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Left-handed Z-DNA and *in vivo* supercoil density in the *Escherichia coli* chromosome

(transposon insertions/methylation inhibition)

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ABSTRACT **A system for studying Z-DNA formation in the *Escherichia coli* chromosome was developed. Prior investigations in recombinant plasmids showed that alternating (Pur-Pyr) sequences can adopt a left-handed Z-DNA conformation both *in vitro* and *in vivo*. We constructed mobile, transposon-based cassettes carrying cloned (Pur-Pyr) sequences containing an *Eco*RI site in the center. These cassettes were subsequently inserted into different locations in the *E. coli* chromosome in a random fashion. A number of stable insertions were characterized by Southern analysis and pulsed-field gel electrophoresis mapping. A cloned temperature-sensitive M*Eco*RI methylase was expressed in trans as the probe to study Z-DNA formation *in vivo*. In this system, the control *Eco*RI sites were quickly methylated when cells were placed at the permissive temperature. Strong inhibition of the methylation was observed, however, only for the *Eco*RI sites embedded in a 56-bp run of (C-G). In contrast, the shorter sequence of 32 bp did not show this behavior. Prior *in vitro* determinations revealed that the longer tract required less energy to stabilize the Z-helix than the shorter block. We conclude that the observed inhibition of methylation is due to Z-DNA formation in the *E. coli* chromosome. *In vitro*, these sequences undergo the B- to Z-DNA transition at a supercoil density of −0.026 for the 56-bp insert and −0.032 for the 32-bp block. Since only the longer (C-G) tract but not the shorter run adopted the left-handed conformation in the chromosome, we propose that these densities establish the boundaries in the different chromosomal loci investigated; these boundaries are in good agreement with the extremes found in plasmids.**

The existence of left-handed Z-DNA *in vivo* (i.e., in viable cells) has been postulated for the last several years. DNA sequence search analyses revealed a broad occurrence of potential Z-DNA-forming sequences in human genes (1). These sequences are nonrandomly distributed with a strong bias toward locations near the sites of transcription initiation. Some naturally occurring purine-pyrimidine tracts (Pur-Pyr) were cloned and shown to form Z-DNA in recombinant plasmids *in vitro* (2–4). Alternating (C-G) stretches are bound strongly by RecA protein and DNA topoisomerase II; they also cause a transcriptional block *in vitro* (5–7). Also, Z-DNA binding proteins have been isolated from *Escherichia coli*, yeast, and *Drosophila* (8–10). However, no functions for Z-DNA in a natural system have been proven to date.

A wide range of physical and biological investigations on left-handed Z-DNA has been conducted (reviewed in ref. 11). Also, at least five lines of investigation have demonstrated the existence of Z-DNA in recombinant plasmids *in vivo* or *in situ* (12). However, no prior work has been conducted in intact chromosomes in living cells.

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Recent attention has been focused on *in vivo* assays in plasmids. In 1987, Jaworski and coworkers (13) discovered, using temperature-sensitive M*Eco*RI methylase, that Z-DNA exists inside *E. coli*. This assay was based on the *in vitro* observation that the recognition site is not methylated by its specific methylase when the site is in or near a left-handed Z-DNA conformation (14, 15). These sequences were capable of adopting left-handed Z-DNA *in vitro* and carried target sites for the M*Eco*RI probe within or at the end(s) of the tracts. Inhibition of methylation *in vivo* was found for the *Eco*RI site embedded in a 56-bp (Pur-Pyr) stretch but not in shorter blocks.

Here, we employed the methylation assay as a part of a system designed to study Z-DNA formation in the *E. coli* chromosome. We inserted (Pur-Pyr) sequences in different locations of the *E. coli* chromosome via transposon mutagenesis. The kinetics of methylation of the *Eco*RI sites were measured. A longer insert (56 bp) but not a shorter (C-G) tract (32 bp) was undermethylated *in vivo* as compared to the control *Eco*RI sites. Thus, we believe this is due to Z-DNA formation. We propose that this system can be used for studying the structure and topology of the chromosomes as well as the existence of other non-B-DNA conformations *in vivo*.

