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Research Articles

Stabilization of Z DNA in Vivo by Localized Supercoiling

A. Rachid Rahmouni and Robert D. Wells

Biological processes such as transcription may generate domains of supercoiling on a circular DNA. The existence of these domains in *Escherichia coli* was investigated by the ability of different lengths of (CG) tracts, cloned upstream or downstream from the tetracycline resistance gene (*tet*) of pBR322, to adopt the Z structure in vivo. Segments as short as 12 base pairs adopt the Z form when cloned upstream from the *tet* gene (Eco RI site), whereas no Z DNA was detected when this sequence was cloned downstream (Sty I site), even with a 74–base pair (CG) tract that requires less supercoiling than shorter tracts for the B-Z transition. Hence the localized supercoil density in pBR322 can be as high as −0.038 and as low as −0.021 at different loci. These data demonstrate the existence of the Z structure for commonly found natural sequences and support the notion of domains of negative supercoiling in vivo.

The polymorphic nature and flexibility of DNA in response to local environmental conditions has been well documented (*1*). Left-handed Z DNA as well as other non–B DNA structures, such as cruciforms and triplexes, are induced by negative supercoiling in recombinant plasmids. These structures have been extensively investigated in vitro (*1*).

Recent studies demonstrated the existence of Z DNA in living cells (*2, 3*). Furthermore, the B-to-Z structural transition causes deletions (*4*). These discoveries contributed substantially to the long-standing hypothesis that DNA structural microheterogeneity plays a key role in cellular processes. However, Z DNA was found in vivo only in alternating CG [(CG)] inserts longer than 40 to 45 bp (*2, 3*), which are uncommon in naturally occurring sequences (*5*).

Transcription can be a major contributor to the level of DNA supercoiling in bacteria (*6–8*) and yeast (*9, 10*). The actual local supercoiling in vivo may be highly positive, highly negative, or negligible, depending on the position of promoters, the rate of transcription, and the efficiency of supercoil removal by topoisomerases (*8, 11*). The waves of negative supercoiling generated behind the transcription machinery can be sufficient to transiently induce non–B DNA structural transitions (*11*).

In this article we describe a more sensitive assay that detects shorter regions of left-handed Z DNA in vivo. Osmium tetroxide (OsO_4) was used to probe the structural distortions at the B-Z or Z-Z junctions at the base pair level, directly inside *Escherichia coli* HB101. Tracts of (CG) as short as 12 bp adopt the Z conformation in vivo when located upstream from the pBR322 *tet* promoter but not when cloned downstream from the *tet* gene. This discovery of short, naturally occurring segments undergoing the B-to-Z transition has significant biological implications. Indeed, long (CG) stretches, which are permanently in the Z form, would not be regulated, whereas the existence of small Z helices, which are more sensitive to slight changes in available free energy, would offer greater opportunities for their involvement in biological regulatory

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processes. In vitro experiments have shown that long (CG) runs are not necessary to effect the B-Z transition since short blocks of this sequence (8 to 12 bp) are sufficient to initiate the transition when the free energy (negative supercoiling) is high enough (*12*).

Osmium tetroxide as a probe for Z DNA in vivo. OsO_4 is a well-known pyrimidine-specific reagent that preferentially modifies the distorted regions of DNA. These include the junctions between B and Z DNA (*13–15*) and out-of-phase blocks of Z DNA (*15–17*), as well as single-stranded regions in cruciforms (*18*) and triplexes (*1, 19*). Therefore we used OsO_4 in the presence of 2,2′-bipyridine (*20*) to probe Z DNA inside *E. coli*. Our in situ assay is based on the observation in vitro that the two consecutive T's of an Eco RI (GAATTC) site, located at the center of a (CG) tract or at the interface with the vector, are hyperreactive to OsO_4 when the insert is in the Z conformation (*16*). The hyperreactive nucleotides were mapped by their ability to terminate the extension of a ^{32}P end-labeled primer with Klenow fragment of DNA polymerase I. This primer extension mapping procedure (*21*) reveals the reactive sites at the nucleotide resolution.

The inserts in the Eco RI or the Sty I sites of pBR322 had (CG) tracts that ranged from 8 to 74 bp, and these tracts had Eco RI (GAATTC) or Bam HI (GGATCC) sites in their interior or at their ends (at the intersection with the vector) (Fig. 1).

