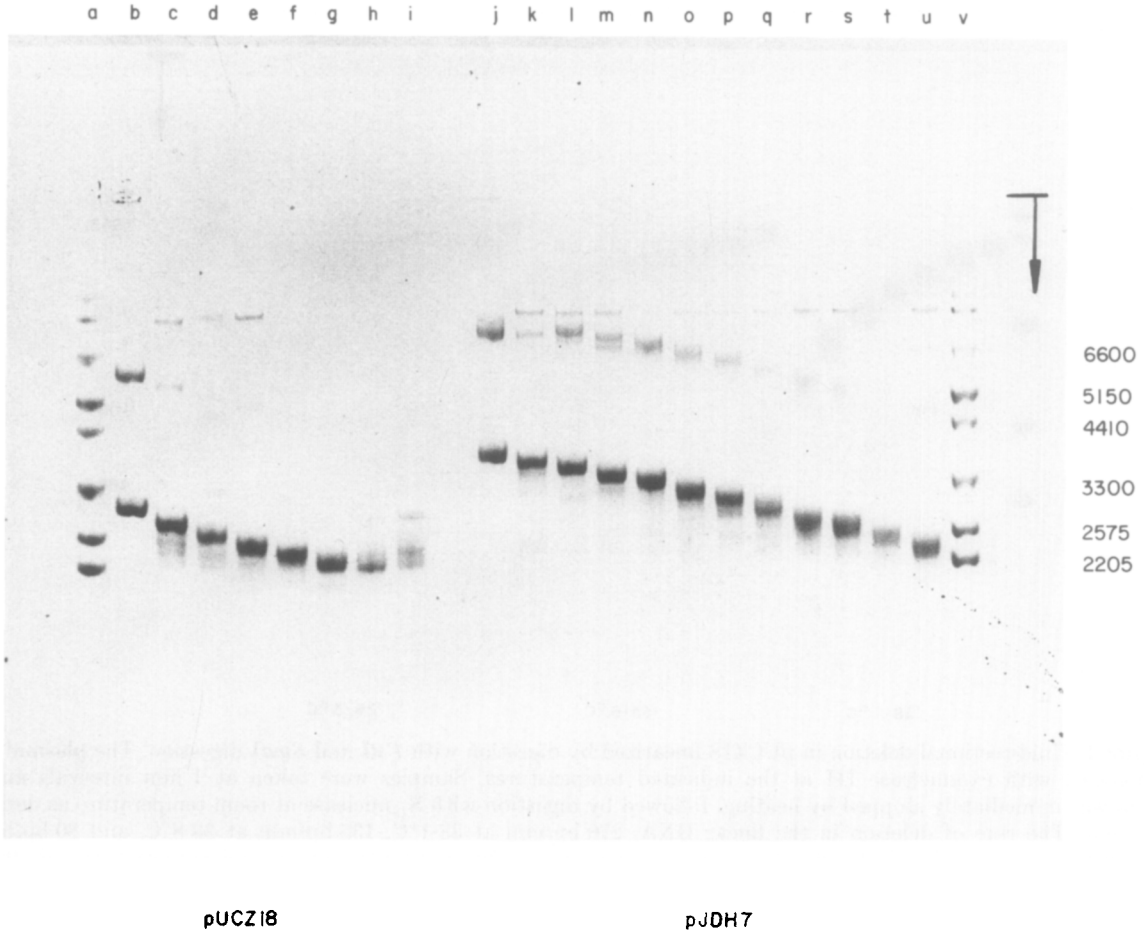

Figure 2. ccc DNA deletion clones. pUCZ18-DNA was digested with *Pst*I and *Sma*I and was unidirectionally digested with exonuclease III at 32°C followed by S_1 nuclease treatment, fill-up with Klenow-enzyme, blunt end ligation, and transformation. The ccc DNA from a plate with all deletion derivatives obtained from 1 portion, the exonuclease III reaction was terminated at 1 min intervals, was separated in an agarose gel (b to i). No truncated derivatives of pUCZ18 were obtained shorter than 2270 bp (g to i); they lost their origin region due to the exonuclease III treatment. Since almost equal total amounts of DNA are applied in each lane, the background of non-uniformly digested plasmids increases in these lanes. The same procedure with an exonuclease III digestion at 30°C was performed with pJDH7, linearized by the restriction nucleases *Pst*I and *Xba*I (j to u). Standards are monomeric and dimeric ccc DNA of plasmids with 3300 bp, 2575 bp, 2205 bp in the monomeric form (a and v).

onto the column and eluted. Fractions (40 µl) were collected and counted.

(f) *Subcloning the plasmid pLC20-30*

pLC20-30 (5·7 µg), a plasmid of about 30,000 bp, was partially digested with 8 units of *Sau*3A in a volume of 30 µl at 37°C for 15 min. The reaction was terminated by the addition of 1 µl of 0·5 M-EDTA and incubation at 68°C for 10 min. The DNA was purified by extraction and precipitation as described above.

*Bam*HI-digested pUC18 (0·9 µg) was incubated with 0·01 unit of calf intestinal alkaline phosphatase (Serva, Heidelberg) in 50 µl of 50 mM-Tris·HCl (pH 9·5), 1 mM-$MgCl_2$, 0·1 mM-$ZnCl_2$, 1 mM-spermidine (Maniatis *et al.*, 1982) at 37°C for 30 min. The incubation was continued for a further 30 min after further addition of 0·01 unit of phosphatase. Then 50 µl of 20 mM-Tris·HCl (pH 8·0), 2 mM-EDTA, 200 mM-NaCl and sodium dodecyl sulfate (final concn 0·1%) were added and the enzyme was inactivated by heating at 68°C for 15 min. The DNA was precipitated with ethanol.

About 0·6 µg of this vector DNA was ligated overnight at 4°C with about 2·8 µg of *Sau*3A-digested pLC20-30 in 30 µl of 30 mM-Tris·HCl (pH 7·6), 10 mM-$MgCl_2$, 5 mM-dithiothreitol, 1 mM-ATP (Serva) by 4 µl of T4 DNA ligase; 6 µl of this ligation was transformed into 200 µl of competent JM83-cells.

(g) *Mapping of Z-DNA*

The principle of the mapping procedure is shown in Fig. 3. Plasmid pUC18 with insert DNA cloned into its *Bam*HI site was digested either with the restriction nucleases *Sst*I and *Sma*I at the "left" side of the insert or with *Xba*I/*Pst*I at its "right". Digestion was performed for several hours at 37°C, to ensure the complete cut of the restriction sites.

Progressively deleted subsets of ccc plasmid DNA were prepared by unidirectional deletion as described above. The exonuclease III reaction was at 30°C to 32°C (90 to 120 nucleotides/min) and portions were removed at intervals of 1 min. The length of deletion in the ccc DNA subsets was checked by agarose gel electrophoresis to avoid gaps in the range of size that was covered by the pool of all subsets. From each set, about 50 ng of DNA

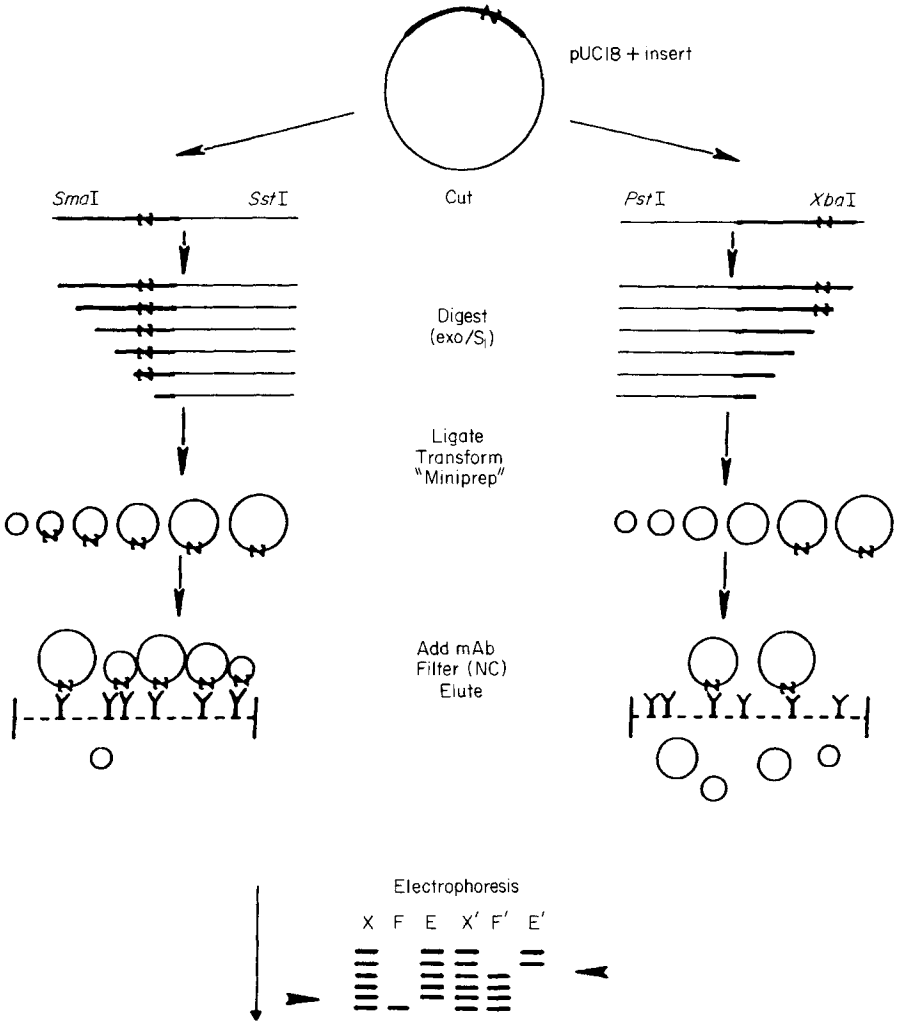

Figure 3. Scheme for Z-DNA mapping (also applicable to map other protein binding sites) within an insert DNA cloned into the *Bam*HI site of the pUC18 polylinker. The plasmid is digested either with the restriction nucleases *Sst*I and *Sma*I or with *Xba*I and *Pst*I, which are flanking the inserted DNA at the "left" and the "right" side, respectively. ccc DNA deletion clones of progressively smaller size are obtained by unidirectional deletion as described. A mixture of them is incubated with monoclonal antibodies (mAb) against Z-DNA. Plasmids that have lost the Z-DNA segment are washed through, while the others are retained by the nitrocellulose membrane (NC) and eluted with ethidium bromide. The DNA of both fractions is electrophoresed. Lane X of the schematic electrophoresis pattern shows the entire pool of truncated DNA, F the DNA in the filtrate, E the eluted DNA.

