Amplified Primer Extension Assay for Psoralen Photoproducts Provides a Sensitive Assay for a $(CG)_6TA(CG)_2(TG)_8$ Z-DNA Torsionally Tuned Probe: Preferential Psoralen Photobinding to One Strand of a B–Z Junction†

Robert W. Hoepfner and Richard R. Sinden*

Department of Molecular Genetics, Biochemistry and Microbiology, College of Medicine, University of Cincinnati, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524

Received February 26, 1993; Revised Manuscript Received April 19, 1993

ABSTRACT: An amplified primer extension assay has been developed for quantitatively mapping the sites of psoralen photoaddition to DNA. This assay was applied to a torsionally tuned Z-DNA-probe that was specifically designed for the primer extension assay. The torsionally tuned Z-DNA forming sequence, $(CG)_6TA(CG)_2(TG)_8$, forms Z-DNA *in vitro* at negative superhelical density: $\sigma = -0.05$. The internal 5′-TA dinucleotide was reactive to psoralen when it existed as B-DNA. Upon the formation of Z-DNA, the internal 5′-TA no longer photobound psoralen. The torsionally tuned sequence was synthesized as an *Eco*RI fragment such that, when Z-DNA formed, the central 5′-AATT of the *Eco*RI sites was part of the B–Z junctions. The 5′-AATT sequence was not reactive with psoralen when it existed as B-DNA. When the 5′-AATT sequence existed as a B–Z junction, one strand of each junction became hyperreactive to psoralen. The TT directly 5′ to the B-DNA–Z-DNA junction photobound psoralen in a strand-specific fashion. Quantitation of the relative rate of psoralen photobinding to the internal 5′-TA and the 5′-AATT at the B–Z junctions provides relationships that are characteristic of the level of supercoiling in DNA.

Human and other eukaryotic genomes contain many sequence motifs that can form cruciforms, intramolecular triplex structures, unwound regions, and left-handed Z-DNA. The frequent association of these sequence motifs with potential control regions of genes, sites of recombination or translocation, or replication origins suggests that these regions may be important biologically. An important question remains the elucidation of the relationship, if any, between alternative helical structures of DNA and their biological function. To answer this question, a number of *in vitro* and *in vivo* assays have been developed that can distinguish between various helical forms of DNA (Rich et al., 1984; Palecek, 1991; Wells et al., 1988).

Our laboratory has utilized 4,5′,8-trimethylpsoralen (Me_3-psoralen)[1] as an *in vivo* probe of cruciforms, Z-DNA (Sinden et al., 1983; Sinden & Kochel, 1987; Kochel & Sinden, 1988, 1989; Zheng et al., 1991; Ussery et al., 1992; Sinden & Ussery, 1992), and intramolecular triplex DNA (Ussery & Sinden, 1993). Sinden and Kochel (1987) originally demonstrated that Me_3-psoralen would cross-link DNA in the B-form but not in the Z-form. The subsequent development of the exoIII/photoreversal assay to map sites of photobinding with base-pair resolution provided a sensitive means to detect the presence of Z-DNA *in vivo* (Zheng et al., 1991). This approach is based on the differential ability of Me_3-psoralen to photobind to a specially designed, torsionally tuned probe sequence that can exist as Z-DNA under the influence of unrestrained negative supercoiling. The probe was designed to provide several diagnostic signals for the formation of Z-DNA. Specifically, Me_3-psoralen photobinding to a central 5′-TA dinucleotide within a $(CG)_n$ or $(TG)_n$ run is abolished when the sequence forms Z-DNA. In addition, certain regions that are nonreactive when they exist as B-DNA show hypersensitive reactivity to Me_3-psoralen photobinding when they exist as B–Z junctions (Kochel & Sinden, 1988, 1989). The $(CG)_6TA(CG)_6$ torsionally tuned Z-DNA probe has been applied to measure superhelical density at specific sites in the chromosomes of living bacterial cells (Zheng et al., 1991).

The exoIII/photoreversal assay for Z-DNA formation within the $(CG)_6TA(CG)_6$ sequence is applicable for plasmid DNA in prokaryotes, but has limitations that impede its application to eukaryotic systems. *In vivo* application of the exoIII/photoreversal assay relies on the analysis of exoIII digestion products of ^{32}P-end-labeled, gel-purified restriction fragments that have been photobound with Me_3-psoralen in living cells. Sensitivity is a major problem with this assay since it requires microgram quantities of plasmid DNA. For application of this approach in eukaryotic systems, a more sensitive mapping procedure is needed. A procedure is needed that can detect photoproducts in low copy number plasmids or in single-copy genes in complex genomes. An additional complication with the analysis of Z-DNA in eukaryotic cells is that the most common Z-DNA-forming sequence found in eukaryotes is of the $(TG)_n$ rather than the $(CG)_n$ type (Schroth et al., 1992). The exoIII psoralen assay for Z-DNA works quite well for $(CG)_n$ Z-DNA-forming sequences. Me_3-psoralen photobinding of both junctions and an internal 5′-TA dinucleotide is sensitive to the conformation of the DNA (Kochel & Sinden, 1988, 1989). Although the reactivity of an internal 5′-TA in a $(TG)_n$ Z-DNA-forming sequence was sensitive to DNA conformation, the hyperreactivity of the B–Z junctions did not parallel the formation of Z-DNA (Kochel & Sinden, 1988). This limited the applicability of $(TG)_n$ Z-DNA-forming sequences as torsionally tuned supercoiling probes *in vivo*. Moreover, it limited the possibility for analysis of the conformation of many naturally occurring

† This work was supported by Grant GM 37677 to R.R.S. from the National Institutes of Health.

* Correspondence should be addressed to this author at the following address: Institute of Biosciences and Technology, Center for Genome Research, Texas A&M University, 2121 West Holcombe Blvd., Houston, TX 77030-3303.

[1] Abbreviations: Me_3-psoralen, 4,5′,8-trimethylpsoralen; exoIII, exonuclease III.

