Left-Handed DNA in Vivo

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Left-handed DNA is shown to exist and elicit a biological response in *Escherichia coli*. A plasmid encoding the gene for a temperature-sensitive Eco RI methylase (MEco RI) was cotransformed with different plasmids containing inserts that had varying capacities to form left-handed helices or cruciforms with a target Eco RI site in the center or at the ends of the inserts. Inhibition of methylation in vivo was found for the stable inserts with the longest left-handed (presumably Z) helices. In vitro methylation with the purified MEco RI agreed with the results in vivo. Supercoil-induced changes in the structure of the primary helix in vitro provided confirmation that left-handed helices were responsible for this behavior. The presence in vivo of left-handed inserts elicits specific deletions and plasmid incompatibilities in certain instances.

THE CONCEPT OF DNA CONFORMATIONAL MICROHETEROgeneity (*1*), that is, neighboring segments with different secondary structures, has emerged over about 20 years (*2–8*) and this heterogeneity is presumed to play a role in key cellular processes. These include replication and recombination, mutagenesis-carcinogenesis and repair, transcription, chromosomal organization, and virus packaging. Substantial progress has been made toward our understanding of the in vitro properties of unusual DNA structures (*2–5*).

Left-handed DNA, proposed in 1970 (*9*), has been the subject of intense investigation since x-ray structural determinations (*10, 11*) identified the Z helix in short DNA oligomers in the late 1970s. Subsequently, numerous in vitro determinations have revealed sequence requirements, conditions for stabilization, thermodynamics and kinetics, the effect of methylation of cytosines, the behaviors of B-Z junctions, immunological properties, characteristics of Z-helix binding proteins, interactions with small ligands, and the like (*2–5*). However, no direct and unequivocal evidence exists regarding the in vivo existence and functions of this or any other unusual DNA structure, although most workers believe that Z-DNA does exist in replicating cells and participates in important biological processes (*2–5*).

In general, structural problems with macromolecules are solved in vitro with the use of crystallographic or spectroscopic determinations. However, these probes are not useful for analyzing the conformations of tiny segments of chromosomes within the complex milieu of a living cell. Since in vivo analyses require genetic studies in order to avoid perturbance of the intracellular environment, we have developed a molecular genetic assay for investigating DNA conformations in vivo.

Genetic assay for DNA conformations. Our in vivo assay is based on the in vitro observations that a target (recognition) site is not methylated by its specific methylase or cleaved by its specific restriction endonuclease when the site is near (*12*) or in (*13, 14*) a left-handed Z helix. However, these enzymes do act on the same target sequences when they exist in a right-handed B structure. For example, when a Bam HI (GGATCC) or an Eco RI (GAATTC) site is located at the interface between a vector (which is right-handed under all conditions) and an alternating $(C\text{-}G)_n$ insert (which may be either right-handed B or left-handed Z depending on the negative supercoil density of the plasmid (*15–18*), these sites are methylated by the respective Bam HI or Eco RI methylases only when the insert is right-handed. Analogous results were found for the homologous restriction endonucleases (*12*).

The in vivo assay for non-B DNA utilizes a clone (pACYC184 derivative) containing the gene for a temperature-sensitive (*19*) Eco RI methylase (MEco RI) (*20, 21*). An *Escherichia coli* strain harboring this plasmid (pRW1602) (Fig. 1) synthesizes the MEco RI in sufficient quantities to fully methylate all GAATTC sites in a brief time period at permissive temperatures (5° to 32°C). However, less than 10 percent of the sites are methylated at the restrictive temperature (42°C). The pRW1602 plasmid was cotransformed with any one of several pBR322 derivative plasmids (Fig. 1) that contain different lengths, orientations, and types of sequences that have varying capabilities in vitro of forming left-handed Z or cruciform structures. Also, the orientation of the GAATTC target site for the MEco RI probe within or at the end or ends of the Z tract or cruciform was varied (Fig. 1). We used a temperature-sensitive MEco RI probe because all sites were readily methylated under all conditions tested when the wild-type MEco RI gene was studied. Presumably, the target sites are methylated during the replication process (*22*), prior to circularization and supercoiling of the DNA. Hence, if negative supercoiling (*15*) is an in vivo stabilizing agent for Z-DNA, the plasmid must be replicated and supercoiled prior to activating the methylase probe.