MATERIALS AND METHODS

Plasmid Constructions. pRW1560 is a pBR322 derivative with *Bgl* II linkers inserted into the filled-in *Eco*RI site. Clones pRW1557 and pRW1561 carry two different (Pur-Pyr) stretches, $(CG)_7AATT(CG)_7$ and $(CG)_{13}AATT(CG)_{13}$, respectively, cloned at the *Bgl* II site of pRW1560. pRW1602 contains the gene for the temperature-sensitive M*Eco*RI methylase. The above plasmids were constructed earlier in this laboratory (13, 16). Transposon Tn*phoA* contained on the suicide plasmid pRT733 (provided by Gunter Schmidt, Forschungsinstitut Borstel, Borstel, Germany) was used to deliver (Pur-Pyr) sequences into the *E. coli* chromosome (17). The cloning strategy was based on the restriction map of the Tn*5* transposon (18). A *Cla* I/*Ava* I fragment of pRW1560 containing the tetracycline-resistance (Tet^R) gene was first removed, producing plasmid pRWI560ΔTet. This plasmid was used as a vector for a 2.7-kb *Bgl* II–*Bgl* II fragment representing an internal part of the Tn*phoA* transposon. Two (Pur-Pyr) stretches, $(CG)_7AATT(CG)_7$ and $(CG)_{13}AATT(CG)_{13}$, were excised from pRW1557 and pRW1561 as *Sst* I/*Ava* I fragments. These fragments, also containing Tet^R markers, were ligated to a *Sal* I linker and cloned between *Sma* I and *Ava* I sites within a *Bgl* II–*Bgl* II part of the Tn*phoA*. Finally, the *Bgl* II fragments with the new *Sal* I site,

Abbreviations: PFGE, pulsed-field gel electrophoresis; CHEF, clamped homogeneous electric field; R, resistance or resistant; Tet, tetracycline; Km, kanamycin.

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(Pur-Pyr) runs, and the *tet* gene were recloned back into pRT733. Constructs pRW2663 and pRW2664 contain the Tn*phoA* transposon carrying 32-bp and 56-bp (Pur-Pyr) tracts, respectively, with *Eco*RI sites in the centers. A schematic representation of the physical maps of these plasmids is shown in Fig. 1.

Transposon Mutagenesis. The suicide behavior of pRT733 results from the presence of an origin of replication from the plasmid RK2. This origin can function only when π protein, the product of a *pir* gene, is provided (19). Therefore, pRW2663 and pRW2664 are stably maintained only in a special host strain *E. coli* SM10 containing the chromosomally inserted *pir* locus (20). However, when introduced by electroporation into competent cells of *E. coli* HB101, plasmids were degraded while transposons carrying (C-G) runs jumped into the chromosome. Mutants were selected for their kanamycin- and tetracycline-resistant phenotype (Km^R, Tet^R). The loss of ampicillin-resistance marker (Amp^R), which is present on the plasmid pRT733 outside the transposon, confirmed the transposition events.

Pulse-Field Gel Electrophoresis (PFGE). Chromosomal insertions of the Tn*phoA*Pur-Pyr segment were mapped using PFGE. Digestion of agarose-embedded DNA in microbeads (21) and separation of *Not* I fragments by clamped homogeneous electric field (CHEF) electrophoresis were carried out as described (22). The CHEF Mapper system with hexagonal array electrodes (Bio-Rad) was used. Concatameres of bacteriophage λ cI857 (48.5 kb) and chromosomes of *Saccharomyces cerevisiae* YPH80 (New England Biolabs) were used as size markers.

Southern Analysis. DNA from pulsed-field gels was transferred to nitrocellulose paper after ultraviolet nicking by the standard capillary blotting protocol (23). A 0.7-kb DNA probe, depicted in Fig. 1, was labeled by the random primer protocol using a nonradioactive chemiluminescent system according to the instructions given by the manufacturer (ECL; Amersham). Hybridization and posthybridization washes also followed directions as recommended by the supplier. Filters were exposed on a Fuji RX x-ray film for 30 min. Negatives were quantitated using a Molecular Dynamics densitometer.