0006-2960/93/0432-7542$04.00/0

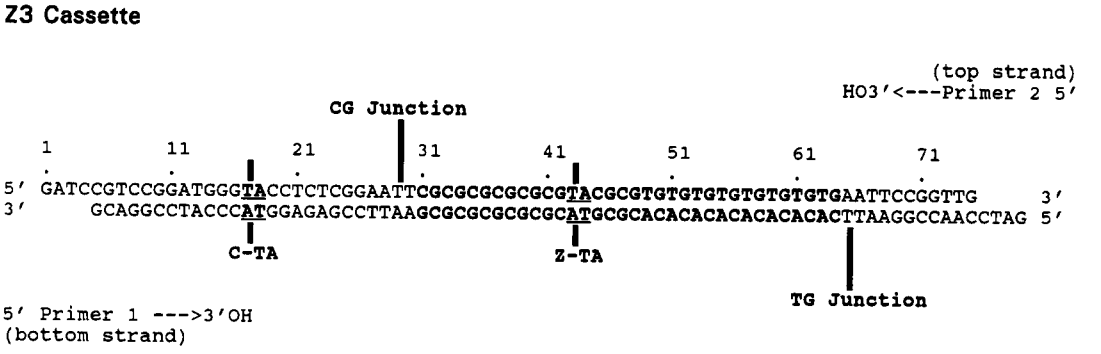

FIGURE 1: Sequence of the Z3 cassette. The 75-bp Z3 cassette was chemically synthesized and cloned into the *Bam*HI site of pUC8. The sequences from bases 11–48, from 5′ to the *Kpn*I (GGTACC, bp 15-20) site past the 5′-TA within the Z-DNA region, are similar to that for the 20-bp control and the 30-bp $(CG)_6TA(CG)_6$-containing sequence in pCGTA-C studied previously (Kochel & Sinden, 1989). The Z-DNA-forming sequence $(CG)_6TA(CG)_2(TG)_8$ is in bold-face type as is the 5′-TA in the control region (bp 17 and 18). The control 5′-TA (C-TA) and the 5′-TA in the Z-DNA region (Z-TA) are underlined and indicated. The positions of the CG junction and the TG junction are also indicated. The bars denote sites of Me_3-psoralen photoreactivity. The Z-TA and C-TA sites are reactive when the sequences exist as B-DNA, whereas only one strand of the junctions is reactive when the $(CG)_6TA(CG)_2(TG)_8$ sequence exists as Z-DNA.

potential $(TG)_n$ Z-DNA-forming sequences in living eukaryotic cells.

An alternate method for mapping sites of photoaddition is to use a polymerase fill-in reaction. Unlike the exoIII/photoreversal assay, which requires specific ends of a restriction fragment for labeling and exoIII digestion, the primer extension method only requires knowledge of the sequence of a primer site. The use of polymerases to map UV photoproducts (Becker & Grossmann, 1992) demonstrates the feasibility of such an assay. A major concern is to identify a polymerase that will stop at both psoralen monoadducts and interstrand cross-links. Thermophilic Taq polymerase stops at a number of covalently modified bases in DNA (Axelrod & Majors, 1989; Govan et al., 1990; Kalinowski et al., 1992) and provides a means to amplify the signal utilizing the APEX method developed by Cartwright and Kelly (1991) and Axelrod and Majors (1989).

Here we describe the development of a linear amplified primer extension assay to map psoralen photoproducts to a torsionally tuned Z-DNA probe specifically designed for this assay. This approach is more sensitive than the exoIII/photoreversal assay. Moreover, unlike the exoIII assay, both strands can be easily analyzed using primers specific for either strand. Analysis of a $(CG)_6TA(CG)_2(TG)_8$ Z-DNA-forming sequence using two different primers demonstrated preferential hyperreactive psoralen photobinding to different strands of the B–Z junction flanking the $(CG)_n$ or $(TG)_n$ Z-DNA regions. The results show that the hyperreactivity of psoralen photobinding to a $(TG)_n$ B–Z junction parallels the formation of Z-DNA *in one strand*. This approach provides an assay sensitive enough for *in vivo* applications in eukaryotes for $(TG)_n$ Z-DNA-forming sequences.

EXPERIMENTAL PROCEDURES

Plasmids, Cloning, and Bacterial Strains. Escherichia coli strain RS2 (*topA10*) is a *recA56* derivative of the original strain (Sternglanz et al., 1981) and was obtained from Dr. Karl Drlica. DH5α contains (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 rel*A1). The 75-bp insert shown in Figure 1 was chemically synthesized on an Applied Biosystems DNA synthesizer. The two strands were hybridized together and the ends were phosphorylated by the addition of ATP and T4 DNA kinase. The insert was ligated into the *Bam*HI site of pUC8 and the plasmid designated pZ3A. The plasmid was transformed into *E. coli* strains DH5α and the topoisomerase I deficient strain RS2 (*topA10*) using the procedure described by Hanahan (1983). The sequence was confirmed by DNA sequencing using a double-stranded DNA Cycle Sequencing System (BRL). Growth of *E. coli* containing pZ3A, purification of plasmid, and generation of topological isomers have been previously described (Sinden et al., 1980). Plasmid topoisomer populations were analyzed using two-dimensional agarose gels to determine superhelical density (Sinden & Kochel, 1987).

Introduction of Me_3-psoralen Photoproducts. The first phase of the linear amplified primer extension assay was to introduce photoproducts into the DNA samples. Me_3-psoralen photobinding conditions have been described previously (Kochel & Sinden 1988, 1989; Ussery et al., 1992). Briefly, purified plasmid samples (50 μg/mL) were treated with 1% (v/v) Me_3-psoralen saturated in ethanol and incubated for 2 min at 4 °C. Upon exposure to 360-nm UV light, 10-μg aliquots were removed at 0.3, 0.6, and 0.9 kJ m^{-2} (incident light intensity 1.2 kJ m^{-1} min^{-1}). The samples were then loaded on ethidium bromide/cesium chloride gradients and centrifuged to equilibrium, and then supercoiled plasmid was purified. This step was omitted for linearized plasmid. The DNA samples were digested with *Pvu*II to generate a 377-bp fragment containing the Z-DNA insert for analysis. Samples were extracted with phenol/chloroform (1:1, v/v), ethanol-precipitated, and then resuspended at a concentration of 100 μg/mL.

Linear Amplified Primer Extension Assay and Analysis. The 20-bp primers used for this assay were synthesized on an Applied Biosystems DNA synthesizer. The 5′-ends of the primers were flush with the 3′-ends of the *Pvu*II fragment. Primer 1 corresponds to sequences beginning 188 bp upstream of the insert (which is in the *Bam*HI site). Primer 2 corresponds to sequences beginning 119 bp downstream of the *Bam*HI site. Primer (1 μg) was end-labeled with 20 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) in a 20-μL reaction volume (Maniatis et al., 1982). The reaction was terminated by the addition of 80 μL of stop buffer (0.375 M sodium acetate and 2.5 mM EDTA) and precipitated with 3 vol of ethanol (EtOH). After the precipitate was dissolved in water, a second EtOH precipitation was performed to remove any unincorporated ATP.