Topoisomeric samples (negative supercoil density $-\bar{\sigma}$ of zero to 0.08, the bar indicates average) of pRW1557, which contained a 32-bp (CG) tract with an Eco RI site in the middle (Fig. 1), were modified with OsO_4 in vitro. The bottom strands of the modified DNA's were then analyzed by primer extension (Fig. 2). For the first two topoisomer populations ($-\bar{\sigma}$ = 0.00 and 0.02), the primer extension analyses show no stop sites for the polymerase. The same patterns were observed when relaxed or native supercoiled ($-\bar{\sigma} \cong 0.054$) unmodified DNA's were used as templates. However, analyses of DNA's modified at $-\bar{\sigma}$ = 0.04, 0.06, and 0.08 showed some termination bands located at the junctions between the insert and the vector and even stronger bands at the center of the (CG) tract. The stops occurred opposite to a thymine or one nucleotide before (as compared to the dideoxy sequencing ladders of the top strand run in parallel). This result implies that thymine glycols, which result from the 5,6–double-bond oxidation with OsO_4, stop the DNA elongation opposite to or one base before, in agreement with previous observations (*22*). This finding was further confirmed when OsO_4-modified DNA's were mapped by hot piperidine cleavage (*14–16*). When the top strands of these DNA's were analyzed, the same reactive sites were observed.

The locations of the reactive sites [the two B-Z junctions and the junction between the two out-of-phase (CG) blocks (Z-Z junction)] indicate that the insert in pRW1557 is in the Z conformation. The insert in pRW1557, as well as most of the inserts used in these studies, is also an inverted repeat sequence and could possibly form a cruciform under superhelical stress. To distinguish between these structures, we extended our investigations with other well-characterized chemical probes including diethylpyrocarbonate (*15, 16, 23*) and bromoacetaldehyde (*24*). In all cases, only Z-type mapping patterns were observed. Also, the B-Z transition in pRW1557 was studied (*3*) by two-dimensional (2-D) gel electrophoresis; the midpoint of this transition was at $-\sigma$ = 0.032. Our in vitro OsO_4–probing experiments agree with these data, since the reactivities were observed only with topoisomeric samples at $-\bar{\sigma}$ = 0.04 and above.

Next, we attempted to detect these reactivities on plasmids modified in vivo, because the T's within the (CG) tract reacted strongly with OsO_4 when the insert was in the Z conformation.

A

Plasmid	Eco RI insert	Base pairs	Sty I insert	Base pairs
pRW2033	GAATT $(CG)_4$ AATTC	8		
pRW2034	GAATT $(CG)_5$ AATTC	10		
pRW2035	GAATT $(CG)_6$ AATTC	12		
pRW1608	AGATC $(CG)_{13}$ AATTC	26		
pRW1557	AGATC $(CG)_7$ AATT $(CG)_7$ GATCT	32		
pRW1556	AGATC $(CG)_9$ AATT $(CG)_9$ GATCT	40		
pRW2056			GGATC $(CG)_9$ AATT $(CG)_9$ GATCC	40
pRW478	GAATT $(CG)_{13}$ GATC $(CG)_{13}$ AATTC	56		
pRW2078			GAATT $(CG)_{13}$ GATC $(CG)_{13}$ AATTC	56
pRW1554	AGATC $(CG)_{22}$ AATT $(CG)_{13}$ GATCT	74		
pRW2054			GGATC $(CG)_{22}$ AATT $(CG)_{13}$ GATCC	74
pRW2036	AGATC $(CG)_9$ AATT $(CG)_9$ GATCT	40	GGATC $(CG)_9$ AATT $(CG)_9$ GATCC	40
pRW2037	AGATC $(CG)_7$ AATTC	14	GGATC $(CG)_9$ AATT $(CG)_9$ GATCC	40