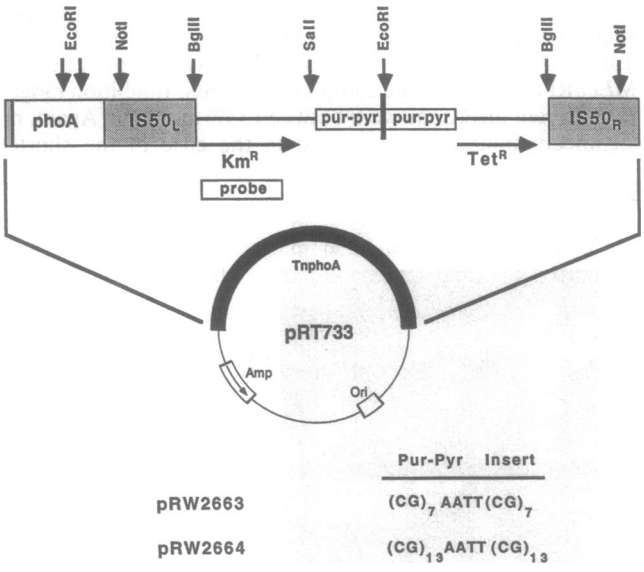

FIG. 1. Schematic representation of the map of pRT733 harboring the Tn*phoA*Pur-Pyr segment. (Pur-Pyr) stretches were cloned between Km^R and Tet^R genes of the transposon. Plasmid pRW2663 contains the 32-bp-long sequence, whereas pRW2664 contains the 56-bp run. A 0.7-kb fragment overlapping the Km^R gene was employed as a DNA probe. Important restriction sites used for cloning, chromosomal mapping, and Southern analyses are shown.

Methylation *in Vivo*. Competent cells of *E. coli* bearing different chromosomal insertions were transformed with pRW1602, which encodes a temperature-sensitive M*Eco*RI (13). Cells were grown under selective pressure with tetracycline (Tn*phoA* inserts) and chloramphenicol (pRW1602) at the nonpermissive temperature, 42°C, until they reached $OD_{600} = 0.55–0.65$. Samples were transferred to a permissive temperature, 22°C, for varying periods of time. Methylation was stopped by adding an equal volume of "ethanol/phenol mixture" (75% ethanol/21 mM sodium acetate, pH 5.3/2 mM EDTA/2% phenol) to the sample, which inactivates enzymatic activities inside the cells (24). Chromosomal DNA was isolated as described (25) and digested by *Eco*RI endonuclease. Each sample then was split into halves and a second digestion with *Bgl* II or *Sal* I was completed. When the *Eco*RI site inside the (Pur-Pyr) sequence is methylated, a 2.7-kb *Bgl* II fragment of the transposon is detected. There is only one *Sal* I site present on the transposon. Therefore, when the control *Eco*RI sites become methylated, a *Sal* I cleavage produces fragments different for each insertion, depending on the position of the nearest chromosomal *Sal* I site. Digested DNA preparations were electrophoresed on the same gel, transferred onto the same membrane, and hybridized together as described.

RESULTS

Construction of Tn*phoA*Pur-Pyr Cassettes. We chose alternating (C-G) stretches of different lengths, with a central interruption of -AATT- because they were well characterized previously in this laboratory both by *in vivo* methylation with a temperature-sensitive M*Eco*RI (13, 16) and by *in situ* chemical probing with osmium tetroxide (OsO_4) on plasmids (26). Transposon Tn*phoA* carries genes conferring resistance to kanamycin (Km^R), phleomycin (Pm^R), and streptomycin (Sm^R) (27, 28) as well as part of the *phoA* gene inserted within insertion sequence $IS50_L$ of the Tn*5* transposon (29). This transposon was chosen because it contains two *Eco*RI sites within the *phoA* gene, which served as control sites in our methylation studies. We replaced a portion of Tn*5* covering Pm^R and Sm^R genes with DNA fragments containing the Tet^R gene and (Pur-Pyr) sequences.