In vivo methylation. *Escherichia coli* HB101 was cotransformed with pRW1602, which encodes a temperature-sensitive MEco RI, and with the six pBR322 derivatives (Fig. 1, right). After cell growth at the nonpermissive temperature (42°C), the temperature was lowered to a permissive temperature (5° or 22°C) for varying time periods. Two of the plasmids (pRW1561 and pRW478) were methylated at approximately half the rate observed for the pBR322 control (Fig. 2). In pRW1561 a methylase target site is at the center of a 56-bp tract which should adopt a Z helix (*12, 23*). In pRW478, Eco RI sites are present at the interfaces between a 56-bp region that can adopt a left-handed helix and the vector. Alternatively, little or no inhibition was observed for the following plasmids: pRW476, which contains a 26-bp tract of potential Z helix with the target site

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at the interface of the vector and the insert; pRW155, which has two 40-bp tracts of (T-G) with methylase target sites at the interfaces of the sequences with a 95-bp segment which does not adopt a Z helix (*1, 15*); pRW1155, which contains an 88-bp segment of (T-G) and (C-A) with the methylase target site in the center; the control pRW465, which contains two Eco RI sites but no neighboring potential Z or cruciform tract; the control pRW1602, which contains the gene for the MEco RI and a single GAATTC site in the pACYC vector. Prior studies (*12, 15, 16, 24*) revealed that longer sequences require less energy than shorter sequences to adopt a left-handed Z helix. Also, substantially less energy is required to cause the transition of a (C-G) sequence into a left-handed Z helix compared to a (T-G) sequence (*2–5*).

The effect of sequence length was further demonstrated with two other plasmids that are related to pRW1561 in which the two tracts of (C-G) were replaced with sequences which were 7 and 7 and 9 and 7 dimer units in length. Neither of these plasmids showed an in vivo inhibition of methylation, whereas the somewhat longer 56-bp sequences (pRW1561 and pRW478) did show substantial inhibition (Fig. 2). Other studies with eight related plasmids are in complete agreement with these concepts.

The length of the sequence that may adopt a left-handed Z helix can be correlated with the extent of inhibition of methylation of a nearby GAATTC target site. The plasmids tested can theoretically also form cruciforms; for example, pRW155 might have a 40-bp stem with a 95-nucleotide loop, whereas pRW1155 would have a 40-bp stem with a 4-nucleotide loop. In these cases the methylation of the Eco RI sites might be inhibited; however, no inhibition was observed (Fig. 2). In fact, the insert in pRW1155 does adopt a cruciform in vitro (Table 1). Also, if the insert in pRW478 adopted a cruciform in vivo, the GAATTC sites would be in the duplex arms and thus may be a substrate for the methylase. This is not consistent with our observed inhibition, and hence supports our notion of a left-handed insert.

The extent (30 to 55 percent) of methylation inhibition observed is substantial considering the inherent slight leakiness of temperature-sensitive mutants, as well as the equilibrium properties of the B-Z transitions (*2–5*). If the B form is methylated during its existence, our experiment will give an underestimate of the amount of Z helix present in vivo. Furthermore, our results are calculated relative to the total plasmid DNA in the cell; undoubtedly, a substantial portion of the DNA in vivo is incapable of forming Z-DNA because of its role in cellular processes and hence may not be supercoiled. Thus, this could contribute to the rates observed (Fig. 2).

