Z DNA and loop structures by immunoelectronmicroscopy of supercoiled pRW751, a plasmid containing left-handed helices

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ABSTRACT

Single and multiple loops were seen when the plasmid pRW751 was allowed to react with anti-Z-DNA or with a Z-specific cross-linking agent. Loop formation was dependent upon negative supercoiling and the presence of Z-specific antibody or cross-linking agent. Restriction enzyme mapping located 18 sites at the bottoms of loops, in addition to the two (dG-dC)_n inserts of pRW751. No more than 5 loops were seen in any of the measured molecules; thus, not all potential Z-sites assume the Z conformation at any particular time. Stretches of alternating purine-pyrimidine sequences occur at all 20 sites. Almost all of the Z sites could be mapped to regions located at the beginnings or ends of reading frames or at various regulatory sites. Our findings support the concept that supercoiling brings distant sequences to within 5Å of one another, allowing joint participation in regulatory processes controlled by DNA-binding proteins.

INTRODUCTION

Since the initial proposal that poly(dG-dC).poly(dG-dC) could assume a novel conformation (1) and the subsequent crystallographic evidence that it was left-handed (2,3), attempts have been made to assign a biological role to left-handed or Z-DNA. Early studies were immunochemical in nature, made possible by the finding that Z-DNA is immunogenic (4). There were reports of Z-DNA in interbands of polytene chromosomes (5) and then in banded regions (6), in nuclei of protozoa (7) and of cells of rat tissue (8), and in metaphase chromosomes (9).

The meaning of these findings became uncertain because of evidence (10) that fixation procedures can induce polynucleotide sequences to assume a Z conformation. This is not a trivial finding since it could imply that local sequences in chromosomal DNA are in a dynamic state, with the potential of assuming a

continuum of conformations, determined by changes in their microenvironment.

Most recently, left-handed DNA has been shown to exist and to elicit a biological response in *E. coli* (10a).

Torsional stress is among the conditions that can induce (or stabilize) the left-handed Z-DNA conformation, as demonstrated by studies on negatively supercoiled plasmids (3,11). We have been using immunoelectronmicroscopic techniques (12,13,14) to study the plasmid pRW751 (15,16), a construct of pBR322 into which a 95 bp lac operator sequence flanked by (dC-dG)₁₆ and (dC-dG)₁₃ has been inserted into the Bam H1 site. The Z-DNA specific antibodies (17,18) were obtained by immunization with acetylaminofluorene-modified poly(dG-dC).poly(dG-dC) (19-21).

This paper presents evidence that foci of Z-DNA in pRW751 tend to occur at boundaries of transcribed sequences and that there is a tendency for certain pairs of these Z foci, although distant from one another in sequence, to be in close proximity in the negatively supercoiled molecule with the resulting formation of loops. These findings may be related to control of transcription, replication and recombination.

EXPERIMENTAL

Materials - pRW751 (16) is a 4520 bp plasmid, a pBR322 (22) derivative containing an insert of 157 bp into the Bam H1 site. The insert consists of a 95 bp *Escherichia coli* lac operator DNA fragment flanked by alternating (dC-dG) stretches of 32 and 26 bp, respectively. The preparation we used has an average negative superhelical density of 0.05, determined by gel electrophoresis; both dC-dG stretches are in a left-handed helical state (16). Pst I was from BRL and Boehringer-Mannheim. Pvu II was from Boehringer-Mannheim. None contained serum albumin. Sepharose CL-4B was from Pharmacia. (In some separations, Elutip-d columns from Schleicher and Schuell, Inc. were used but were found to result in a less satisfactory background for electronmicroscopy than Sepharose CL-4B.) DL-diepoxybutane (DEB) was from Sigma.

The antibodies were prepared as previously described

(17,18). Globulin fractions were obtained by $(\text{NH}_4)_2\text{SO}_4$ precipitation of preimmune sera or of immune sera taken after the fifth and sixth booster injections. The precipitate was redissolved in 0.01M KH_2PO_4 - Na_2HPO_4 , 0.15M NaCl, pH 7.4 (PBS) and dialyzed against the same buffer.