Thus, we obtained mobile transposon-based cassettes bearing DNA tracts that serve as reporters for left-handed Z-DNA (Fig. 1). Two suicide plasmids harboring Tn*phoA*Pur-Pyr segments were obtained: pRW2663 carrying a $(CG)_7AATT(CG)_7$ block and pRW2664 with a longer insert of $(CG)_{13}AATT(CG)_{13}$. These constructs have the following important features that make them suitable for our studies: (*i*) the two *Eco*RI sites within the *phoA* gene (30) can be used as a control for the *Eco*RI site embedded in the alternating (C-G) sequence, (*ii*) Km^R and Tet^R markers provide good selections in a broad range of bacteria, (*iii*) the suicidal nature of pRT733 ensures a high frequency of transposition, (*iv*) the insertion sites of Tn*5* exhibit little or no similarity to the sequence element into which it inserts in a random manner and therefore a large number of target sites can be accessed (31), and (*v*) two *Not* I sites are present within the IS sequences flanking the transposon (32), which can be used for restriction map analysis of the insertion sites.

Transposon Mutagenesis and Mapping. Chromosome targeting can be achieved by homologous recombination using cloned and sequenced genes as carriers for a sequence of interest. Unfortunately, along with location, other variable factors are being changed, which must be taken into account when DNA conformation is studied. These include different GC content of the flanking sequence, orientation and strength of the promoter(s), or type of the targeted proteins in regard to their membrane attachment.

Therefore, we employed a nonhomologous random insertion method via transposon Tn*phoA*. Advantage was taken of

always having the same transcriptional–translational organization of the cassette and the same control *Eco*RI sites. Both pRW2663 and pRW2664 were introduced into *E. coli* HB101 by electroporation. Km^R colonies were isolated, although the efficiency of transposition seemed to be lower than when pRT733 (Tn*phoA* without Pur-Pyr block) was used. All Km^R colonies were also ampicillin sensitive (Amp^S), which implies that Km^R mutants result from the transposition events, not recombination of the whole plasmid as reported earlier with this system (33).

The locations of the insertion sites were tested in two ways by PFGE. Fig. 2 shows an example of the analysis of eight mutants (numbered 801–808). These transpositions were obtained with pRW2664 so they carry (Pur-Pyr) inserts of total length 56 bp. Genomic DNA inside the agarose beads was digested by *Xba* I followed by fragment separation using CHEF electrophoresis. Fragments then were blotted onto the membrane and hybridized with a transposon-specific probe (Fig. 2*A*). Alternatively, DNA was cut with *Not* I and gels were stained with ethidium bromide after electrophoresis (Fig. 2*B*). Most of the insertions lie on different *Xba* I fragments except nos. 802 and 806. However, even these two give different *Not* I patterns indicating different chromosomal locations. Therefore, we conclude that our (Pur-Pyr) sequences were delivered into the chromosome in a random fashion.

The placement of these insertions on a *Not* I physical map of the *E. coli* genome (22) was conducted. We analyzed three insertions located within either the 1000-kb *Not* I fragment (58–80 min on the chromosomal map, no. 803), 275 kb (0–6 min, no. 807), or 191 kb (49–53 min, no. 805). Similar mutagenesis and PFGE mapping were performed with pRW2663 carrying 32 bp of alternating (C-G), and insertion no. 827 was chosen for analysis by methylation *in vivo*. However, some *Not* I bands of similar size cannot be separated in a gel and thus any insertions in these fragments could not be studied further.

Methylation *in Vivo*. Earlier studies using recombinant plasmids revealed strong undermethylation *in vivo* of the *Eco*RI site flanked by the 56-bp (C-G) tract (13). However, no inhibition was observed for the *Eco*RI site within a shorter (Pur-Pyr) insert of 32 bp. The B- to Z-DNA transition points for these sequences were $\sigma = -0.026$ and $\sigma = -0.032$, respectively, as measured *in vitro* by two-dimensional gel electrophoresis (16). Since $\sigma = -0.026$ is in the range of superhelical density found *in vivo* in *E. coli* HB101 (34, 35), methylation inhibition by the longer sequence was correlated with Z-DNA formation. However, we assume, but have not proven, that the two-dimensional gels and methylation analyses measure the same feature of the B-to-Z transition. Structure formation was further proven by chemical probing *in situ* for both the B–Z and Z–Z junctions on this sequence (36).