B

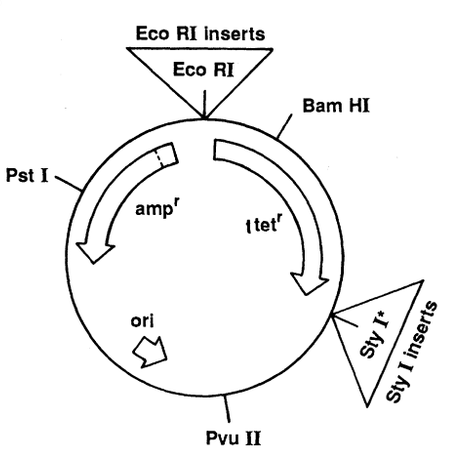

Fig. 1. (**A**) Plasmids used in these studies. The plasmids pRW2033, pRW2034, and pRW2035 were made by cloning synthetic oligonucleotides having sticky ends into the Eco RI site of pBR322. The construction of the remaining plasmids with the inserts in the modified Eco RI site of pRW1560 was described (*2, 3*). Plasmids pRW2056, pRW2054, and pRW2078 were obtained by recloning the 48- and 82-bp Xho II fragments and the 64-bp Eco RI fragment from pRW1556, pRW1554, and pRW478, respectively, into the filled-in Sty I site of pBR322. In the same manner, pRW2036 and pRW2037 were constructed by recloning the 48-bp Xho II fragment from pRW1556 into the filled-in Sty I site of pRW1556 and pRW1567 (*3*), respectively. In general, all of the cloning and screening was carried out according to standard procedures (*39*). The columns marked base pairs indicate the length of the (CG) tract for each insert; when present, the central 4-bp (AATT or GATC) are added to the total length. Only the top-strand sequences are presented. All of the plasmids encode resistance to both ampicillin and tetracycline. (**B**) The restriction map of pBR322 shows the location of the inserts in the plasmids described above. The asterisk (*) refers to the modification of the Eco RI site and the filling in of the Sty I site in the plasmids.

After transformation of *E. coli* HB101 with pRW1557, the growing cells were treated either with 3 m*M* 2,2′-bipyridine as a control or with 3 m*M* 2,2′-bipyridine plus 3 m*M* OsO_4 for 5 minutes at 37°C. The plasmids were then extracted, and the bottom strands were analyzed by primer extension as for the in vitro experiments. For the control (Fig. 2, lane M), no significant stop sites were observed in the insert region. However, for the DNA isolated from cells treated with OsO_4, the primer extension pattern (Fig. 2, lane E) resembles precisely the one observed with the in vitro modified DNA's. Thus strongly reactive T's were observed in the center of the (CG) stretch and at the 5′ end junction. Also, the 3′ end junction was modified but weakly. These reactive sites were also observed when the top strand was mapped. The differences in reactivity between the 5′ and the 3′ junctions were also observed with the in vitro modified DNA's (compare to sample at $-\bar{\sigma} = 0.04$) and were previously reported (*15, 16*). In summary, these in vivo OsO_4 modifications indicate that some of the plasmid molecules have their inserts in the Z conformation or at least the (CG) tracts are in a dynamic B-Z equilibrium state.

Possible secondary modification of the plasmid outside the cells during the isolation was excluded. *E. coli* containing no plasmid was treated with OsO_4 and 2,2′-bipyridine under standard conditions and then mixed with either 2 μg of unmodified pRW1557 or with untreated cells harboring the plasmid. The primer extension patterns of the isolated DNA's showed no detectable reactivity. These controls indicate that the OsO_4 modifications arise inside the cells, as shown also by the results (described below) with the supercoil-density–indicator plasmids (plasmids that contain two inserts cloned at different loci).

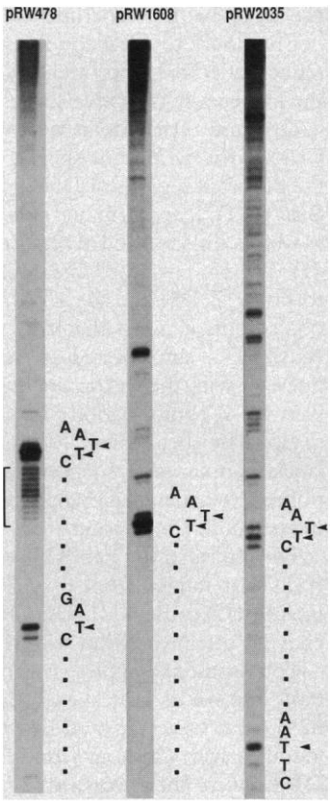

Fig. 3. In vivo OsO_4 reactivity on plasmids containing different lengths of (CG) inserts. *Escherichia coli* HB101 harboring pRW478, pRW1608, or pRW2035 were treated with OsO_4 and 2,2′-bipyridine, the DNA's isolated, and the bottom strands mapped by primer extension as in Fig. 2. The letters at the right of each lane show the bottom-strand sequences flanking the (CG) tracts represented by dots; 3′ to 5′ is lower to upper. The arrowheads indicate the OsO_4 reactive sites. The bracket in the left lane shows nonspecific stop sites that were also observed for the control. The in vivo OsO_4 reactivity patterns were run in parallel with the mock controls in order to identify the nonspecific stop sites. However, some reactive T's outside the insert were also observed; these could be a result of the high negative supercoiling in this region. These observations also pertain to Figs. 2, 5, and 6.

