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A domain that assumes a Z-conformation includes a specific deletion in some cloned variants of a complex satellite

(Recombinant DNA; DNA conformations; topoisomers; antibody binding; land crab; *Gecarcinus lateralis*)

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SUMMARY

Sequence analyses show that deletions of 10 and 12 bp occur at homologous sites in a domain that is rich in alternating purines and pyrimidines (Pu/Py) in B42 and EXT, two cloned variants of a complex satellite DNA. A 3-bp deletion occurs 27 bp upstream from the site of the specific deletions in B42 and RU, a third cloned satellite variant that has not suffered the 10-bp deletion. Under torsional stress, the Pu/Py-rich domain adopts a Z-conformation as shown by (*i*) inhibition of cutting at a *Bss*HII site that accounts for 2/5 of a 15-bp tract of pure Pu/Py in the domain; (*ii*) binding of polyclonal and monoclonal anti-Z-DNA antibodies to the domain; and (*iii*) antibody stabilization and subsequent relaxation of the Z-region.

INTRODUCTION

We have explored the potential for the adoption of a Z-conformation by domain V, a segment of a complex satellite DNA of the Bermuda land crab *Gecarcinus lateralis* that is rich in alternating Pu/Py (Skinner, 1967; LaMarca et al., 1981; Skinner et al., 1982; 1983). The reasons for our interest follow: (*1*) Domain V is associated with major changes in primary sequence of several otherwise very closely related cloned variants of the satellite; segments of Z-DNA appear to be involved in recombination in other systems (Klysik et al., 1982; Jovin et al., 1983; Kmiec et al., 1985; Kmiec and Holloman, 1986). (*2*) The domain contains a 9- and either a 14- or

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Abbreviations: aZ-Ab, anti-Z-DNA immunoglobulins; bp, base pair(s); CQ, chloroquine; EtdBr, ethidium bromide; EXT, see INTRODUCTION; kb, kilobase(s) or 1000 bp; N, non-alternating purines and pyrimidines; NAT, native plasmids isolated from cells ($-\sigma$ = approx. 0.055 ± 0.01); nt, nucleotide(s); Pu, purine; Py, pyrimidine; Pu/Py, blocks of alternating purines and pyrimidines; pBRZ1-4, segments of Pu/Py in pBR322 that adopt a Z-DNA conformation; REL, relaxed plasmids; RU, see INTRODUCTION; $-\sigma$, negative superhelical density; VHS, very highly supercoiled plasmids; Z-DNA, left-handed alternative to B-DNA.

15-bp segment of uninterrupted Pu/Py (Fowler et al., 1985; see Fig. 1); with the exception of d(T-A) (Ellison et al., 1986), Pu/Py of 9 or more bp adopt a Z- as opposed to the usual B-DNA conformation under certain conditions including high levels of torsional stress (reviewed in Rich et al., 1984). (*3*) Domains I–IV in the crab satellite comprise unrelated repetitive sequences (Fowler et al., 1985); at high levels of torsional stress domains I–IV also adopt altered conformations, albeit different from Z (Fowler et al., 1985; Fowler and Skinner, 1986); they are also the sites of major changes in primary sequence in different cloned variants of the crab satellite. Thus, it appears that domains of the satellite that are susceptible to alterations in higher-order structure are also susceptible to sequence modifications.

We describe here experiments on domain V from RU, a cloned satellite variant of 2089 bp, representative of the average-sized repeat unit (Fowler et al., 1985). The domain is a 53-bp Pu/Py-rich segment. To study it free from the influence of other domains of unusual sequences nearby in the satellite repeat unit, we used a subclone that contained domain V from RU (Stringfellow et al., 1985). Two other full-length cloned variants, EXT, which is extended by an amplified segment outside domain V (Bonnewell et al., 1983; Fowler et al., 1985), and B42, a satellite variant inserted into a different restriction site of pBR322 (Stringfellow et al., 1985), have deletions of 12 and 10 bp, respectively (see Fig. 1) that include most of a 9-bp Pu/Py segment near the 5′-end of domain V. RU, B42 and one other variant also contain a 3-bp deletion 27 bp upstream from the site of the 10- and 12-bp deletions in B42 and EXT; EXT and six other variants do not (not shown).

Segments of DNA rich in Pu/Py can be induced to flip to a Z-conformation by a number of treatments (Peck et al., 1982; Kilpatrick et al., 1984; Rich et al., 1984; Singleton et al., 1984; Wang et al., 1984; 1985; Hayes and Dixon, 1985). Segments of interrupted Pu/Py also adopt a Z-conformation (Nordheim et al., 1982; Nordheim and Rich, 1983; Singleton et al., 1983; Barton and Raphael, 1985; Feigon et al., 1985; Konopka et al., 1985). In our experiments, we have induced Z-DNA formation by torsional stress.

A single site for the restriction enzyme *Bss*HII (5′–GCGCGC–3′) occurs in the longest stretch of Pu/Py that constitute domain V (Fig. 1) in all 75 variants of the satellite tested. Since *Bss*HII cleaves DNA in the B- but not Z-conformation (Azorin et al., 1984), the site serves as a specific probe for the adoption of the Z-conformation in the satellite insert. We have used both polyclonal and monoclonal anti-Z-DNA immunoglobulins (aZ-Ab) to confirm that this domain is in a Z-conformation under high levels of torsional stress. Monoclonal antibody binding also revealed that the length of the altered conformation encompasses the entire 53-bp Pu/Py segment despite several interruptions in alternation. Thus the domain in RU may be represented by the formula $Z_6N_3Z_9Z_4Z_5NZ_{15}NZ_5Z_4$ where Z_n indicates the length of a Pu/Py segment and N_n indicates the length of interruptions in Pu/Py.

MATERIALS AND METHODS

(a) Plasmids

B42, a full-length satellite repeat unit inserted into the *Pst*I site of pBR322, and three subclones containing selected fragments of RU inserted at the *Cla*I site of pBR322 were studied (Figs. 1 and 2 and see Stringfellow et al., 1985). The subclones were (*1*) pZE15-4.53kb containing domain V of RU plus 105 bp upstream and 13 bp downstream; (*2*) pZE15-2.66kb, constructed by deleting 1881 bp from the *Eco*RV to the *Pvu*II site from pZE15-4.53kb (Stringfellow et al., 1985) to remove one of the Z-DNA segments identified in pBR322 (Nordheim et al., 1982) and (*3*) pDEL, a spontaneous deletion of approximately the same size (viz. 2.6 kb) which (*a*) has no crab satellite DNA, (*b*) lacks one of the Z-DNA segments in pBR322, but (*c*) includes three other potential Z-DNA segments detected by Barton and Raphael (1985); the latter are described in RESULTS AND DISCUSSION, section **c**.