We found similar results with these sequences when they were inserted into the *E. coli* chromosome. However, as compared to the studies in plasmids, the complexity of our system was greater by 1000-fold in size, and about 800 times more *Eco*RI sites are present in the chromosome.

Fig. 3 shows the results of the methylation experiments with insertion no. 803 containing the 56-bp block of alternating (C-G) (Fig. 3 *A* and *B*) and insertion no. 827 carrying the 32-bp run (Fig. 3 *C* and *D*). Only the longer (Pur-Pyr) sequence inhibited the *in vivo* methylation by M*Eco*RI. After 15 min of methylation, the control *Eco*RI sites were completely methylated since no hybridization product could be detected (Fig. 3*B*, lane 15; solid arrow). At the same time, about half of the *Eco*RI sites embedded in the (Pur-Pyr) sequence were not methylated and therefore digested by the *Eco*RI endonuclease (Fig. 3*B*, lane 5; open arrow). The differences in the kinetics of methylation of these *Eco*RI sites are shown (Fig. 3 *A* and *C*). Methylation of the control sites was characterized by the steep slope (Fig. 3*A*). In contrast, the *Eco*RI site inside a 56-bp (C-G) block significantly slowed the methylation process. Note that Fig. 3*A* reveals only a lower estimate of the amount of Z-DNA *in vivo*. The dynamic B- to Z- and Z- to B-DNA transitions result in an accumulation of the methylated *Eco*RI sites whenever the (Pur-Pyr) sequence exists transiently in a right-handed B conformation. As for plasmids, after prolonged incubation at the permissive temperature (22°C), complete methylation of all *Eco*RI sites was found. This is due to the rapid nature of B- to Z-DNA transition (16) as well as cellular processes that may absorb unrestrained supercoils making the B form of DNA accessible for the methylase.

No inhibitory effect was observed when the shorter insertion [32-bp (C-G)] no. 827 was tested (Fig. 3 *C* and *D*). Both *Eco*RI sites, located inside the (Pur-Pyr) tract, and the control sites were fully methylated within 30 min and no significant differences in the kinetics of methylation of these sites were detected (Fig. 3*C*).

Other insertions (nos. 805 and 807) carrying the 56-bp-long sequences also caused undermethylation *in vivo* by M*Eco*RI. Table 1 summarizes the results showing an inhibitory effect on M*Eco*RI reactions at the 15-min time points. Inhibition ranges for different insertions were between 38% and 43%. Again, no significant inhibition was noted in the case of the shorter

