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Genetics

Z-DNA-forming sequences are spontaneous deletion hot spots

(DNA structure and mutagenesis/frameshift mutations/DNA topology)

ANNE-MARIE FREUND, MARC BICHARA, AND ROBERT P. P. FUCHS

Groupe de Cancérogénèse et de Mutagénèse Moléculaire et Structurale, Institut de Biologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique, 15, rue Descartes, 67084 Strasbourg Cedex, France

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ABSTRACT **Z-DNA-forming sequences are shown to elicit a biological response in *Escherichia coli*. Plasmids containing sequences capable of adopting the Z conformation (GC and CA/GT) are shown to be hot spots for spontaneous deletions. All the deletions involve an even number of base pairs. The distribution of the deletion events shows that the process ends when the Z-DNA-forming sequence has been reduced to a size no longer capable of adopting the Z conformation at natural superhelical density.**

The left-handed DNA structure called Z-DNA was first determined in 1979 (1). Since that time, there have been numerous *in vitro* studies on sequence requirements for Z-DNA, conditions for its stabilization, and chemical modifications that favor or disfavor its formation. There also have been theoretical studies of the B → Z transition and experimental studies of B/Z junctions by chemical and enzymatic probes. There also has been speculation about possible functions of Z-DNA *in vivo*, including roles in transcription (2) and recombination (3). The existence of the Z conformation *in vivo* has been demonstrated convincingly in plasmids in *Escherichia coli* (4, 5). Wells and his collegues (6, 7) have noted the relative instability of $(CG)_n$ inserts cloned in plasmids. Being interested in mechanisms of mutagenesis, we have investigated the stability of Z-DNA-forming sequences.

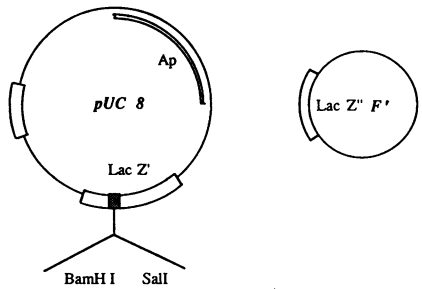
MATERIALS AND METHODS

Plasmids. Plasmid pUC-$(GC)_2$ was constructed by inserting the *Bam*HI–*Sau*3AI restriction fragment of a pBR322 mutant carrying a −2 frameshift mutation at position 434 into the *Bam*HI site of the polylinker region of plasmid pUC8. This plasmid was used to construct a series of pUC-$(GC)_n$ plasmids by extending the GC repeat by cycles of *in vitro* restriction enzyme cutting and polymerase fill-in reactions. The restriction enzymes used were *Nar* I for the construction of plasmid pUC-$(GC)_4$ and *Bss*HII for the other plasmids in the series. Plasmids carrying GT or AT inserts were obtained by cloning synthetic oligonucleotides into the *Sal* I site of the pUC8 polylinker.

Analysis of the Mutation Events. *Phenotypic analysis.* Each spectrum represents the analysis of 50 independent mutant colonies. The 50 clones were pooled, and plasmid DNA was prepared. Plasmid DNA from the 50 pooled clones was digested with *Bam*HI, 5′-end-labeled with phage T4 kinase, and digested with *Sal* I in the case of GC-containing plasmids. The pool of the plasmid containing AT or GT inserts was digested with *Hin*dIII, 5′-end-labeled, and redigested with *Eco*RI. The labeled fragments were loaded on an 8% sequencing gel, and the relative intensities of autoradiogram bands were measured by densitometric scanning. These intensities were used to calculate the frequencies of the different mutational events.

Biochemical analysis. About 10^4 transformants containing plasmid pUC-$(GC)_{13}$ were pooled, and their plasmid DNA was analyzed on sequencing gels as described for the phenotypic analysis. The autoradiogram revealed a high intensity band of starting plasmid and several lighter bands. We have only quantified the bands corresponding to the "long deletion events."