The brominated-poly(dG-dC).poly(dG-dC)-Affigel 102 column was prepared according to a method of K. Smith and B.D. Stollar (personal communication), described in refs. 17 and 18. The Z-depleted antibody was prepared as described (17,18).

Reactions of pRW751 with anti-Z antibodies and hydrolysis by restriction enzymes - The standard reaction solution (19 ul) was at pH 8.0 and contained 10mM triethanolamine-HCl, 1mM EDTA ("buffer") and 0.4 ug pRW 751. After addition of 1 ul of the globulin fraction of anti-Z antibody (25 mg/ml protein, approximately 35 ug/ml antibody in PBS), the mixture was incubated in a 37° water bath for 1 hr. The reactions of the plasmid with pre-immune globulin and with the globulin fraction of the serum depleted of Z-antibody by passage through the brominated poly(dC-dG) columns were carried out in the same way.

Separation of the free DNA and the DNA-antibody complexes was by chromatography on a column of Sepharose CL-4B. The column, packed to a height of 5 cm, was prepared in a Pasteur pipette; I.D. 0.5 cm. Before use, the column was equilibrated with the same buffer used for the DNA-antibody reaction (above), and the void volume determined with Blue Dextran (Bio-Rad). The sample (20 ul) was gravity-fed through the column and eluted with "buffer" at a rate of about 0.2 ml/min. Fractions of 60 ul were collected and examined by electronmicroscopy, as described below.

Prior to hydrolysis of peak fractions by restriction enzymes, MgCl_2 was added to a concentration of 10mM. The enzyme was then added to a final concentration of 15 units/ml and the reaction mixture was allowed to incubate for 1 hr at 37°C. The samples were then stored on ice for electronmicroscopy.

In the reaction of antibody with Pst I cleaved pRW751, the concentration of EDTA was increased to 0.02M prior to reaction with antibody to inhibit DNase.

Reaction of pRW751 with DEB - The solution of "buffer" (171 ul) contained 5.2 ug of pRW751. After addition of 19 ul of DEB, the mixture was incubated for 1 hr in a 37°C water bath, followed by dialysis against 500 ml of "buffer" in a cold room overnight. MgCl₂ was added to a concentration of 10mM. Pst I was then added to a final concentration of 15 units/ml and the mixture was incubated for 1 hr at 37°C. The solution was diluted with "buffer" to 4 ug/ml DNA for immediate preparation of the grids as described below. For a final 100 ul drop, 2 ul of the 4 ug/ml solution was used. The alkylation seems to be somewhat inhibitory toward the restriction enzyme. Only 70% of these molecules were cleaved while, under the same conditions, 90% of the unalkylated molecules were cleaved (Figs. 1F and 1G).

Kang et al. (23) found 80% relaxation of supercoiled DNA in 1 hour under several reaction conditions. Under our conditions, we have also found destruction of the molecules with time. However, if the material is put on the grids within less than 24 hours from initiation of the alkylation, the supercoiled structure remains intact (Figs. 1E, 1F, 1G and 1K).

Electronmicroscopy - The procedures were generally as reported previously (12,13,24). Briefly, the fractions were diluted about 1:20 in "buffer" and brought to $2 \times 10^{-4}\%$ concentration of benzyldimethylalkonium chloride (BAC) (24,25) by a 1:100 dilution of 0.02% BAC in formamide. Drops (100 ul) were placed on a parafilm surface for 10 minutes. CAM grids, i.e. partially polymerized butyl methacrylate in collodion (26) (we will provide copies of our revised preparation procedure, if requested), were floated for an hour on a 0.005% solution of ethidium bromide in water (27) and briefly blotted on filter paper prior to application to the surface of drops of the diluted complexes.