For the primer extension assay, each DNA sample (100 ng) was mixed with 20 ng of labeled primer (>5 molar excess) in 50 μL of primer extension reaction buffer (10 mM Tris (pH 8.3), 10 mM KCl, and 4 mM $MgCl_2$). All four deoxynucleotides were added to the reaction buffer at 0.5

mM each. To this mixture, 5 units of AmpliTaq Stoffel fragment (Cetus) was added, and a layer of mineral oil was overlaid on each sample. The primer extension reactions were performed in a thermal cycling unit under the following conditions: 96 °C for 2 min (to denature the strands), 60 °C for 30 s (to allow primer hybridization), and finally 80 °C for 5 min (for DNA polymerization). For this amount of DNA, 10 cycles were sufficient to provide a strong signal on a DNA sequencing gel. This cycling program was optimized for the GC-rich Z-DNA torsionally tuned Z3A probe.

When the thermal cycling was completed, the oil was removed and the samples were extracted once with phenol and once with chloroform/isoamyl alcohol (24:1). Glycogen (5 μg) was then added, the sample was adjusted to 0.3 M potassium acetate, and the DNA was precipitated by the addition of 4 vol of ethanol. The samples were resuspended and separated by electrophoresis on a 5% polyacrylamide denaturing gel with accompanying sequence markers extended from the same primer. The gel was dried and analyzed using a Molecular Dynamics PhosphoImager to quantitate the primer extension stops at Me_3-psoralen photoproducts.

RESULTS

Design of the Z3 Torsionally Tuned Probe. Previously, we have designed torsionally tuned Z-DNA probes containing $(CG)_n$ Z-DNA-forming sequences, which form Z-DNA at a lower superhelical density than $(TG)_n$ Z-DNA-forming sequences of similar length (Kochel & Sinden, 1988, 1989). We designed the Z3 Z-DNA torsionally tuned probe for application in eukaryotic cells where a sensitive primer extension assay could be utilized. Preliminary experiments with a $(CG)_6TA(CG)_6$ sequence showed that the CG-rich region presented read-through problems for Taq polymerases, in that chain elongation stopped at the center of the palindromic sequence. In addition, the palindromic nature of long $(CG)_n$ sequences appeared to lead to genetic instability of the sequence in *E. coli* and possibly in eukaryotic cells (Klysik et al., 1982; R. W. Hoepfner & R. R. Sinden, unpublished observations). The Z3 Z-DNA cassette containing the 34-bp nonpalindromic Z-DNA-forming region shown in Figure 1 was designed with a $(CG)_6TA(CG)_2$ region, in which the reactivity of the 5′-TA should mimic that found in the $(CG)_6TA(CG)_6$ probe used previously (Kochel & Sinden, 1988, 1989; Zheng et al., 1991). The sequence $(TG)_8$ was added 3′ to the Py·Pu sequence to provide a 20-bp region of Z-DNA 3′ to the TA dinucleotide. Analysis on two-dimensional agarose gels showed that this region formed Z-DNA at $\sigma = -0.05$ (data not shown). The relaxation of about six negative superhelical turns was indicative of the Z-DNA conformation.

Analysis of Z-DNA Formation in the Z3 Cassette Using the exoIII/Photoreversal Assay. Naturally supercoiled pZ3A with a superhelical density of $\sigma = -0.05$ was treated with Me_3-psoralen and light, and the existence of Z-DNA was analyzed using the exoIII/photoreversal assay. At this superhelical density, about 50% of the topoisomers contained the Z3 insert in the Z-DNA conformation. A standard exoIII/photoreversal assay was performed on this construct, and representative data are shown in Figure 2. The pattern of exoIII stops shown in Figure 2 was expected on the basis of our previous results for a sample containing a mixture of B and Z forms of the Z3 Z-DNA-forming region (Kochel & Sinden, 1988, 1989). The center 5′-TA (Z-TA) generated an exoIII stop from photobinding in plasmid molecules in which the insert existed as B-DNA. The *Eco*RI site on the CG side of the insert (termed CG junction) was hypersensitive because

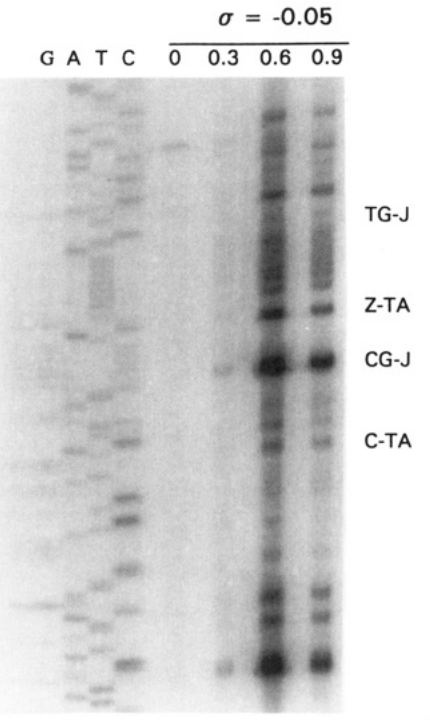

FIGURE 2: exoIII/photoreversal assay on psoralen-treated naturally supercoiled pZ3A DNA. The exoIII/photoreversal assay was performed as described (Kochel & Sinden, 1989). Briefly, purified DNA was treated with psoralen and exposed to increasing doses of 360-nm light, and then supercoiled plasmid was purified from a cesium chloride/ethidium bromide density gradient. Samples were then digested with *Pvu*II, end-labeled with ^{32}P, and digested with *Hind*III, and the *Pvu*II–*Hind*III fragment was purified on a 5% acrylamide nondenaturing gel. The fragment was then treated with exoIII, the cross-links were photoreversed, and the samples were analyzed on a 5% acrylamide denaturing gel. The autoradiograph shows that the exonuclease stops on the right and the DNA sequence marker lanes on the left. At a superhelical density of $\sigma = -0.05$, about 50% of the molecules in the plasmid preparation will maintain the Z3 sequence in the Z-DNA conformation. The sample lanes were treated with psoralen and 0, 0.3, 0.6, and 0.9 kJ m^{-2} of 360-nm light, as indicated. The following exoIII stops are indicated: TG-J, the B–Z junction flanking the $(TG)_n$ motif; Z-TA, the 5′-TA in Z-DNA sequence; CG-J, the B–Z junction flanking the $(CG)_n$ motif; and C-TA, the control 5′-TA in the *Kpn*I site that cannot form Z-DNA.