Purified plasmids were enriched in supercoiled forms by phenol extraction at pH 4.0 (Zasloff et al., 1978). Such NAT plasmids had negative superhelical densities ($-\sigma$) of 0.055 ± 0.01. Populations of topoisomers were prepared by relaxation with topoisomerase I (wheat germ, Promega Biotec, or calf thymus, Bethesda Research Laboratories) in the absence of EtdBr (REL/covalently-closed;

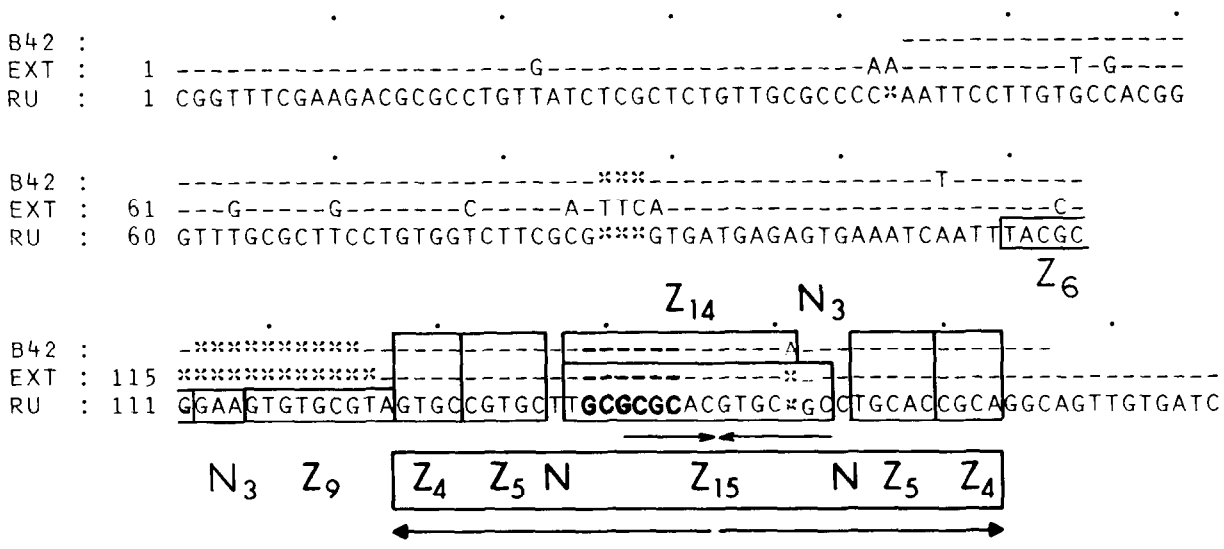


Fig. 1. Sequence of subcloned 171-bp *Msp*I fragment of RU containing the 53-bp Pu/Py-rich domain V (bottom line) and homologous regions of two other satellite variants. Numbers on left margins: nt positions within RU and EXT (for complete sequences of RU and EXT, see Fowler et al., 1985). Clone B42 is not numbered because the sequence of the entire repeat unit has not been determined. Blocks of Pu/Py segments in domain V are boxed. Between some blocks the Pu/Py alternation is out of phase (i.e., Z_9 abuts Z_4 which abuts Z_5). Dashes, homologous residues; bold letters and dashes, *Bss*HII site; multiplication symbols, deletions; Z, blocks of Pu/Py; N, non-Pu/Py; head-to-head arrows, 12-bp inverted repeat in RU and EXT, 10-bp inverted repeat in B42. Tail-to-tail arrows, global dyad axis of symmetry.

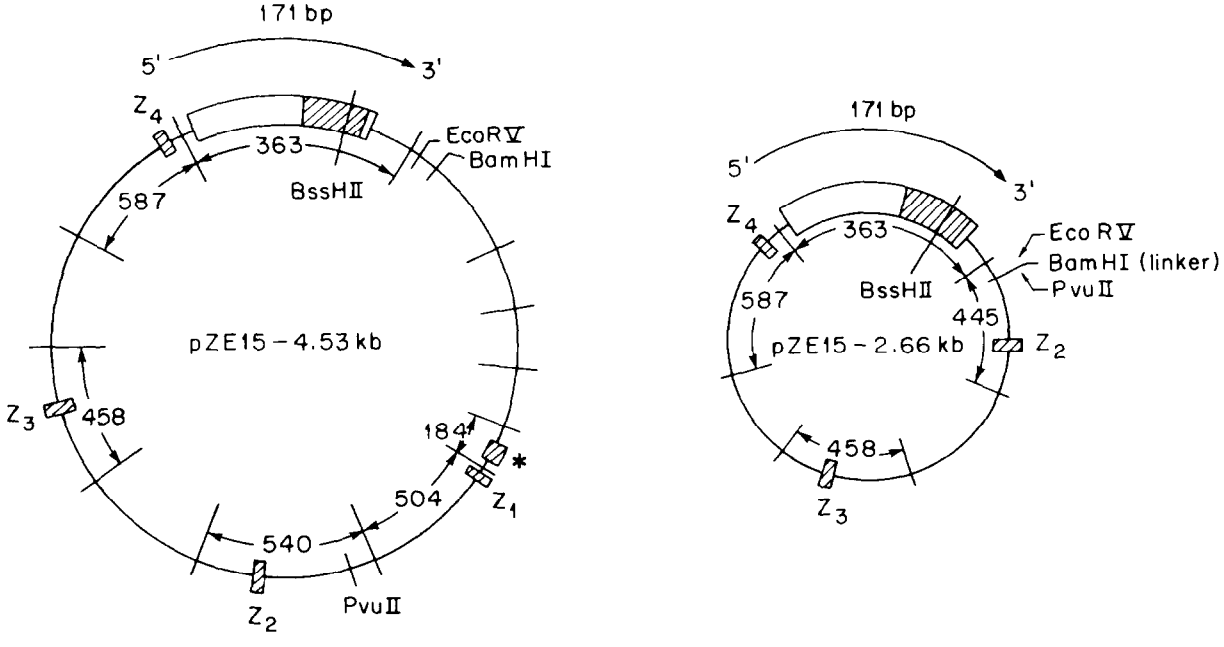


Fig. 2. Diagrams of hybrid plasmids pZE15-4.53kb and pZE15-2.66kb. The 171-bp *Msp*I fragment (open segment; not drawn to scale) shown in Fig. 1 was inserted into the *Cla*I site of pBR322. Large hatched segment, Pu/Py-rich segment of satellite; small hatched segments, Z-DNA segments in pBR322 at nt positions 1447–1460 (Z_1, Nordheim et al., 1982), 2315–2328, 3265–3277, and 4254–4264 (Z_2, Z_4, respectively, Barton and Raphael, 1985), and 1349–1360, 1388–1393, and 1410–1423; asterisk, Z-DNA segment(s) first detected here; pBRZ1 has been removed from pZE15-2.66kb. Bent arrows, *Eco*RV, *Pvu*II, restriction sites deleted during construction of pZE15-2.66kb. Named restriction sites were used for constructing derivatives or for assays. *Hae*III sites, used for determining sites of antibody binding, are indicated by lines; those that produce fragments smaller than 100 bp are not shown; those fragments whose sizes are shown contain Z-DNA.

$-\sigma = 0 \pm 0.01$) or in the presence of 17.5 μg/ml EtdBr (VHS; $-\sigma = 0.11 \pm 0.01$) at 37°C in buffers specified by the manufacturers. Topoisomerase was inactivated by heating to 65°C (wheat germ) or adding SDS to 1% (calf thymus) and topoisomers were purified by ethanol precipitation. The $-\sigma$ was determined by the band-counting method (Keller, 1975). The sequence of the domain V insert of pZE15-4.53 kb was determined (Maxam and Gilbert, 1980) as was that of a segment of B42 by a strategy used for homologous fragments of RU and EXT (Fowler et al., 1985).