were used, at most a total of 600 ng. It was incubated with 2 μg of Z-D11 for about 1 h at room temperature. The bound and the free DNA were separated by filtration through nitrocellulose and the two fractions were applied to a 1·5% agarose gel, run in 40 mM-Tris-acetate (pH 8·2), 1 mM-EDTA, 0·5 μg ethidium bromide/ml for about 4 h at 7·5 V/cm in the presence of appropriate standard plasmids. After electrophoresis, the gel was photographed under long-wave ultraviolet illumination; the distance of the binding site from the restriction sites that were used for linearizing the original recombinant plasmid was determined by the position of the break in the 2 DNA patterns in the gel.

(h) *Plasmid DNA sequencing*

A method of sequencing within plasmid DNA, proposed by Smith (1979), using exonuclease III to create the single-stranded template for the enzymatic chain terminator method (Sanger *et al.*, 1977), was adapted to the pUC18-system.

For each experiment, 0·3 pmol of recombinant pUC18 with insert DNA ligated into the *Bam*HI site was digested with 2 restriction enzymes, *Sst*I and *Sma*I for sequencing with the commercial universal 15 bp primer, *Xba*I and *Pst*I for sequencing the other strand with the 17 bp reverse primer (from Boehringer-Mannheim or synthesized by the phosphite triester method). The linear plasmid was digested with exonuclease III at 37 °C as described above, removing 1 strand unidirectionally. After the enzyme had worked about 500 bases downstream from the primer binding site of the remaining strand, boiling for 3 min in a volume of 12·5 μl containing 1 μl of 70 mM-Tris · HCl (pH 7·5), 70 mM-$MgCl_2$, 500 mM-NaCl, 0·8 pmol of primer and water to the final volume terminated the reaction. The sample was slowly cooled to room temperature for primer annealing. Dithiothreitol (1 μl of 0·1 M: Sigma, Munich) was then added and 7·5 μCi of dried [^{32}P]dATP (Amersham; 3000 mCi/mmol) were taken up in the sample. The dideoxy-reactions were performed according to the recipes given by Hindley & Staden (1983) at room temperature. The annealed DNA

was added to the nucleotide mixture and the reaction started upon addition of the large fragment of DNA polymerase I (Boehringer-Mannheim). The reaction was quenched by adding 1 vol. 20 mM-EDTA, 0·03% xylene cyanol, 0·03% bromphenol blue in 100% formamide. Electrophoresis with direct blotting to Nytran membrane (Schleicher & Schüll) or Biodyne-A membrane (Pall) as immobilizing matrix was as described (Beck & Pohl, 1984).

All sequences were determined several times in both directions.

(i) *Two-dimensional electrophoresis*

Plasmid topoisomers were prepared by relaxation of 1 μg of DNA with about 20 units of calf thymus topoisomerase I (isolated as described by Pulleyblank & Morgan, 1975), in the presence of 0 to 20 μM (steps of 5 μM) ethidium bromide in 50 μl of 10 mM-Tris·HCl (pH 8·0), 2 mM-EDTA, 0·2 M-NaCl for about 30 min at 37 °C (Peck & Wang, 1981). The reaction mixture was extracted with phenol/chloroform and then precipitated with ethanol. The DNA was diluted to a concentration of about 0·1 mg/ml. The superhelical density was determined by agarose gel electrophoresis (Keller, 1975). Several samples were pooled to get a broad distribution of topoisomers.

Two-dimensional electrophoresis was performed in 1% agarose, dissolved in 90 mM-Tris-borate (pH 8·3), 2·5 mM-EDTA (Peck & Wang, 1983); 0·5 to 0·8 μg of DNA was applied to the gel. Different mixtures of topoisomers were applied in slots, which were displaced in the x and y directions by a known distance. The 1st-dimension electrophoresis was run for 24 to 30 h at 4·5 V/cm at a temperature of 18 °C. The gel slice with DNA was soaked in buffer containing 0·88 μM-chloroquine. It was turned by 90° and a fresh gel with chloroquine poured around. Electrophoresis in the 2nd dimension was similar to the 1st but in the presence of chloroquine. After the run, the gel was stained in 1 μg ethidium bromide/ml for 1 h, destained in water for the same time, and then photographed using either positive or negative Polaroid film, with an excitation wavelength of 366 nm and a cut-off filter.

3. Results

(a) *Isolation of Z-DNA*

Antibodies to *Z*-DNA (Lafer *et al.*, 1981; Malfoy & Leng, 1981) have become an important instrument for investigating this DNA structure. Precipitation experiments with labeled *Z*-form DNA in the presence of a large excess of right-helical calf thymus DNA using monoclonal antibodies indicated the selectivity and sensitivity of recognition (Pohl, 1983). Affinity chromatography with antibodies to *Z*-DNA allows the isolation and purification of plasmids from genomic libraries (Thomae *et al.*, 1983). The large capacity of such columns allows their use for preparative isolation purposes. Chemical linkage between column beads and antibody, and the time-intensive recovery procedure are disadvantages. But the major drawback is the size discrimination by the matrix material, which limits the range of effective usage by exclusion of large molecules.

Therefore, the solid support of the column beads was replaced by nitrocellulose, commonly used to select DNA fragments bound to proteins (Riggs *et al.*, 1968). The two steps of isolation (detection and selection) are thereby separated in space and time. So, it is possible to regulate the stringency of isolation in two ways, by means of incubation as well as filtration, e.g. by varying either ion concentration, which influences the affinity (Azorin *et al.*, 1983), or the concentration of DNA intercalating dyes. Since the amount of antibody and the incubation time are not determined by the column size, DNA with a low potential to adopt the *Z*-conformation can be isolated easily. Normally, the DNA was incubated with an excess of antibodies. Complexation changes the *B*/*Z* equilibrium of DNA towards *Z*-formation, increasing the final amount of *Z*-positive molecules.

Elution was achieved by intercalating ethidium bromide, which unwinds the DNA. The supercoiling is reduced and *Z*-DNA converts to the *B*-structure, dissociating the antibody–DNA complex and allowing the DNA to pass the filter.

Various experiments (Fig. 4) show the high selectivity of this simple procedure for the particular plasmids with *Z*-DNA, as known from other experiments. Only ccc DNA (monomers and multimers) with *Z*-DNA tracts are retained, whereas open circular and linear forms pass the filter.

We examined plasmid DNA up to the size of about 30,000 bp for non-specific retention on the filter. All the double-stranded DNA, independent of the particular form, was able to pass the filter (pore size of 0·45 μm), and was not affected by bovine serum albumin, added to the buffer in various concentrations.

A high percentage of ethidium bromide was bound to the nitrocellulose, preventing reuse of the same filter well.

(b) *Screening of Z-DNA in recombinant* E. coli *DNA*

E. coli chromosomal DNA exists in a state of structural organization (Worcel & Burgi, 1972) causing torsional stress, which is apparently essential for biological processes. Therefore, *Z*-DNA, a conformation favored by negative supercoiling, should be included in discussions about functional regulations. Since the possibility of transition between the *Z* and *B*-forms exists, it might encode variable information, a requirement for any structure involved in regulation.

To examine the frequency and location of potential left-helical DNA within the entire *E. coli* genome, the Clarke–Carbon library (Clark & Carbon, 1975, 1976) was screened. *E. coli* K12 chromosomal DNA was sheared mechanically into pieces with an average size of 14,000 bp. These segments were cloned into the unique *Eco*RI site of ColE1 by AT-tailing. Recombinant plasmids have lost their colicinogeneity but not their immunity. The approximately 2100 clones comprising this library contain, with