The grids were floated on double-distilled H₂O for 10 min and subsequently dehydrated by a dip into 90% methanol. Rotary shadowing was as previously reported except that the shadowing angle was 9.8°. Microscopy and calibration of the Hitachi HU-11C transmission electronmicroscope were as reported previously (24). Micrographs were photographed at magnifications ranging from 30,000X to 70,000X. Measurements

were from micrographs projected at 5.6X magnification using techniques described earlier (24).

The average total magnification was about 400,000. Molecules and antibody were traced on paper. Nucleic acid molecular lengths and positions of antibody were measured with a Numonics Graphics Calculator. The antibody binding sites in each molecule were analyzed in both directions by computer. Orientation of the molecules was relative to the two dC-dG tracts (32 and 26 bp), the site at 1447-1460 (28,29), and two additional map sites at 960 and 230 (29) reported previously. Only full size molecules were analyzed.

Programming - Calculations were from programs written by H.C.

Sequence and hypothetical protein analyses were performed with the nucleic acid sequence database and associated programs from the National Biomedical Research Foundation, Georgetown University Medical Center. These programs are part of the Pronuc facility for protein and nucleic acid sequence manipulation at the Columbia University Cancer Center Computing Facility.

RESULTS

Reaction with anti-Z-DNA - pRW751, with an average negative superhelical density of 0.05, was allowed to react with a globulin fraction of rabbit anti-Z-DNA (17,18), followed by cleavage by Pst I or by Pvu II. After separation of the antibody-DNA complexes on a Sepharose CL-4B column, the complexes were examined by electronmicroscopy.

Fig. 1A shows a widefield view of a preparation cleaved by Pst I. On scanning about 500 molecules on a grid, approximately 65% were observed to have single or multiple loops (see below).

Several controls were examined. They included a) cleavage with Pst I prior to reaction with antibody (Fig. 1B), b) reaction with preimmune globulin followed by Pst I cleavage (Fig. 1C) and c) reaction with an anti-Z-DNA globulin fraction that had been passed through a brominated poly(dG-dC).poly(dG-dC) column to remove anti-Z antibody; cleavage was by Pst I (Fig. 1D). Very few loop structures were observed in the control preparations: about 1% under conditions of Figs. 1B and 1C; about 4% under

Fig. 1A. Widefield view of pRW751 treated with anti-Z globulin and then cleaved with Pst I. Note frequency and variety of loops (see arrows).

conditions of 1D. These frequencies can be considered as the level of chance loops that are observed when the BAC procedure (14,24,25) is used in spreading of the DNA molecules (see Experimental).

Reaction with DEB - It had been shown earlier (13,14,23) that DEB could crosslink regions of Z-DNA. Treatment of pRW751 with DEB, followed by Pst I cleavage, gave fields similar to that shown in Fig. 1A. Figs. 1E, 1F and 1G show details of some loop structures. In Fig. 1K, which is an enlargement of a molecule seen in Fig. 1G, a supercoiled region appears to be trapped by DEB crosslinking. A similar molecule is shown in Fig. 1H; in this case, however, crosslinking was by anti-Z-DNA. In Fig. 1J,

Fig. 1B. Widefield view of pRW751 cleaved first with Pst I and then treated with anti-Z globulin.

an enlarged portion of the looped strands in Fig. 1I, there is a molecule in which it appears that interwrapped strands of DNA are trapped by antibody (cf. 30), also see below).

Location of the loops - In the total set of experiments, 316 molecules with loops were examined: 78 molecules in the antibody-Pst I experiments, 139 molecules in the antibody-Pvu II experiments and 99 molecules in the DEB-Pst I experiments. Of the total, 187 molecules had one loop, 83 had two loops, 30 had three loops, 11 had 4 loops and 5 had 5 loops.