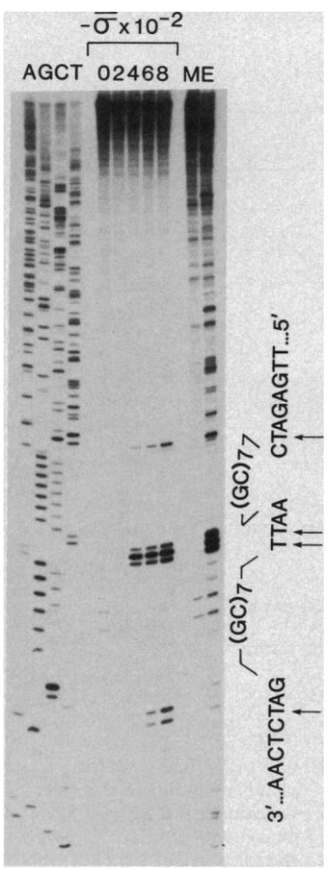

Fig. 2. Primer extension analyses of OsO_4-reactive sites on the bottom strand of pRW1557 modified in vitro and in vivo. Topoisomeric samples (*40*) of pRW1557 at the indicated average supercoil densities ($-\bar{\sigma}$) were modified in vitro with OsO_4 (*41*). Lanes M and E show the primer extension analyses of the plasmid extracted from cells treated either with 3 m*M* 2,2′-bipyridine (mock control) or 3 m*M* 2,2′-bipyridine plus 3 m*M* OsO_4 (experiment), respectively (*41*). Primer extension analyses were performed essentially as described (*21*), except that dGTP (deoxyguanosine triphosphate) was substituted by 7-deaza dGTP (BMB). To map the Eco RI inserts, two primers were used, namely pBR Eco RI clockwise primer and pBR Hind III counterclockwise primer (New England Biolabs) for the bottom and the top strands, respectively. The products of the primer extension were analyzed in a 10 percent acrylamide gel containing 7*M* urea and 30 percent formamide. These denaturants, along with the use of 7-deaza dGTP, reduce the compression of the bands on the gel. Lanes A, G, C, and T indicate the sequencing pattern for the top strand obtained on unmodified DNA using the same primer as for the modified samples, except that the corresponding dideoxynucleotide triphosphates (ddNTP's, BMB) were added in the reaction mixtures. The sequence of the bottom strand, mapped in this experiment, is indicated at the right side. The arrows show T's on the template strand, which are stop sites for the polymerase and hence are modified with OsO_4.

The in vivo existence of the Z structure for the 32-bp (CG) tract in pRW1557 is surprising, since previous experiments with either the Eco RI methylase (MEco RI) (*2*) or the linking number (*3*) assays showed Z DNA formation in vivo only in (CG) inserts longer than 40 to 45 bp. This result is likely to be due to the higher sensitivity of the OsO_4 assay. If the insert is in a dynamic B-Z equilibrium, the OsO_4 probe positively detects the transient Z state; alternatively, the MEco RI assay probes the insert while in the B state and thus was a "negative" assay for left-handed DNA. However, the OsO_4 assay, which we consider to be more sensitive since it detects shorter Z helices, does not easily enable an estimate of the percentage of the inserts which are in the non-B conformation.

Minimum size of insert to form Z DNA in vivo. These in vivo analyses were extended to a series of plasmid inserts varying in length from 8 to 74 bp (CG) tracts; all of the inserts were in the Eco RI site of pBR322 (Fig. 1). *Escherichia coli* harboring the plasmids was treated with OsO_4, and the reactivity patterns of the DNA's were compared with the results obtained in vitro. As expected, OsO_4 hyperreactive sites were detected on pRW478 (Fig. 3), which contains a long (CG) insert (56 bp). The most reactive T's were located within the Eco RI site at the B-Z junction at the 5′ end. These hyperreactive sites were also observed for pRW1608, which contains a 26-bp (CG) tract (Fig. 3). In addition, substantial reactivities were observed at the B-Z junctions of the 12-bp (CG) insert in pRW2035 (Fig. 3). However, when plasmids with shorter inserts were assayed, no reactivities were detected, either with pRW2034 or pRW2033 that contained 10- and 8-bp (CG) inserts, respectively. These results indicate that for this family of plasmids (inserts in the Eco RI site of pBR322) a minimum insert length of $(CG)_6$ is essential for stabilizing a Z helix in vivo.