Two-Dimensional Agarose Gel Technologies. The topoisomers were run on a 2% agarose gel, 10 cm long, in TAE buffer (30). The first direction was run without intercalating agent at 3 V/cm for 17 hr. In the second direction, the gel (turned 90°)



Plasmids :	Insert	length (bp)	Phenotype	
pUC-(GC)13	...(GC)13..	111 (3n)	Lac +	blue
pUC-(GC)12	...(GC)12...	109 (3n+1)	Lac -	white
pUC-(GC)9	...(GC)9...	103 (3n+1)	Lac -	white
pUC-(GC)8	...(GC)8..	101 (3n+1)	Lac -	white
pUC-(AT)12	...(AT)12..	58 (3n+1)	Lac -	white
pUC-(GT)12	...(GT)12..	58 (3n+1)	Lac -	white
pUC-(GT)16	...(GT)16..	66 (3n)	Lac +	blue
pUC-(GT)24	...(GT)24..	82 (3n+1)	Lac -	white

Mutation Assays

Lac⁻ ->Lac⁺ : (3n+1) bp insert : the loss of (3p+1) bp or the addition of (3p+2) bp are detected
(3n+2) bp insert : the loss of (3p+2) bp or the addition of (3p+1) bp are detected

Lac⁺ ->Lac⁻ : (3n) bp insert : the loss or the addition of (3p+1) bp or (3p+2) bp are detected

FIG. 1. Strategy for the frameshift mutation assay. Various lengths of alternating purine-pyrimidine bases (such as GC, GT, or AT) have been cloned in the early part of the *lacZ* gene of plasmid pUC8. As shown, these plasmids were used in the β-galactosidase α complementation assay to monitor the frameshift mutation frequency within the inserted sequence.

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Abbreviations: SADE, small addition/deletion event; LDE, long deletion event; AAF, 2-acetylaminofluorene.

was run for 6 hr at 7 V/cm in the presence of ethidium bromide at 20–30 μg/liter.

RESULTS

Frameshift Mutation Assay. We constructed plasmids that contain alternating GC sequences of defined lengths early in the *lacZ* gene and used the β-galactosidase α complementation assay to measure the frameshift mutation frequency in the inserted sequence (Fig. 1). On plates containing 5-bromo-4-chloro-3-indolyl (X-Gal), LacZ$^+$ cells form blue colonies. Insertions within the early part of the *lacZ'* fragment do not interfere with β-galactosidase activity provided that the insert is $3n$ base pairs (bp) and does not introduce a stop codon. The frameshift mutation assays used here involve inserted sequences containing $3n$, $3n + 1$, or $3n + 2$ bp. The $3n + 1$ or $3n + 2$ insertions cause the colonies to be white (Lac$^-$), and frameshifts that restore the reading frame grow as blue colonies (Lac$^+$). In contrast to this Lac$^-$ → Lac$^+$ assay, plasmids containing an insertion of $3n$ bp can be used to detect frameshifts that disrupt the correct reading frame, (Lac$^+$ → Lac$^-$). These assays were used to measure the frequency of spontaneous mutations within $(GC)_n$ sequences. Similar constructions containing $(AT)_n$ or $(GT/CA)_n$ inserts were used to evaluate the contribution of Z-DNA-forming structures relative to cruciform-forming sequences (Fig. 1).

Frequency of Spontaneous Frameshift Mutations Increases with the Size of $(GC)_n$ Insertions. The frequency of spontaneous frameshift mutations increased with the size of the $(GC)_n$ insert (with n between 6 and 13; Table 1). These assays scored the change from the Lac$^-$ to the Lac$^+$ phenotype except for plasmid $(GC)_{13}$, which detected the change from Lac$^+$ to Lac$^-$. The high spontaneous mutation frequency

Table 1. Mutation frequencies ($\times 10^{-4}$) in plasmids containing different $(GC)_n$ inserts (n ranging from 6 to 13) in strain JM103