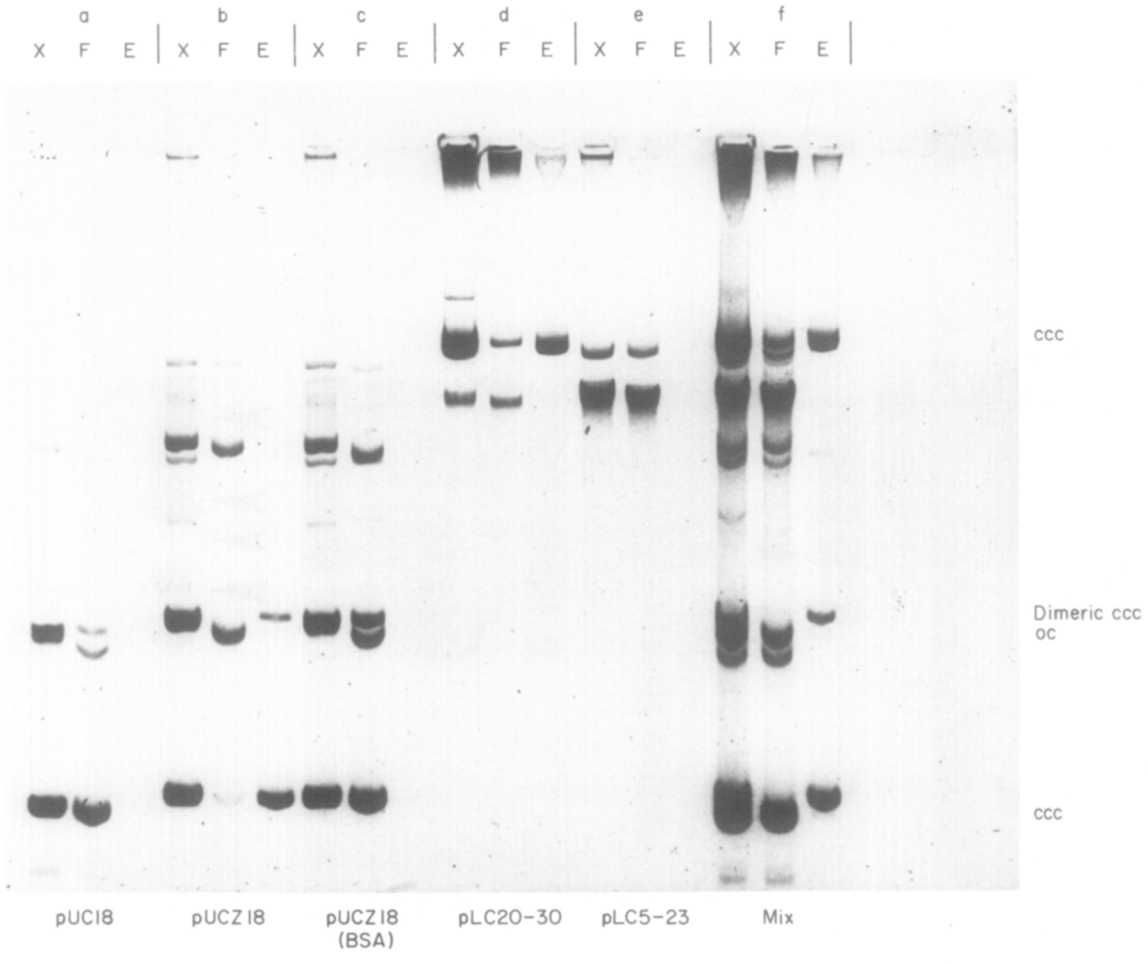

Figure 4. Isolation of *Z*-DNA-containing plasmids by binding of the monoclonal antibody Z-D11 and retention on nitrocellulose filters. About 100 ng of plasmid DNA was incubated with Z-D11, filtered, and eluted with ethidium bromide. DNA in the filtrate and eluate, respectively, were precipitated and then electrophoresed in an agarose gel (the smiling effect is due to the tRNA added to enhance the DNA precipitation). Three lanes per assay are used: untreated DNA as a control (X), the DNA in the filtrate (F), the DNA eluted with ethidium bromide (E). Plasmids containing *Z*-DNA segments (pUCZ18 (b and c), pLC20-30 (d)) and those without (pUC18 (a), pLC5-23 (e)) were assayed. A mixture of them was used in the 6th (f) experiment; the 3rd experiment (c) was identical with the 2nd but bovine serum albumin was substituted for Z-D11. No DNA bound to the filter in (a), (c) or (e).

high probability, the entire genome. Handling them was difficult due to the small and staggering rate of transformation and a relatively poor yield in preparation of about 1·5 μg of ccc DNA from a plate with about 100 large bacterial colonies, in comparison to pUC18 derivatives with a similar yield from just a few colonies.

For screening, 15 to 45 clones were grown overnight on an agar plate. The cells were scraped from the plate and the plasmid DNA was prepared in one tube. After incubation with the antibody Z-D11, it was filtered through nitrocellulose and rinsed well. The bound DNA was eluted, precipitated, washed with 70% ethanol to remove the ethidium bromide, transformed and spread on selection plates, parallel with untreated, non-selected DNA from the same source. Plasmid DNA obtained from both transformation plates was compared by gel electrophoresis (e.g. see Fig. 5). While ColE1 plasmids were lost by this treatment, in most cases, no particular change in the band pattern occurred. Only 14% of the bands disappeared and 1·5% were significantly reduced in intensity after repetition of the whole procedure. About 80% showed no change compared with the control lane, while about 4·5% of the screened plasmids were enriched in concentration.

This means that about 15 to 16% of the screened plasmids have no *Z*-DNA at the supercoil density of the recombinant plasmid DNA, or only at a very low potential of formation.

Since this is a rather high percentage of *Z*-DNA sequences, the amount of *Z*-DNA was determined for some particular plasmids. About 10% of the entire *E. coli* genome is covered by the insert DNA, whose location in the chromosome is known (plasmids pLC3-22, 3-24, 3-33, 3-46, 4-5, 4-6, 4-14, 4-21, 5-14, 5-23, 5-31, 7-18, 7-19, 8-29, 9-34, 10-4, 15-12, 17-22, 17-43, 20-30, 26-8, 34-34 and 37-40; Neidhardt *et al.*, 1983). An excess of ^{125}I-labeled antibody was incubated with the plasmid DNA of a supercoil density as prepared from *E. coli*. Bound

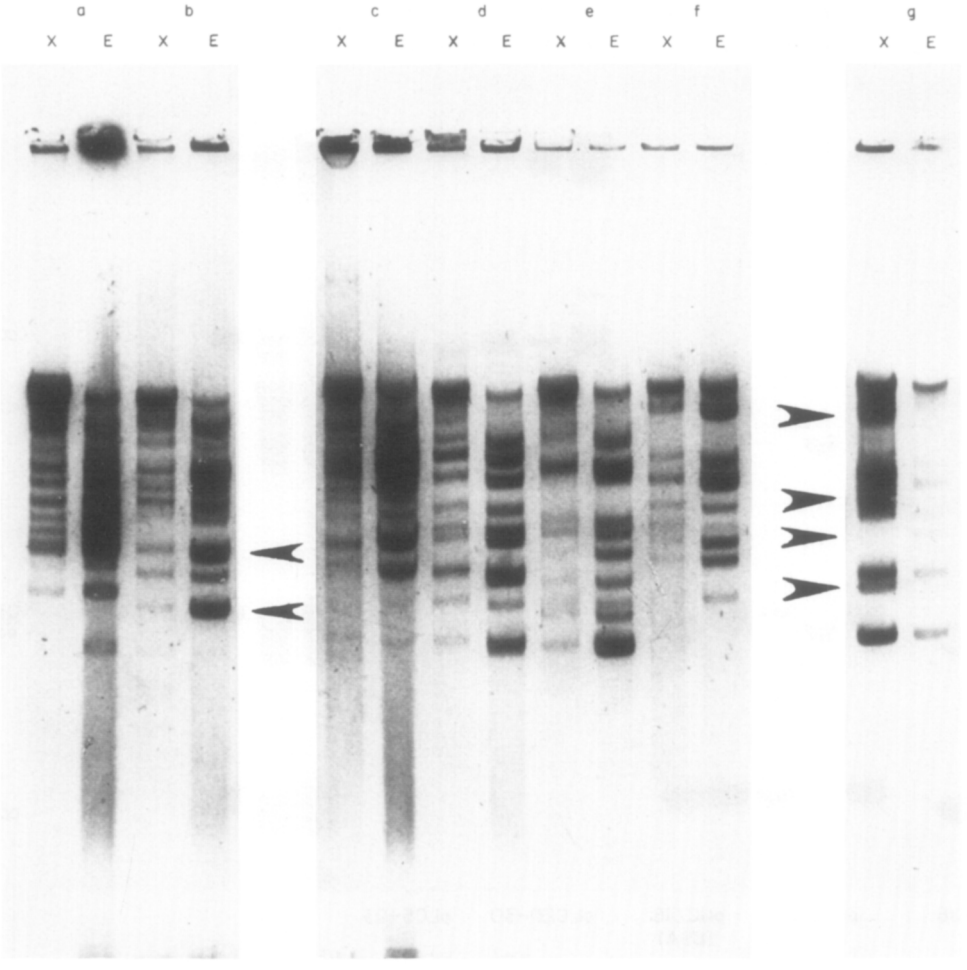

Figure 5. Screening for *Z*-DNA in *E. coli*. Mixtures of plasmids containing recombinant *E. coli* DNA were selected for *Z*-DNA by antibody binding and nitrocellulose filtration. The eluate from the filter (E) was transformed in parallel to DNA applied to the filter (X). The DNA preparations of this parallel transformation plates were compared by electrophoresis; (X) the pattern obtained with untreated DNA, (E) that obtained with DNA selected for *Z*-DNA. The arrows indicate some particular differences of the DNA patterns. The clones used were: (a) pLC5-1 to pLC5-36, (b) pLC17-1 to pLC17-37, (c) pLC26-1 to pLC26-48, (d) pLC27-1 to pLC27-47, (e) pLC36-1 to pLC36-48, (f) pLC39-1 to pLC39-48, and (g) pLC15-25 to pLC15-48.

antibodies were crosslinked to the DNA by glutaraldehyde and the plasmid DNA and the free antibodies were separated almost completely in a 500-μl column of Sepharose CL-4B (Fig. 6).

The elution-profile of Z-D11 obtained with linear DNA or form I of pUC18, even in a 20-fold excess, was identical with that of free antibodies. No radioactivity moved with the DNA.