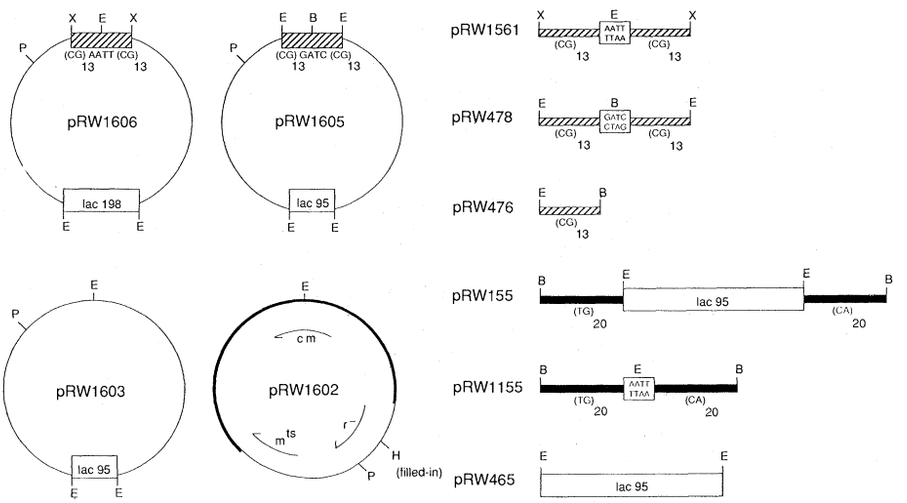

Fig. 1. Plasmids used in these studies. The inserts were cloned into the Eco RI site (or a modified Eco RI site) of pBR322 except for pRW155 and 1155 which had their inserts in the Bam HI site of pRW460 (*27*). The *E. coli lac* 95-bp fragment was inserted into the Pvu II site of plasmid pRW1561 to form 1606, into 478 to form 1605, and into pBR322 to form 1603. Plasmid pRW1602 was derived from pLV59 (*19*), a pACYC184 plasmid (chloramphenicol resistant) containing the coding sequences for both the Eco RI restriction enzyme and the temperature-sensitive Eco RI methylase. The Eco RI restriction enzyme gene was destroyed by filling in the single Hind III site with four dNTP's and DNA polymerase I Klenow fragment. In general, all plasmids were screened according to standard procedures. All pBR322 derivatives are resistant to both ampicillin and tetracycline except pRW476, pRW155, and pRW1155, which are ampicillin-resistant only. The exact lengths and sequences of the inserts of pRW1561 and pRW478 were verified by chemical sequencing (*27*). Other clones were confirmed by restriction analyses with several different enzymes. X, Xho II site (PuGATCPy); E, Eco RI site (GAATTC); B, Bam HI site (GGATCC); P, Pst I (CTGCAG); H (filled-in) in pRW1602 is a filled-in Hind III site; r^-, inactivated Eco RI restriction endonuclease; m^{ts}, temperature-sensitive MEco RI; cm, chloramphenicol resistance gene.

