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# Reduced 4,5′,8-Trimethylpsoralen Cross-Linking of Left-Handed Z-DNA Stabilized by DNA Supercoiling†

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ABSTRACT: Z-DNA-forming sequences, $(GT)_{21}$, $(GT)_{12}ATGT$, and $(CG)_6TA(CG)_6$, were cloned into plasmids. These sequences formed left-handed Z-DNA conformations under torsional tension from negative supercoiling of DNA. 4,5′,8-Trimethylpsoralen, on absorption of 360-nm light, forms monoadducts and interstrand cross-links in DNA that exists in the B-helical conformation. Trimethylpsoralen cross-links were introduced into the potential Z-DNA-forming sequences in relaxed DNA when these sequences existed as B-form DNA. In supercoiled DNA when these sequences existed in the Z conformation, the rate of cross-linking was greatly reduced, and trimethylpsoralen did not form monoadducts appreciably to Z-DNA. As an internal control in these experiments, the rates of cross-linking of the Z-DNA-forming sequences were measured relative to that of an adjacent, cloned sequence that could not adopt a Z conformation. The initial relative rates of cross-linking to Z-DNA-forming sequences were dependent on the superhelical density of the DNA, and the rates were ultimately reduced by factors of 10–15 for Z-DNA in highly supercoiled plasmids. This differential rate of cross-linking provides a novel assay for Z-DNA. Initial application of this assay in vivo suggests that a substantial fraction of $(CG)_6TA(CG)_6$, which existed as Z-DNA in plasmid molecules purified from cells, existed in the B conformation in vivo.

Psoralen derivatives have been widely used as probes of DNA and RNA structure [see Cimino et al. (1985)]. Psoralens intercalate into double-stranded DNA and form monoadducts and interstrand cross-links to DNA on absorption of 360-nm light. The rate of 4,5′,8-trimethylpsoralen ($Me_3$-psoralen)[1] photobinding to DNA is proportional to the level of unrestrained superhelical tension in DNA (Sinden et al., 1980). The rate of $Me_3$-psoralen photobinding to naturally supercoiled

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[1] Abbreviations: $Me_3$-psoralen, 4,5′,8-trimethylpsoralen; bp, base pair(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; kbp, kilobase pair(s).

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DNA is twice that observed for photobinding to relaxed DNA. Although this difference is not large, it has been used successfully to probe for unrestrained torsional tension in living cells (Sinden et al., 1980; Sinden & Pettijohn, 1981, 1982). Unrestrained superhelical tension is known to introduce a number of alternate helical conformations in DNA, including cruciforms (Mizuuchi et al., 1982; Sinden et al., 1983), left-handed Z-DNA (Singleton et al., 1982; Haniford & Pulleyblank, 1983a), and unusual structures within runs of homopurine/homopyrimidine sequences (Pulleyblank et al., 1985). DNA that exists in the B conformation in cruciform arms can be cross-linked with $Me_3$-psoralen, thus providing an assay for cruciform formation (Sinden et al., 1983). It is not clear, however, that $Me_3$-psoralen would cross-link other alternate helical forms of DNA that are stabilized by torsional tension in DNA. $Me_3$-psoralen cross-linking of the B form of DNA is facilitated by the near-precise alignment of the photoreactive 3,4-pyrone and 4′,5′-furan double bonds of the intercalated psoralen and the 5,6 double bond of pyrimidine bases in opposite strands (Cole, 1970). From analysis of the structure of Z-DNA (Wang et al., 1979), it appears that, if psoralen even intercalates into Z-DNA, the bond angles and distances between the 5,6 double bond of pyrimidines and the reactive bonds of psoralen would not be conducive to cross-link formation. Here we show that $Me_3$-psoralen cross-links regions of potential Z-DNA when they exist in the B-helical conformation but that cross-links are formed poorly or not at all when these sequences exist in the Z-helical form. This differential rate of cross-linking provides a novel assay for Z-DNA and should be useful as a sensitive measure of unrestrained torsional tension in DNA in vivo.

## Materials and Methods

*Plasmids, Cloning, and Bacterial Strains.* The 50 bp $(GT)_{21}$ *Eco*RI fragment [AATTCC$(GT)_{21}$GG] was made from poly[d(GT)·d(AC)] (P-L Biochemicals) by treatment with DNase I and S1 nuclease and subsequent ligation of *Eco*RI linkers. The 72 bp "control" fragment consisted of the 64 bp *Hae*III fragment of pBR322 cloned with *Eco*RI linkers. These fragments were cloned contiguously into plasmid pUR222 (≈2700 bp) (Boerhinger Mannheim Inc.) and designated pUR222ZH3. $(GT)_{12}$ATGT was constructed from ligation of two d$(GT)_5$d$(AC)_5$ polymers (P-L Biochemicals) and one 5′GTGTATGT polymer. This was cloned into the *Sma*I site of pUC8 (plasmid pUCTA-1, 2693 bp). AATT$(CG)_6$TA-$(CG)_6$ and AATTCGATGGGTACCATCGG *Eco*RI fragments were manually synthesized by using phosphite triester chemistry according to the procedure described by Caruthers (1985) as modified by Hagerman (1985) and cloned contiguously into the *Eco*RI site of pUC8 (plasmid pCGTA-C, 2715 bp). All sequences were determined by Maxam–Gilbert sequencing (Maxam & Gilbert, 1980). Plasmids were cloned into *Escherichia coli* strains HB101 and JTT1. The *recA* strain of JTT1 was obtained from K. Drlica (Pruss et al., 1982). Plasmid purification and preparation of various topological isomers were as described previously (Sinden et al., 1980). Superhelical densities were determined from two-dimensional agarose gel analysis.