The locations of the loops were determined by two different methods. In one, location of the site or sites was by plotting each site twice, once in each direction along the DNA strands (29,31). In the second procedure, use was made of three previously located antibody binding sites (28,29), as well as the known positions of the two (dG-dC)_n sequences of the insert. In the second method, analysis was of data derived from the antibody-Pst I, antibody-Pvu II and DEB-Pst I experiments. In

the first procedure, data from the antibody-Pst I and antibody-Pvu II experiments were correlated. In both methods, histograms were constructed from which geometric means were calculated. Average locations were then determined. The results of both methods were in good agreement. Each showed 20 loop forming sites (including the two (dG-dC)_n sites) whose average locations were in good agreement. However, method 1, because of the doubling, distorts the probabilities of each site, increasing some and decreasing others. Without marker sites, method 1 must be used. For greater accuracy, however, we recommend reanalyzing the data by method 2 using as markers the sites determined by method 1.

The results in Table I, which were derived by the second procedure, are presented as calculated probabilities (29,31,33). For a total of 20 sites, they ranged as high as 0.074. As noted above, no measured molecule had more than five loops, most had one or two. The 20 sites, therefore, are considered to have the potential of forming Z structures although only a few do at any particular time (see Discussion). Fig. 3 locates the sites on the biological map.

Site pairs that are crosslinked to form loops - The pairs of sites that were crosslinked by antibody and by DEB to form loops are given in Fig. 2. It is apparent that they are not selected at random. For example, although site 15 is paired with 16 other sites, 35 of 54 loops were formed by pairing site 15 with only five sites: 11,12,13,16 and 17. Of these, the nearest is about 300 bp away; the most distant ones are about 1000 bp away.

Another striking finding is the high frequency of bridging of sites 2 and 3, the sites that flank the insert of pRW 751.

DISCUSSION

The reaction of supercoiled pRW751 with Z-specific antibody (17,18) or with DEB, a Z-specific crosslinking reagent

Fig. 1C. Widefield view of pRW751 treated with pre-immune globulin and then cleaved with Pst I.

Fig. 1D. Widefield view of pRW751 treated with a globulin depleted of anti-Z antibody and then cleaved with Pst I.

Figs. 1E, F and G. PRW751 treated with DEB and then cleaved with Pst I. Arrows point to loops. Fig. 1H. PRW751 treated with anti-Z globulin and cleaved with Pst I. Arrow on right points to loop. Arrow on left points to supercoiled region trapped by antibody binding to base of loop. 1I. Same as 1H. Arrow head points to highly interwapped portion of loop. 1J. Enlargement of interwapped portion of loop seen in 1I. 1K. Enlargement of molecule designated by arrowhead in Fig. 1G. Note supercoiled and interwapped regions.

[illegible]

Fig. 2. Pairs of sites forming loops crosslinked by antibody or DEB.

(13,14,23), resulted in the formation of loops whose locations could be analyzed after cleavage by restriction enzymes. Control reactions showed that loop formation was dependent, first of all, on supercoiling of the plasmid; very few loops were seen when the linearized plasmid was allowed to react with the Z-specific antibody. Secondly, loop formation required the presence of Z-DNA-specific antibody; very few loops were seen where non-immune globulin or globulin depleted of anti-Z antibody was allowed to react with the supercoiled plasmid. Our results are consistent with the findings of others that torsional stress caused by supercoiling can induce (or stabilize) the left-handed Z-conformation (reviewed in refs. 3 and 11).

Loops have been noted by others after treatment of supercoiled DNA with anti-Z antibodies (29,31,32,33) but no analysis of their size and location has been presented.