of photobinding to molecules containing the insert in the Z-DNA form. The TG junction gave a weak signal. We have previously shown that a $(TG)_n$ *Eco*RI junction only displayed a hypersensitive signal at very high negative superhelical densities (Kochel & Sinden, 1988). As shown in Figure 2, strong stops for psoralen photobinding at 5′-TAs and weak stops at 5′-ATs were observed. There were many stops in the $(TG)_8$ strand, as observed previously for a $(TG)_6TA(TG)_6$ sequence (Kochel & Sinden, 1988). In relaxed DNA ($\sigma = 0$) when the Z3 cassette existed as B-DNA, there was no reactivity at the CG junction. In highly supercoiled DNA ($\sigma = -0.073$) when the Z3 cassette in all topoisomers existed as Z-DNA, reactivity was not observed at the internal 5′-TA dinucleotide (data not shown).

The intensity of individual exoIII stops was measured relative to a control 5′-TA (C-TA) that cannot form Z-DNA. The 5′-TA within the *Kpn*I site of the Z3 cassette (bp 17 and 18 in Figure 1) was used as the control 5′-TA. This is the same restriciton site that has been used successfully for analysis of other Z-DNA probes (Sinden & Kochel, 1987; Kochel & Sinden, 1988, 1989). Moreover, this site is 2 bp farther from the end of the Z-DNA-forming sequence than in previous probes. exoIII stop ratios were measured for various times of irradiation with Me_3-psoralen and light, and zero dose values

were determined. As discussed previously (Kochel & Sinden 1989), photobinding of Me_3-psoralen appears to drive the Z-DNA conformation back to B-DNA, which will change the relative rate of photobinding to individual photobinding sites. Therefore, it is necessary to extrapolate (by linear regression) the photobinding ratios to zero dose to obtain values that are independent of any potential variability in reaction conditions. The average zero dose ratios of the exoIII stops for three independent experiments on pZ3A with $\sigma = -0.05$ were 0.2 for the TG junction (TG-J/C-TA), 2.3 for the Z-TA (Z-TA/C-TA), and 6.9 for the CG junction (CG-J/C-TA).

Analysis of Z-DNA Using the Primer Extension Assay. Primers 1 and 2, which hybridize to the ends of the *Pvu*II fragment, were used for the analysis of Z-DNA in pZ3A. Primer 1 was used for generating the DNA sequencing markers for the exoIII analysis, and it hybridizes to the end of the *Pvu*II site that is end-labeled in the exoIII assay. However, it hybridizes to the unlabeled or bottom strand and detects sites of Me_3-psoralen photobinding in this strand. Primer 2 hybridizes to the top strand at the other end of the *Pvu*II fragment with respect to primer 1. Primer 2 detects sites of photobinding in the top strand shown in Figure 1, the strand analyzed in the exoIII assay.

Figure 3 shows a primer extension assay using primer 2 on three pZ3A plasmid populations with different superhelical densities: relaxed, naturally supercoiled, and highly supercoiled (DNA purified from RS2). The results are qualitatively and quantitatively similar to those from the exoIII assay. As the plasmid becomes more supercoiled, reactivity at the center 5′-TA (Z-TA) disappears, the CG junction becomes hypersensitive, and the TG junction produces only a weak signal.

The quantitation of primer stops for representative experiments is shown in Table I as a ratio of photobinding. The ratios represent the intensities of Me_3-psoralen photobinding sites relative to the intensity of the stop at the 5′-TA in the control region. The 5′-TA within the *Kpn*I site in the Z-DNA cassette is 12 bp away from the alternating purine-pyrimidine stretch and provides a convenient control TA (C-TA) since it will not form an alternate DNA conformation (Sinden & Kochel, 1987; Kochel & Sinden, 1988, 1989). Because Me_3-psoralen photobinding can drive the Z-DNA conformation to B-DNA, the ratios are extrapolated to zero dose (Kochel & Sinden, 1988, 1989). The zero dose values for the Z-TA and both the CG and TG B–Z junctions are shown in Table I.

Figure 4 shows the primer extension analysis of naturally and highly supercoiled DNA samples using primer 1. Primer 1 detects sites of psoralen photobinding in the opposite strand compared to that analyzed by the exoIII assay (as applied in pZ3A) and by primer 2. The reactivity of the 5′-TA within the Z-DNA-forming region decreased upon Z-DNA formation, as expected. The behavior of both junctions, however, was unexpected. Sequences within the B–Z junction flanking the $(CG)_n$ sequence, which exhibited the characteristic marked hyperreactivity using the exoIII assay and analysis with primer 2, showed rather weak reactivity on the formation of Z-DNA. In contrast, the sequences at the site of the $(TG)_n$ B–Z junction showed hyperreactivity to Me_3-psoralen photobinding upon the formation of Z-DNA. The relative intensities of the primer extension stops for a representative experiment are quantitated in Table I.

Application of the Z3 Cassette as a Torsionally Tuned Probe. The relationships between superhelical density and the average zero dose relative intensities of the primer extension stops within the Z-DNA-forming region and at the (CG) and (TG) B–Z junctions are shown in Figure 5. The behavior of