*Gel Electrophoresis.* Denaturing polyacrylamide–urea gels used to quantitate DNA cross-linking were in TBE buffer (100 mM Tris–boric acid and 1 mM EDTA, pH 8.3). Two-dimensional agarose gel electrophoresis was used to determine the superhelical density at which Z-DNA formed in plasmid DNA and was basically the procedure described by Haniford and Pulleyblank (1983a) using a 22 × 22 × 0.45 cm gel. Two-dimensional gels were electrophoresed at room temperature for 24 h at 80 V (3.6 V/cm) in TAE buffer (40 mM Tris, 25 mM sodium acetate, and 1 mM EDTA, pH 8.3) in the first dimension. The second dimension (run 90° to the first) was run 24 h, 80 V in the presence of 30 μg/mL chloroquine. Buffer was recirculated during electrophoresis.

*4,5′,8-Trimethylpsoralen Photobinding and Cross-Link Analysis.* 4,5′,8-Trimethylpsoralen ($Me_3$-psoralen), [$^3$H]-$Me_3$-psoralen, and photobinding conditions have been described in detail previously (Sinden et al., 1980). Briefly, DNA samples (50 μg/mL) in a saturated solution of $Me_3$-psoralen (≈0.6 μg/mL) in TEN buffer (10 mM Tris, 50 mM NaCl, and 1 mM EDTA, pH 7.6) were irradiated at 4 °C at an incident intensity of 1.2 kJ·m$^{-2}$·min$^{-1}$ using two General Electric BLB20 bulbs. Additional $Me_3$-psoralen was added at 6 kJ$^{-2}$ intervals. For cross-linking in *E. coli* JTT1 and RS2, strains were permeabilized by EDTA treatment as described previously (Sinden et al., 1983). For analysis of the extent of cross-linking, supercoiled DNAs were reisolated from CsCl–ethidium bromide gradients to avoid contamination from nicked molecules present in plasmid preparations. Following cross-linking and repurification, supercoiled plasmid DNAs were cut exhaustively with an excess of *Eco*RI to liberate the potential Z-DNA and the "control", B-DNA fragments. Samples were then treated with alkaline phosphatase, end labeled with [$^{32}$P]ATP as described previously (Sinden & Pettijohn, 1984), and run on denaturing polyacrylamide gels.

The rationale for the quantitation of the percent of cross-links in DNA is that individual strands of a restriction fragment separate and migrate at a unique position in denaturing polyacrylamide gels whereas covalently cross-linked strands run at a different position. We have found that the migration position of the cross-linked band relative to the individual single strands varies in gels containing different acrylamide concentrations. We have also observed that for DNA strands <100 bp the cross-linked band migrates more slowly than the individual strands, as may seem intuitively obvious. Above approximately 100 bp, however, the cross-linked DNA fragment runs faster than the individual single strands (as shown in Figure 1A). This migration behavior was consistent for both denaturing urea–polyacrylamide gels and denaturing formamide–polyacrylamide gels. The average number of cross-links per DNA fragment, $F_B$ or $F_Z$ equals $-(1 - \ln X)$, where $X$ = the fraction of the total radioactivity of a DNA fragment that migrates as cross-linked DNA. This calculation assumes a Poisson distribution of cross-links. For DNA fragments containing a single TA preferential cross-link site, the "percent fragment cross-linked", rather than $F_B$ or $F_Z$ is presented. Densitometric analyses of autoradiograms were done with an EC densitometer (E.C. Apparatus Corp., St. Petersburg, FL) equipped with a 3390 Hewlett Packard integrator.

## Results

*Rates of Cross-Linking of $(GT)_{21}$ in the B and Z Conformations.* Trimethylpsoralen cross-linking to the 50 bp $(GT)_{21}$ *Eco*RI fragment in the B- or Z-DNA conformation was measured on denaturing urea–polyacrylamide gels as described under Materials and Methods. To compare the rate of cross-linking to $(GT)_{21}$ in the B or Z form, cross-linking was performed on three different DNA plasmid populations: relaxed DNA ($\sigma = 0$); naturally supercoiled DNA ($\sigma = -0.058$); and highly supercoiled DNA ($\sigma \simeq -0.12$). In relaxed DNA, the $(GT)_{21}$ sequence existed in the B form, while in the naturally and highly supercoiled DNAs $(GT)_{21}$ existed as Z-DNA as determined by two-dimensional agarose gel electrophoresis (data not shown). The $(GT)_{21}$ sequence formed Z-DNA at topological isomer number 13, or $\sigma = -0.046$. The highly