Molecules with one or two loops dominated the fields examined by electron microscopy, although as many as 5 loops could be seen on occasion. The distribution of the loops among the DNA strands examined indicated that as many as 20 sites

Table I. Potential Z-DNA Sites in pBR322 (pRW751)

Site No.	Probability	Average Position bp ^a	pu-py Sequences ^b
1	0.074	27 [±] 52	<u>ATGCG</u> , <u>CGTGTATG</u> 37 81
2	0.053	221 [±] 49 ^C	<u>GCGCTATATGCGT</u> 233 <u>CTATGCGCAC</u> (<u>CGGTTC</u>) 258
3	0.056	551 [±] 95 ^C	<u>CATCTCCT</u> <u>TGCATGCAC</u> 553 561 <u>CG</u> <u>GCGTG</u> <u>GATG</u> <u>GTG</u> 505 <u>GCAG</u> <u>GCCCCGTG</u> <u>GCCG</u> 521
4	0.047	771 [±] 47	<u>CATGCA</u> 748 <u>GTGC</u> <u>CG</u> <u>GCAGCGC</u> 767
5	0.062	943 [±] 60 ^C	<u>ACGCGC</u> <u>TGGGC</u> <u>TACGT</u> 945 <u>GCGTTCGCG</u> <u>ACGCG</u> AG 968
6	0.057	1168 [±] 63 ^C	<u>TCG</u> <u>GCGAGC</u> <u>AC</u> <u>ATG</u> GA 1169
7	0.037	1406 [±] 56 ^C	<u>CACGGGTGCGCATG</u> <u>AT</u> 1448 <u>GC</u> <u>CGCACGCGCGCAT</u> 1409
8	0.041	1576 [±] 40 ^C	<u>CAC</u> <u>CG</u> <u>ATACGCGAGCG</u> 1529 <u>ACGCGAGCGAACGTGA</u> 1536
9	0.026	1719 [±] 55 ^C	<u>TACAT</u> , <u>TGTAT</u> 1710 1716
10	0.023	1905 [±] 34	<u>TACACG</u> 1900 <u>ACATG</u> 1945
11	0.027	2052 [±] 39 ^C	<u>GCA</u> <u>GC</u> <u>TGCCTCGCGCG</u> 2065
12	0.024	2227 [±] 58 ^C	<u>GGGCGCGT</u> <u>CA</u> <u>GCGGGT</u> 2177 <u>GTGT</u> <u>CGGGGCGCA</u> <u>GCC</u> 2201 <u>CACGTAGCG</u> , <u>GTGTATAC</u> 2227 2244 <u>TATGCG</u> 2261
13	0.023	2509 [±] 67	<u>ACATGTG</u> 2475 <u>ACGAGCAT</u> 2559
14	0.042	2767 [±] 58 ^C	<u>GC</u> <u>TGTGTGCACG</u> <u>AACC</u> 2784

Table I. (continued)

15	0.057	3024 ⁺⁶⁹ ^c	GAT <u>TACGCGCA</u> AAAA 3097 <u>GTATGTA</u> 2917
16	0.054	3318 ⁺⁶¹	<u>GTATATATG</u> , <u>CGTGTA</u> 3267 3373
17	0.070	3999 ⁺⁴⁹ ^c	<u>TA ACCCACTCGTGCA</u> , 4025 <u>CTCGTGC ACCCA AC TG</u> 4032
18	0.065	4209 ⁺⁵³ ^c	<u>AGGGGTTCCGCGCAC</u> 4249
19	0.064	383 ⁺⁴⁰ ^d	Contains (dG-dC) ₁₆
20	0.073	517 ⁺⁴³ ^d	Contains (dG-dC) ₁₃

^aOrigin is defined as EcoRI restriction site in pBR322. Sites 1 to 18 are mapped for pBR322 according to Sutcliffe (22), incorporating a sequence correction (57,58).

^bPosition number is for first base of sequence. Continuous pu-py sequences are underlined. Discontinuities are not.

^cIndicates that the site falls within predictions of a computer aided thermodynamic approach (36, and personal communication). In those cases, sequence is as given in those references.

^dThe numbering is for pRW751 from the EcoRI cleavage site. The precise location of sites 19 and 20 are 378-410 and 505-531, respectively.