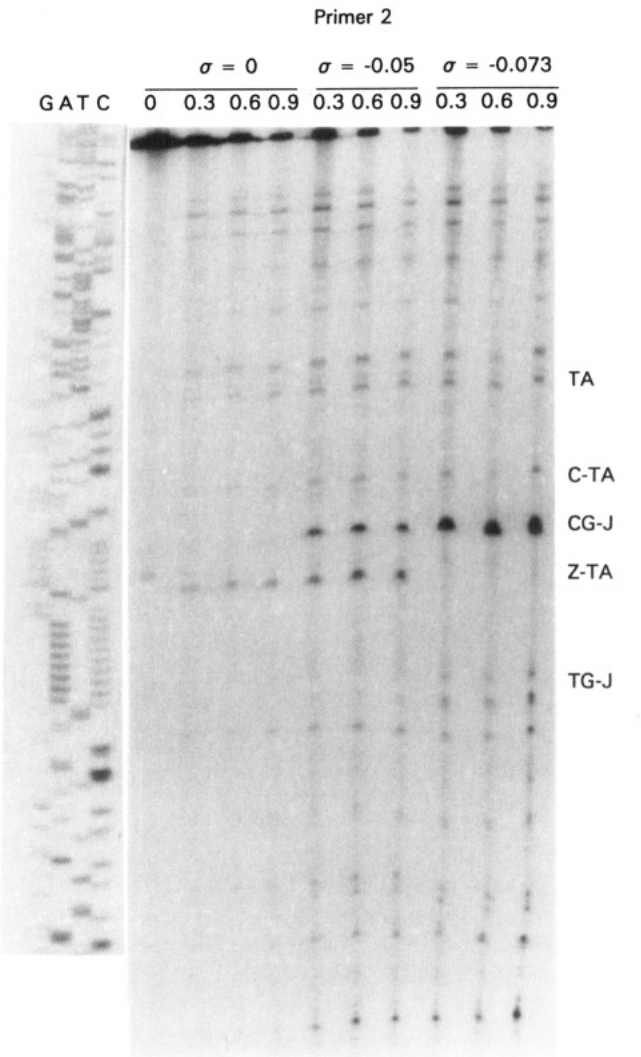

FIGURE 3: Linear amplified primer extension assay on psoralen treated pZ3A using primer 2. Purified DNA was treated with Me_3-psoralen and light as described in the Experimental Procedures. Plasmid purified from a cesium chloride/ethidium bromide gradient was digested with *Pvu*II, primer-extended for 10 cycles, extracted with phenol and chloroform, and analyzed on a 5% acrylamide denaturing gel. The DNA sequence lanes are on the left. Lane 1 labeled 0 corresponds to a control *Pvu*II fragment with no Me_3-psoralen treatment. The next three sets (three samples each) are primer extension patterns for relaxed pZ3A ($\sigma = 0$), naturally supercoiled DNA ($\sigma = -0.05$), and highly supercoiled DNA ($\sigma = -0.073$) treated with psoralen and 0.3, 0.6, and 0.9 kJ m^{-2} of 360-nm light, respectively. The positions of the C-TA, Z-TA, CG-J, TG-J, and one other 5′-TA are indicated.

the 5′-TA within the Z-DNA-forming region was expected from previous analysis of Z-DNA sequences using the exoIII assay (Kochel & Sinden, 1988, 1989). As Z-DNA formed, the intensity of Me_3-psoralen photobinding to the Z-TA decreased to zero. The midpoint of the curve occurred at approximately $\sigma = -0.05$, the superhelical density at which the Z3 sequence forms Z-DNA. The behavior of this internal 5′-TA was similar when probed in either strand. The increase in reactivity of the (CG) B–Z junction paralleled the formation of Z-DNA using primer 2, and the increase in reactivity of the (TG) B–Z junction paralleled Z-DNA formation using primer 1. There was little reactivity detected by primer 1 at the CG junction or by primer 2 at the TG junction. These curves provide relationships by which the superhelical density of DNA can be estimated from the relative intensities of the primer stops determined for a DNA sample. Application of this Z3 torsionally tuned probe can be used to estimate the

Table I: Intensities of Primer Extension Stops

		primer 1			primer 2		
σ	light (kJ m^{-2})	TG-J/ C-TA[a]	Z-TA/ C-TA[a]	CG-J/ C-TA[a]	TG-J/ C-TA	Z-TA/ C-TA	CG-J/ C-TA
0	0	1.18	3.15	0.25	0	4.84	0
	0.3	0.98	3.03	0.21	0	4.59	0
	0.6	1.45	2.98	0.22	0.28	4.04	.03
	0.9	0.92	2.83	0.15	0.29	3.96	0.23
–0.03	0	1.03	2.54	0.27	0.14	3.0	0.86
	0.3	0.98	2.74	0.22	0.1	2.95	0.74
	0.6	0.68	2.31	0.16	0.03	3.12	0.5
	0.9	0.76	2.82	0.11	0	3.04	0.44
–0.05	0	1.13	2.14	0.44	0	3.44	3.56
	0.3	0.98	1.91	0.37	0	3.52	3.15
	0.6	0.88	1.75	0.27	0	4.15	4.12
	0.9	0.7	1.48	0.22	0.1	3.96	3.02
–0.054	0	2.42	2.45	0.65	0.01	2.04	3.24
	0.3	1.86	2.13	0.56	0.08	2.24	3.07
	0.6	1.39	2.22	0.65	0.05	2.82	3.13
	0.9	0.79	1.69	0.47	0.17	2.83	2.85
–0.057	0	1.7	1.58	0.65	0.21	1.54	4.57
	0.3	1.58	1.53	0.67	0.23	1.64	4.45
	0.6	1.83	1.77	0.62	0.28	1.6	3.8
	0.9	1.52	1.57	0.68	0.28	1.77	3.99
–0.073	0	3.82	0.27	1.45	0.7	0	9.14
	0.3	4.0	0.23	0.12	1.03	0	11.4
	0.6	3.75	0.26	0.64	0.81	0	10.08
	0.9	4.91	0.18	0.55	1.42	0.18	14.13

[a] TG-J/C-TA, Z-TA/C-TA, and CG-J/C-TA ratios were determined as described in the text. The zero dose (0 kJ m^{-2}) values were determined by linear regression as described previously (Kochel & Sinden, 1989). Data are shown for one representative experiment.

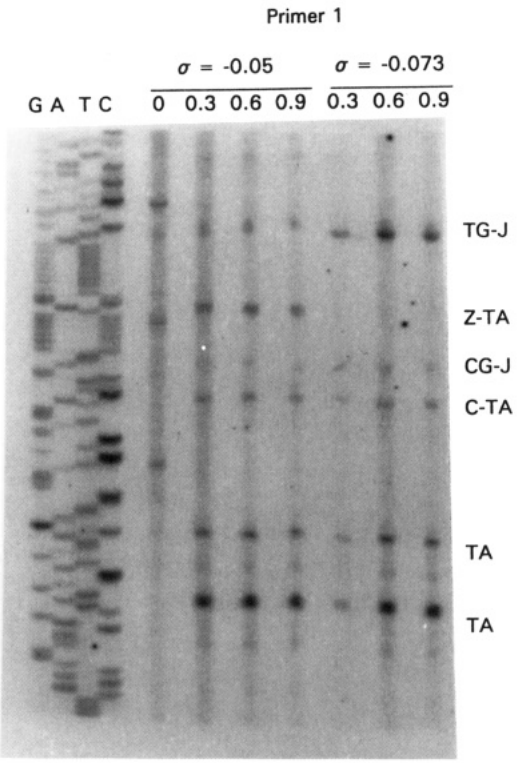

FIGURE 4: Linear amplified primer extension assay on psoralen-treated pZ3A using primer 1. Conditions for this experiment are as described in the legend to Figure 3. Primer extension products utilizing primer 1, which detects photoproducts in the top strand, are shown on the right. The sample lane labeled 0 is a primer extension on an untreated *Pvu*II fragment. Primer extension patterns for naturally supercoiled pZ3A (σ = –0.05) and highly supercoiled DNA (σ = –0.073) treated with psoralen and 0.3, 0.6, and 0.9 kJ m^{-2} of 360-nm light are shown. The positions of the TG and CG junctions, C-TA and Z-TA, and two other 5′-TAs are indicated.

superhelical density at a defined location in a DNA molecule in living cells (Zheng et al., 1991).