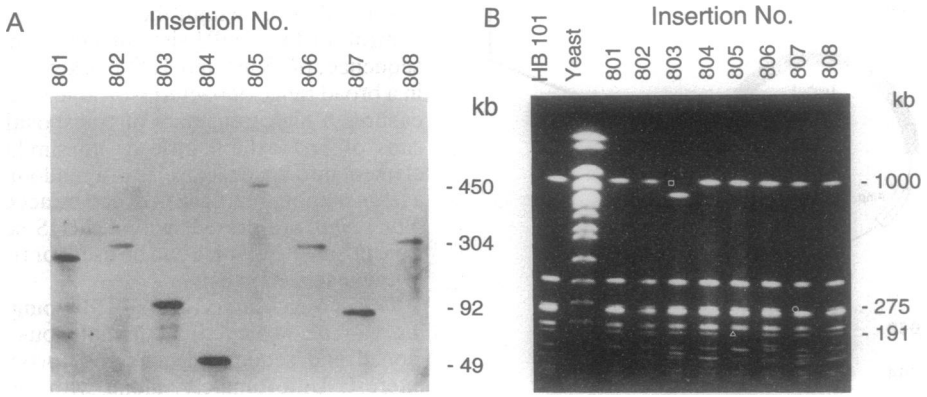

FIG. 2. Analysis of different insertions of Tn*phoA*Pur-Pyr in the *E. coli* chromosome. (*A*) Hybridization of a transposon-specific probe to *Xba* I fragments of genomic DNA prepared from cells carrying the insertions numbered 801–808. Transposons were detected as single insertions targeting different chromosomal loci. (*B*) Ethidium bromide-stained *Not* I fragments of corresponding insertions, nos. 801–808. *Not* I restriction patterns of the mutants were compared with that of the parental *E. coli* HB101 strain. Some insertions, which can be mapped within distinct single *Not* I bands, are marked by symbols: 803 (□), 805 (△), and 807 (○). Lane labeled Yeast is a size marker and contains yeast chromosomes. PFGE was run using a CHEF Mapper apparatus in 1% agarose/0.5× TBE (1 × TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) at 5 V/cm with pulses as follows: (*A*) 5–90 sec with ramping factor Rf = −1.821 for 18 hr and (*B*) 5–150 sec with Rf = −2.378 for 30 hr.