(including the (dG-dC)_n sites) had the potential of assuming the Z conformation (Table I). This number exceeds that reported by others in experiments in which other pBR322 derived plasmids containing inserts were allowed to react with Z-DNA-specific antibody (28,29). These earlier reports, taken together, identified 4 Z sites, including a (dG-dC)_n insert. In a more recent report (34), a Z specific reagent identified four major Z sites that correspond to, or are close to, our sites 7,12,16 and 18. Thus, six sites, corresponding to sites 2,5,7,12,16 and 18, have been identified by others.

The DNA-antibody reaction in our studies was allowed to occur at a much lower salt concentration than in the experiments of DiCapua *et al.* (29) and Nordheim *et al.* (28). It has been documented (13,35) that decreases in ionic strength can cause increased Z-DNA formation in pBR322 and in plasmids derived from it and, moreover, low salt favors Z-DNA formation at lower negative superhelicities. Thus, under our conditions, more

Fig. 3. Biological map of pBR322 (pRW751) with the anti-Z reactive sites indicated (1-20). The map was adapted from Sutcliffe (22); incorporating the sequence correction (57,58), adding promoters, RNAs and proteins subsequently identified. The 157 bp insert of pRW751 and the two Z sites are above the BamHI insertion site. The length in amino acids of each peptide is indicated on the arrow whose tail is at the amino-terminal end. RNA lengths are in nucleotides.

The following is a list of the hypothetical proteins (some of which have been isolated) starting at the EcoRI site and proceeding clockwise:

No. of amino acids	location (bp)	No. of amino acids	location (bp)
396	86-1273	72	1593-1808
127	259-639	96	1818-2105
80	565-326	69	2011-2217
116	789-442	60	2300-2479
152	1081-626	286	4155-3298
112	1180-1515	88	3425-3688
132	1913-1518	56	3698-3865
66	1523-1720		
For pRW751, 396, 127 and 80 are not present. Four new proteins are predicted:			
114	86-427	92	722-447
62	259-244	261	648-1430

potential Z regions (see below) can presumably assume the Z conformation. The possibility of non-specific interactions between DNA and our globulin preparations is eliminated by our controls, i.e., little significant looping occurred with linearized DNA or with globulin preparations lacking Z-DNA-specific antibody.

Additional confidence comes from the presence of alternating purine-pyrimidine sequences at the sites identified as being in the Z conformation (Table I). Moreover, 13 of the 18 sites seen by us (not including the two (dG-dC)_n sites in pRW 751), coincide with regions in pBR322 predicted by a computer aided thermodynamic approach (36, and personal communication) to be capable of assuming the Z conformation at superhelical densities of -0.04719 to -0.05589.

We find, therefore, that, under the conditions of our experiments, as many as 20 sites in pRW751 may have the potential to assume the Z conformation but that, at any particular time, only a small number do so. It is possible that a similar situation exists in vivo, with the number and location of conformational changes being controlled by local ionic and torsional changes that occur during the replicative cycles of cells and their plasmids (see below).

Figure 3 shows the biological map of pBR322 with the various Z sites indicated by numbers. Except for site 5, sites crosslinked by antibody to form loops coincide with locations either at the beginning or the end of reading frames. For example, site 10 is at the site of initiation of the RNA of "rop" protein (37) (position 1917) and site 11 is at its termination (position 2108). Site 15 is at the initiation of primer RNA II (38). Other sites are at the points of initiation and termination of RNA for a number of "hypothetical" proteins (see Fig. 3 and legend). Thus, regions of Z-DNA can be seen as sites that delineate transcribable regions. Barton and Raphael (34) came to a similar conclusion concerning two of the sites they found using a Z-specific cleavage reagent, sites that correspond to our sites 16 and 18.

Other sites fall near regulatory regions. For example, sites 1,12,15,18 are in regions of known strong promoter

sequences (39-41). Sites 1,14,15 and 16 are in regions of S1 nuclease-sensitive sites (42). Revet *et al.* (32,43) and Hagen *et al.* (31) have also reported Z sites in regions controlling expression in ϕ X 174 RFI and in SV40, respectively. If Z-DNA sequences are involved in the regulation of transcription, they might do so by being targets of Z-DNA binding proteins (44) which, like anti-Z-DNA, might cause looping out of regions where transcription is to be regulated.