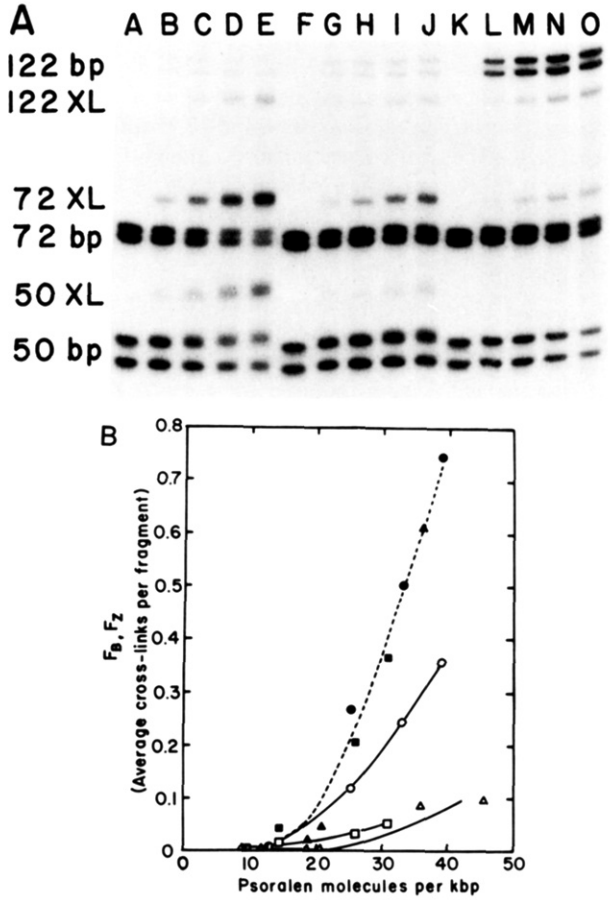


FIGURE 1: [$^3$H]$Me_3$-psoralen cross-linking of $(GT)_{21}$ in the B- or Z-helical conformation. pUR222ZH3 was cross-linked, repurified from CsCl–ethidium bromide gradients to remove any nicked DNAs, cut exhaustively with *Eco*RI, end labeled with $^{32}$P, and analyzed on urea–polyacrylamide gels. (A) 7% polyacrylamide–urea gel showing resolution and cross-linking of the 50 bp $(GT)_{21}$ and the 72 bp control *Eco*RI fragments. Lanes A, F, and K, non-cross-linked samples. Lanes B–E, relaxed DNA, $\sigma = 0$, treated with $Me_3$-psoralen and with doses of 12, 24, 36, and 48 kJ·m$^{-2}$ 360-nm light, respectively. Lanes G–J, naturally supercoiled DNA, $\sigma = -0.058$, with 6, 12, 18, 24 kJ·m$^{-2}$ 360-nm light, respectively. Lanes L–O, $\sigma = -0.12$, with 3, 6, 9, and 12 kJ·m$^{-2}$ light, respectively. Migration positions of 50, 72, and 122 bp and of cross-linked 50, 72, and 122 bp bands (50 XL, 72 XL, and 122 XL) are indicated. Doublets for non-cross-linked bands represent separate DNA strands. (B) This figure shows the average cross-links per fragment as a function of $Me_3$-psoralen molecules per kilobase pair. $F_B$ and $F_Z$ represent average cross-links per fragment (72 bp, B fragment; or 50 bp, Z fragment, respectively) calculated as described under Materials and Methods. Psoralen molecules per kilobase pair were calculated from specific activity measurements of [$^3$H]$Me_3$-psoralen incorporation. (○) $F_Z$; (●) $F_B$ for relaxed DNA. (□) $F_Z$; (■) $F_B$ for naturally supercoiled DNA. (△) $F_Z$; (▲) $F_B$ for highly supercoiled DNA.

supercoiled topological isomer preparation provided a sample in which $(GT)_{21}$ was maintained extensively in the *Z* conformation.

Figure 1A shows that cross-links were introduced into the 72 bp control fragment at all superhelical densities. This sequence should exist in the B conformation at all superhelical densities. Lanes B–E, G–J, and L–O correspond to sets of four increasing cross-linking doses for the relaxed, natural, and highly supercoiled DNAs, respectively. The levels of cross-linking in these three sets of samples were not equivalent, and data are normalized to cross-links per kilobase pair in Figure 1B. Cross-links were introduced into the 50 bp $(GT)_{21}$ sequence in relaxed DNA (Figure 1A, lanes B–E) and at much lower levels in a naturally supercoiled DNA sample (lanes G–J). At the low levels of cross-linking used for the highly supercoiled DNA sample (lanes L–O), cross-linking of the Z fragment was not detectable. At very high levels of $Me_3$-psoralen, photobinding cross-linking of the Z fragment was observed in the highly supercoiled DNA sample. Figure 1B shows quantitation of the above and other results plotted as $F_B$ or $F_Z$, the average number of cross-links per B or Z fragment, respectively, as a function of the number of psoralen molecules per kilobase pair. Although rates of photobinding are dependent on superhelical density (Sinden et al., 1980), Figure 1B shows that the rate of cross-linking of the 72 bp B-DNA fragment ($F_B$) (plotted as a function of photobound psoralen molecules) is independent of superhelical density. However, the rate of cross-linking to $(GT)_{21}$, $F_Z$, is dependent on superhelical density. In the two DNA samples in which $(GT)_{21}$ existed as Z-DNA, the rate of cross-linking was reduced significantly compared to relaxed DNA in which $(GT)_{21}$ existed as B-DNA. These results suggest that cross-links do not form readily in $(GT)_n$ when it exists in the Z conformation.

The higher molecular weight 122 bp band in Figure 1A results from an inhibition of restriction at the center *Eco*RI site [between the $(GT)_{21}$ and 72 bp control fragment]. This is due to the presence of psoralen adducts in the DNA (Carlson et al., 1982). We have consistently observed that inhibition of cutting at the center of *Eco*RI site is most pronounced in the very highly supercoiled DNA samples. As mentioned under Discussion, in the very highly supercoiled DNA samples there appears to be preferential photobinding to DNA sequences very near the center *Eco*RI site (T. J. Kochel and R. R. Sinden, unpublished observations) which may be in or near an extended B–Z junction.