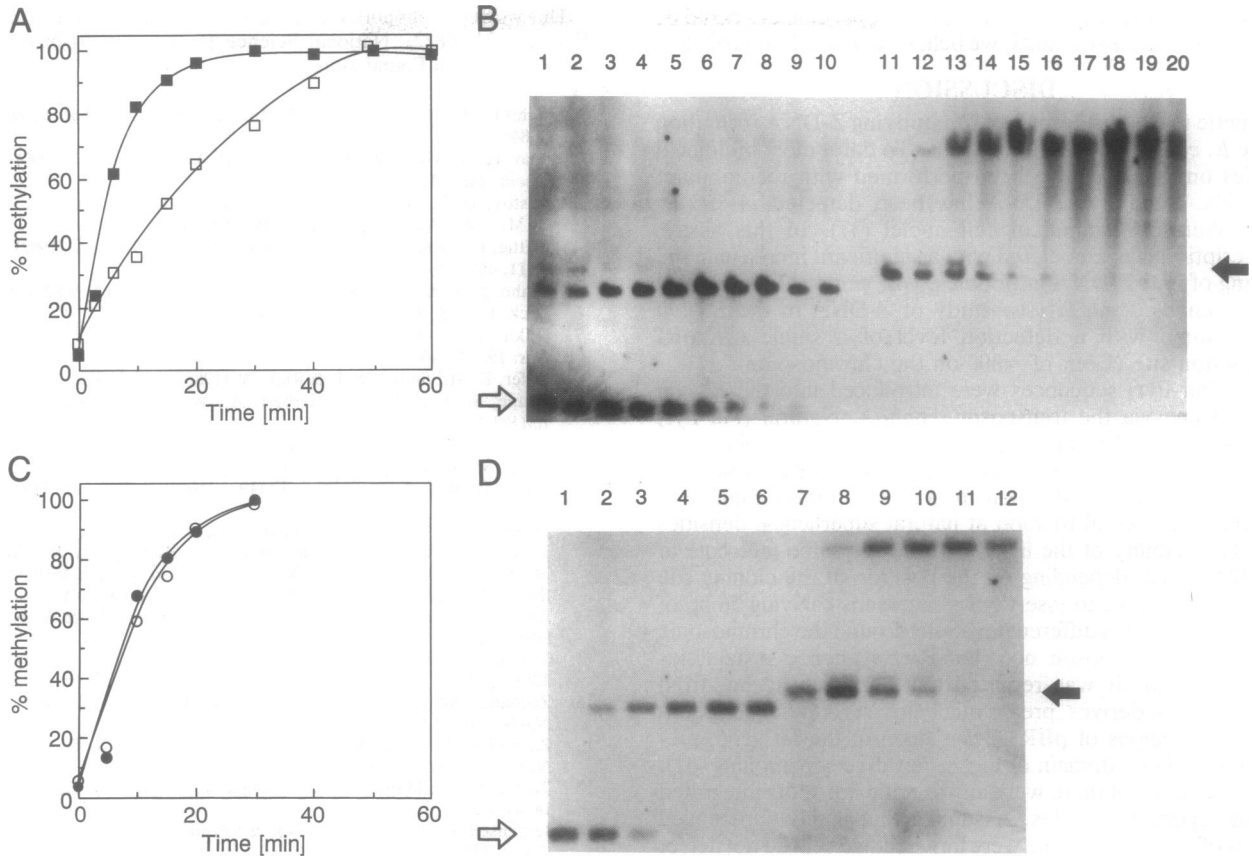

FIG. 3. Kinetics of *in vivo* methylation by M*Eco*RI. Hybridization products from the methylation experiment with insertion no. 803 are shown in *B*, and the results are plotted in *A*. Samples were double-digested with *Eco*RI/*Bgl* II (lanes 1–10) or with *Eco*RI/*Sal* I (lanes 11–20). Methylation was stopped at different periods of time as follows: 0 min (lanes 1 and 11); 3 (lanes 2 and 12); 6 (lanes 3 and 13); 10 (lanes 4 and 14); 15 (lanes 5 and 15); 20 (lanes 6 and 16); 30 (lanes 7 and 17); 40 (lanes 8 and 18); 50 (lanes 9 and 19); and 60 (lanes 10 and 20). When the *Eco*RI site located in the center of the (Pur-Pyr) tract becomes methylated, it disappears (band indicated by open arrow) and the larger hybridization product corresponding to a 2.7-kb *Bgl* II fragment of the Tn*phoA* is detected. Similarly, methylation of the control *Eco*RI sites resulted in the disappearance of the band depicted by the solid arrow and the detection of a larger-sized product (DNA fragment between *Sal* I site on Tn*phoA* and first chromosomal *Sal* I site). The results are plotted as a percentage of methylation of the *Eco*RI sites as a function of time. Methylation of the *Eco*RI sites embedded in the 56-bp (Pur-Pyr) sequence (□) was strongly inhibited in comparison to the control sites (■). (*C* and *D*) Similar data obtained for insertion no. 827 containing the 32-bp stretch. Since all *Eco*RI sites are fully methylated in 0.5 hr, subsequent experiments were carried out for only 30 min. Samples were double-digested with *Eco*RI/*Bgl* II (lanes 1–6) or *Eco*RI/*Sal* I (lanes 7–12). Methylation was stopped at the following time periods: 0 min (lanes 1 and 7); 5 (lanes 2 and 8); 10 (lanes 3 and 9); 15 (lanes 4 and 10); 20 (lanes 5 and 11); and 30 (lanes 6 and 12). No significant difference between the kinetics of methylation of the *Eco*RI sites flanked by the (Pur-Pyr) block (○) and the control sites (●) was detected.

insertion no. 827. Importantly, the kinetics of methylation of the control *Eco*RI sites were the same for all insertions. This means that different chromosomal locations did not change the specificity of the M*Eco*RI recognition. The control *Eco*RI sites are located close to the chromosomal sequences, whereas the target *Eco*RI sites are separated from the chromosomal DNA by nearly 3 kb of Tn*phoA* DNA. Thus, it is very unlikely that the observed undermethylation effects were caused by chromosomal sequences flanking the transposon. To further exclude this possibility, an *in vitro* control experiment was performed. Chromosomal DNA (sheared linear DNA fragments of about 50–100 kb) was isolated from the mutant no. 803 and *in vitro* methylation by the M*Eco*RI was conducted. No difference in the kinetics of methylation between the *Eco*RI sites embedded in the (Pur-Pyr) sequence and the control sites was found (data not shown).

Table 1. Methylation inhibition by insertions at different chromosomal locations

Insertion no.	Chromosomal location in min (*Not* I fragment)	% inhibition of methylation at 15 min
803	58–80 (1000 kb)	43
805	49–53 (191 kb)	39
807	0–6 (275 kb)	38
827	ND	5

Kinetics of methylation were measured for different chromosomal insertions (nos. 803, 805, 807, and 827). The control *Eco*RI sites were saturated for all insertions after 15 min of methylation at this time point; the *Eco*RI sites inside the long (Pur-Pyr) stretches were methylated by 55–60%. The differences in the methylation rates between the experimental and the control *Eco*RI sites are presented. Only insertions containing longer (C-G) blocks showed a significant inhibitory effect on the methylation by M*Eco*RI *in vivo*. % inhibition is expressed as the difference between methylation of the control *Eco*RI sites (at 96–100%, Fig. 3 *A* and *C*) and the *Eco*RI sites inside the (Pur-Pyr) runs at the 15-min time point. ND, not determined.