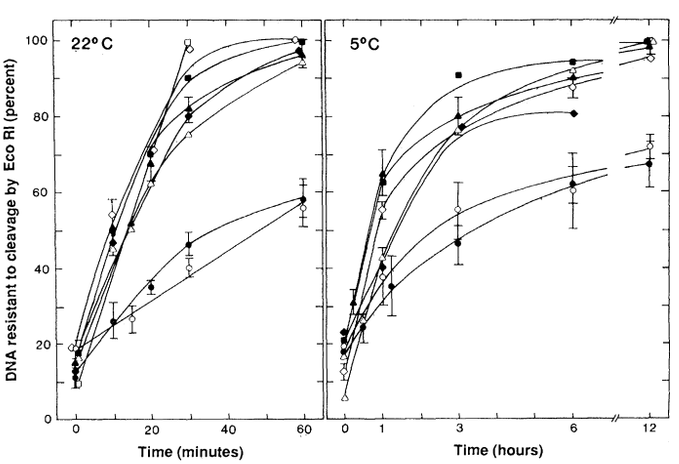

Fig. 2. The kinetics of in vivo methylation by MEco RI. *Escherichia coli* (HB101) cells harboring stable pRW1602 transformants were cotransformed with pRW1561 (●), pRW478 (○), pRW476 (◇), pRW155 (□), pRW1155 (◆), pRW465 (△), and pBR322 (■). Data for the control pRW1602 is shown as ▲. The cotransformants were grown in L broth medium containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) at the nonpermissive temperature (42°C) to an OD_{600} of 0.7 to 0.8. The culture temperatures were then rapidly lowered to 22°C and 5°C. Similar results were obtained at 32°C. Samples (10 to 20 ml) were taken at intervals and immediately returned to 42°C. Plasmid purification (*41*), restriction analyses, electrophoresis, and quantitation were as described (*38*). Change to permissive temperatures is recorded as zero time. The relative copy number of the pBR322 derivatives (Fig. 1) to the pACYC184 derivative (pRW1602) was 1.8 ± 0.25. The integrity of the plasmids was determined by restriction mapping after all cotransformation studies to ensure that deletions or other changes had not occurred. Control studies revealed that little or no (<10 percent) methylation of GAATTC sites occurred if the cells were maintained at the nonpermissive temperature (42°C). Also, 30 minutes at the permissive temperature of 22°C was required to activate the MEco RI to the extent that most or all (>90 percent) of the target sites on cotransformed control plasmids were fully methylated (not cleaved by Eco RI). Virtually identical results were found regardless of whether the pBR derivatives were transformed into HB101 cells harboring pRW1602 or vice versa.

Test for selective compartmentalization. Our results could conceivably be explained by a specific inaccessibility in vivo to the MEco RI of the pBR322 derivatives which have the highest capacity to form Z helices. In order to test this hypothesis, we constructed three plasmids (pRW1606, pRW1605, and pRW1603) (Fig. 1) that contain two additional Eco RI sites at locations distant from the sequence which has the Z-helix potential. The methylation of the GAATTC sites within or near the potential Z helix ("12 o'clock" for pRW1605 and pRW1606) is substantially inhibited, whereas the distant target sites (near "6 o'clock") in the three genomes are not inhibited (Fig. 3). For the control, pRW1603, which does not contain sequences that can adopt Z helices, both the Eco RI sites at "12 o'clock" and the ones near "6 o'clock" were rapidly methylated at similar rates.

The results of Fig. 3 lend further support to the concept that the marked inhibition in vivo of the MEco RI is due to the adoption of a left-handed helix (probably Z) by the longest (C-G) sequences rather than a specific compartmentalization within the cell.

In vitro methylation. Negative supercoiling stabilizes Z-DNA and cruciforms (*1–5, 15–18*). In general, Z helices form at lower supercoil densities than cruciforms. However, in certain special cases (*23*), a delicate balance influenced by environmental conditions exists between the types of structures formed. Also, more energy is required to convert (T-G) sequences into a left-handed helix compared to (C-G) sequences (*2–5*). We have determined the relation between negative supercoil density and the in vitro kinetics of MEco RI methylation of the plasmids shown in Fig. 1.

Nearly complete inhibition of the methylation of the Eco RI sites in pRW1561 and pRW478 was observed compared to the control pBR322 when these plasmids were at the superhelical density as isolated from *E. coli* (Fig. 4). When pRW1561 and pRW478 were relaxed with topoisomerase, the kinetics were similar to that of the control pBR322. No inhibition of methylation was observed for pRW155, pRW1155, and pRW465 as expected.

Plasmid pRW1561 readily forms a structure that is unsuitable for methylation at the GAATTC site whereas more negative supercoiling is required for pRW1155 (Fig. 4, inset). Parallel studies with pRW478 showed the inhibition at the same supercoil density as for pRW1561, as expected. Additional experiments with nuclease P1 confirmed the conclusions from Fig. 4. Thus, the results from the in vitro methylations by the MEco RI are in agreement with the results found in vivo.

Z helices or cruciforms? The repetitive sequences for the inserts shown in Fig. 1 are both direct and inverted repeats. In principal, these sequences could adopt left-handed helices (presumably Z), cruciforms, or slipped structures (*2–5*). However, earlier studies (*1, 12–18, 23–25*) indicated that the inserts in the first four plasmids would adopt Z helices, but there are too few studies with similar molecules to be able to predict whether pRW1155 would form a Z helix or a cruciform.