The supercoil-induced B-to-Z transitions for these plasmids were analyzed in vitro by 2-D gel electrophoresis (Fig. 4). This determination provides important information on the nature and energies of the B-to-Z transitions (*1–3, 12, 16*). As expected (*2–4*), $-\sigma$ values

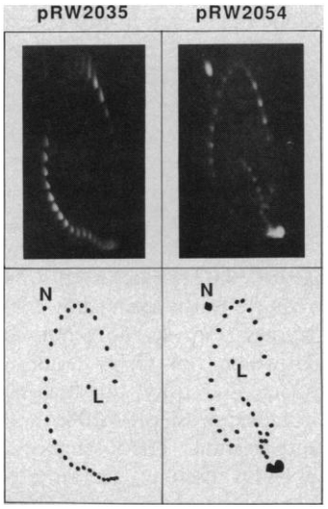

Fig. 4. Two-dimensional gel-electrophoretic analyses of topoisomers of pRW2035 and pRW2054. Topoisomer populations were prepared for each of the plasmids (*40*) and subjected to electrophoresis as described (*2, 3*), except that pRW2035 was run in 1.5 percent agarose gels and with 1 μM chloroquine for the second dimension. The direction of electrophoresis is top to bottom for the first dimension and left to right for the second dimension. The positions of the nicked (N) and linear (L) DNA are designated. The values for $-\sigma$ at the midpoint of the transition were corrected for one-half the amount of total relaxation at the complete B-Z transition (*3*). The principal conclusions for the transition in pRW2054 were derived from the first transition; the shape of the second transition was influenced by the relaxation in the second dimension due to the concentration of chloroquine used (*2*). Two-dimensional gels were run on all of the plasmids used herein; the analyses on these two plasmids are shown as representative data because of their importance to the interpretation of these results.

at the midpoint of the transitions decrease as the length of the (CG) tract increases. For the small inserts, these values were 0.04 and 0.038 for 10- and 12-bp (CG) tracts, respectively (Fig. 4). Hence we conclude from these results and the in vivo OsO_4 probing that the negative supercoil density inside *E. coli* can be as high as 0.038.

Supercoil domains in pBR322 in vivo. Our value of the in vivo supercoil density (-0.038) differs significantly from previous results (-0.025 and -0.027) (*3, 25, 26*). This difference may be attributed to our assay measuring transient local supercoil density (at the Eco RI site of pBR322 in this case), whereas the earlier methods (*3, 25, 26*) were based on more static supercoil measurements. The Eco RI site in pBR322 is located between two divergent transcription units (ampicillin and tetracycline genes) (Fig. 1). Thus, according to the model proposed by Liu and Wang (*6*), our value could reflect the existence of a highly negatively supercoiled domain, or at least transient negative supercoils generated upstream from the transcription units.

To test this hypothesis, we compared the in vivo OsO_4 reactivity for a given (CG) insert when cloned upstream or downstream from the *tet* gene. The 40-bp (CG) tract of pRW1556 was inserted in the Sty I site of pBR322 to give pRW2056 (Fig. 1). Thus the two plasmids differ only by the sites of insertion of the (CG) tract.

The plasmids were first probed in vitro at native supercoil density (~ -0.054) and each one was mapped with the appropriate primer. The primer extension patterns showed similar reactivities (B-Z and Z-Z junctions) for both plasmids (Fig. 5), indicating that the insert was in the Z conformation whether cloned upstream or downstream from the *tet* gene under these conditions. However, when the plasmids were probed in vivo (Fig. 5), only pRW1556 showed strongly reactive T's at the center of the (CG) tract (Z-Z junction). The absence of reactivities for pRW2056 (Fig. 5) indicates that the B-to-Z transition of the insert in this plasmid does not occur in vivo.

The supercoil-induced B-to-Z transitions for both pRW1556 and pRW2056 were analyzed by 2-D gel electrophoresis (*3*). The results show that the transitions occur at the same supercoil density; other features of this transition were described (*3*). Thus. the in vivo behavior of the insert in pRW2056 cannot be explained by a flanking sequence effect alone that might increase the free energy for the B-Z transition.

To further investigate this finding, we cloned a longer (CG) insert (56 bp from pRW478) into the Sty I site of pBR322 to give pRW2078 (Fig. 1). Again, when pRW2078 was probed with OsO_4 in vivo, no reactive sites were observed, an indication of no Z DNA. However, under the same conditions, the 56-bp (CG) tract underwent a B-Z transition in vivo when cloned in the Eco RI site of pBR322 (pRW478 in Fig. 3).