(b) Binding of monoclonal or polyclonal aZ-Ab to specific segments of DNA

NAT and VHS plasmids (1–3 μg DNA) were treated with a 10:1 mass ratio of goat polyclonal aZ-Ab G10cDE2 (gift of B.D. Stollar, Department of Biochemistry and Pharmacology, Tufts University School of Medicine; Lafer et al., 1981, 1985a) as described (Nordheim et al., 1982), or with a 4.7:1 mass ratio of monoclonal aZ-Ab Z22B (gift of B.D. Stollar) for 30 min at 37°C in 50 mM NaCl, 40 mM triethanolamine (pH 7.5) and crosslinked with 0.1% glutaraldehyde during 2-h incubation at room temperature (polyclonal aZ-Ab; Nordheim et al., 1982) or 30 min at 37°C (monoclonal aZ-Ab; Nordheim et al., 1986). Excess glutaraldehyde was quenched with 20 mM glycine for 1–2 h at room temperature after which samples were diluted five-fold into restriction enzyme reaction mixtures. Because of the unfavorable reaction conditions for subsequent restriction digests, high levels of enzymes (10 units/μg DNA) and long digestion times were used. Twice as much VHS DNA was used in some experiments to compensate for heavy losses of the VHS DNA-aZ-Ab complex during purification. After purification, samples were electrophoresed on 7% polyacrylamide gels. Gels were stained with EtdBr and photographed. Negatives were scanned with an LKB UltroScan soft laser densitometer.

(c) Stabilization of Z-DNA by high concentrations of monoclonal aZ-Ab

Samples containing 1 to 3 μg VHS plasmids in topoisomerase I buffer were treated with 10 to 100 μg/ml monoclonal aZ-Ab Z22B for 30 min at 37°C. Plasmids containing Z-DNA stabilized by aZ-Ab binding were relaxed by treatment with calf thymus topoisomerase I (20 units/μg DNA) at 23°C for 24 h after which another aliquot of topoisomerase was added and the incubation continued for 24 h (Lafer et al., 1985a). The topoisomerase and aZ-Ab were digested with 300 μg/ml proteinase K in the presence of 0.5% Sarkosyl at 37°C for 1 h following which samples were electrophoresed directly on agarose gels containing 10 μM CQ. Gels were stained, photographed, and negatives scanned as described in MATERIALS AND METHODS, section **b**.

RESULTS AND DISCUSSION

(a) B42 contains a 10-bp deletion at the same site as EXT

The sequence of domain V in B42 has a 3-bp deletion at the same site as in RU. This deletion occurs in three of ten cloned satellite variants (not shown) but not in EXT. The sequence of B42 differs from that of RU by only 2 bp, an A → T transversion and an insertion, and by a 10-bp deletion. This larger deletion is shared by EXT which has lost an additional bp on either end. Evidence that the differences in the cloned satellite inserts were not due to recombination in the bacterial host was the identity of three different restriction enzyme digests of recloned RU to those of the original RU (Stringfellow et al., 1985). Even more directly, no changes in the sequence of domain V from pZE15-4.53kb were detected on resequencing.

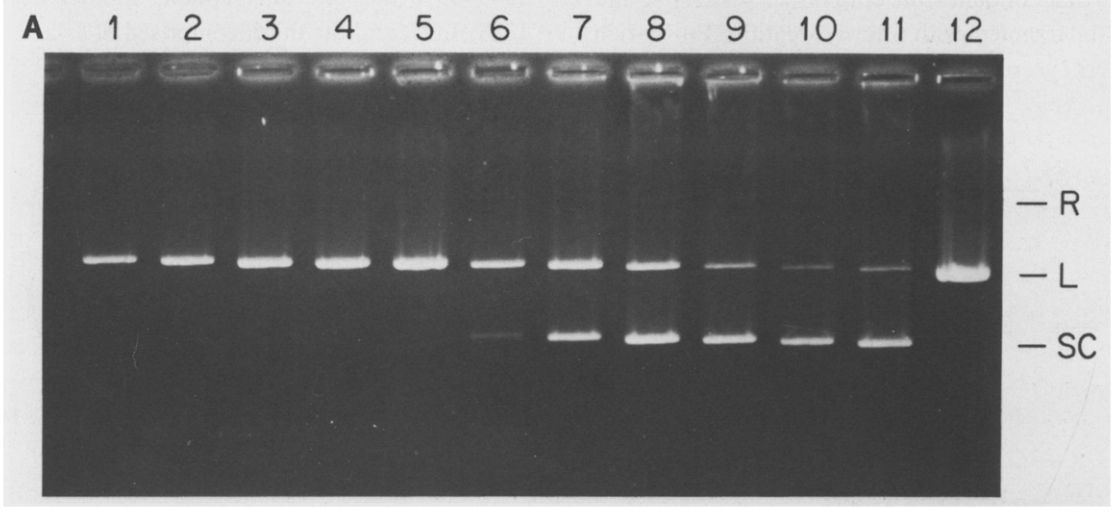
(b) Demonstration of an altered DNA conformation in domain V by inhibition of restriction enzyme cleavage

Populations of topoisomers of pZE15-4.53kb with $-\sigma$ ranging from 0 to 0.14 were digested with *Bss*HII, ethanol-precipitated, and electrophoresed on agarose gels. Supercoiled plasmids under torsional strain insufficient to drive a structural transition were linearized (Fig. 3A, lanes 1–6 and 12), whereas those with higher $-\sigma$ were not (lanes 7–11), indicating inhibition of *Bss*HII cleavage. Linearized DNA in samples in which cleavage of the supercoiled species

was inhibited is due to the presence of nicked molecules, approx. 20% of the total. Quantification of the amount of linear DNA in each sample by densitometry showed that cutting occurred at $-\sigma$ lower than 0.08, but decreased significantly as $-\sigma$ increased. The transition had a midpoint of $-\sigma = 0.095$ (Fig. 3B). Inhibition of *Bss*HII cleavage is most likely due to structural alterations induced by negative supercoiling rather than by EtdBr remaining in the samples. This was demonstrated by control experiments (not shown) in which aliquots of the same topoisomer samples of pZE15-4.53kb were linearized by *Eco*RI. Both *Bss*HII and *Eco*RI linearized pZE15-4.53 kb after exposure to the same range of EtdBr concentrations but without topoisomerase I.

(c) Localization of Z-DNA by antibody binding to two subclones containing domain V

The first Z-DNA segment identified in pBR322 at nt positions 1447–1460 (Nordheim et al., 1982) and three additional Z-DNA segments at nt positions 2315–2328, 3265–3277, and 4254–4264, all cleaved



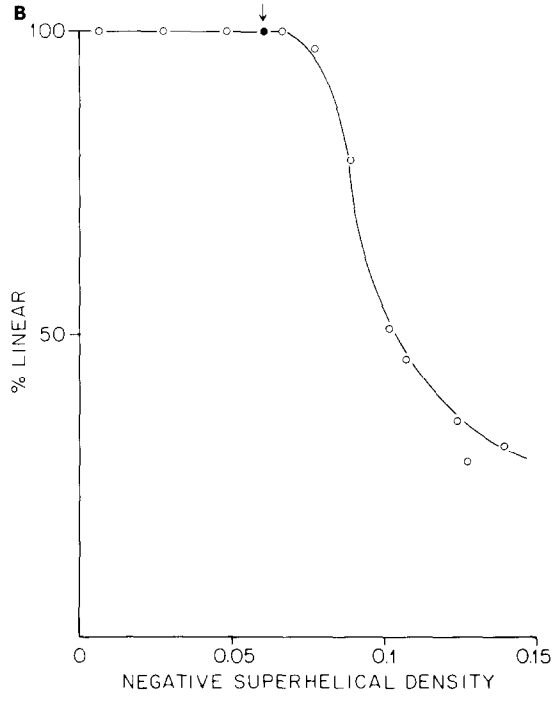


Fig. 3. pZE15-4.53kb under different $-\sigma$ treated with *Bss*HII. (Panel **A**) Agarose gel (1.5%) showing *Bss*HII digests of topoisomers of pZE15-4.53kb with $-\sigma$ of 0.007 (lane 1), 0.027 (lane 2), 0.047 (lane 3), 0.063 (lane 4), 0.081 (lane 5), 0.089 (lane 6), 0.100 (lane 7), 0.107 (lane 8), 0.124 (lane 9), 0.127 (lane 10), 0.139 (lane 11), and 0.055 (native, lane 12). SC, supercoiled; R, relaxed, L, linear forms of DNA. (Panel **B**) Plot of *Bss*HII cleavage as a function of $-\sigma$. Fraction of linear DNA in each lane of A was determined by densitometry and plotted against $-\sigma$. Native pZE15-4.53kb is indicated by a solid circle.

by cobalt(III)[Tris-(diphenylphenanthroline)]$^{3+}$ (Barton and Raphael, 1985) are designated here as pBRZ1-Z4 in a clockwise direction from the *Eco*RI site (Fig. 2). At the time we constructed pG1E15-2.66kb, the only Z-DNA segment of pBR322 that had been described was pBRZ1 (Nordheim et al., 1982). Accordingly, we deleted 1.88 kb from pZE15-4.3kb which included only that segment and did not attempt to delete other Pu/Py-rich regions.