The long binding left-handed tract in pUCZ18, with $(dC\text{-}dG)_{16}$ in *Z*-form, is indicated by a large peak of radioactivity in the DNA fractions. To date, Z-forming regions in pBR322 are detected only in artificial, highly supercoiled plasmids (Nordheim *et al.*, 1982*a*). In the assay shown here, the small but reproducible peak of bound antibodies, increasing linearly with the amount of DNA, shows the *Z*-DNA in pBR322, prepared at "natural" superhelicity.

There is also a small affinity of Z-D11 to ColE1, the vector of the library.

Data obtained from a few hydrid plasmids are listed in Table 1. The clones assayed had an average size of about 17,100 bp. About 13% of the plasmids showed the same binding capacity as the pure vector ColE1. The others bound, on the average, about 0·63 antibody per 10,000 bp of insert DNA under these conditions used. Including the hybrids with no affinity, it was roughly estimated from this data that there is one binding site per 18,000 bp of *E. coli* DNA detectable with our antibodies. The ratio is in agreement with 85% of library clones, which were retained by the nitrocellulose. This led to a value of about one binding site per 16,500 bp. Z-D11 shows preferential binding to dC-dG segments in the *Z*-form. Another antibody, called Z-H3 which shows less sensitivity to methylation of cytosine (Thomae, 1984), was used for some binding assays. The results obtained are similar to those with Z-D11 (Fig. 6).

(c) *Mapping of Z-DNA tracts*

For further investigation of potential *Z*-DNA, the vector and selection system of the Clarke–Carbon

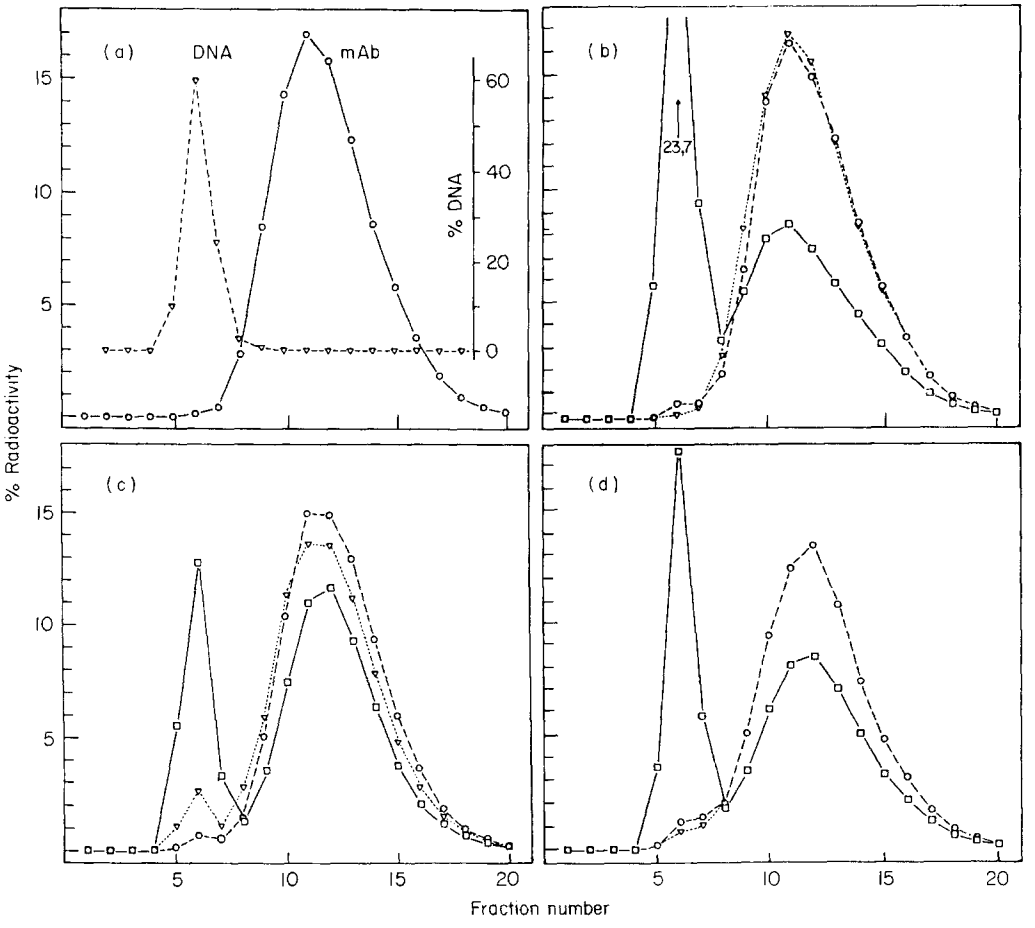

Figure 6. Determination of Z-DNA tracts by binding and crosslinking ^{125}I-labeled monoclonal antibodies against Z-DNA. (a) Free antibody (○) and the plasmid-DNA (▽) were separated on a Sepharose CL-4B column as described in Materials and Methods. (b) and (c) The elution profiles of Z-D11 obtained with various plasmids ((b) pUCZ18 (□), pBR322 (○), pUC18 (▽); (c) pLC20-30 (□), pLC5-23, ColE1 (○), pLC4-6 (▽)). (d) Results with a different monoclonal antibody, Z-H3 (pLC20-30 (□), pLC5-23 (○), ColE1 (▽)). The amount of radioactivity moving with the DNA is proportional to the amount of Z-DNA–antibody complex.

bank is rather inconvenient and the inserted DNA is too long. For a more precise examination, the plasmid pLC20-30, which showed the highest antibody binding of all hybrids, was subcloned into pUC18. *Z*-forming plasmids were isolated and amplified by three cycles of antibody binding, nitrocellulose filtration and transformation of the eluted DNA (Fig. 7). The complete loss of the original vector DNA after the first selection process served as an internal standard for the selectivity. Twelve plasmids were isolated from single clones obtained by transforming the DNA bands cut out of a low-melting-point agarose gel (Crouse *et al.*, 1983). They were analyzed by a *Sau*3A digestion. Subsequent treatment with *Bss*HII changed the *Sau*3A pattern. For each plasmid, one specific band

Table 1
Binding of monoclonal antibody Z-D11 to supercoiled plasmid DNA

Plasmid	bp	Radio-activity (%)	Bound mAb/ 10,000 bp *E. coli* DNA	Location in *E. coli* (min)	Genes
pLC20-30	30,000	21·7	0·66	8	*lac*I, Y, Z
pLC4-6	21,000	5·8	0·24	28	*trp*B, C, D, E, *top*A, *cys*B
pLC5-23	19,500	1·4	0·00	28	*trp*B, C, D, E, *top*A, *cys*B
pLC8-29	15,000	9·4	0·72	49	*glp*T
pLC3-22	9750	5·5	1·00	92	*dgk*A, *pls*B
pLC7-18	9050	2·1	0·22	42	*uvr*C, *tyr*P, *fla*D
ColE1	6650	1·4	—	—	—
pUCZ18	2953	39·2	—	—	—

^{125}I-labeled antibodies to the plasmid DNAs were counted as described in Materials and Methods.