DISCUSSION

We have utilized a sensitive primer extension assay for mapping sites of psoralen photobinding with base-pair resolution. As presently performed, this assay will permit detection of psoralen photoproducts in DNA of low copy number in eukaryotic extrachromosomal elements or in single-copy sequences within genomes the size of *Drosophila* (Cartwright & Kelly, 1991). Excellent signals can be obtained using as little as 10 ng of plasmid DNA at levels of psoralen photobinding around one psoralen per thousand base pairs for 10 cycles of primer extension. The assay is both more rapid and more sensitive than the Me_3-psoralen-based exoIII/photoreversal assay developed previously (Kochel & Sinden, 1988, 1989). In addition, the use of primers allows facile analysis of both strands of the DNA and does not rely on the judicious placement or fortuitous location of appropriate restriction enzyme sites in DNA for the analysis of particular regions of DNA. With internally labeled primers, linear amplification can be applied to the analysis of single-copy genes in higher eukaryotes (Becker et al., 1989; Saluz & Jost, 1989).

A torsionally tuned Z-DNA-forming sequence, containing both $(CG)_n$ and $(TG)_n$ Z-DNA-forming regions, was designed to provide a torsionally tuned probe for the level of supercoiling in DNA using the primer extension assay. Application of the primer extension assay on a $(CG)_6TA(CG)_6$ torsionally tuned Z-DNA probe (Sinden & Kochel, 1987) showed that Taq polymerase stops at the center of the probe sequence. We tested several thermophilic DNA polymerases, and all had difficulty transversing the GC-rich region. This was in spite of the elevated temperature of polymerization that should facilitate the melting of any C·G base pairs within a hairpin arm. The GC-rich torsionally tuned probe is also sensitive to compression on gels and hairpin formation within the single-stranded products of DNA sequencing reactions. Hairpin formation can lead to a strong band at the center of the palindromic $(CG)_6TA(CG)_6$ Z-DNA-forming region. The Z3 Z-forming sequence was designed to form Z-DNA at a relatively low superhelical density (σ = –0.05). There are no inherent stops in chain elongation through this sequence under the conditions of amplification described using the Stoffel fragment DNA polymerase.

As shown in Figure 5, primer extension analysis of the Z3 probe revealed a characteristic pattern of psoralen photobinding that was sensitive to the level of DNA supercoiling (and the extent of Z-DNA formation). The decrease in reactivity of the 5′-TA within the Z-DNA-forming region paralleled the fraction of the Z-DNA-forming sequence that existed as Z-DNA in the plasmid population. The Z-TA was reactive when in the B-form but not in the Z-form. Measurement of the Z-TA/C-TA ratio of DNA photobound *in vivo* will reflect the superhelical density present in the DNA of the cells at the time of psoralen photobinding. Moreover, the hyperreactivity of the 5′-AATT sequences at the B–Z junctions provides a positive signal for the presence of Z-DNA. The relative levels of photobinding at the two junctions also reflect the amount of Z-DNA formed in a DNA topoisomer population. This will provide two independent indicators of the level of existence of Z-DNA in cells. Coupled with the sensitivity of the primer extension assay, the Z3 probe should allow analysis of the level of unrestrained torsional tension in specific sites in the DNA of eukaryotic cells. Such analyses

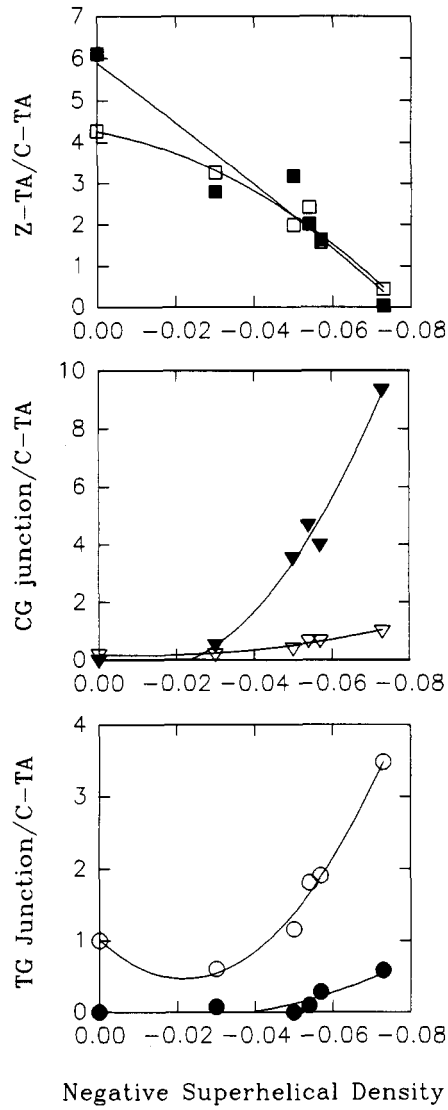

FIGURE 5: Superhelical density dependence of the psoralen reactivity of the Z-TA and B–Z junctions. The zero dose ratios of the psoralen reactivity at the Z-TA and two B–Z junctions relative to the reactivity of the C-TA are plotted as a function of superhelical density. In the top panel, □ and ■ represent the Z-TA/C-TA ratios. In the middle panel, ▽ and ▼ represent the (CG junction)/C-TA ratios. In the bottom panel, ○ and ● represent (TG junction)/C-TA ratios. The open symbols correspond to ratios determined for primer 1, which detects photoproducts in the bottom strand, whereas the filled symbols refer to primer 2, which detects photoproducts in the top strand. The data for $\sigma = 0, -0.03, -0.05, -0.054, -0.057$, and -0.073 are averages of 5, 2, 3, 2, 2, and 4 independent experiments for primer 1 and averages of 2, 2, 3, 2, 2, and 3 independent experiments for primer 2, respectively. In some, but not all, experiments a diffuse band near the position expected for a TG junction signal was observed in the relaxed DNA samples (data not shown). We are not certain of the origin of this diffuse band. It may reflect a Me_3-psoralen photoproduct at or near the *Eco*RI site flanking the $(TG)_n$ region that is present in some relaxed DNA samples. This band was also weakly present in some non-Me_3-psoralen-treated samples, which may reflect a weak polymerase pause site. Quantitation of the (TG) junction included analysis of this area, which led to the average TG-J/C-TA ratio of 1 for relaxed DNA.

are underway in our laboratory (R. W. Hoepfner & R. R. Sinden, unpublished results).