A complication with cross-link analysis of $(GT)_n$ sequences is that large numbers of total photoproducts must be introduced since this sequence is devoid of 5′TA preferential cross-link sites (Sinden & Hagerman, 1984; Gamper et al., 1984). An additional complication from high levels of photobinding is that at 30 psoralens per 1000 bp about 6.5 turns would be removed from the 2.8 kbp plasmid [assuming 28° unwinding per photobound psoralen (Wiesehahn & Hearst, 1978)], producing a change of $\sigma = +0.023$. This relaxation of superhelical tension should result in a destabilization of Z-DNA. In addition, psoralen adducts at a rate of 1 per 33 bp may begin to influence the helical structure of the DNA being measured.

*Rate of Cross-Linking to $(CG)_6TA(CG)_6$ in the B and Z Conformations.* The sequence 5′AATT$(CG)_6$TA$(CG)_6$ was synthesized to contain the 5′TA preferential cross-link site in the center of a Z-forming GC sequence (the two strands hybridize to form a 30 bp *Eco*RI fragment). By placing a cross-linkable TA in the center of a Z-forming sequence, it should be possible to measure the decrease in the rate of cross-linking of Z-DNA at low levels of $Me_3$-psoralen photobinding. The *Eco*RI fragment 5′AATTCGATGGGTACCATCGG which cannot form Z-DNA was also synthesized to provide a control B-DNA fragment of similar size (20 bp) with a single center TA sequence.

The superhelical density at which transitions between B- and Z-DNA occur can be determined from two-dimensional agarose gel electrophoresis. Analysis of the B to Z transition using two-dimensional gels has been described in detail (Haniford & Pulleyblank, 1983a; Peck & Wang, 1983). Briefly, the formation of left-handed Z-DNA by a right-handed B-DNA sequence within a closed topological domain, such as a negatively supercoiled plasmid molecule, results in the relaxation of superhelical turns in DNA. Z-DNA formations result in a decrease in helical turns, *T*, in plasmid DNA, with

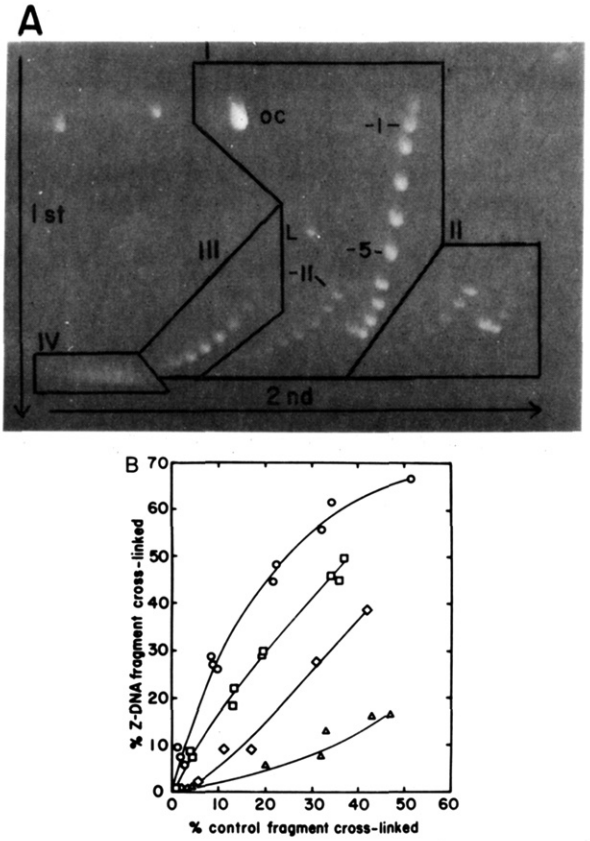


FIGURE 2: Cross-link analysis of $(CG)_6TA(CG)_6$ in supercoiled DNAs. (Panel A) A single two-dimensional gel containing four topological isomer populations of pCGTA-C. Box I shows a mixture of topological isomer numbers 0 to −18. Z-DNA forms at isomer number −11 as described in the text. In topological isomers 0 to −10, $(CG)_6TA(CG)_6$ exists as B-DNA, and in topological isomers <−11, the sequence exists as Z-DNA. Positions of open circular (nicked) plasmid, OC, and that of linear DNA, L, are indicated as are isomer numbers −1, −5, and −11. Box II, topological isomer population with $\sigma = -0.036$ in which Z-DNA exists in about half the DNA population. In box III, $\sigma = -0.055$, and box IV, $\sigma = -0.087$ in which Z-DNA exists in all DNA topoisomers. (Panel B) Percent cross-linking of AATT-$(CG)_6TA(CG)_6$ plotted relative to the percent cross-linking of the 20 bp control fragment. The experiment was performed essentially as described for Figure 1, but samples were electrophoresed on a 15% polyacrylamide–urea gel. Samples were in general treated with $Me_3$-psoralen and between 1.2 and 12 kJ·m$^{-2}$ 360-nm light for data points shown. (○) Relaxed DNA sample; (□) $\sigma = -0.036$ (shown in box II); (◇) $\sigma = -0.055$ (box III); (△) $\sigma = -0.087$ (box IV).

no change in the linking number, $L$. This results in a decrease in negative superhelical turns in DNA or the writhing number, $W$ (since $L = T + W$). The relaxation of superhelical turns in DNA results in a decreased electrophoretic mobility in the first dimension of the agarose gel. The second dimension is run at 90° in the presence of sufficient chloroquine to introduce positive supercoils into topological isomers $L - L_0 > -20$ (where $L_0$ is the linking number of relaxed DNA). This converts the Z-DNA sequence back to B-DNA and allows resolution of the faster migrating (more negatively supercoiled) topoisomers on the basis of $L - L_0$.