Plasmids	Assay	Mutation frequency $\times 10^4$
pUC-$(GC)_6$	Lac$^-$ → Lac$^+$	<0.3
pUC-$(GC)_8$	Lac$^-$ → Lac$^+$	0.7
pUC-$(GC)_9$	Lac$^-$ → Lac$^+$	2.3
pUC-$(GC)_{12}$	Lac$^-$ → Lac$^+$	48.6
pUC-$(GC)_{13}$	Lac$^+$ → Lac$^-$	300

(≈0.5%) found with plasmid $(GC)_{12}$ underestimated the actual mutation frequency because the selection procedure scored Lac$^-$ → Lac$^+$ events; mutations that did not restore the reading frame were not detected. Indeed, plasmid pUC-$(GC)_{13}$, which detected Lac$^+$ → Lac$^-$ events, showed a spontaneous mutation frequency close to 3%.

Spontaneous Mutagenesis Reduces the Lengths of $(GC)_n$ Inserts from Their Original Size to a Distribution of Sizes Centered Around $(GC)_6$. Pooled plasmid DNA from 50 independent mutants was analyzed by measuring at nucleotide resolution the length of a restriction fragment that contains the inserted $(GC)_n$ sequence. When 50 independent blue colony-forming mutants from plasmid pUC-$(GC)_{12}$ were analyzed, bands corresponding to plasmids with $(GC)_4$, $(GC)_7$, $(GC)_{10}$, and $(GC)_{13}$ inserts were found. Events that could restore the correct reading frame were insertions of $3p + 2$ bp or deletions of $3p + 1$ bp. The predominant event was the deletion of 10 bp leading to the $(GC)_7$ plasmid (67% of the mutants; Fig. 2*A*). The deletions involved an even number of base pairs: deletions of 4, 10, and 16 bp occurred at relative frequencies of 10%, 67%, and 14%, respectively. None of the