VHS pZE15-4.53kb, a subclone containing domain V (Fig. 2), was treated with monoclonal aZ-Ab at pH 7.5 and digested with *Hae*III. *Hae*III was selected because it produced restriction fragments that contained the crab insert, pBRZ1-4, and a 184-bp fragment with a newly identified Pu/Py-rich segment in pBR322 that bound aZ-Ab (Fig. 2, asterisk in left map), each in an individual fragment larger than 180 bp. Considerable aZ-Ab binding had occurred to the crab insert as shown by marked reduction in the amount of a 363-bp fragment that contained it (Fig. 4,A and B). Two fragments of 504 and 540 bp containing pBRZ1 and pBRZ2, respectively, were noticeably reduced; a 587-bp fragment containing pBRZ4 was slightly reduced, and a 458-bp fragment containing pBRZ3 was not affected. The 184-bp fragment that contains heretofore undetected segments of Z-DNA in pBR322 had disappeared. That fragment has three segments of Pu/Py-rich DNAs: one extending from nt 1349–1360 has one interruption, another from nt 1388–1393, and the third, comprised of 8- and 6-bp contiguous blocks, extends from nt 1410–1423.

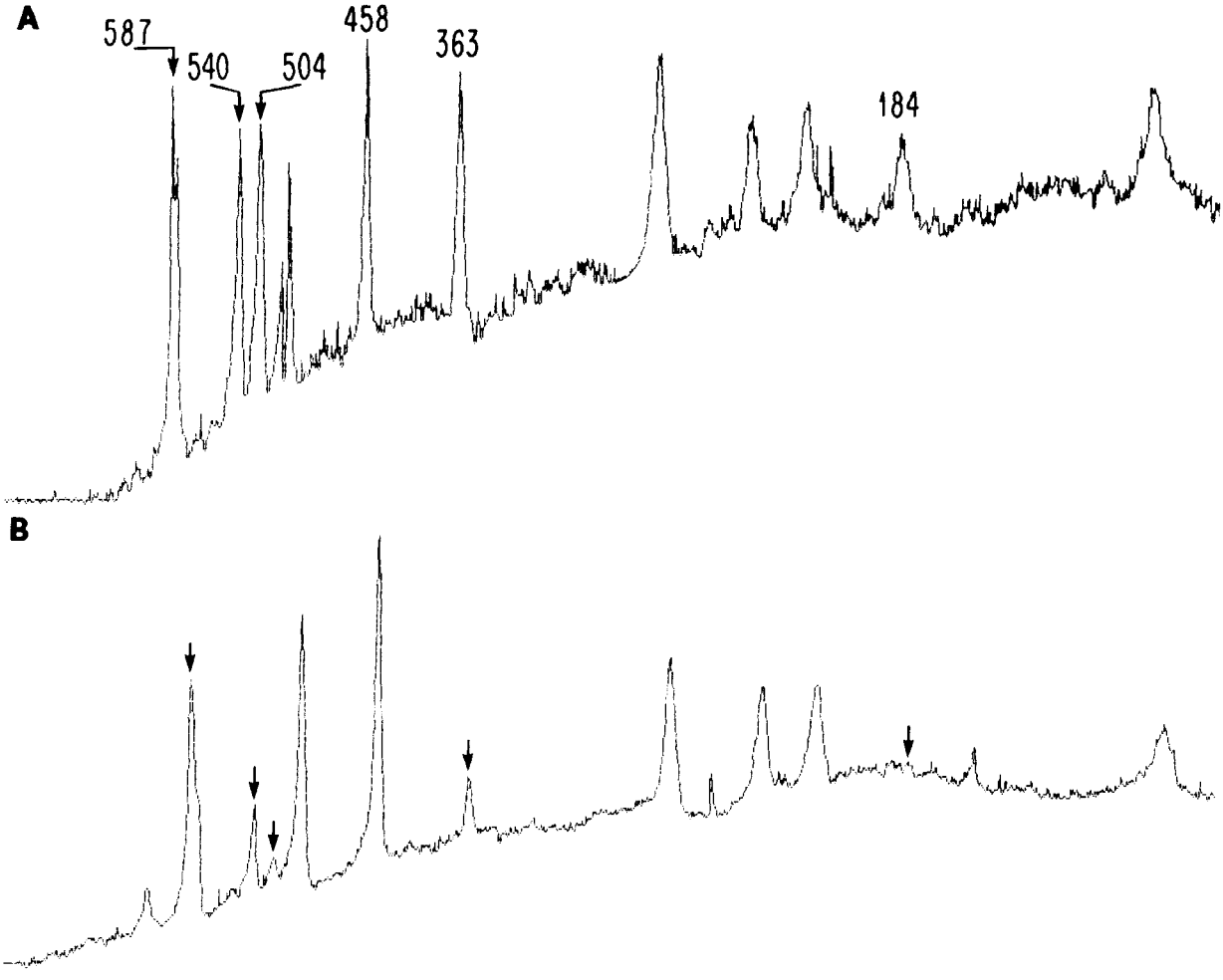


Fig. 4. Scans of 7% polyacrylamide gel showing REL (**A**) and VHS (**B**) forms of pZE15-4.53kb bound and cross-linked to monoclonal aZ-Ab and digested with *Hae*III. Sizes of fragments that contain satellite DNA (363 bp) or potential Z-DNA of pBR322 are shown. Arrows in (**B**) point to fragments reduced by aZ-Ab binding.