Figure 2A shows a two-dimensional gel containing four different topological isomer populations of pGCTA-C DNA. To facilitate analysis, boxes are drawn to distinguish separate DNA samples. Box I contains a distribution of topological isomers from isomer numbers 0 to −18, where the topological isomer number equals $L - L_0$. Boxes II, III, and IV show distributions of pCGTA-C with increasing superhelical density (see figure legend). Formation of Z-DNA from B-DNA for the 26 bp alternating pyrimidine/purine tract should remove about 4.7 superhelical turns from pCGTA-C. This assumes a helical repeat of 10.5 for B-DNA and 11.6 for the $(CG)_n$ Z-DNA sequence (Peck & Wang, 1983). In the first dimension, topological isomer number −11 migrated between the positions for topological isomers −6 and −7, a difference of 4.5 (see box I). The reduced electrophoretic mobility is diagnostic for the B to Z transition occurring at $\sigma = -0.040$. Consequently, $(CG)_6TA(CG)_6$ exists as Z-DNA at isomer numbers ≤−11. This is a slightly higher superhelical density for Z-DNA formation in $(CG_6TA(CG)_6$ than required for $(CG)_{12}$ (Peck & Wang, 1983) although different buffer and agarose concentrations were used. Apparently, the introduction of TA in the center of the CG stretch has not dramatically increased the superhelical density required for Z-DNA formation of $(CG)_6TA(CG)_6$ compared to a pure $(CG)_n$ sequence.

For the supercoiled plasmid populations shown in Figure 2A, the percent cross-linking of the 30 bp AATT$(CG)_6$TA-$(CG)_6$ Z-forming sequence is plotted relative to the percent cross-linking of the 20 bp control fragment in Figure 2B. The percent cross-linking is plotted rather than $F_Z$ or $F_B$ since there is essentially only one preferential cross-link site in these molecules. From Figure 1, it was evident that the extent of cross-linking to a B-DNA fragment as a function of $Me_3$-psoralens per kilobase pair was independent of superhelical density. Thus, the use of a neighboring non-Z-DNA-forming fragment provides an "internal control" with which to measure *relative rates of cross-linking* of the Z-DNA fragment. The cross-linkable TA sequences in Z-DNA and control *Eco*RI fragments are only 25 bp apart. As the superhelical density of pCGTA-C increases, there is a concomitant decrease in the rate of cross-linking of the TA in the Z-DNA-forming sequence relative to that in the control sequence. The initial relative rate (i.e., initial slope) of cross-linking the Z-DNA fragment in the highly supercoiled sample is more than 10 times lower than that in the relaxed DNA sample. Compared to the initial relative rate observed for relaxed DNA, the initial relative rates of cross-linking to DNA samples of $\sigma = -0.036$, −0.055, and −0.087 were reduced by factors of 0.5, 0.2, and 0.07, respectively. The superhelical density dependence of the initial relative rate of cross-linking to $(CG)_6TA(CG)_6$ is shown in Figure 3. The data points are determined from the initial slopes shown in Figure 2B and from analyses of other topological isomer populations of pCGTA-C (data not shown). There is a somewhat sigmoidal decrease in the initial relative rate of cross-linking as negative superhelical tension increases. A decrease of 50% in the relative rate of cross-linking is observed at $\sigma = -0.04$ which is the superhelical density at which Z-DNA forms in pCGTA-C.

Cross-linking analysis of $(CG)_6TA(CG)_6$ can be performed at low levels of photobinding. At 30% cross-linking of this 30 bp fragment in relaxed DNA, we calculated there were about 5 psoralens per kilobase pair or 14 per plasmid. This was only sufficient to unwind about one superhelical turn. At the highest levels of cross-linking in Figure 2B, about four superhelical turns could have been removed. An additional advantage of the $(CG)_6TA(CG)_6$ sequence [compared to $(GT)_{21}$] was that there was less inhibition of restriction at the center *Eco*RI site. This is probably due in part to the inability of $Me_3$-psoralen to photobind $(CG)_n$ sequences and the very reduced photobinding to 5′AT sequences compared to the 5′TA cross-linkable sequence [see Sinden & Hagerman (1984)]. In the experiments shown in Figure 2B, less than 5–10% of the *Eco*RI fragments ever appeared as a 50 bp fragment following restriction. The percent cross-linking of the 50 bp fragment was approximately equal to the sum of the percent cross-