Differential Psoralen Photobinding to Opposite Strands of $(CG)_n$ or $(TG)_n$ B–Z Junctions. The application of two primers from either side of the Z3 sequence revealed a novel feature of psoralen reactivity at the B–Z junctions within this torsionally tuned probe. The two strands of the DNA had markedly different reactivities to psoralen photobinding. Although photobinding is occurring at an inverted repeat (a symmetrical) sequence (AATT)·(AATT) clearly within the structure of these B–Z junctions, an asymmetry exists between the regions of B-DNA and Z-DNA. In the upstream [$(CG)_n$ end] 5′-AATT on the 5′ side of the $(Pu{\cdot}Py)_n$ region (the 5′-B-DNA–Z-DNA-3′ junction), the top strand was hyperreactive to psoralen photobinding. The reactive region of the CG junction is indicated by the bar over the TT dinucleotide in Figure 1. The opposite strand of this region represents a 5′-Z-DNA–B-DNA-3′ junction, which was only weakly reactive to Me_3-psoralen. The reactive 5′-B-DNA–Z-DNA-3′ junction in the top strand is identical to that which was hyperreactive to Me_3-psoralen photobinding in the $(CG)_6$-$TA(CG)_6$ palindromic Z-DNA sequence (Kochel & Sinden, 1988, 1989). The 5′-Z-DNA–B-DNA-3′ junction in the top strand of the symmetrical $(CG)_6TA(CG)_6$ Z-DNA sequence was reactive. However, the reactivities of the junctions in the complementary strand of this palindromic sequence have not been investigated.

The downstream [$(TG)_n$ end] 5′-AATT on the 5′ side of the strand containing the $(CA)_8$ sequence motif was hyperreactive to psoralen photobinding. This, like the hyperreactivity on the opposite strand, represents a 5′-B-DNA–Z-DNA-3′ junction. The 5′-AATT on the opposite strand containing the $(TG)_8$ motif was only weakly reactive to psoralen. Previous analysis of the $(TG)_n$ strand of the $(TG)_6TA(TG)_6$ probe sequence did not show hyperreactivity at B–Z junctions until a very high level of supercoiling was present in the DNA (Kochel & Sinden, 1988). In our previous analysis, the 5′-AATT sequences adjacent to a $(CA)_n$ motif were not analyzed for psoralen hyperreactivity. In summary, the Z3 probe demonstrates strand-preferential Me_3-psoralen photobinding to 5′-AATT sequence in a single strand at both 5′-B-DNA–Z-DNA-3′ junctions.

The B–Z junction is the region of the DNA sequence that flanks the bases that have made the transition to Z-DNA. Analyses using CD and NMR have characterized the junction as an undetermined structure of about 3 bp that exhibits neither B-DNA nor Z-DNA characteristics (Sheardy & Winkle, 1989). Thermodynamic measurements have shown that the B–Z junctions flanking $(CG)_n$ or $(TG)_n$ Z-DNA tracts are different (O'Connor et al., 1986). Various chemical DNA probes such as osmium tetraoxide, hydroxylamine, and diethyl pyrocarbonate have revealed distorted base pairing at B–Z junctions (Nejedlý et al., 1985; Johnston & Rich, 1985; Herr, 1985; Galazka et al., 1986; Vogt et al., 1988b; Palaček et al., 1987; Kohwi-Shigematsu et al., 1987). Chloroacetaldehyde and bromoacetaldehyde have also been used to demonstrate unpaired bases in the junction (Palaček et al., 1987; Vogt et al., 1988a; Kohwi-Shigematsu et al., 1987). In many of these chemical analyses differential reactivity of the junctions was observed.

Our previous results showed that the structures of $(CG)_n$ B–Z junctions and $(TG)_n$ B–Z junctions were different with respect to Me_3-psoralen reactivity (Kochel & Sinden, 1988, 1989). The results presented here show an asymmetry to a 5′-AATT sequence that exists as a B–Z junction. The Me_3-psoralen reactivity is strongest at the 5′-B-DNA–Z-DNA-3′ junction that represents the strand containing the TT dinucleotide adjacent to the Z-DNA-forming region. This half of the junction, adjacent to the Z-DNA region, is either in a conformation more conducive to the intercalation of psoralen or in a conformation that facilitates the photobinding reaction of psoralen. The hyperreactivity of Me_3-psoralen presumably reflects the unwinding of the bases at the junctions, which

promotes intercalation. The uniqueness of the reactivity pattern for the Z3 sequence and the ability to accurately quantitate the level of Me_3-psoralen photobinding to this sequence provide a powerful torsionally tuned probe for detecting Z-DNA in living eukaryotic cells.

ACKNOWLEDGMENT

The authors thank James Lacobelle, Philip Kramer, and Jan Klysik for critically reading this manuscript and Beverly Domingue for assistance in its preparation.

REFERENCES

Axelrod, J. A., & Majors, J. (1989) An improved method for photofootprinting yeast genes *in vivo* using Taq polymerase. *Nucleic Acids Res. 17*, 171–183.

Becker, M. M. & Grossmann, G. (1992) Footprinting DNA *In Vitro*. *Methods Enzymol. 212*, 262–272.

Becker, M. M., Wang, Z., Grossmann, G., & Becherer, K. A. (1989) Genomic footprinting in mammalian cells with ultraviolet light. *Proc. Natl. Acad. Sci. U.S.A. 86*, 5315–5319.

Cartwright, I. L., & Kelly, S. E. (1991) Probing the nature of chromosomal DNA-protein contacts by *in vivo* footprinting. *Biotechniques 11*, 188–203.

Galazka, G., Palecek, E., Wells, R. D., & Klysik, J. (1986) Site-specific OsO_4 Modification of the B-Z Junctions Formed at the $(dA–dC)_{32}$ Region in Supercoiled DNA. *J. Biol. Chem. 261*, 7093–7098.