Populations of topoisomers of pZE15-2.66kb, a construct containing domain V and pBRZ2-4 but not pBRZ1 (Fig. 2, right panel), and of pDEL, a spontaneous deletion of 2.6 kb that is missing the crab satellite DNA but has pBRZ2-4, were also treated with monoclonal Z22B, crosslinked, and digested with *Hae*III. Again, the satellite insert had bound aZ-Ab: as $-\sigma$ increased, the 363 fragment to which antibody had bound was reduced in amount in restriction patterns (Fig. 5,A and B). Fragments

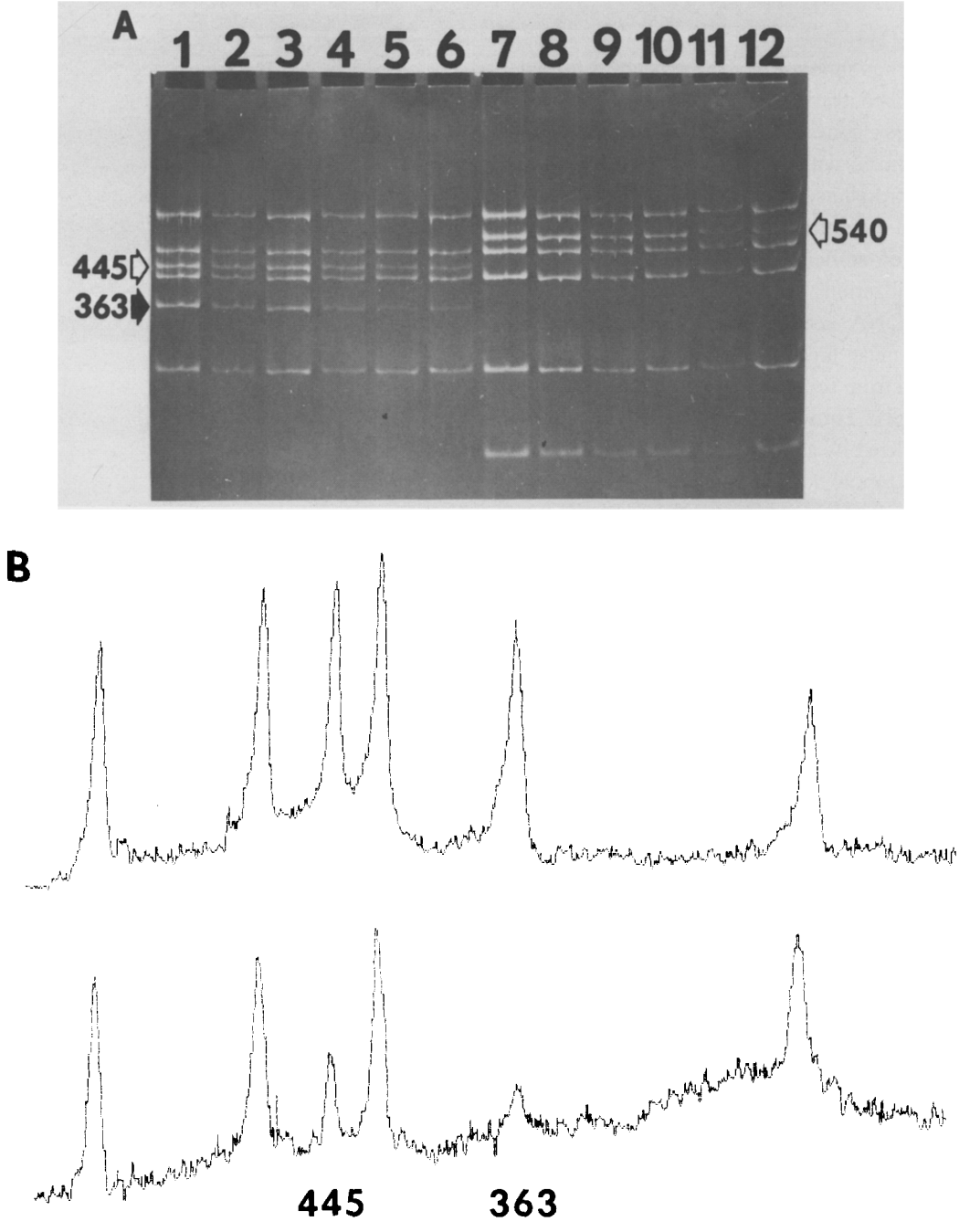


Fig. 5. Binding and crosslinking of monoclonal aZ-Ab to pZE15-2.66kb and pDEL. (Panel **A**) Polyacrylamide gel showing *Hae*III digests of topoisomers of pZE15-2.66kb (lanes 1–6) or pDEL (lanes 7–12) with $-\sigma$ of approx. 0.005 (lanes 2, 8), 0.010 (lanes 3, 9), 0.050 (lanes 4, 10), 0.085 (lanes 5, 11), or 0.130 (lanes 6, 12). Controls (lanes 1, 7), *Hae*III digests of plasmids without monoclonal aZ-Ab treatment. (Panel **B**) Densitometer scans of lanes 1 and 6 in panel **A**. Numbers indicate the size of fragments reduced in amount by aZ-Ab binding.

that contained pBRZ2, 445 bp in pZE15-2.66kb and 540 bp in pDEL (Fig. 5A), were markedly depleted, while fragments containing pBRZ3 and pBRZ4 were not affected in either plasmid.

(d) Estimation of the length of the Z-DNA segment in domain V

The equilibrium in favor of Z-DNA can be forced by very high concentrations of aZ-Ab. In such experiments, plasmids containing Z-DNA stabilized by aZ-Ab are relaxed with topoisomerase I and then treated with proteinase K to digest both the topoisomerase and the antibody (Lafer et al., 1985b). In the absence of sufficient superhelical stress and without stabilization by the antibody, Z-DNA reverts to B-DNA accompanied by a measurable change in the mean linking number (Lk), which can be determined from the change in positions of topoisomers on gels. Thus, the length of the Z-DNA segment stabilized by aZ-Ab can be estimated from changes in the topological properties of the products.

VHS forms of pZE15-2.66kb, pDEL, and pZE15-4.53kb were treated with up to 100 μg/ml monoclonal antibody Z22B. Following relaxation by topoisomerase I and digestion with proteinase K, samples were electrophoresed on agarose gels containing 10 μM CQ which permits the resolution of topoisomers with higher numbers of superhelical turns by decreasing the number of turns present. Both pZE15-4.53kb (not shown) and pZE15-2.66kb (Fig. 6A, lanes 7–10) exhibited a significant decrease in the mean linking number over the range of aZ-Ab concentrations, reaching a maximum at approx. 100 μg/ml aZ-Ab. By contrast, control plasmid pDEL exhibited no detectable shift in the mean $-\sigma$ (Fig. 6A, lanes 2–5). Although pZE15-4.53kb exhibits a distinct change under these conditions, it is not quantitatively comparable to pZE15-2.66kb because the molar ratio of plasmid to antibody is different. To demonstrate that the binding was specific, we used a monoclonal IgG (IgGp3 × 63Ag8) from a control source (gift of J. Hotchkiss and S. Kennel, Biology Division, Oak Ridge National Laboratory); there was no binding with either pZE15-2.66kb or pDel (not shown).

Densitometric analyses (Fig. 6B) of the gel shown in Fig. 6A were used to determine the approximate centers of the distributions of topoisomer populations of pZE15-2.66kb resulting from relaxation in the presence of 1 μg/ml aZ-Ab (indistinguishable from samples relaxed in the complete absence of aZ-Ab) and 100 μg/ml aZ-Ab. At low aZ-Ab (lane