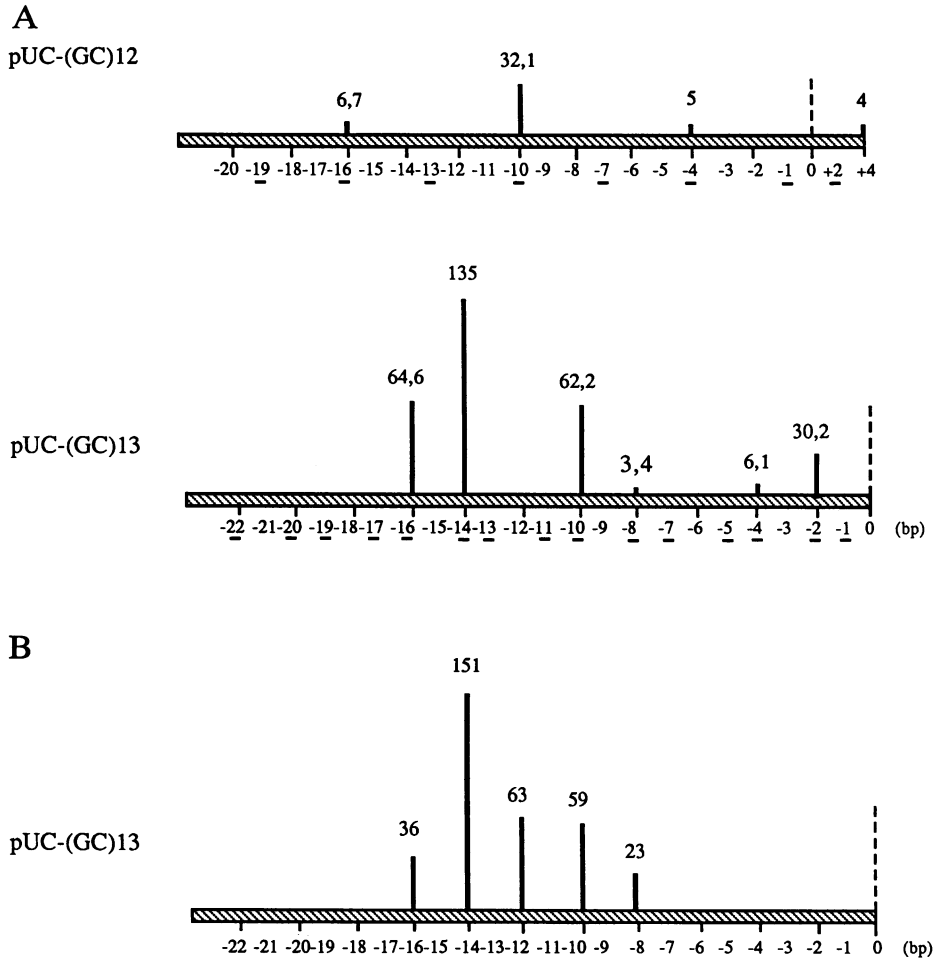

FIG. 2. Spectrum of spontaneous mutations (mutation frequency $\times 10^4$) arising in plasmids pUC-$(GC)_n$ (n = 12 and 13). Data were obtained in strain JM103. (*A*) Phenotypic analysis. The mutation spectrum is shown as vertical bars whose lengths are proportional to the frequency of occurrence of each event. The frequency of each class of mutation is indicated above the bar. The position of the bars along the axis designates the length of the insert in the mutant, + and − designating the number of base pairs added or deleted relative to the size of the starting plasmid. All of the mutation events that can be phenotypically detected are underlined. (*B*) Biochemical analysis. For plasmid pUC-$(GC)_{13}$, the high spontaneous mutation frequency permitted a direct analysis of the mutations. The representation is similar to that in *A*.

detectable deletions of odd numbers of base pairs (i.e., 1, 7, 13...bp) were found. Only one insertion event, the addition of 2 bp, was observed and represented 8% of the observed mutants.

With plasmid pUC-$(GC)_{13}$, the frequency of $Lac^+ \rightarrow Lac^-$ mutations was about 3%, and the distribution of mutants was centered around $(GC)_6$ (i.e., loss of 14 bp) (Fig. 2*A*). Only deletions of even numbers of base pairs were found (Fig. 2*A*). The high mutation frequency observed with this plasmid made it possible to analyze the mutation spectrum without identifying mutants by phenotype. For this analysis (biochemical analysis), about 10^4 pUC-$(GC)_{13}$-transformed clones were pooled. When the plasmid DNA was analyzed on a sequencing gel, one saw a distribution of bands corresponding to the mutation spectrum and a major band of unmutated pUC-$(GC)_{13}$. The distribution of mutants was centered around $(GC)_6$ (Fig. 2*B*). For plasmid pUC-$(GC)_{12}$, by assuming a distribution of mutation events that is similar to that measured for plasmid pUC-$(GC)_{13}$, one could estimate that the mutation frequency observed with the phenotypic detection represented only about 20% of the actual mutation frequency. This accounts in part for the difference in the observed phenotypic mutation frequency between plasmids pUC-$(GC)_{12}$ and $(GC)_{13}$ (Table 1).

The mutation spectrum includes two components: (*i*) "small addition/deletion events" (SADEs), which are additions or deletions of one or two repeats, and (*ii*) "long deletion events" (LDEs), which are deletions of more than two repeats. For pUC-$(GC)_{13}$, SADEs represented about 10% and LDEs about 90% of the detected mutations (Fig. 2).