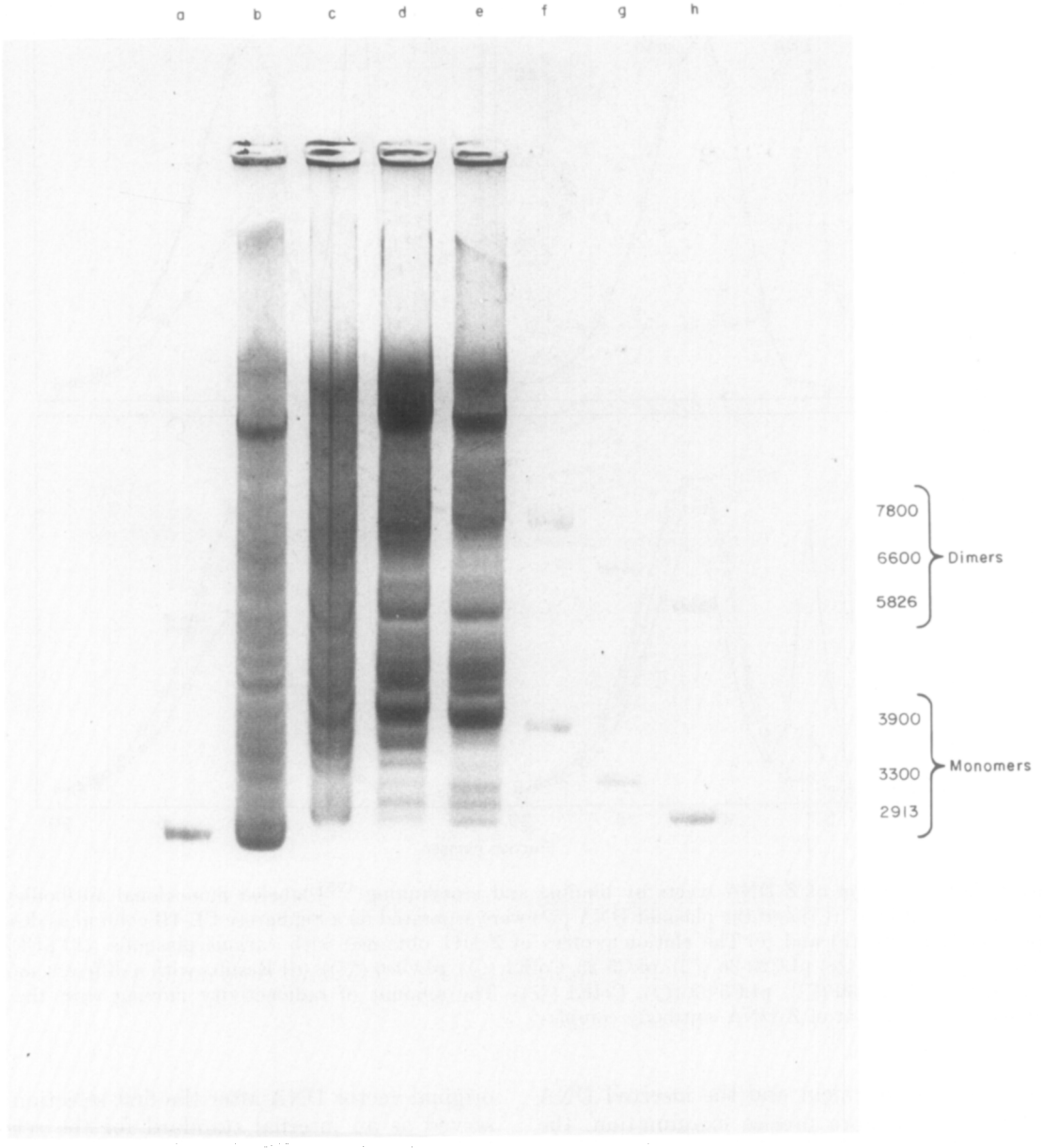

Figure 7. Subcloning of the plasmid pLC20-30, which was partially digested by the restriction nuclease *Sau*3A and subcloned into the *Bam*HI site of pUC18; (a) the vector pUC18, (b) the entire sub-library. Z-DNA-containing plasmids were isolated by 3 cycles of affinity filtration with Z-D11 and transformation (c) to (e). (f) to (h) pJDH7 (3900 bp), pJDH6 (3300 bp), and pJDH1 (2913 bp), 3 particular plasmids isolated after the 3rd selection cycle.

disappeared in the gel (for 9 of the 12 clones, see Fig. 8). From this restriction analysis it was concluded that three distinct segments of pLC20-30 were isolated, suggesting three binding sites. Three plasmids, pJDH1, pJDH7 and pJDH9, containing these segments were chosen for further investigation.

Unidirectional deletion with exonuclease III allows a simple and rapid mapping of *Z*-DNA tracts: the DNA is digested either with *Sst*I and *Sma*I, for deletion from left to right, or with *Xba*I/*Pst*I for right–left deletion. DNA sets of progressively smaller sizes are obtained after treatment with exonuclease III and S_1 nuclease, followed by blunt end ligation and transformation. A pool of these sets covers the entire range of deletions from the original recombinant plasmid down to the pure vector. This mixture is subjected to antibody binding and filtration through nitrocellulose as described. Molecules that have lost their binding site by the unidirectional deletion are washed through the filter. Those retained on the membrane are eluted in a buffer containing ethidium bromide. The DNA of the wash fraction and that of the eluate are applied to an agarose gel and electrophoresed in the presence of appropriate standard DNA. The position of the binding site is determined redundantly by the break in the two DNA patterns. Repeating the mapping from the other side make it possible either to prove and verify the position of the binding site or to detect a second one in the same plasmid.

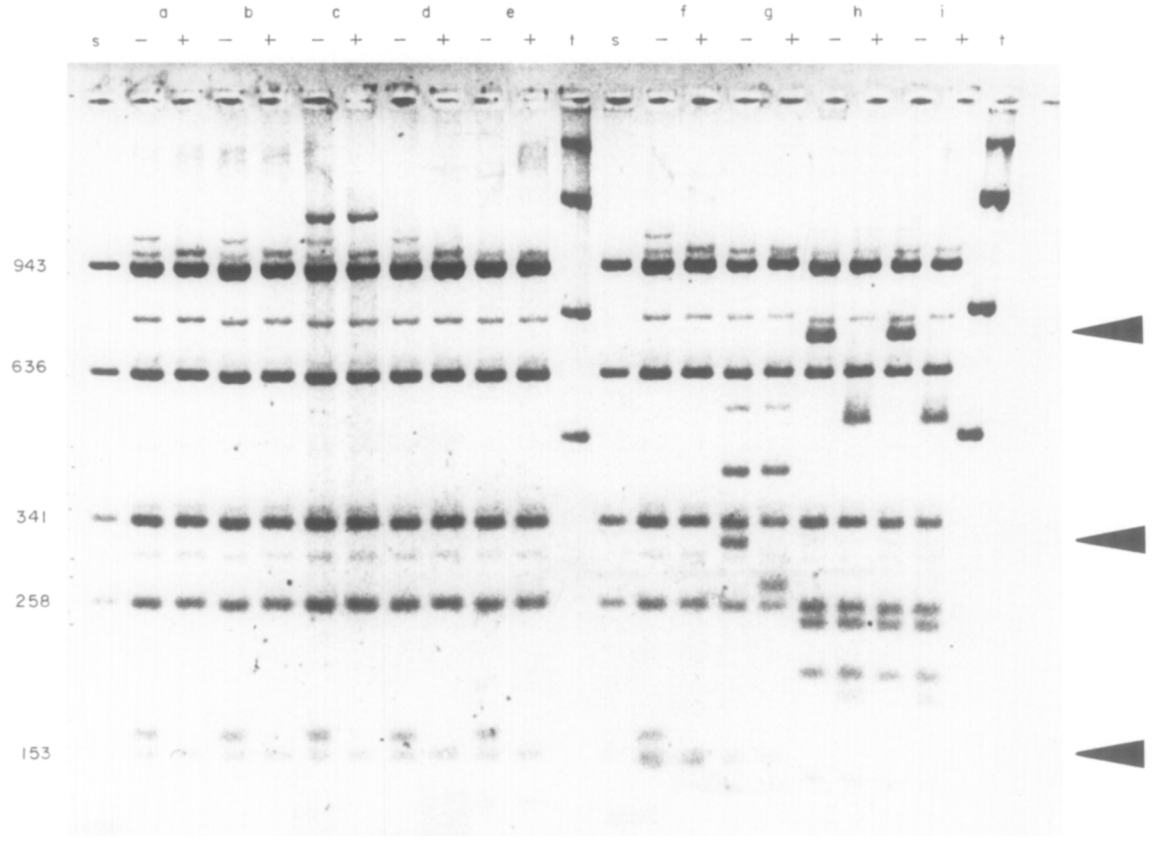

Figure 8. Restriction analysis of pJDH1 to pJDH9, (a) to (i). The plasmids were randomly isolated from the pLC20-30 sub-library after selection for *Z*-DNA-containing plasmids. The DNAs were completely digested with the restriction nuclease *Sau*3A (−) followed by a subsequent digestion with *Bss*HII (+). Standard (s) is the *Sau*3A-fragment pattern of the vector pUC18 (943 bp, 636 bp, 341 bp, 258 bp, 153 bp, and some smaller fragments); standard (t) that of a *Taq*I digestion mixed with linear pUC18. Electrophoresis was in 3% low-melting-point agarose. The arrows mark the 3 *Sau*3A fragments that disappear after *Bss*HII digestion.

To test this approach, the plasmid pUCZ18 was used as a model system. It was digested with *Pst*I, creating the 3′ protrusion, and *Sma*I, followed by the deletion towards its *Z*-forming $(dC\text{-}dG)_{16}$ segment. No unidirectional-digested derivatives were obtained shorter than 2270 bp (Fig. 2), although there was no stoppage of deletion in the linear DNA (Fig. 1). Shorter DNA had lost the origin region by the digestion and could therefore not replicate. All those plasmids smaller than 2270 bp, which were isolated and assayed by restriction analysis, are bidirectionally digested (data not presented).

The sets of truncated pUCZ18 were pooled, incubated with Z-D11 and selected by filtration through nitrocellulose. The two fractions of DNA were electrophoresed in an agarose gel (Fig. 9). The binding site was mapped 330 bp from the *Pst*I site with a confidence interval of 15 bp. The distances of the ends of the $(dC\text{-}dG)_{16}$ fragment from the *Pst*I site are 313 bp and 345 bp, respectively, known from the sequence. This indicated the high precision of the procedure.

The position of the *Z*-adopting sequences in the recombinant *E. coli* DNAs was determined from both sides of the inserted DNA; e.g. for pJDH7 it was 305(±30) bp from the "right" *Pst*I site and 870(±30) bp from the "left" *Sst*I site (Fig. 10). The sum of 1175 bp compares well with the insert size of 1160 bp determined by restriction analysis. The accuracy of this result was confirmed by sequencing. The distance between the *Pst*I site and the *Z*-forming sequence is 281 bp.

If the distance of the *Z*-fragment from the restriction site is small, like the 82 bp in pJDH1, the precision of mapping can be doubled by using bidirectionally deleted plasmid derivatives.

For further examination of the three *Z*-DNA tracts, single deletion clones of different sizes were picked from the appropriate deletion sets. They facilitated the determination of the *Z*-formed segments, e.g. by sequencing (Henikoff, 1984).