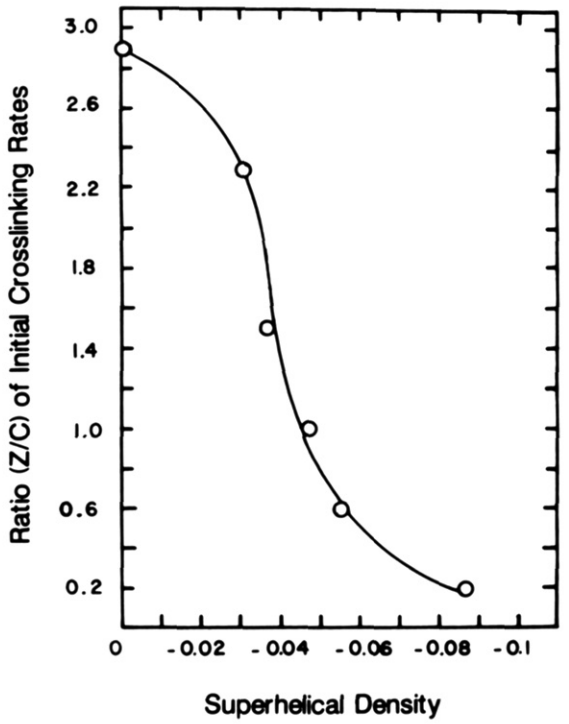


FIGURE 3: Relationship between the initial relative rate of cross-linking to $(CG)_6TA(CG)_6$ and superhelical density. The initial relative rates of cross-linking to the Z-DNA forming $(CG)_6TA(CG)_6$, relative to cross-linking the control 20 bp fragments, were determined from the initial slopes, in the linear range, from Figure 2B. Additional relative rates were determined from pCGTA-C populations with $\sigma = -0.03$ and $-0.047$ (data not shown). These are plotted as the percent cross-linking of the 30 bp fragment/percent cross-linking of the 20 bp fragment. These ratios are plotted as a function of superhelical density which was determined from two-dimensional agarose gel electrophoresis as in Figure 2A. From measurement of the initial relative rate of cross-linking, the corresponding superhelical density can be determined.

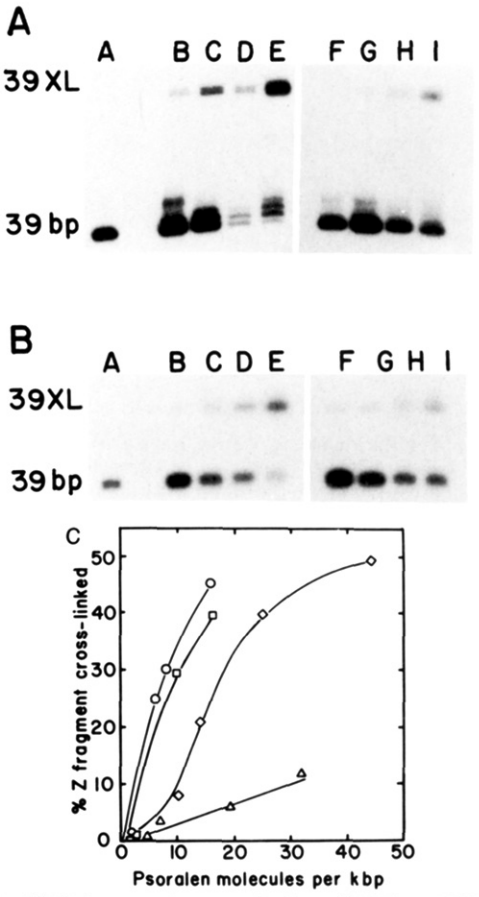


FIGURE 4: [$^3$H]$Me_3$-psoralen cross-linking of $(GT)_{12}ATGT$. pUC-TA-1 was cross-linked, repurified, and cut with *Bam*HI and *Eco*RI to liberate a 39 bp fragment which was analyzed on a 20% polyacrylamide–urea gel. (A) Samples selectively labeled at the *Eco*RI end to show the $(GT)_{12}ATGT$ strand. (B) Samples labeled at the *Bam*HI end to show the $ACAT(AC)_{12}$ strand. For (A) and (B): (lane A) non-cross-linked 39 bp *Eco*RI–*Bam*HI fragment; (lanes B–E) relaxed DNA treated with [$^3$H]$Me_3$-psoralen and 1.8, 3.6, 9, and 18 kJ·m$^{-2}$ 360-nm light, respectively; (lanes F–J) $\sigma \simeq -0.12$, treated with [$^3$H]$Me_3$-psoralen and doses of 1.2, 2.4, 6, and 12 kJ·m$^{-2}$ light. Migration positions of the 39 bp *Bam*HI–*Eco*RI fragment and of the cross-linked band, 39 XL, are indicated. (C) Quantitative densitometric analysis of the percent cross-linking to $(GT)_{12}ATGT$. (○) Relaxed DNA; (□) naturally supercoiled DNA, $\sigma = 0.057$; (◇) $\sigma = -0.08$; (△) highly supercoiled DNA, $\sigma \simeq -0.12$.

linking of the individual fragments, suggesting the 50 bp fragment did not represent an unusual or unique population of the sample. It is reasonable that a percent of the 30 bp fragment approximately equal to that not restricted by *Eco*RI at the center site may not be cut at the *Eco*RI site adjacent to pUC8 sequences. This fraction will not be detected in our analysis. Since this fraction represents less than a few percent, it would not significantly change the results presented.