SADEs most likely reflect slippage events (8) whose mechanism involves slipped-strand mispairing of the two DNA strands within repeated sequences. The most frequent event involves the addition or deletion of a single repeat (8). The mutation spectrum in a strain carrying a mutation in a mismatch repair gene (strain w7118, *mutL*) suggests that SADEs are indeed slippage events; the frequency of the +2-bp event was increased 4-fold compared to the wild-type strain. The ratio of the frequencies in the *mutL* strain to the wild-type strain for all of the other mutational events was close to 1 (exact value = 0.8), indicating that all of the mutations except the 2-bp additions are *mutL*-independent. The *mutL* dependence of the +2-bp event is in favor of the slippage model for this event. Indeed, it is known that the so-called long patch mismatch repair pathways is able to

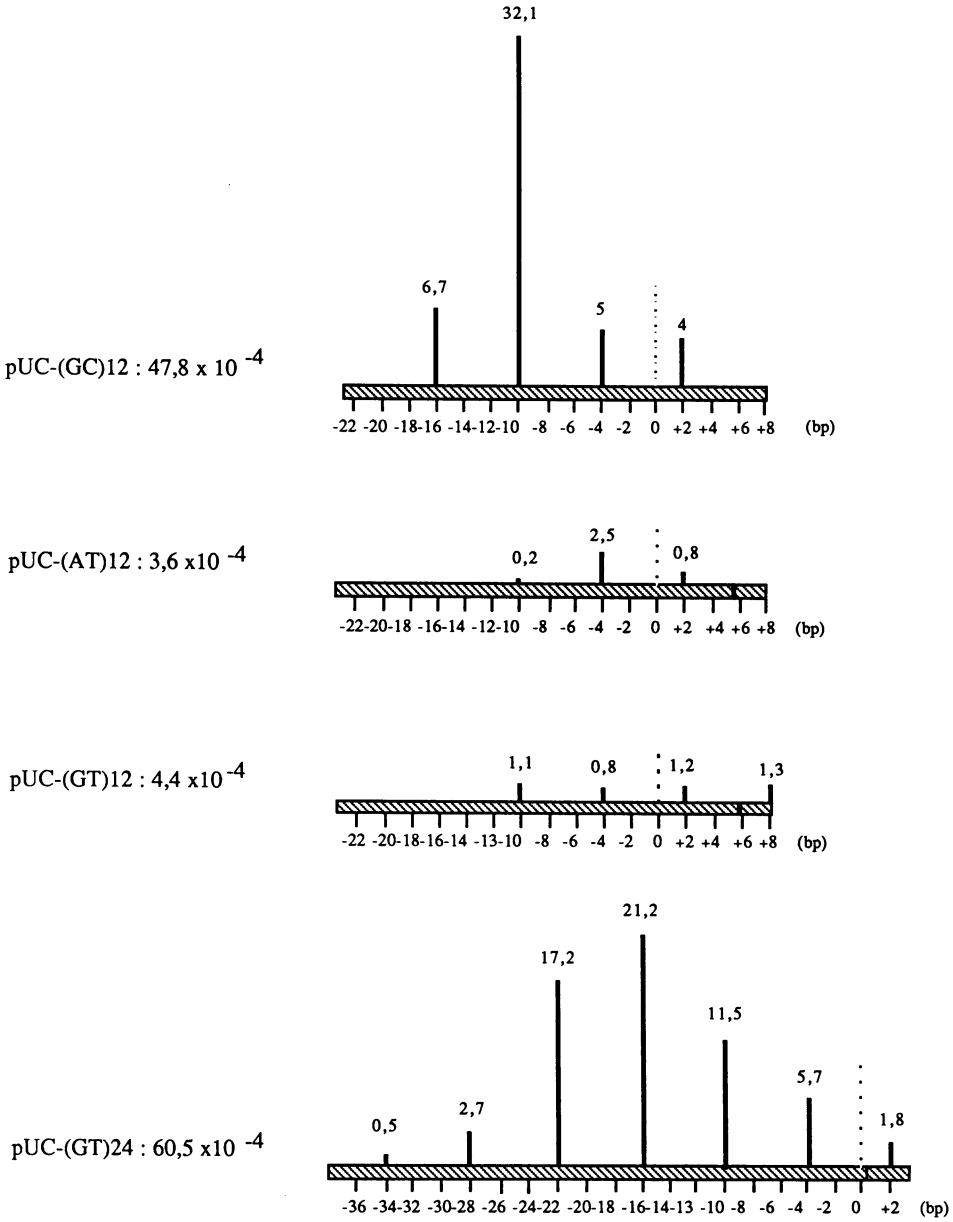

FIG. 3. Phenotypic analysis of the mutations. Spectrum of spontaneous mutations arising in plasmids containing GC, AT, and GT inserts shows the total mutation frequency × 10^4. The phenotypic analysis was performed essentially as described.

correct small loops (9). The independence of all of the other mutational events (i.e., the long deletions) suggests that either these events are slippage events that cannot be corrected by mismatch repair or that they arise by a totally different mechanism.