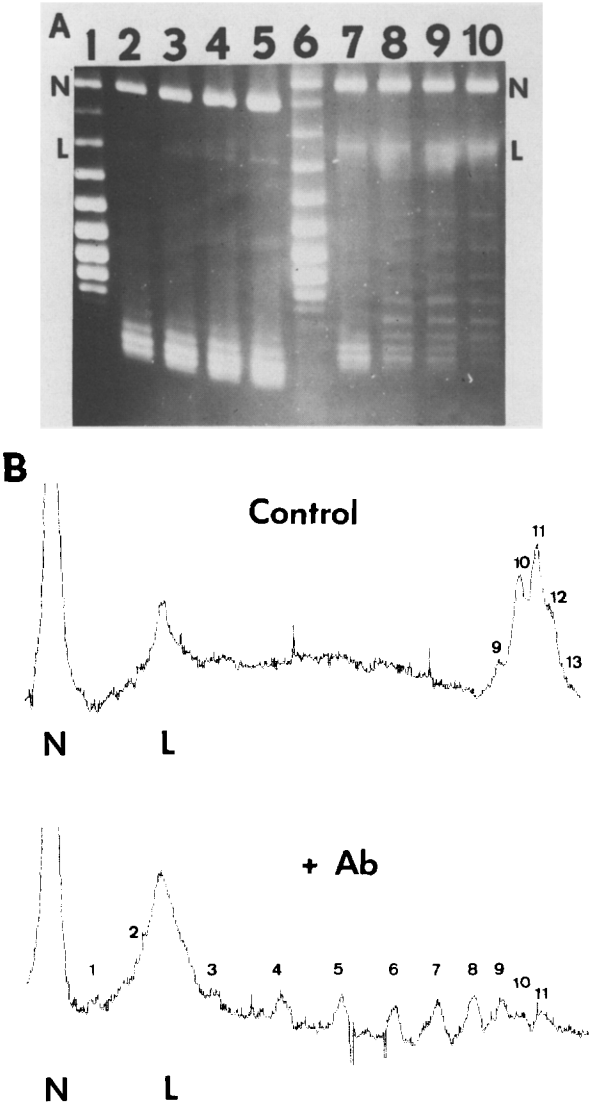


Fig. 6. Estimation of length of Z-DNA segment. (Panel **A**) Agarose gel (1.5% with 10 μM CQ) of pZE15-2.66kb and pDEL following stabilization in a Z-conformation by high levels of monoclonal aZ-Ab, treatment with topoisomerase, then proteinase K (positively supercoiled under these gel conditions). pDEL (lanes 2–5), pZE15-2.66kb (lanes 7–10) treated with monoclonal aZ-Ab at concentrations of 1 (lanes 2, 7), 10 (lanes 3, 8), 20 (lanes 4, 9), or 100 μg/ml (lanes 5, 10). Lanes 1 and 6 show native pDEL and pZE15-2.66kb, respectively; they are negatively supercoiled and illustrate the appearance of a typical distribution of topoisomers. (Panel **B**) Top and bottom panels, respectively, densitometer scan of lanes 7 (control) and 10 (100 μg/ml aZ-Ab) (see panel **A**). Numbers, positions in topoisomer ladder; N, nicked; L, linear forms of DNA.

7), populations were centered at approximately 11.5 superhelical turns under the gel conditions used (running buffer containing 10 μM CQ). The topoisomers relaxed in the presence of 100 μg/ml aZ-Ab (lane 10) were shifted considerably. The broad and non-Gaussian distribution of individual topoisomeric species suggested that a heterogeneous population of conformational isomers had been produced. The approximate center of the distribution was measured as 6.5, indicating an average change of approx. five superhelical turns and corresponding to the formation of a Z-DNA segment of approx. 29 bp, calculated as shown below:

$\Delta Lk = l/(10.5 \text{ bp per turn of B-DNA}) + l/(12 \text{ bp per turn of Z-DNA})$
$5 = l/10.5 + l/12$
$l = 28.6$

where l is the length of the Z-DNA segment (Klysik et al., 1981) and ΔLk is the change in the linking number.

The broad and non-Gaussian distribution of the 100 μg/ml-treated samples (Fig. 6A, lane 10) indicates heterogeneity in the length of the Z-DNA segments formed. It is reasonable to consider the lengths of Z-DNA segments corresponding to each individual topoisomer species in the distribution. Position 1 reflects the maximal shift of approx. 10.5 superhelical turns, corresponding to a Z-DNA segment of approx. 60 bp that would include the entire Pu/Py-rich domain and possibly reflect some contribution from pBR322 Z-DNA segments. Each successive topoisomer species in the distribution corresponds to a decrease in the length of Z-DNA by 6-bp increments. Position 5 reflects a shift of 6.5 superhelical turns and best approximates the population of molecules with a 35-bp stretch of Z-DNA, possibly the $Z_4Z_5NZ_{15}NZ_5Z_4$ segment. The Z_9 and Z_{15} blocks would be expected to form Z-DNA most readily, and they would correspond to positions between 8 and 10. Thermal fluctuations in the plasmid molecules produce a Gaussian distribution for each distinct molecular species (Pulleyblank et al., 1975), thus the broad distribution seen with pZE15-2.66kb most likely consists of overlapping Gaussian distributions contributed by several distinct conformational isomers.

The $-\sigma$ required to drive a B-Z transition in the crab insert is large when compared to that required to drive Z-DNA in tracts of polyd(G-C) polyd(G-C) or polyd(C-A)·polyd(T-G) of similar lengths, but it is comparable to values reported for other naturally occurring sequences containing mixed A : T and G : C base pairs with interruptions in alternation (Rich et al., 1984). In naturally occurring sequences this may reflect the relative instability of A : T base pairs (Wang et al., 1984) and interruptions in Pu/Py, but it is also consistent with the hypothesis that this region of the crab satellite undergoes a cooperative transition to a Z-DNA structure that is destabilized by interruptions in alternation and by the fact that some of the Pu/Py are in short contiguous blocks. Furthermore, methylation facilitates a B-Z transition (Behe and Felsenfeld, 1981). Primary sites of methylation are CpG dinucleotides (Cooper, 1983). Domain V contains nine CpG pairs (Fig. 1), an unusually high number for a 53-bp segment of eukaryotic DNA (Swartz et al., 1962). Although methylation is uncommon in the DNAs of other arthropods (Urieli-Shoval et al., 1982), preliminary evidence indicates that the crab satellite is methylated (P.M. Biesiot and D.M.S., unpublished). Thus, $-\sigma$ required for the B-Z transition may be considerably lower in vivo.

When the sequence $d(C\text{-}G)_6d(T\text{-}A)_4d(C\text{-}G)_6$ is under torsional stress, $d(T\text{-}A)_4$ was reported to be in an underwound state rather than a left-handed helix (Ellison et al., 1986). The nucleotides that comprise the downstream segment following the *Bss*HII site in the Pu/Py-rich segment of the crab satellite are 75% GC and the two A : T pairs are scattered therefore it is unlikely that the segment would unwind as did the $d(T\text{-}A)_4$ segment bordered on each side by $d(C\text{-}G)_6$.

Domains V of two variants of the crab satellite, RU and EXT, are not only rich in Pu/Py with an arrangement that would permit the adoption of a Z-DNA structure with dyad symmetry along the phosphodiester backbone, they also contain a perfect 12-bp inverted repeat that could adopt a cruciform structure extending in either direction from its center to include the entire 35-bp $Z_4Z_5NZ_{15}NZ_5Z_4$ region with only a few mismatches (Fig. 7). B42 has one additional interruption which trims its longest Pu/Py segment from 15 to 14 nt (Fig. 1). Similar G + C-rich 12-bp perfect inverted repeats are found at regular intervals in a minor cryptic satellite of another crab (Fowler and Skinner, 1985). The sequence of the

Fig. 7. Diagram of possible cruciform in domain V of RU and EXT. Although pairing is perfect over the outermost 12 bp, a 4-bp loop, the smallest permitted (Scheffler et al., 1970), is shown. The hypothetical cruciform includes the entire $Z_4Z_5NZ_{15}NZ_5Z_4$ structure indicated for RU and EXT in Fig. 1. N3 separating Z_{14} from Z_5 on the 3′-end of B42 (Fig. 1) would shorten the 5-bp uppermost part of the stem by 1 bp.