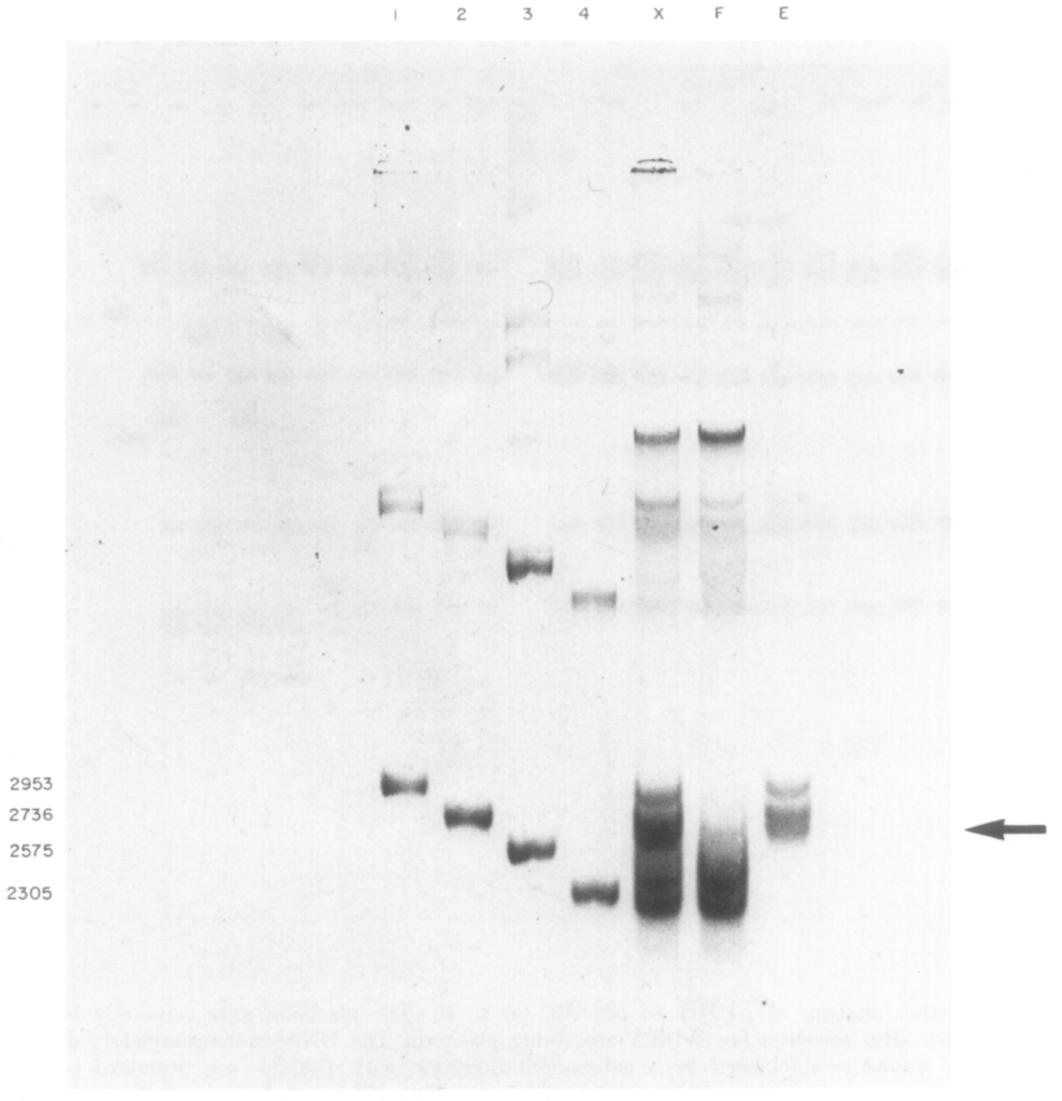

Figure 9. Mapping of the antibody binding site in pUCZ18. The subsets of unidirectionally deleted ccc DNA of pUCZ18 (see Fig. 2) were mixed (X), incubated with Z-D11, and selected by filtration on nitrocellulose as described. The DNA in the filtrate (F) and in the eluate (E) were precipitated and applied to an agarose gel. The position of the binding site is to be determined by the break of the DNA pattern in the gel as indicated by the arrow. Lanes 1 to 4 are standard plasmids of 2953 bp, 2736 bp, 2575 bp and 2305 bp.

(d) *Characterization of the Z-DNA tracts*

Two-dimensional electrophoresis is a convenient method of measuring the extent of the *B* to *Z* transition. In its first dimension, topoisomers of a superhelical state sufficient to induce left-handed DNA reduce their negative superhelicity and hence their electrophoretic mobility (Klysik *et al.*, 1981). Upon binding of chloroquine, the negative supercoiling decreases, producing a transition of the *Z*-DNA into right-handed structure and a corresponding mobility change (Peck & Wang, 1983). This shows up as a break within the trace of topoisomeric DNA (Fig. 11).

From the two-dimensional gel electrophoresis, the changes in the number of superhelical turns ($\Delta\tau$) produced by the *Z*-DNA tracts isolated from *E. coli*-DNA were determined. $\Delta\tau$ for pJDH1 was found to be $-3{\cdot}0(\pm 0{\cdot}3)$, for pJDH7 $-3{\cdot}3(\pm 0{\cdot}5)$, for pJDH9 $-2{\cdot}9(\pm 0{\cdot}2)$. With the assumption of $\Delta\tau = -0{\cdot}4$ per *B–Z* junction, and $-\Delta\tau = (1/10{\cdot}5) + (1/12)$ per base-pair in *Z*-conformation (Peck & Wang, 1983), it was possible to estimate the size of the left-helical DNA segments as being about 12 to 14 bp (see Table 2).

The supercoil density, a quotient of supercoil number and the number of right-helical turns in the relaxed molecule (Vinograd *et al.*, 1968), at which the *B* to *Z* transition occurred was $-0{\cdot}044$ for pJDH1, $-0{\cdot}042$ for pJDH7 and $-0{\cdot}043$ for pJDH9.

The range of negative superhelicity in plasmid DNA as prepared from the bacteria was examined simultaneously with the artificial topoisomer mixtures in the same experiment. There are differences in the degree of supercoiling. In pJDH1, the supercoil density was $-0{\cdot}036$ to $-0{\cdot}068$ (average $-0{\cdot}052$). Only a fraction of the "natural

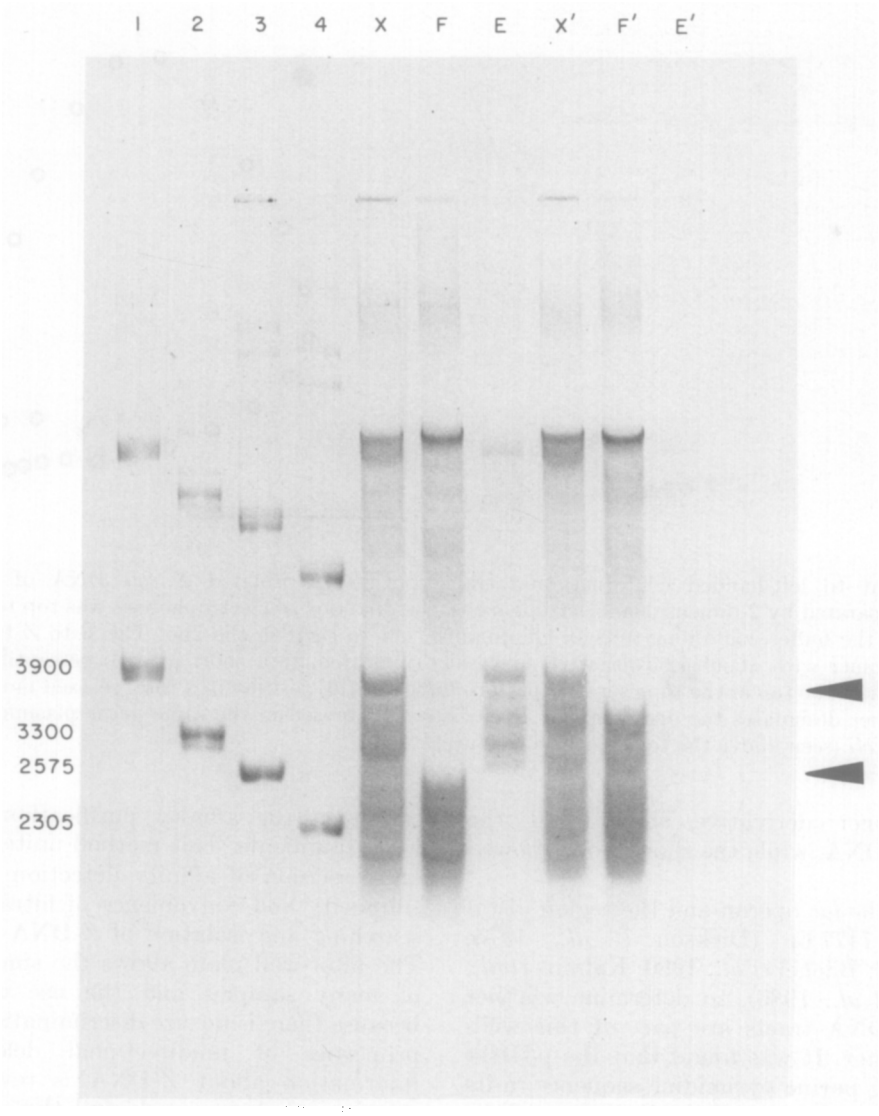

Figure 10. Mapping of *Z*-DNA in pJDH7. The plasmid was unidirectionally degraded from the left (*Sma*I/*Sst*I sites; X to E) and the right side (*Xba*I/*Pst*I sites; X′ to E′), respectively. Twelve subsets of deletion ccc DNA were prepared. A mixture of them (X) was incubated with Z-D11, filtered through nitrocellulose (F) and eluted (E). The DNA was applied to an agarose gel. The break in the DNA pattern allows the mapping of the antibody binding site as indicated by the arrows. Lanes 1 to 4; standard plasmids of 3900 bp, 3300 bp, 2575 bp and 2305 bp.

supercoiled" plasmids were actually carrying the potential *Z*-formed sequence in this conformation (Fig. 11).

pJDH9 was prepared in a supercoil density of −0·047 to −0·072 (average −0·060), pJDH7 in high superhelical forms of −0·056 to −0·077 (average −0·066), values revealed from two-dimensional electrophoresis with 1·8 μM in place of 0·88 μM-chloroquine in the second dimension to separate the single DNA topoisomers (not shown). There is *Z*-DNA in the whole pools of plasmid, prepared under "natural" conditions.