Comparison Between $(GC)_n$-, $(GT/CA)_n$-, and $(AT)_n$-Containing Plasmids. The following experiments were performed to discriminate between potential roles of Z-DNA and of cruciform structures in the induction of LDEs. We constructed plasmids containing either $(GT/CA)_{12}$ or $(AT)_{12}$. These plasmids formed mutant colonies (i.e., blue colonies) at a frequency (≈ 3–5×10^4) similar to each other but at least 1/10th that in plasmid pUC-$(GC)_{12}$ ($\approx 50 \times 10^4$). The spectrum of spontaneous mutations was determined by analyzing 50 independent mutant clones (Fig. 3). Only SADEs were found for the $(AT)_{12}$ plasmid. In addition to these events, +8- and −10-bp events were also found for pUC-$(GT/AC)_{12}$. The $(GC)_{12}$, $(GT/CA)_{12}$, and $(AT)_{12}$ plasmids showed a similar frequency of SADEs; the 10-fold greater mutability of the $(GC)_{12}$ plasmid is due to the high frequency of the LDEs that are unique to this plasmid. The difference between the $(AT)_{12}$ and $(GC)_{12}$ plasmids makes the involvement of cruciform structures in the generation of LDEs seem unlikely, because the $(AT)_{12}$ plasmid should be as susceptible to cruciform formation as the $(GC)_{12}$ plasmid, but only the $(GC)_{12}$ plasmid produced LDEs.

Both $(GC)_n$ and $(GT/CA)_n$ sequences can undergo the B→Z transition (10). To test the hypothesis that the Z conformation is involved in the LDEs in the GC-containing plasmids, we constructed $(GT/CA)_n$ plasmids with longer inserts (n = 16 and 24). For n = 16, the mutation frequency was low (2×10^{-4}), but for n = 24, the frequency ($\approx 60 \times 10^{-4}$) was similar to that of pUC-$(GC)_{12}$. Molecular analysis of the mutants revealed a low frequency of SADEs and a high frequency of LDEs centered around −16 bp (Fig. 3). Levinson and Gutman (11) showed that (GT/CA) repeats exhibit a high frequency of short frameshift mutations. In their study, $(GT/CA)_n$ repeats (with n = 11, 19, 20, 21, and 22) were cloned in the polylinker region of phage M13mp18. About 90% of the mutations resulted from the deletion or addition of a single GT/CA repeat (deletion/addition ratio = 3). The frequency of frameshift events was about 1% in a wild-type strain and 10-fold higher in mismatch-repair-deficient strains. Our finding that plasmids containing $(GT/CA)_n$ insertions (n = 12, 16, and 24) produce SADEs is qualitatively similar to the results of Levinson and Gutman (11), but the frequency of these events was much lower in our assay. The basis for this quantitative difference is unclear but may depend on the assay (phage versus plasmid). They (11) did not observe the LDEs that we see with plasmid $(GT/CA)_{24}$; since LDEs depend on the length of the insert (Table 1), it is possible that their longest insert (n = 22) was too short to exhibit this component of the mutation spectrum or that the requisite length depends on the sequence context or topology.