Conceivably, the absence of reactivity in vivo of the pRW2078 insert could be explained by the inaccessibility of the target nucleotides to OsO_4 when the insert was cloned in the Sty I site of pBR322. To test this hypothesis, we performed a different analysis for Z DNA, the linking number analysis (*3*), on both plasmids. Whereas pRW478 revealed two topoisomer populations indicating the presence of Z DNA in vivo as previously reported (*3*), pRW2078 showed only one population, and hence no Z DNA. Thus the good agreement between the linking number assay and the OsO_4 probe suggests that the absence of Z DNA in vivo for the 56-bp (CG) tract, when cloned downstream from the *tet* gene, is due to the low negative supercoil density in this region rather than to a specific inaccessibility of the target sites.

To determine the supercoil density value ($-\sigma$) in this domain, we inserted the longest (CG) tract (74 bp) cloned to date in this laboratory (*3, 4*) from pRW1554 in the Sty I site of pBR322, which gave pRW2054. The two related plasmids (pRW1554 and pRW2054) were probed with OsO_4 in vivo; only the pRW1554 pattern showed hyperreactive sites and hence Z DNA. The in vitro B-to-Z transition of the insert occurred at the same supercoil density for both plasmids with a midpoint at $-\sigma = 0.021$ (*3*) (Fig. 4). Thus these data indicate that the $-\sigma$ value in the Sty I region of pBR322 is lower than 0.021.

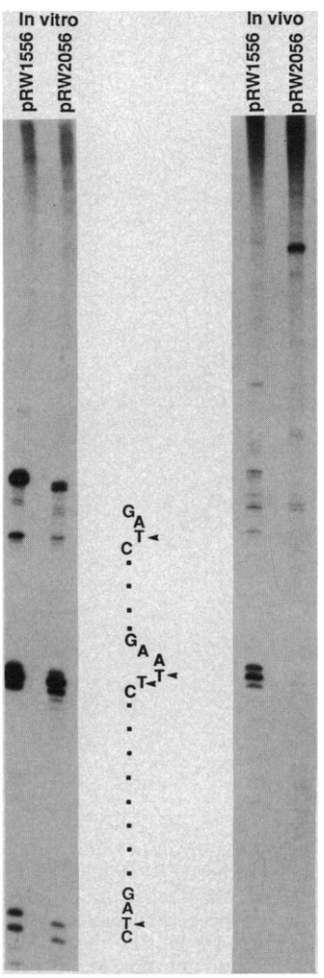

Fig. 5. Comparison of OsO_4 reactivity on a 40-bp (CG) tract when cloned into the Eco RI or the Sty I sites of pBR322. The plasmids pRW1556 and pRW2056 (containing the 40-bp insert cloned either in the Eco RI or the Sty I sites of pBR322, respectively) were modified in vitro at native supercoil density ($-\bar{\sigma} = 0.054$) or in vivo as in Fig. 2. The bottom strand of pRW1556 was mapped using Eco RI clockwise primer. The top strand of pRW2056 was mapped using Sty I counterclockwise primer, which hybridizes downstream from the Sty I restriction site between nucleotides 1384 and 1400 of pBR322. In both cases, the complementary strands were also mapped and gave similar results. The symbols are the same as in Fig. 3.

In vivo OsO_4 reactivity studies were also performed in the presence of rifampicin (*27*) to determine the effect of transcription on the capacity of various lengths of (CG) tracts (40, 56, or 74 bp) in the Eco RI or the Sty I sites of pBR322 to adopt Z structures. No hyperreactive sites were found in all cases. Therefore transcription apparently generates the two supercoil domains observed in the absence of rifampicin. Hence, in the presence of rifampicin, both the Eco RI and Sty I sites have a $-\sigma$ value lower than 0.021.

Supercoil-density–indicator plasmids in vivo. To further evaluate the existence of supercoil domains on a plasmid in vivo, we constructed a pair of recombinant plasmids, each containing two inserts; pRW2036 contains two 40-bp (CG) tracts, one inserted in the Eco RI and the other in the Sty I sites of pBR322, whereas pRW2037 contains a shorter (CG) block (14 bp) in the Eco RI site and a longer one (40 bp) in the Sty I site (Fig. 1). After in vivo probing with OsO_4 as usual, the two inserts of each plasmid were mapped simultaneously with the use of the appropriate primers. The primer extension patterns of the Eco RI inserts are shown in Fig. 6; hyperreactive T's were found at the center of the (CG) tract in pRW2036 (Z-Z junction) and at the junction with the vector in pRW2037 (B-Z junction). On the other hand, no reactivities were detected in the Sty I inserts for both plasmids (Fig. 6).

These results indicate that in the same plasmid, the (CG) tract cloned upstream from the *tet* gene (even a run as short as 14 bp) undergoes a B-Z transition in vivo, whereas the segment inserted downstream from the gene (even as long as 40 bp) does not.