The antibody binding sites were directly sequenced using plasmid DNA. The polylinker of pUC18 permits unidirectional deletion by exonuclease III and S_1 nuclease, followed by creation of the single-stranded template DNA in the deletion clones from the other side of the polylinker using exonuclease III and sequencing by the chain termination method. Since very few clones of the deletion set had lost their primer binding site by an exonuclease activity in the S_1 nuclease, all derivatives used for sequencing were digested with *Hin*dIII and *Eco*RI to ensure the presence of the binding sites, which are beyond these restriction sites flanking the polylinker.

All the mapped positions for *Z*-DNA have alternating purine-pyrimidine sequences (Table 2), suggesting their responsibility in *Z*-formation. To check this, deletion clones that had just lost these tracts were used. The loss corresponds to the loss of the antibody site as well as with the loss of the *Z*-DNA-induced break in the pattern of the two-dimensional electrophoresis (Figs 12 and 13). Comparing unidirectional-deleted clones of pJDH9, which differ in 9 of the 13 alternating purine-pyrimidine base-pairs, indicated significantly the left-handed structure of this tract in supercoiled ccc

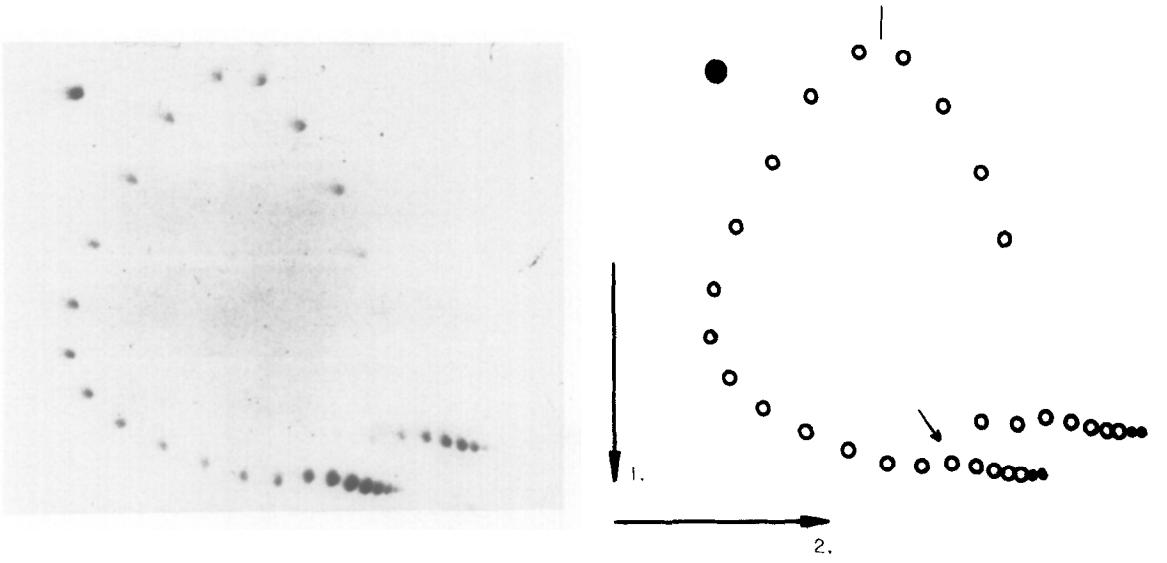

Figure 11. Right to left-handed conformational transition in the isolated *E. coli* DNA of pJDH1. Plasmid topoisomers are separated by 2-dimensional electrophoresis; the direction of electrophoresis was top to bottom in its 1st dimension and, in the buffer containing 0·88 μM-chloroquine, left to right in the 2nd. The *B* to *Z* transition occurred around the topoisomer with about 12·5 negative supercoils (indicated by a short arrow), perceptible by its reduced mobility. The mark at the top of the topoisomeric pattern indicates the position of a total relaxed molecule. The spot in the left upper corner designates the open circular DNA. Electrophoresed in the same gel is plasmid DNA pJDH1 as prepared from *E. coli* (seen above the topoisomeric mixture).

DNA. The longer derivative showed all the evidences for *Z*-DNA, while the shorter one showed none.

We analyzed the *lac* operon and the region distal to it, in all 7477 bp (Dickson *et al.*, 1975; Farabaugh, 1978; Büchel *et al.*, 1980; Kalnins *et al.*, 1983; Hediger *et al.*, 1985), to determine whether the isolated *Z*-DNA tracts are part of this well-known gene cluster. It was found that the pJDH1 15 bp alternating purine-pyrimidine sequence in its *Sau*3A fragment to be almost in the center of the *lac*Z gene, 1503 bp distant from the start codon and 1551 bp from the stop codon.

The two other isolated segments are located outside the *lac* operon in the residual 15,000 bp of pLC20-30.

4. Discussion

(a) *Affinity filtration and its use in mapping protein binding sites*

Monoclonal antibodies as detector molecules and the nitrocellulose filter as separation element simplified the affinity purification of *Z*-adopting DNA fragments. This method unites the sensitivity and precision of affinity detection with the speed, simplicity and convenience of filtration. Screening, searching and isolation of *Z*-DNA are accelerated. The filter-well plate allows the simultaneous assay of many samples and the use of large DNAs, because there is no size discrimination. Utilizing the properties of unidirectional deletion, mapping information about *Z*-DNA is revealed with the same procedure used in the isolation. This confirmed that the mapped segment is responsible for isolation. Exonuclease III removes nucleotides of each 3′ end in a processive and, when in excess, synchronous manner (Wu *et al.*, 1976). An important exception are four-base 3′-protruding ends, which are digested very inefficiently. This property of the enzyme allows, in combination with S_1 nuclease which digests the remaining single strand, the creation of unidirectional-deleted derivatives of double-stranded DNA. Cloning into the polylinker of the plasmid pUC18, especially the *Bam*HI site at its centre, simplifies a number of applications; for example, deletion clones were used

Table 2

Z-DNA-forming sequences isolated from the E. coli *genome*

Plasmid	Sequence	Length Z-DNA† (bp)
pJDH1	TTTGCCCG ATGTACGCGCGCGTG GATGAAGA	12
pJDH7	ACCCTCTT TGCGCGCGCGCA GAATGCGT	14
pJDH9	TGTGGCAA ACACGCGCGCACG GTCCTTGT	12

† The length of the Z-DNA tracts is estimated from the 2-dimensional gels.

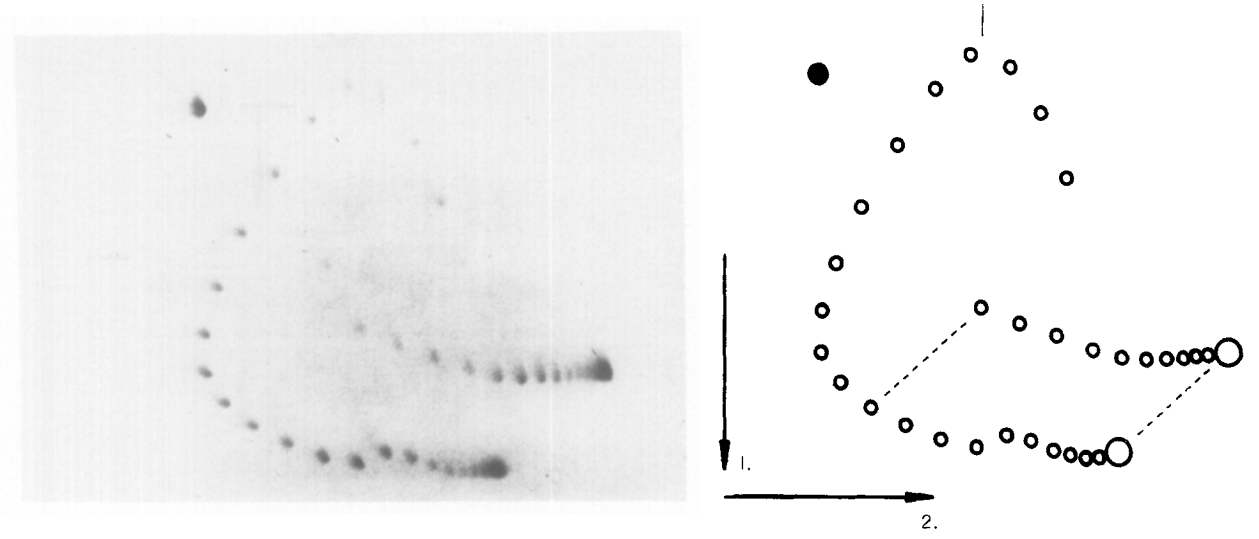

Figure 12. A topoisomer mixture of a pJDH7 deletion clone containing the alternating purine-pyrimidine tract was separated by 2-dimensional electrophoresis, top to bottom in its 1st, left to right in its 2nd dimension. In the same gel, a subset of topoisomers with more than 8 negative supercoils of another deletion clone, just missing this tract, was electrophoresed. The loss corresponds to the disappearance of the *Z*-DNA-induced break in the DNA pattern at the topoisomer with about 12 negative supercoils. Corresponding topoisomers are connected by broken lines.

for non-random sequencing (Henikoff, 1984); genetic markers in recombinant DNA can be mapped by simple phenotypic analysis, as was demonstrated with the origin region of pUCZ18.