We examined supercoil induced changes in the primary helix (*1*) by two-dimensional gel electrophoresis (*26*) in order to characterize the unusual secondary structures adopted by the inserts and to better understand the structural basis of the methylase inhibitions (Figs. 2 to 4). The relation between the negative free energy of supercoiling and the structural transitions of appropriate regions of duplex

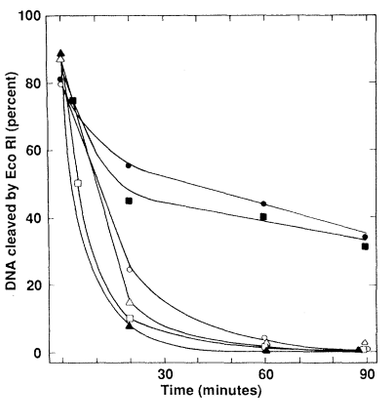

Fig. 3. Kinetics of in vivo methylation by MEco RI of plasmids containing additional target sites. *Escherichia coli* HB101 cells containing pRW1602 were cotransformed (see Fig. 2) with each of the circular plasmids shown in Fig. 1. The experiments were performed with 22°C, as the permissive temperature. Calculations of percent DNA cleaved by Eco RI at the "12 o'clock" and "6 o'clock" GAATTC sites are shown as a function of time; zero is the time when cultures reached 22°C (permissive temperature). The sites at "6 o'clock" are ~2000 bp distal from the "12 o'clock" sites in these plasmids. pRW1606 (●), 12:00; ○, 6:00); pRW1605 (■, 12:00; □, 6:00); and pRW1603 (▲, 12:00; △, 6:00).