*Triethylpsoralen Cross-Linking of $(GT)_{12}ATGT$ in the B or Z Conformation.* $(GT)_{12}ATGT$ was constructed to contain a TA preferential cross-link site near the end of a potential Z-DNA-forming sequence. Control experiments showed that this sequence formed Z-DNA at $\sigma = -0.048$. Figure 4 shows results of an experiment similar to that described in Figure 1 for the $(GT)_{21}$ sequence. $(GT)_{12}ATGT$ was blunt-end cloned into the *Sma*I site of pUC8 and is therefore flanked by *Eco*RI and *Bam*HI sites which, on restriction, will produce a 39 bp fragment. Cross-linking of the 39 bp *Eco*RI–*Bam*HI fragment is shown in Figure 4A,B. The samples shown in Figure 4A were selectively labeled at the *Eco*RI end (by *Eco*RI restriction, end labeling, and subsequent *Bam*HI restriction), and only the $(GT)_{12}ATGT$ strand is being detected. Figure 4B shows the $ACAT(AC)_{12}$ strand, selectively labeled at the *Bam*HI end. The rates of cross-linking of the 39 bp fragment primarily reflect cross-linking at the TA in $(GT)_{12}ATGT$ since this Z-forming sequence contains the only 5′TA in the *Eco*RI–*Bam*HI fragment. As shown in Figure 4C, cross-links form readily in relaxed and naturally supercoiled DNA ($\sigma = -0.057$). Cross-links form at an intermediate rate in DNA with $\sigma = -0.08$ and at a much reduced rate in the highly supercoiled DNA sample ($\sigma \simeq -0.12$). It is evident that the initial rates of cross-linking (i.e., initial slopes) decrease as a function of increasing superhelical density. The initial rate of cross-linking to the *Eco*RI–*Bam*HI fragment in relaxed DNA is about a factor of 15 higher than that in highly supercoiled DNA in which $(GT)_{12}ATGT$ should be maintained in the Z conformation. In the naturally purified sample ($\sigma = -0.057$), two-dimensional gel analysis showed that Z-DNA existed in >90% of the topological isomers. However, the rate of cross-linking of the Z-DNA sequence in naturally purified DNA was reduced only slightly compared to that for the relaxed DNA sample. One interpretation of this result is that the TA preferential cross-link site which is near the end of a potential Z-DNA sequence was substantially in more of a B-DNA than Z-DNA conformation in naturally supercoiled DNAs and that unusually high levels of supercoiling were needed to maintain the TA in a Z-DNA conformation.

*4,5′,8-Trimethylpsoralen Does Not Form Monoadducts Appreciably with Z-DNA.* Since the rate of $Me_3$-psoralen cross-linking to Z-DNA is reduced compared to that for B-DNA, it was of interest to determine if $Me_3$-psoralen formed

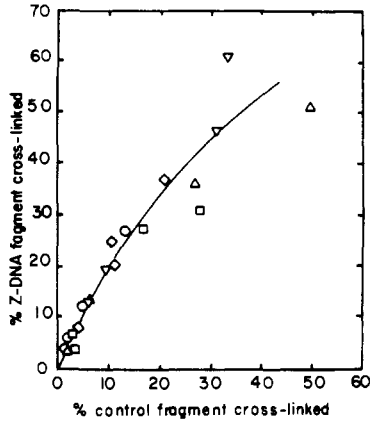


FIGURE 5: Application of the $Me_3$-psoralen cross-linking assay for Z-DNA in vivo. pCGTA-C in JTT1 was cross-linked in vivo, and the percent cross-linking to the 30 bp Z-forming fragment and the 20 bp control fragments was determined. For in vivo cross-linking, JTT1 was grown to late log phase and amplified with 100 μg/mL chloramphenicol for 20 h (experiments A–D) and, in one experiment, 5 h (experiment E). Cells were permeabilized by treatment with EDTA and quickly chilled to 0–4 °C in a dry ice EtOH bath, and $Me_3$-psoralen was photobound to DNA in cells. Supercoiled DNAs were purified from CsCl–ethidium bromide gradients, and the extent of cross-linking was analyzed. pCGTA-C in JTT1 cross-linked in vivo: experiment A (○); experiment B (□); experiment C (◇); experiment D (▽); experiment E (△). Although the superhelical density of DNA purified from different cultures of JTT1 varied slightly, the superhelical density of all samples was $\sigma = -0.058 \pm 0.003$. By use of the standard curve in Figure 3, the superhelical density suggested from the initial slope was $\sigma = -0.035$.

monoadducts with Z-DNA. Psoralen adducts increase the length of double-strand DNA by the equivalent of about 1 bp per adduct (Sinden & Hagerman, 1984). Monoadducts to a single strand of DNA retard migration in 20% urea–polyacrylamide gels (Gamper et al., 1984). Formation of both $Me_3$-psoralen cross-links and monoadducts can be seen in the 39 bp *Eco*RI–*Bam*HI fragment shown in Figure 4A, lanes B–E. Single-strand molecules containing one, two, and three monoadducts appear as discrete bands just above the position for the non-cross-linked sample. In a highly supercoiled DNA sample, few cross-links are formed, and monoadducts are not significantly detected (lanes F–I). Some samples shown in Figure 4A contained a contaminating (possibly RNA) diffuse band observed at about the position of 42–43 bp. Since $Me_3$-psoralen preferentially binds to thymine, fewer psoralen monoadducts would be expected in the $ACAT(AC)_{12}$ strand. As shown in Figure 4B, monoadducts were not observed in this strand, even in the sample shown in Figure 4A, lane E, which contained up to three monoadducts in the opposite strand (lane I). These results suggest that psoralen does not form monoadducts appreciably with Z-DNA.

*Demonstration of the Applicability of the Cross-Linking Assay for Z-DNA in Vivo.* pCGTA-C DNA was cross-linked in vivo in chloramphenicol-amplified JTT1 cells. Plasmid DNA purified from JTT1 in these experiments had superhelical densities of $\sigma = -0.058$ to $-0.062$ which on two-dimensional gel electrophoresis showed all topological isomers containing Z-DNA. The rate of cross-linking of the 30 bp Z-forming sequence in vivo relative to the rate of cross-linking the 20 bp control, B-DNA sequence is shown in Figure 5 for five separate experiments. Data for pCGTA-C in JTT1 were very similar to the relative rate expected for DNA of $\sigma = -0.036$ (shown in Figure 2B) in which about half of the topological isomers contained Z-DNA as determined from two-dimensional gel analysis. The actual superhelical density suggested from the initial relative rate of cross-linking and the analysis shown in Figure 3 was $\sigma = -0.035$. The relative rates of cross-linking of the Z-forming sequence in vivo were significantly higher than that for in vitro cross-linking of purified DNA of the same superhelical density . These results are consistent with the interpretation that the potential Z-forming sequence, $(CG)_6TA(CG)_6$, existed substantially in the B conformation in vivo. As mentioned under Discussion, experiments are in progress to accurately quantitate in vivo the existence of Z-DNA.