Hanahan, D. (1983) Studies on Transformation of *Escherichia coli* with Plasmids. *J. Mol. Biol. 166*, 557–580.

Herr, W. (1985) Diethyl pyrocarbonate: A chemical probe for secondary structure in negatively supercoiled DNA. *Proc. Natl. Acad. Sci. U.S.A. 82*, 8009–8013.

Johnston, B. H., & Rich, A. (1985) Chemical Probes of DNA Conformation: Detection of Z-DNA at Nucleotide Resolution. *Cell 42*, 713–724.

Kalinowski, D. P., Illenye, S., & Van-Houten, B. (1992) Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay. *Nucleic Acids Res. 20*, 3485–3494.

Klysik, J., Stirdivant, S. M., & Wells, R. D. (1982) Left Handed DNA. Cloning, characterization, and instability of inserts containing different lengths of (dC–dG) in *Escherichia coli*. *J. Biol. Chem. 257*, 10152–10158.

Kochel, T. J., & Sinden, R. R. (1988) Analysis of trimethylpsoralen photoreactivity to Z-DNA provides a general *in vivo* assay for Z-DNA: Analysis of the Hypersensitivity. *Biotechniques 6*, 532–543.

Kochel, T. J., & Sinden, R. R. (1989) Hyperreactivity of B-Z junctions to 4,5′,8-trimethylpsoralen photobinding assayed by an exonuclease III/photoreversal mapping procedure. *J. Mol. Biol. 205*, 91–102.

Kohwi-Shigematsu, T., Manes, T., & Kohwi, Y. (1987) Unusual conformational effect exerted by Z-DNA upon its neighboring sequences. *Proc. Natl. Acad. Sci. U.S.A. 84*, 2223–2227.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Nejedlý, K., Kwinkowski, M., Galazka, G., Klysik, J., & Palaček, E. (1985) Recognition of the structural distortions at the junctions between B and Z segments in negatively supercoiled DNA by osmium tetroxide. *J. Biomol. Struct. Dynam. 3*, 467–477.

O'Connor, T. R., Kang, D. S., & Wells, R. D. (1986) Thermodynamic parameters are sequence-dependent for the supercoil-induced B to Z transition in recombinant plasmids. *J. Biol. Chem. 261*, 13302–13308.

Paleček, E. (1991) Local supercoil-stabilized DNA structures. *CRC Crit. Rev. Biochem. Mol. Biol. 26*, 151–226.

Paleček, E., Boublíková, P., Galazka, G., & Klysik, J. (1987) Inhibition of restriction endonuclease cleavage due to site specific chemical modification of the B-Z junction in supercoiled DNA. *Gen. Physiol. Biophys. 6*, 327–341.

Rich, A., Nordheim, A., & Wang, A. H.-J. (1984) The Chemistry & Biology of Left-Handed Z-DNA. *Annu. Rev. Biochem. 53*, 791–846.

Saluz, H., & Jost, J.-P. (1989) A simple high-resolution procedure to study DNA methylation and *in vivo* DNA-protein interactions on a single-copy gene level in higher eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* 86, 2602–2606.

Schroth, G. P., Chou, P.-J., & Ho, P. S. (1992) Mapping Z-DNA in the human genome: Computer-aided mapping reveals a non-random distribution of potential Z-DNA-forming sequences in human genes. *J. Biol. Chem. 267*, 11846–11855.

Sheardy, R. D., & Winkle, S. A. (1989) Temperature-dependent CD and NMR studies on a synthetic oligonucleotide containing a B-Z junction at high salt. *Biochemistry 28*, 720–725.

Sinden, R. R., & Kochel, T. J. (1987) Reduced 4,5′,8-trimethylpsoralen crosslinking of left-handed Z-DNA stabilized by DNA supercoiling. *Biochemistry 26*, 1343–1350.

Sinden, R. R., & Ussery, D. W. (1992) Analysis of DNA Structure *In Vivo* Using Psoralen Photobinding: Measurement of Supercoiling, Topological Domains, and DNA-Protein Interactions. *Methods Enzymol. 212*, 319–335.

Sinden, R. R., Carlson, J. O., & Pettijohn, D. E. (1980) Torsional tension in the DNA double helix measured with trimethylpsoralen in living *E. coli* cells: Analogous measurements in insect and human cells. *Cell 21*, 773–783.

Sinden, R. R., Broyles, S. S., & Pettijohn, D. E. (1983) Perfect palindromic lac operator DNA sequence exists as a stable cruciform structure in supercoiled DNA *in vitro* but not *in vivo*. *Proc. Natl. Acad. Sci. U.S.A. 80*, 1797–1801.

Sternglanz, R., DiNardo, S., Voelkel, K. A., Nishimura, Y., Hirota, Y., Becherer, K., Zumstein, L., & Wang, J. C. (1981) Mutations in the gene coding for *Escherichia coli* DNA topoisomerase I affect transcription and transposition. *Proc. Natl. Acad. Sci. U.S.A. 78*, 2747–2751.

Ussery, D. U., & Sinden, R. R. (1993) Environmental influences on the *in vivo* level of intramolecular triplex DNA in *Escherichia coli*. *Biochemistry* (in press).

Ussery, D. W., Hoepfner, R. W., & Sinden, R. R. (1992) Probing DNA Structure with Psoralen *In Vitro*. *Methods Enzymol. 212*, 242–262.

Vogt, N., Marrot, L., Rousseau, N., Malfoy, B., & Leng, M. (1988a) Chloroacetaldehyde reacts with Z-DNA. *J. Mol. Biol. 201*, 773–776.

Vogt, N., Rousseau, N., Leng, M., & Malfoy, B. (1988b) A study of the B-Z transition of the AC-rich region of the repeat unit of a satellite DNA from cebus by measuring chemical probes. *J. Biol. Chem. 263*, 11826–11832.

Wells, R. D., Collier, D. A., Hanvey, J. C., Shimizu, M., & Wohlrab, F. (1988) The chemistry and biology of unusual DNA structures adopted by oligopurine·oligopyrimidine sequences. *FASEB J. 2*, 2939–2949.

Zheng, G., Kochel, T., Hoepfner, R. W., Timmons, S. E., & Sinden, R. R. (1991) Torsionally Tuned Cruciform and Z-DNA Probes for Measuring Unrestrained Supercoiling at Specific Sites in DNA of Living Cells. *J. Mol. Biol. 221*, 107–129.