Z sites are also found in regions that regulate processes other than transcription. For example, site 13 is at the origin of replication. Site 14 is in a region in which replication is controlled by "rop" protein and RNA I (37). Gamas *et al.* (45) have recently reported that an *E. coli* host factor that binds to a transposable element, IS1, also binds to four regions in pBR 322 that are hot spots for the recombinational insertion of IS1 into the plasmid. Site 17 is within one of the hot spots; site 18 is 6 bp beyond it.

Our attempts and the attempts of others (31,32,34,41) to relate Z regions with regulatory processes must be considered speculative if only because the extent of superhelicity in the cell is unknown. Estimates have ranged from -0.025 (46) to -0.048 (47), with changes in superhelicities occurring with changes in growth conditions (47). Interestingly, the best match between levels of expression *in vitro* vs. *in vivo* was reported (47) to occur at -0.048 ± 0.005 , which is within range of our conditions.

The physical process of loop formation by antibody requires that certain specific Z loci in supercoiled molecules are within 120Å of each other (the approximate distance between the two combining sites of antibody) for periods of time sufficient to allow crosslinking by antibody. That the distance between juxtaposed Z regions can be even shorter is indicated by our experiments using DEB to crosslink the Z regions. Following linearization with Pst I, the same frequency of loops was seen in positions identical to that found after treatment with antibody. These findings indicate that certain Z regions in the supercoiled molecule are within 3.5-5Å of one another (the

approximate length of DEB) for periods of time sufficient to allow crosslinking.

The ability of two regions of DNA, distant from one another in the primary sequence, to associate and be crosslinked by a DNA-binding protein (anti-Z-DNA) may have important functional significance, e.g. to demarcate domains whose transcription is to be turned on or off during particular phases of a cell cycle. Regulation of the foci of Z conformations could be by systematic changes in torsional stress (cf. 48) mediated by DNA binding proteins, by enzymes such as topoisomerases and by locally induced changes in ionic strength. The tendency of Z-DNA to self-associate (12,14,31,42,49) may also play a part in bringing distant Z foci together. The interwrapping (30) at the base of the loops (Figs. 1J and 1K) may be a reflection of this.

Our observations might also be relevant to the numerous recent reports suggesting that topological factors can influence gene expression. This has been inferred because of evidence that regulatory regions far removed from one another along the primary sequence appear to cooperate in controlling expression. Examples include:

- (a) Enhanced repression of the lac promoter by upstream operators (50).
- (b) Repression of the araBAD operon by the binding of AraC protein at two distant sites (51).
- (c) The stimulating activity of enhancers even when distant from promoter regions of SV 40 virus (52).
- (d) Synergism between immunoglobulin enhancers and promoters (53).
- (e) Evidence for long range cooperativity between gene regulatory sequences in E. coli (54).

Loop formation has been invoked to explain (a), (b) (c) and (e). Moreover, Wasserman and Cozzarelli (55) have suggested that loops and other topological forms might be intermediates in recombination.

More recently, DNA loops have been shown, by electron microscopy, to form when progesterone receptor binds to regulatory elements that are distant from one another on DNA (56).

Our findings provide definitive evidence for loop formation in supercoiled DNA, mediated by a DNA-specific protein. Whether the antibody reacts with each site simultaneously or in a stepwise fashion, is unknown and is not a crucial consideration. We demonstrated that supercoiling can bring two distant sites in DNA to close proximity so that DNA binding proteins can interact with both sites. In fact, this may be one of the major functions of supercoiling. Another function might be to allow programmed changes in torsional stress to regulate DNA conformation locally so as to provide regions capable of binding conformation-specific regulatory proteins (cf. 48). As illustrated in Figs. 1H and 1K, the overall (average) superhelicity may not reflect the superhelicity in local regions, some of which will be more highly supercoiled than others.

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