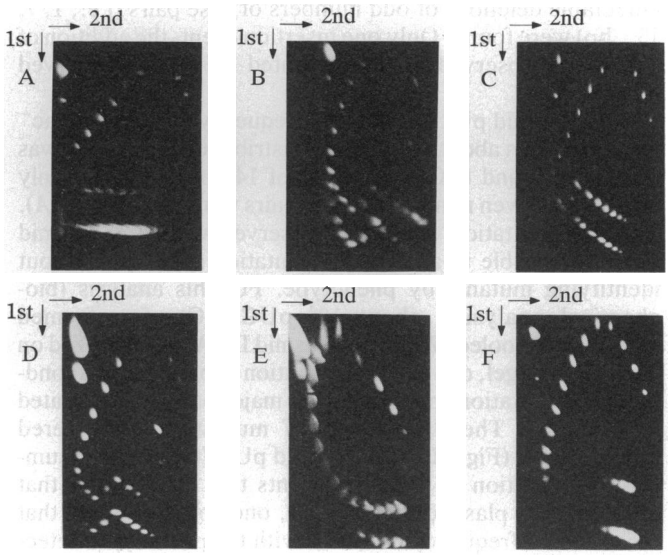

FIG. 4. Two-dimensional agarose gel electrophoresis analysis of topoisomers of plasmid pUC-$(GC)_6$ (*A*), pUC-$(GC)_{12}$ (*B*), pUC-$(GC)_{13}$ (*C*), pUC-$(AT)_{12}$ (*D*), pUC-$(GT)_{12}$ (*E*), and pUC-$(GT)_{24}$ (*F*). The directions of migration are indicated by the arrows. In each panel, the upper portion shows the different topological isomers that were generated *in vitro* by using topoisomerase I in the presence of various concentrations of ethidium bromide, and the lower part represents the topoisomers as they were isolated from the JM103 strain that was used in the mutation experiments.

Spontaneous Mutation Frequencies Parallel the Potential of the Different Inserts To Adopt a Z-DNA Conformation. We used two-dimensional agarose gel electrophoresis (12, 13) to analyze the presence of Z-DNA in the plasmids (Fig. 4). In such gels, the topoisomers of a plasmid migrate along a smooth curve if the plasmid contains neither a region of Z-DNA nor a cruciform structure. In contrast, there is a break in the migration pattern of the topoisomers that contain a portion of their DNA in the Z conformation or in a cruciform structure. The magnitude of this break permits calculation of the length of the DNA portion that has adopted the unusual conformation (12, 13). For each plasmid, we analyzed on the same gel the distribution of topoisomers isolated from a wild-type strain and the collection of topoisomers generated *in vitro* (14). The topoisomers of plasmid pUC-$(GC)_6$ migrated along a smooth curve, whereas a break was seen in the migration pattern of the topoisomers of plasmid pUC-$(GC)_{12}$

Table 2. Predicted and observed extents of supercoil relaxation as determined by analysis of two-dimensional agarose gel electrophoresis

Plasmid	Length of the insert, n	Theoretical relaxation		Observed relaxation	Structure of the insert	$-\sigma$ of the transition	$-\sigma$ *in vivo*
		Cruciform*	Z				
puC-$(GC)_6$	12	1.3	2.3	—	—	—	0.047–0.070
puC-$(GC)_{12}$	24	2.5	4.3	4.2	Z	0.038	0.027–0.070
puC-$(GC)_{13}$	26	2.7	4.6	4.5	Z	0.038	0.031–0.065
puC-$(AT)_{12}$	24	3.2	—	4.2	Cruciform	0.044	0.039–0.075
puC-$(GT)_{12}$	24	—	4.5	—	—	—	0.039–0.059
puC-$(GT)_{16}$	32	—	5.7	4.4	Partial Z	0.047	0.039–0.063
puC-$(GT)_{24}$	48	—	8.6	6	Partial Z	0.052	0.056–0.076

The theoretical supercoil relaxation was calculated by assuming 12 bp per turn for Z-DNA and 10.5 bp per turn for B-DNA. The σ *in vivo* corresponds to the range of supercoil density that is calculated for topoisomers as they are isolated from the bacteria.