Pu/Py-rich region of the land crab satellite might permit an equilibrium between two symmetrical altered conformations within the same stretch of DNA. Similar structures in other systems are stabilized by negative superhelical stress (Bauer, 1978; Lilley, 1980; Lee and Bauer, 1985). Thus, domain V presents a situation where one or both axes of symmetry might be binding sites for specific proteins: one dimeric protein might recognize the dyad symmetry of a cruciform structure (Fig. 7) while another might recognize a different dyad symmetry in the same stretch of DNA in a Z-configuration (Fig. 1). There are many examples of dimeric DNA binding proteins that recognize dyad symmetries in nucleotide sequence. Notable among them are most restriction enzymes, the cyclic AMP receptor (McKay and Steitz, 1981) and the lambda *cro* repressor (Anderson et al., 1981). All of these apparently bind to a region of symmetry in the B-DNA conformation or a distorted version of B-DNA. Other proteins in addition to aZ-Ab that specifically bind Z-DNA have been identified in eukaryotic systems (Kmiec et al., 1985; Lafer et al., 1985b; Kmiec and Holloman, 1986). Particularly noteworthy in the present context, some of the aZ-Abs, and presumably other proteins, recognize the Z-DNA phosphodiester backbone without regard to nucleotide sequence (Lafer et al., 1985b; Nordheim et al., 1986).

The 15-bp segment of Pu/Py-rich DNA containing the *Bss*HII site in the crab satellite is the longest uninterrupted stretch of complex Pu/Py DNA yet described in eukaryotic DNA. Longer runs of Pu/Py are confined to simple sequences such as runs of d(T-A) associated with histone and globin genes in *Xenopus* (Greaves and Patient, 1985), which do not form Z-DNA (Ellison et al., 1986), or d(A-C), adjacent to protamine genes of the trout, which do (Aiken et al., 1985; see also Hamada and Kakunaga, 1982). Other naturally occurring more complex Pu/Py-rich segments that adopt the Z-conformation are shorter than 15 bp and/or contain an occasional interruption.

(e) Conclusions

Under the influence of relatively high levels of negative superhelical stress, a Pu/Py-rich domain of the crab satellite and three Pu/Py-rich segments of pBR322 adopt altered conformations with properties similar to Z-DNA as shown by binding to aZ-Ab. Inhibition of cutting by *Bss*HII localized the site of Z-conformation in the subclone containing the crab satellite insert to the longest stretch of Pu/Py in domain V. In regions immediately adjacent to, but not including, the $Z_4Z_5NZ_{15}NZ_5Z_4$ segment (Fig. 1), a 3-bp deletion has occurred in homologous sites of two of three cloned variants and specific 10- and 12-bp deletions have occurred in a homologous site in two of the three; the latter deletion includes an entire Z_9 segment in EXT and 7/9 nt of that segment in B42. If these deletions are omitted from calculations of homology, the segment of >500 bp 3′ to domain V is 96% conserved between RU and EXT, two variants that have been completely sequenced. Furthermore, 300 bp upstream are 97% homologous between those two. In addition, 235 bp of TRU, a cloned variant that has been truncated near its 3′ end, is 95% conserved with respect to RU and EXT upstream from the site of its truncation. Partial sequences of ten other cloned variants indicate that they, too, share greater than 90% homology (not shown). Thus, these deletions are confined to two specific loci in segments of otherwise very highly conserved DNA as are other major sequence changes in the satellite (Fowler et al., 1985; Fowler and Skinner, 1986). Given the evidence that Z-DNA is implicated in recombination (Klysik et al., 1982;

Jovin et al., 1983; Kmiec et al., 1985; Kmiec and Holloman, 1986), and that symmetries in DNA primary or secondary structure are potential binding sites for proteins, this region of the crab satellite appears to be a mutation hotspot or regulatory element that might be recognized by hypothetical proteins by virtue of its dual potential for symmetrical organization either as a conventional cruciform or as a symmetrical Z-DNA structure.

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REFERENCES

Aiken, J.M., Miller, F.D., Hagen, F., McKenzie, D.I., Krawetz, S.A., van de Sande, J.H., Rattner, J.B. and Dixon, G.H.: Tandem repeats of a specific alternating purine-pyrimidine DNA sequence adjacent to protamine genes in the rainbow trout that can exist in the Z form. Biochemistry 24 (1985) 6268–6276.

Anderson, W.F., Ohlendorf, D.H., Takeda, Y. and Matthews, B.W.: Structure of the *cro* repressor from bacteriophage λ and its interaction with DNA. Nature 290 (1981) 754–758.

Azorin, F., Hahn, R. and Rich, A.: Restriction endonucleases can be used to study B-Z junctions in supercoiled DNA. Proc. Natl. Acad. Sci. USA 81 (1984) 5714–5718.

Barton, J. and Raphael, A.L.: Site-specific cleavage of left-handed DNA in pBR322 by λ-tris (diphenylphenanthroline)-cobalt(III). Proc. Natl. Acad Sci. USA 82 (1985) 6460–6464.

Bauer, W.R.: Structure and reactions of closed duplex DNA. Annu. Rev. Biophys. Bioeng. 7 (1978) 287–313.

Behe, M. and Felsenfeld, G.: Effects of methylation on a synthetic polynucleotide: The B-Z transition in poly(dG-m^{5}dC)·poly(dG-m^{5}dC). Proc. Natl. Acad. Sci. USA 78 (1981) 1619–1623.

Bonnewell, V., Fowler, R.F. and Skinner, D.M.: An inverted repeat borders a fivefold amplification in satellite DNA. Science 221 (1983) 862–865.

Cooper, D.N.: Eukaryotic DNA methylation. Hum. Genet. 64 (1983) 315–333.

Ellison, M.J., Feigon, J., Kelleher III, R.J., Wang, A.H.-J., Habener, J. and Rich, A.: An assessment of the Z-DNA forming potential of alternating dA-dT stretches in supercoiled plasmids. Biochemistry 25 (1986) 3648–3655.

Feigon, J., Wang, A.H.-J., van der Marel, G.A., van Boom, J.H. and Rich, A.: Z-DNA forms without an alternating purine-pyrimidine sequence in solution. Science 230 (1985) 82–84.

Fowler, R.F. and Skinner, D.M.: Cryptic satellites rich in inverted repeats comprise 30% of the genome of a hermit crab. J. Biol. Chem. 260 (1985) 1296–1303.

Fowler, R.F. and Skinner, D.M.: Eukaryotic DNA diverges at a long and complex pyrimidine·purine tract that can adopt altered conformations. J. Biol. Chem. 261 (1986) 8994–9001.

Fowler, R.F., Bonnewell, V., Spann, M.S. and Skinner, D.M.: Sequences of three closely related variants of a complex satellite DNA diverge at specific domains. J. Biol. Chem. 260 (1985) 8964–8972.

Greaves, D.R. and Patient, R.K.: $(AT)_n$ is an interspersed repeat in the *Xenopus* genome. EMBO J. 4 (1985) 2617–2626.

Hamada, H. and Kakunaga, T.: Potential Z-DNA forming sequences are highly dispersed in the human genome. Nature 292 (1982) 396–398.

Hayes, T.E. and Dixon, J.E.: Z-DNA in the rat somatostatin gene. J. Biol. Chem. 260 (1985) 8145–8156.

Jovin, T.M., McIntosh, L.P., Arndt-Jovin, D.J., Zarling, D.A., Robert-Nicoud, M., van de Sande, J.H., Jorgenson, K.F. and Eckstein, F.: Left-handed DNA: from synthetic polymers to chromosomes. J. Biomol. Struct. Dyn. 1 (1983) 21–57.

Keller, W.: Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. Proc. Natl. Acad. Sci. USA 72 (1975) 4876–4880.