Our results in the chromosome are in complete agreement with data obtained earlier with recombinant plasmids as reported by both the *in vivo* and the *in situ* assays (13, 16). We conclude that the supercoil-dependent undermethylation effect, observed with three different insertion numbers, 803, 805, 807, located in different chromosomal regions, is due to Z-DNA formation of the 56-bp alternating (C-G) stretch. A shorter sequence of 32 bp present on insertion no. 827 does not form Z-DNA *in vivo*, at least at a level detectable in our system.

Whereas other types of DNA conformational changes could be responsible for these results, we believe that this is improbable.

DISCUSSION

A genetic system is described for studying Z-DNA formation in the *E. coli* genome in living cells. To date, most biological studies on Z-DNA have been performed with recombinant plasmids. Recently, Z-DNA has been detected *in situ* in permeabilized mammalian cell nuclei (37). In this assay, transcription was associated with a significant increase in the binding of anti-Z-DNA antibodies. Our genetic/biochemical system enabled the *in vivo* study of Z-DNA in the *E. coli* chromosome with a detection level of a single M*Eco*RI recognition site (1 out of ≈800 on the chromosome).

The (Pur-Pyr) sequences were introduced into the *E. coli* chromosome via the transposon Tn*phoA*. Natural (Pur-Pyr) stretches are underrepresented in the genome of prokaryotes (38). Long (Pur-Pyr) sequences in recombinant plasmids underwent spontaneous deletions to a size no longer capable of adopting the Z conformation at natural superhelical densities (39). The stability of the inserts within the same molecule of pBR322 varied, depending on the position of the cloning site (40). We were able to insert our structosons carrying 56 bp of alternating (C-G) in different locations around the chromosome and neither transposon nor (Pur-Pyr) sequence instabilities were observed. It was reported that Tn*5* transposon, from which Tn*phoA* derives, preferentially inserts into transcriptionally active regions of pBR322 upstream of the *tet* gene, presumably within a domain of higher negative supercoiling (41). We do not know if there were any specific hot spots integrated by our reporter cassettes. Assuming that highly supercoiled regions of the chromosome were targeted, we still did not detect a B- to Z-DNA transition of the shorter 32-bp sequence [$\sigma = -0.032$ for its transition is only slightly above the physiological level of supercoiling (34, 35)].

The *E. coli* genome consists of a single chromosome compacted into multiple, independently supercoiled domains (42). The number of these domains has been estimated to be between 40 and 50 (43). Different approaches have been undertaken to measure the level of supercoiling in the bacterial chromosome *in vivo*, including *lac* expression (44), λ Int recombination (35), and RNA polymerase *rpoB* mutants (45). Our measurements are based on the fact that inhibition of M*Eco*RI was observed only for insertions containing a 56-bp-long (Pur-Pyr) sequence (*in vitro* transition at $\sigma = -0.026$) but not a 32-bp tract ($\sigma = -0.032$). Since both Z-DNA and cruciforms have been employed for measuring the supercoiling of plasmids inside living cells (26, 46), we believe that this is a suitable alternate method of studying the supercoiling in chromosomes.

Two recent papers have addressed the question of the difference in the level of supercoiling between chromosomal domains in *E. coli* and *Salmonella typhimurium* (47, 48). Both systems were based on the activity of the supercoiling-sensitive promoters linked to reporter genes and delivered into the chromosomes using Tn*10*. No significant differences in the level of supercoiling (expression of the reporter genes) were found between different chromosomal locations. Likewise, we did not observe significant differences in the kinetics of methylation of insertions 803, 805, and 807, although many more locations should be investigated.

Z-DNA exists in the *E. coli* chromosome *in vivo* as detected by these M*Eco*RI inhibition studies. This genetic/biochemical system is significant since it is conducted inside living cells without external perturbations; however, it may not be the most sensitive determination. Since we were able to detect Z-DNA, other non-B-DNA structures (triplexes, cruciforms, nodule DNA, direct repeat sequences) may also be amenable to study using various reporter cassettes in chromosomes *in vivo*.

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