*Due to the constructions of the plasmids, the length of the DNA stretches susceptible of being in cruciform structure is 14 bp for pUC-$(GC)_6$, 26 bp for pUC-$(GC)_{12}$, 28 bp for pUC-$(GC)_{13}$, and 34 bp for pUC-$(AT)_{12}$.

(Fig. 4). The magnitude of the break is in agreement with the whole $(GC)_{12}$ insert being flipped into Z conformation (Table 2). Similarly, no transition was seen in the case of plasmid pUC-$(GT/CA)_{12}$, whereas some of the topoisomers of plasmid pUC-$(GT/CA)_{24}$ had undergone a conformational transition (Fig. 4). The magnitude of the transition suggests that only part of the (GT/CA) region was in Z conformation (Table 2) (10, 15). The topoisomers of plasmid pUC-$(AT)_{12}$ presented a discontinuity in their migration pattern that could be explained by the extrusion of a cruciform (16–19).

The high spontaneous frequency of LDEs in plasmids $(GC)_{12}$, $(GC)_{13}$, and $(GT/CA)_{24}$ parallels the potential of these inserted sequences to undergo a B → Z transition. All LDEs involve an even number of base pairs, a fact reminiscent of the Z-DNA conformation being formed of dinucleotide units. We hypothesize that the mechanism responsible for LDEs recognizes the B/Z junction or the Z-DNA region and reduces the length of the inserted sequence by the stepwise elimination of units of 2 bp; the process ends when the insert is too short to adopt the Z conformation.

Recent studies on the Z-DNA *in vivo* have shown that the average superhelical density *in vivo* is approximately half of the value measured *in vitro* on deproteinized DNA (4, 5, 20, 21). According to Wells and colleagues (4, 5), a GC region must be at least 40–45 bp long to have half the topoisomers be in Z conformation *in vivo*. The GC inserts studied here are not longer than 26 bp [pUC-$(GC)_{13}$]. If the molecular determinant that triggers the LDEs were indeed Z-DNA, one would have to suppose that a small proportion of the plasmid molecules, perhaps undetectable in the assay of Wells and colleagues, contains a region of Z-DNA, at least transiently. $(GT/CA)_n$-containing plasmids exhibited a similar instability to that of shorter $(GC)_n$ regions, reflecting the difference in the energetics of the B → Z transition between GC and GT/CA.

DISCUSSION

We initiated the present work after observing that the chemical carcinogen 2-acetylaminofluorene (AAF) induces at high frequency −2 frameshift mutations within sequences containing short stretches of alternating GCs (22–24). Neither a bias in the binding of the carcinogen (25) nor in the recovery of the mutants can explain these mutation hot spots (23). We propose that the −2 mutations result from the processing of an "unusual" DNA structure that we hypothesize to be a local Z-DNA-like conformation that is induced by the binding of AAF within such sequences. Indeed, it has been shown that AAF favors the B → Z transition in alternating GC or GT/CA polynucleotides *in vitro* (26–29).

A common structural determinant between AAF-induced mutations at alternating GCs and the spontaneous mutations observed within stretches of alternating GC or GT/CA sequences might be Z-DNA. We show that sequences that can adopt a Z conformation are subject to high rates of spontaneous deletions that reduce the length of the Z-DNA-forming region until it is too short to be in the Z conformation at natural superhelical density.

The deletion process does not appear to be SOS dependent, and it is not affected by a *recA* mutation (*recA1* allele). The topology of the DNA undoubtedly plays a role in the deletion process. The topology of a given DNA molecule might be highly regulated *in vivo*, so that the superhelical density of the DNA may vary with such aspects of the physiological state as times and rates of transcription, replication, and repair. Moreover, the superhelical density may not be uniformly distributed within a DNA molecule so that certain domains, defined by specific protein-DNA interactions, might have higher superhelical densities than others. The instabilities found in the present work may depend on all these parameters, and it will be of interest to see whether they depend upon the specific locations of the Z-DNA-forming sequences.

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