Kilpatrick, M.W., Klysik, J., Singleton, C.K., Zarling, D.A., Jovin, T.M., Hanau, L.H., Erlanger, B.F. and Wells, R.D.: Intervening sequences in human fetal globin genes adopt left-handed Z helices. J. Biol. Chem. 259 (1984) 7268–7274.

Klysik, J., Stirdivant, S.M., Larson, J.E., Hart, P.A. and Wells, R.D.: Left-handed DNA in restriction fragments and a recombinant plasmid. Nature 290 (1981) 672–677.

Klysik, J., Stirdivant, S.M. and Wells, R.D.: Left-handed DNA. J. Biol. Chem. 257 (1982) 10152–10158.

Kmiec, E.B. and Holloman, W.K.: Homologous pairing of DNA molecules by *Ustilago rec1* protein is promoted by sequences of Z-DNA. Cell 44 (1986) 545–554.

Kmiec, E.B., Angelides, K.J. and Holloman, W.K.: Left-handed DNA and the synaptic pairing reaction promoted by *Ustilago rec1* protein. Cell 40 (1985) 139–145.

Konopka, A.K., Reiter, J., Jung, M., Zarling, D.A. and Jovin, T.M.: Concordance of experimentally mapped or predicted Z-DNA sites with positions of selected alternating purine-pyrimidine tracts. Nucleic Acids Res. 13 (1985) 1683–1701.

Lafer, E.M., Moller, A., Nordheim, A., Stollar, B.D. and Rich, A.: Antibodies specific for left-handed Z-DNA. Proc. Natl. Acad. Sci. USA 78 (1981) 3546–3550.

Lafer, E.M., Sousa, R., Rossen, B., Hsu, A. and Rich, A.: Isolation and characterization of Z-DNA binding proteins from wheat germ. Biochemistry 24 (1985a) 5070–5076.

Lafer, E.M., Sousa, R. and Rich, A.: Anti-Z-DNA antibody binding can stabilize Z-DNA in relaxed and linear plasmids under physiological conditions. EMBO J. 4 (1985b) 3655–3660.

LaMarca, M.E., Allison, D.P. and Skinner, D.M.: Irreversible denaturation mapping of a pyrimidine-rich domain of a complex satellite DNA. J. Biol. Chem. 256 (1981) 6475–6479.

Lee, F.S. and Bauer, W.R.: Temperature dependence of the gel electrophoretic mobility of supercoiled DNA. Nucleic Acids Res. 13 (1985) 1665–1682.

Lilley, D.M.J.: The inverted repeat as a recognizable structural feature in supercoiled DNA molecules. Proc. Natl. Acad. Sci. USA 77 (1980) 6468–6472.

Maxam, A.M. and Gilbert, W.: Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65 (1980) 499–560.

McKay, D.B. and Steitz, T.A.: Structure of catabolite gene activator protein at 2.9 Å resolution suggests binding to left-handed B-DNA. Nature 290 (1981) 744–749.

Nordheim, A. and Rich, A.: Negatively coiled simian virus 40 DNA contains Z-DNA segments within transcriptional enhancer sequences. Nature 303 (1983) 674–679.

Nordheim, A., Lafer, E.M., Peck, L.J., Wang, J.C., Stollar, B.D. and Rich, A.: Negatively supercoiled plasmids contain left-handed Z-DNA segments as detected by specific antibody binding. Cell 31 (1982) 309–318.

Nordheim, A., Pardue, M.L., Weiner, L.M., Lowenhaupt, K., Scholten, P., Moller, A., Rich, A. and Stollar, B.D.: Analysis of Z-DNA in fixed polytene chromosomes with monoclonal antibodies that show base sequence-dependent selectivity in reactions with supercoiled plasmids and polynucleotides. J. Biol. Chem. 261 (1986) 468–476.

Peck, L.J., Nordheim, A., Rich, A. and Wang, J.C.: Flipping of cloned d(pCpG)n.d(pCpG)n DNA sequences from right- to left-handed helical structure by salt, Co(III), or negative supercoiling. Proc. Natl. Acad. Sci. USA 79 (1982) 4560–4564.

Pulleyblank, D.E., Shure, M., Tang, D., Vinograd, J. and Vosbert, H.-P.: Action of nicking-closing enzyme on supercoiled and non-supercoiled closed circular DNA: formation of a Boltzmann distribution of topological isomers. Proc. Natl. Acad. Sci. USA 72 (1975) 4280–4284.

Rich, A., Nordheim, A. and Wang, A.H.-J.: The chemistry and biology of left-handed Z-DNA. Annu. Rev. Biochem. 56 (1984) 791–846.

Scheffler, I.E., Elson, E.L. and Baldwin, R.L.: Helix formation by "The submitted manuscript has been authored by a contractor of the U.S. Government under contract No. DEAC05-84OR21400. Accordingly, the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this contribution, or allow others to do so, for U.S. Government purposes." d(TA) oligomers II. Analysis of the helix-coil transitions of linear and circular oligomers. J. Mol. Biol. 48 (1970) 145–171.

Singleton, C.K., Klysik, J. and Wells, R.D.: Conformational flexibility of junctions between contiguous B- and Z-DNAs in supercoiled plasmids. Proc. Natl. Acad. Sci. USA 80 (1983) 2447–2451.

Singleton, C.K., Kilpatrick, M.W. and Wells, R.D.: S1 nuclease recognizes DNA conformational junctions between left-handed helical (dT-dG)·(dC-dA) and contiguous right-handed sequences. J. Biol. Chem. 259 (1984) 1963–1967.

Skinner, D.M.: Satellite DNA's in the crabs *Gecarcinus lateralis* and *Cancer pagurus*. Proc. Natl. Acad. Sci. USA 59 (1967) 103–110.

Skinner, D.M., Bonnewell, V. and Fowler, R.F.: Sites of divergence in the sequence of a complex satellite DNA and several cloned variants. Cold Spring Harbor Symp. Quant. Biol. 47 (1982) 1151–1157.

Skinner, D.M., Fowler, R.F. and Bonnewell, V.: Domains of simple sequences or alternating purines and pyrimidines are sites of divergences in a complex satellite DNA. UCLA Symp. Mol. Cell. Biol. 10 (1983) 849–861.

Stringfellow, L.A., Fowler, R.F., LaMarca, M.E. and Skinner, D.M.: Demonstration of remarkable sequence divergence in variants of a complex satellite DNA by molecular cloning. Gene 38 (1985) 145–152.

Swartz, M.N., Trautner, T.A. and Kornberg, A.: Enzymatic synthesis of deoxyribonucleic acid, XI. Further studies on nearest neighbor base sequences in deoxyribonucleic acids. J. Biol. Chem. 237 (1962) 1961–1967.

Uricli-Shoval, S., Gruenbaum, Y., Sedat, J. and Razin, A.: The absence of detectable methylated bases in *Drosophila melanogaster* DNA. FEBS Lett. 146 (1982) 148–152.

Wang, A.H.-J., Hakoshima, T., Van der Marel, G., Van Boom, J.H. and Rich, A.: AT base pairs are less stable than GC base pairs in Z-DNA: the crystal structure of d(m^5CGTAm^5CG). Cell 37 (1984) 321–331.

Wang, A.H.-J., van der Marel, G., van Boom, J.H. and Rich, A.: Crystal structure of Z-DNA without an alternating purine-pyrimidine sequence. Proc. Natl. Acad. Sci. USA 82 (1985) 3611–3615.

Zasloff, M., Ginder, G.D. and Felsenfeld, G.: A new method for the purification and identification of covalently closed circular DNA molecules. Nucl. Acids Res. 5 (1978) 1139–1151.

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