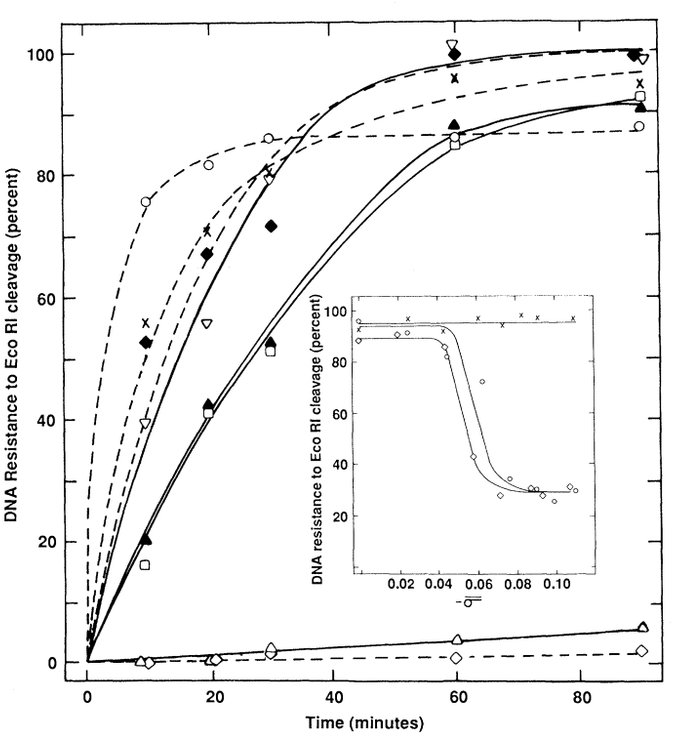

Fig. 4. Influence of DNA structure on in vitro methylation. Unless indicated otherwise, the supercoil density (about −0.06) of the plasmids was as isolated from *E. coli*. Plasmid DNA (5 μg) in 40-μl reactions containing 100 m*M* NaCl, 100 m*M* tris-HCl (*p*H 8.0), 1 m*M* EDTA, 1.2 m*M* *S*-adenosylmethionine, and 40 units of Eco RI methylase (New England Biolabs) was incubated at 37°C; (5 μl) were taken at intervals and heated to 75°C for 10 minutes. After being cooled, the mixtures were treated with 10 units of Eco RI and 10 units of Pst I (BMB) at 37°C for 2 hours in 20-μl reactions to which was added 50 m*M* tris-HCl, 100 m*M* NaCl, 20 m*M* $MgCl_2$, and 1 m*M* DTT. Electrophoresis and quantitation were as described (*27*). The plasmids are pRW1561, ◇; pRW478, △; pRW155, ▽; pRW1155, ○; pRW465, □; and pBR322, X. A filled symbol indicates that the plasmid was relaxed with calf thymus topoisomerase (*25*) before methylation. Plasmids containing two Eco RI sites are designated with a solid line; those with one Eco RI site by a dashed line. The inset shows the influence of negative supercoil density (sigma) on the in vitro methylation by MEco RI. Topoisomer populations were generated as described (*42*). Each DNA (0.5 μg) was treated with 20 units of MEco RI in100 m*M* NaCl, 2 m*M* *S*-adenosylmethionine, 1 m*M* EDTA, and 100 m*M* tris-HCl, *p*H 8.0. The reactions (20-μl volumes) were carried out at 37°C for 60 minutes and then stopped by heating at 65°C for 10 minutes. After the addition of $MgCl_2$ to 20 m*M*, 5 units of Pst I and 5 units of Eco RI were added and the reactions were continued for 90 minutes at 37°C. Electrophoresis and quantitation were standard. The superhelix densities required for these inhibitions should not be directly compared to the values that stabilize Z-DNA or cruciforms (Fig. 5 and Table 1) because of different conditions. The observation that the inhibitions in the inset level off at 30 percent and not zero, as in the main panel, is due to differences in experimental procedures, such as fivefold higher MEco RI to DNA ratio in the inset.

primary helix into either a Z structure or a cruciform has been investigated (*2–5, 8, 12, 15–18, 23–27*), for example, for pRW1561 and pRW478 (Fig. 5); the results for all plasmids studied are summarized in Table 1. The (C-G) tracts of the first three DNA's form left-handed Z helices and the extents of relaxations are in almost perfect agreement with the values expected. The entire (C-G) tracts adopt left-handed helices and thus explain why the GAATTC sites are inhibited for methylation. The DNA (pRW476) with a 26-bp segment of (C-G) also adopts a left-handed helix but showed no inhibition of methylation in vivo. We presume that its shorter length elicits this property which may be of a kinetic or equilibrium nature. The last two rows in Table 1 show the results for the two (T-G) containing DNA's (pRW155 and pRW1155). We expect the $(T\text{-}G)_{20}$ tracts in pRW155 to form Z-DNA, but at superhelical density values greater than for (C-G) sequences (*2–5*). The presence of the 95-bp nonsymmetrical sequence in the center of the insert for pRW155 should inhibit the formation of a cruciform; the insert in pRW155 formed a Z helix, as expected (*25*). Table 1 also indicates that the insert in pRW1155 adopts a cruciform.