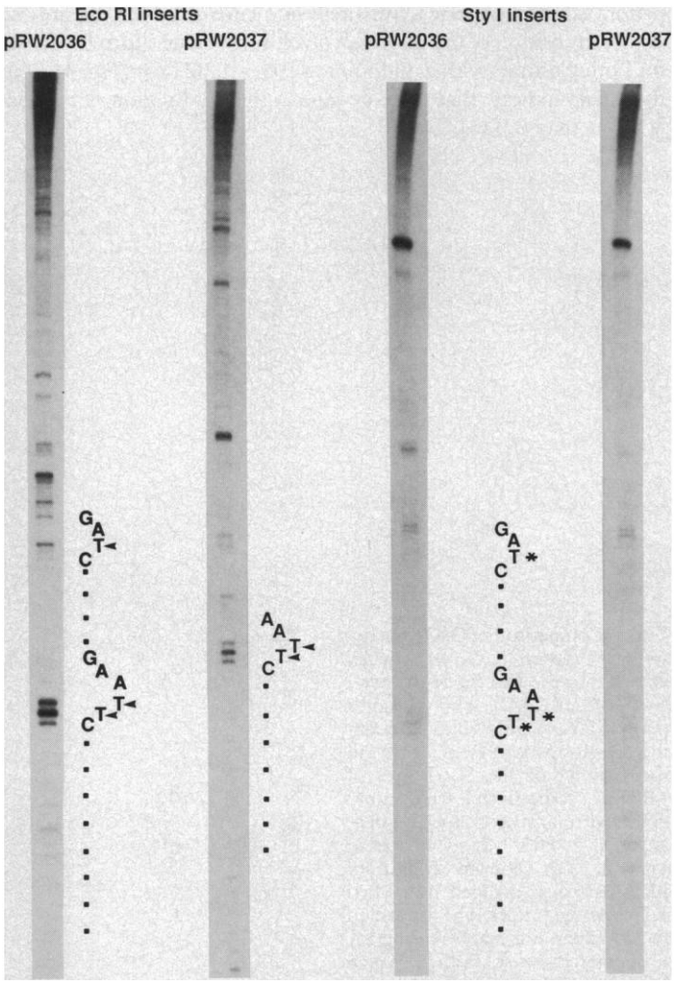

Fig. 6. In vivo OsO_4 reactivity on two (CG) inserts in the same plasmid. Cells harboring pRW2036 and pRW2037 were treated with OsO_4 as in Fig. 2. Following DNA isolation, the Eco RI and the Sty I inserts of each plasmid were mapped simultaneously using the appropriate primers as in Fig. 5. The asterisks (*) indicate the T's which did not react in vivo, and the other symbols are the same as in Fig. 3.

Implications. In summary, our data implicate the existence of two supercoil domains for pBR322 inside *E. coli*. First, a highly negatively supercoiled domain exists in the Eco RI region where the density can reach -0.038, a value estimated by the midpoint of the B-Z transition for 12 bp in pRW2035 (Fig. 4). The second domain, in the Sty I region, has a lower negative supercoil density ($-\sigma < 0.021$) since even a 74-bp (CG) tract (pRW2054), which requires much less energy for Z formation as revealed by in vitro studies (Fig. 4), does not undergo a B-to-Z transition in vivo. However, our OsO_4 results do not necessarily give an average supercoil density, but may reveal a maximum value. The results obtained with pRW2036 and pRW2037 (Fig. 6) confirm these interrelations. The absence of reactivity at a 40-bp (CG) insert while a 14-bp insert on the same plasmid reacted well agrees with the control experiments (described above) that the OsO_4 reactions took place within the bacteria and were influenced by steady-state biological processes.

Z DNA, which is stabilized by negative supercoiling, is induced in *E. coli* on (CG) sequences cloned upstream but not downstream from the *tet* gene. Therefore, these results support the twin supercoiled domain model (*6*). The model predicts that the translocation of an RNA polymerase elongation complex along the double helix generates positive supercoil waves ahead of and negative supercoil waves behind the transcription ensemble. Our results agree with this prediction with the more negatively and the less negatively supercoiled domains in the Eco RI and the Sty I regions of pBR322, respectively. Moreover, our data implicate the transcription process in generating the two supercoiled domains, since the rifampicin treatment (*27*) renders the two domains equivalent.