Mapping information about *Z*-DNA has been obtained by a number of methods. The effectiveness of digestion with restriction nucleases is reduced or eliminated in the left-handed helix (Azorin *et al.*, 1984, and our own unpublished results); the junction between the *B* and the *Z*-form is sensitive to single-strand-specific nucleases (Kilpatrick *et al.*, 1983; Singleton *et al.*, 1984); the binding of antibodies to the DNA protects it from nucleases (Nordheim *et al.*, 1982*a*); the position of crosslinked antibodies was screened by electron microscopy (DiCapua *et al.*, 1983) and chemical probes of the *Z*-conformation (Herr, 1985; Johnston & Rich, 1985) were developed. Some advantages of the exonuclease III mapping protocol, besides its similarity to the isolation procedure, are the following: (1) large fragments of DNA are assayed in a single experiment in a minimum of time; (2) no restriction site in or near the *Z*-DNA tract is necessary. (3) Mapping occurs in a vector DNA, which facilitates further application, for example sequencing. (4) Concomitant with the mapping information, deletion clones of different length but with the identical *Z*-segment are established, which may allow study of the influence of flanking regions. (5) Either the information is obtained redundantly by using clones unidirectionally deleted from both sides of the insert or the loss of this redundance indicates two or more *Z*-DNA sites within one fragment.

This protocol allows isolation and the rapid detection of the specific binding site of any DNA-binding protein under appropriate conditions, especially if they exist only in supercoiled DNA, like *Z*-DNA.

The polylinker of pUC18 permits preparation of plasmid sets with unidirectional-deleted insert DNA and sequencing particular plasmids of the sets after template creation from the other side of the insert. The use of the different restriction sites of the polylinker enables the sequencing of both strands in long DNA fragments without subcloning into M13 phage DNA, the whole procedure being done in one vector.

(b) *Z-DNA in* E. coli

For screening of potential *Z*-adopted segments in the *E. coli* genome, a recombinant library was used. Nevertheless, the discrepancies in supercoiling, an important effector for *Z*-DNA formation, should not be very big between real genomic and the recombinant DNA. Recombinant genes transfected into living bacteria showed expression of their proteins (Neidhardt *et al.*, 1983). Therefore, it is concluded that the state of the recombinant DNA is not very different from that of the genome within the cell.

From the data obtained, the number of potential *Z*-segments is estimated to be more than 230 within the entire genome. Two or more *Z*-forming sequences in tandem arrangement within one plasmid perhaps do not all switch into the energetically unfavored left-handed structure, because of an inadequate degree of superhelicity. Therefore, there could be more sites of potential *Z*-DNA than are detectable in this way. Z-D11, the antibody used in isolation, preferentially binds to *Z*-DNA of alternating dC-dG, a sequence favored in *Z*-DNA formation, suggesting that the detected binding sites are tracts of alternating purine-pyrimidine sequence that contain segments of alternating dC-dG, an assumption that is supported by the base sequence of the isolated *E. coli*

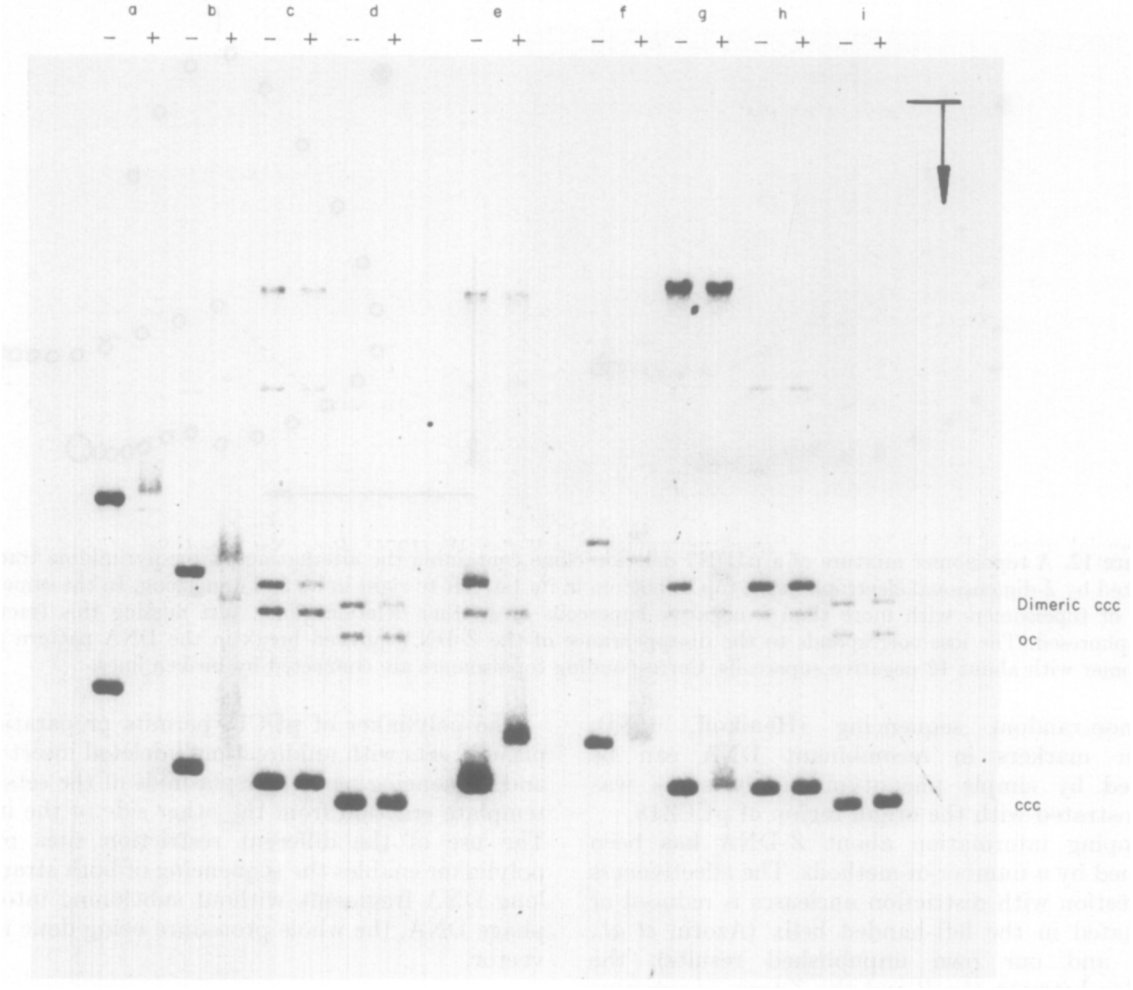

Figure 13. Loss of antibody binding after deletion of the alternating purine-pyrimidine segment. About 100 ng of ccc DNA was incubated with 100 ng of Z-D11 for about 30 min and separated in an agarose gel (+); each sample ran parallel to one incubated without antibody (−). DNA–antibody complexes are retarded in the gel. Plasmids were: (a to c) pJDH7; (e) pUCZ18 as a control of retardation; and (f to h) pJDH9/1, a deletion derivative of pJDH9. The entire recombinant plasmid (a and f), a unidirectionally deleted derivative containing the alternating purine-pyrimidine segment (b and g), one which just lost this segment (c and h), and the vector pUC18 (d and i) were assayed. The open circular DNA serves as an internal control of unspecific retardation.

fragments. Statistical analysis of available prokaryotic base sequences (Trifonov *et al.*, 1985) suggests the presence of about 600 alternating purine-pyrimidine segments in a length of 11 to 16 bp within the entire *E. coli* genome. The limitation in base composition for *Z*-DNA segments detectable with Z-D11 diminishes this number to a value comparable with the estimate from the data presented here.

Since, in the column assay, glutaraldehyde crosslinks the antibody covalently to the DNA, no quantitative information on the strength of the antibody binding is available.

Potential *Z*-DNA seems to be spread rather than clustered along the genome, pointing either to a frequent function of this conformation or to random occurrence.

All three isolated *Z*-forming sequences convert to the *Z*-conformation at a relative low superhelical density of about −0·042 to −0·044 (in electrophoresis buffer) compared to a value of about −0·03 necessary for a $(dC\text{-}dG)_{16}$ insert (Peck & Wang, 1985). It seems to be low enough that it might occur locally within the living bacterium. The *Z*-DNA region of pJDH7 appears to include non-alternating nucleotide sequences (Table 2). Such an effect was observed in a synthetic sequence using chemical modification as a probe of DNA conformation (Johnston & Rich, 1985). It was shown in crystals and in solution (Wang *et al.*, 1985; Feigon *et al.*, 1985; Ellison *et al.*, 1985) that such an alternation is not an absolute requirement for the formation of *Z*-DNA. But, up to now, no *Z*-DNA fragment has been observed that lacks the alternation entirely.

The lack of *Z*-DNA in the plasmid pLC5-23 agrees with the lack of sequences within the *trp* operon having a high potential for *Z*-DNA formation.

The position of a potential *Z*-forming segment within the *lac* operon, located in the center of the *lacZ* gene, suggests a possible biological role for *Z*-DNA in the regulation of transcription. It is the

only segment with a very high Z-potential within the entire *lac* operon, as estimated from the sequence. Peck & Wang (1985) showed that, *in vitro*, *E. coli* RNA polymerase stops at the left-handed DNA formed by alternating dC-dG in a supercoiled plasmid, providing a model for a downstream regulation of mRNA synthesis. Further biological experiments will be necessary to show whether such a possibility is realized within the *lacZ* gene.

The Z-DNA conformation induced by supercoiling and the antibody binding serves as a model system for the interaction of proteins with particular DNA structures. The methods developed for isolation and mapping such sites from genomic DNA should be helpful in other systems involving protein–DNA interactions.

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