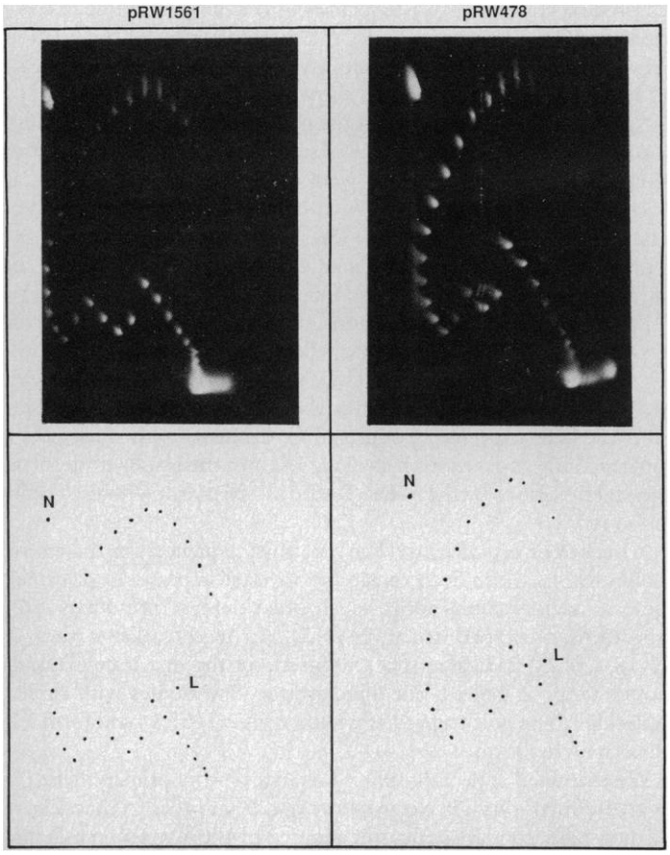

Fig. 5. Two-dimensional gel-electrophoresis analysis of topoisomers of pRW1561 and pRW478. Topoisomer populations ranging in negative supercoil density from 0 to 0.10 were prepared for each of the plasmids (*42*); a portion (1.5 to 2.0 μg) was subjected to electrophoresis on a 30-cm 1 percent agarose gel at 3 V/cm for 44 hours at room temperature in tris-borate EDTA (TBE) buffer. For the second dimension, the gel was turned 90° and run another 24 hours at 3 V/cm in TBE containing 0.75 μ*M* chloroquine. The direction of electrophoresis on the photographs is top to bottom for the first dimension and left to right for the second dimension. Other details were as described (*23, 25, 26*). The positions of nicked (N) and linear (L) DNA are designated. The data are quantitated in Table 1. Upper panels, gel photographs; lower panels, schematic representations of gel photographs.

Thus, the inhibitions of the MEco RI in vivo and in vitro are due to the formation of a left-handed helix rather than a cruciform since pRW1155 shows no methylase inhibition in vivo but does form a cruciform in vitro. Also, the in vivo inhibition of methylation of pRW478 agrees with its adoption of a Z helix but not a cruciform.

Biological effects. The stability of the inserts was influenced by their location in the plasmids. For example, insertion of the 56-bp insert, capable of forming a left-handed helix, into the Bam HI site of pRW460 (pRW477) was not stable, that is, deletions in the insert were observed. When the same 56-bp insert was cloned into the Eco RI site of pRW1560 (pRW1561) (Fig. 1), the insert was quite stable. Deletion of (C-G) inserts in the Bam HI site of pBR322 was reported earlier (*23, 27, 28*).

This behavior may be due to the existence in vivo of left-handed DNA and may be a consequence of transcription since the Bam HI site is in the tetracycline resistance gene. Alternatively, the effect may be due to an influence of neighboring sequences; others have observed (*29*) an apparent telestability effect (*6, 30*) of neighboring sequences on the in vitro properties of cruciforms.

Also, the presence of MEco RI influences the stability of the inserts in these plasmids. Cotransformation of pRW477 with pJC1 [a 5655-bp derivative of pACYC184 encoding the active (wild-type) MEco RI] or pRW1602 (at the permissive temperature) substantially increases the frequency of deletions. This increased frequency was also observed with the cotransformation of pRW1602 or pJC1 with pRW483, a plasmid containing a single 95-bp *lac* operator fragment flanked by $(C\text{-}G)_{22}$ and $(C\text{-}G)_{13}$.

Implications. Our data indicate the existence of an unusual duplex (non-B) DNA structure in a living cell. Hence, other unusual

Table 1. Predicted and observed extents of supercoil relaxation. Two-dimensional agarose gel electrophoresis analyses were performed on the plasmids as described in Fig. 5. The rationale of the calculations of the theoretical total supercoil relaxation for left-handed Z-DNA and for cruciforms was described previously (*12, 15–18, 23, 25, 43*) and is based on 12.0 bp per turn as well as the cruciform arms extending through the full length of the inverted repeat sequence. The control plasmid was pBR322.