The differences between our OsO_4 probe data and the two in vivo assays previously reported (*2, 3*) suggest that the short (CG) inserts are in a transient rather than a static Z-form. This finding is consistent with the idea of waves of negative supercoils upstream from the *tet* and *amp* genes, which have slow diffusional pathways, as recently suggested (*11, 28*). Presumably, our assay detects the dynamic state of transcriptionally induced supercoiling, rather than a frozen supercoiled domain at the junction of two divergent transcription units (*11, 28*). Therefore OsO_4 may modify the non–B DNA structure on a plasmid while it is undergoing biological processes such as transcription.

Our data show also that (CG) sequences as short as 12 bp can undergo a B-Z transition in vivo when localized in a negatively supercoiled domain. Depending on the level and distribution of the topoisomerases, the strength of the promoters, and the length of the transcripts, negative supercoiling in localized regions of the chromosome could become quite substantial (*8, 29*); thus we have no reason to believe that our value of -0.038 is an upper limit in all circumstances. These data show that lengths of (CG) that are commonly found naturally (*30*) can adopt the Z conformation. Indeed, the *E. coli* chromosome contains a numerous 8- and a few 10- and 11-bp (CG) tracts (*30*). Also, long runs of (TG) sequences (*31*) or (CG) islands (*32*) are found abundantly in mammalian genomes. Just as we do not yet know the upper limit of supercoil density transiently formed in plasmid domains, we do not know the lower length of an appropriate sequence [reviewed in (*12*)] to form the Z structure in living cells.

Z DNA was detected with antibodies in metabolically active mammalian cell nuclei (*33*) and was localized in transcriptionally active chromosomes (*34*). The level of Z DNA on chromosomes seems to be regulated by torsional stress (*33*) and hence by the dynamic balance between the biological processes (transcription and

replication) an the action of topoisomerases. Also, in addition to supercoiling, the binding of specific proteins [reviewed in (*1, 35*)] or other ligands such as carcinogens and mutagens (*36*) may play an important role in stabilizing the Z conformation. Many recent experiments have shown the importance of DNA supercoiling and therefore topoisomerase action in gene activation (*37*), replication (*38*), and recombination (*26, 35*). These findings along with the results described herein enhance the likelihood of the involvement of Z DNA and other non–B DNA structures in biological processes.

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41. The in vitro OsO_4 reaction was performed with 2 μg of DNA in 0.2*M* NaCl, 25 m*M* tris-HCl buffer, *p*H 7.5, 2.5 m*M* EDTA, 2 m*M* 2,2′-bipyridine, and 2 m*M* OsO_4 in a final volume of 25 μl. After incubation at 37°C for 15 minutes, the samples were brought to 100 μl with water and extracted twice with ether, and then centrifuged through a Sephadex G50 column equilibrated in water as described (*39*). The samples were then analyzed by primer extension. The in vivo OsO_4 modification was done as follows: *E. coli* HB101 harboring the plasmids was grown in L broth containing ampicillin at 100 μg/ml to an optical density at 600 nm of 0.8. The 10-ml samples were centrifuged and the cells were resuspended in 2 ml of 0.15*M* NaCl, 50 m*M* phosphate buffer, *p*H 7.5. At this time, OsO_4 and 2,2′-bipyridine were added to a final concentration of 3 m*M*. After 5 minutes at 37°C with shaking, the cells were quickly diluted (1:10) with cold buffer and centrifuged; the plasmid was then isolated (by the boiling method) as described (*21*). After the DNA was precipitated, the samples were passed through a G50 column as for the in vitro modified samples and then analyzed by primer extension.

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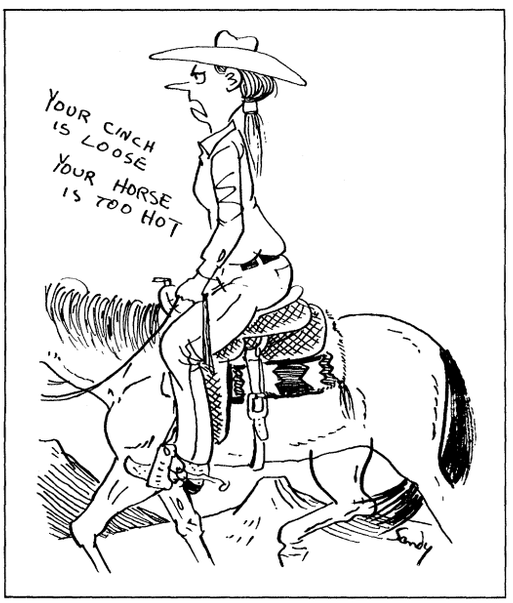

" The computer age has gone too dang far."