Plasmid	Length of insert* (bp)	Total supercoil relaxation			Structure of insert (from cell)
		Theoretical		Observed†	
		Z helix	Cruciform		
pRW1561	56 (80)‡	10.0	7.6	10.0 (4.6, 5.4)§	Left-handed
pRW478	56 (68)‡	10.0	6.5	10.0 (4.5, 5.5)§	Left-handed
pRW476	26	4.6	2.5	4.5	Left-handed
pRW155	187	15.0 (7.5, 7.5)§	‖	14.0 (7.0, 7.0)§	¶
pRW1155	88 (98)‡	15.7	9.3	7.0	Cruciform

*Does not include sticky ends of restriction sites between vector and insert. †Similar types of supercoil relaxation studies under different conditions revealed the existence of cruciforms in addition to the left-handed structures for some DNA's. ‡Total length of perfect inverted repeat sequence (in parentheses) includes the restriction sites between the inserts and the vectors as well as vector sequences, where appropriate. §Two transitions as shown in Fig. 5 were found for all plasmids except pRW476 and 1155. The relaxations for each of the transitions are shown in parentheses. ‖Due to the 95-nucleotide loop in the case of a cruciform, this structure would be unlikely. ¶The B to Z transition of the insert is only partially completed.

DNA structures (*2, 3*) which have been demonstrated in vitro may also be found in vivo. Thus, DNA microheterogeneity may play an important role in genetic regulation and expression. This concept, postulated for a number of years (*4–7*), is now amenable to experimental attack.

The resistance to methylation by the MEco RI in vivo demonstrates that the target substrate is in a non-B conformation. When coupled with the study of a family of recombinant plasmids with different lengths and types of sequences that have varying capabilities to adopt left-handed or cruciform structures (or both), the conclusion of left-handed [Z or Z family (*31, 32*)] DNA in vivo is apparent.

The in vivo inhibition of methylation by the MEco RI was found for the first two plasmids shown in Fig. 1, which have 56-bp left-handed blocks. However, shorter lengths of (C-G) (26 bp or less) as well as (T-G) sequences as long as 84 bp in length did not show the inhibition. Alternatively, the (C-G) tracts cannot be substantially longer than those shown in Fig. 1 since the plasmids were found to be incompatible when cotransformed with pRW1602.

Segments of Z-DNA were detected in *E. coli* DNA by the binding of antibodies raised against Z-DNA to isolated plasmids harboring portions of the chromosome (*33*). Previous investigations have proposed that the in vivo negative superhelical density is within the range of 0.025 to 0.038 (*34–37*). Our results demonstrate that sufficient superhelical density existed in vivo to stabilize the left-handed helices for pRW1561 and pRW478, thus agreeing with previous predictions. These data show that left-handed DNA can exist in plasmids in *E. coli*; other determinations will be required to demonstrate its existence and physiological functions in the *E. coli* chromosome.

This assay should be useful for evaluating the role of conformations in vivo, a subject of much speculation (*2–5*). Notably, several studies infer a role for Z-DNA in genetic recombination (*28, 38, 39*). The recA protein of *E. coli* binds and utilizes left-handed Z-DNA (*38*). Hence, our experiments were performed in the HB101 strain which is recA$^-$. Thus, the in vivo inhibitions cannot result from specific Z-DNA binding by the recA protein. However, we cannot exclude the possibility that specific protein binding to the Z-DNA helices is responsible for the results obtained in Figs. 2 and 3. Nevertheless, such an interpretation also supports the conclusion that Z-DNA exists in vivo. The unlikely possibility exists that our results are due to the binding of a factor to (C-G) sequences which are longer than 26 bp in the B-form.

Our substantial knowledge (*2–5*) of the in vitro properties of unusual DNA structures and this development of an in vivo assay for non-B DNA should facilitate various types of functional studies.

Note added in proof: Panayotatos and Fontaine (*40*) have recently demonstrated the presence of a native cruciform structure in *E. coli*.

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