In the experiments shown in Figure 5, samples were from cells amplified for either 5 or 20 h after addition of chloramphenicol. In preliminary experiments using a modified Holmes and Quigley (1981) rapid purification procedure, we observed an increase in the linking number of DNA at 4–8 h following amplification (compared to 0 and 20 h). This was consistent with the observations of Haniford and Pulleyblank (1983b, 1985). However, in the experiment shown in Figure 5, the superhelical densities of the 5- and 20-h samples were identical. We do not know if this reflects differences due to EDTA treatment or differences in plasmid purification procedures.

## Discussion

The results shown here demonstrate that the rate of $Me_3$-psoralen cross-linking to DNA is dependent on the helical conformation of the DNA. The relative rate of cross-linking to DNA that exists in the Z-helical form is reduced by up to 10–15 times that observed for cross-linking the same sequence when it exists in the B-helical form in vitro. The initial rate of cross-linking of potential Z-DNA sequences relative to a B-DNA, control fragment is dependent on the superhelical density of the DNA. Analysis of the rate of cross-linking of a Z-forming DNA sequence relative to that of an appropriate internal control fragment that can exist only as B-DNA provides an indication of the helical configuration of potential Z-DNA sequences. This provides a novel assay for Z-DNA with which the applicability of $Me_3$-psoralen in vivo can be used to analyze the conformation of potential Z-DNA sequences in living cells.

At relatively low levels of photobinding to $(GT)_{21}$, no cross-linking was observed in highly supercoiled DNA ($\sigma \simeq -0.12$) when $(GT)_{21}$ was clearly maintained as Z-DNA. At levels of more than one psoralen per 50 bp, the $(GT)_{21}$ sequence was cross-linked in this highly supercoiled DNA sample. Levels of cross-linking to $(GT)_{21}$ in naturally supercoiled DNA, in which $(GT)_{21}$ existed as Z-DNA in >90% of the topoisomers, were reduced compared to that in relaxed DNA. Cross-linking of $(CG)_6TA(CG)_6$ was reduced (and dependent on superhelical density) in topological isomer populations in which the $(CG)_6TA(CG)_6$ sequence was maintained completely in the Z conformation. At the levels of photobinding used for $(CG)_6TA(CG)_6$, the superhelical density of the plasmid populations should still be significantly above that required to maintain Z-DNA. If $Me_3$-psoralen molecules were not influencing the formation or stability of Z-DNA, the cross-linking of the Z-DNA form of $(CG)_6TA(CG)_6$ might be nonexistent. Although there is some evidence for binding of intercalating drugs to forms of left-handed DNA (van de Sande & Jovin, 1982), it appears that ethidium bromide does not stably intercalate into Z-DNA (Pohl et al., 1972; Walker et al., 1985). DNA intercalating drugs have been shown to reduce the rate of Z-DNA formation (Mirau & Kearns, 1983). As originally shown by Pohl et al. (1972), ethidium bromide drives poly[d(G-C)], which exists as Z-DNA in 4.4 M NaCl, back to the B-helical form. Reversal of salt-induced Z-DNA by intercalating drugs has been confirmed by a number of

investigators (van de Sande & Jovin, 1982; Rio & Leng, 1983; Walker et al., 1985a,b; Chaires, 1985). In the Z conformation of poly[d(G-C)] in 4.4 M NaCl, one ethidium bromide molecule reverses 3–4 bp back to B-DNA, whereas in less stable forms of Z-DNA, ethidium bromide will reverse as many as 25 bp per intercalated molecule (Walker et al., 1985). The binding of the intercalater daunomycin to poly[d(G-C)] is dependent on ionic strength. Increasing concentrations of daunomycin are required to reverse Z-DNA formed in increasing concentrations of NaCl (Chaires, 1985). Thus, in general, it appears that the more stable the Z-DNA conformation the less this structure is disrupted by intercalating drugs. Our results with psoralen intercalation and subsequent cross-linking of Z-DNA in supercoiled DNA are consistent with these observations. That is, the rates of cross-linking of $(GT)_{21}$, $(CG)_6TA(CG)_6$, and $(GT)_{12}ATGT$ in supercoiled plasmids were reduced as the superhelical density increased. Although we do not know if $Me_3$-psoralen is intercalating into Z-DNA, it is clear that monoadducts are not formed appreciably in Z-DNA. The observed cross-linking of Z-DNA may result from trapping and subsequent reaction with B-form DNA during equilibrium transitions between the B and Z conformations. By binding intercalatively, $Me_3$-psoralen molecules are probably acting to drive the Z conformation back to the B conformation. This effect is probably in addition to reversal from relaxation of supercoils due to the intercalation of $Me_3$-psoralen. It seems reasonable that the $Me_3$-psoralen-induced reversal to B-DNA should decrease as Z-DNA is stabilized by increasing torsional tension. Cross-linking of DNA adjacent to the Z-DNA region may also be favoring B-DNA formation in the potential Z-DNA regions. If Z-DNA is in an equilibrium with the B form and the B–Z junctions are mobile or dynamic, the covalent "clamping" of a flanking B-form helix by a cross-link may favor extending the B conformation through the region of Z-DNA.

It is known that B–Z junctions can move in response to superhelical density and that in highly supercoiled DNA the junctions extend beyond the end of the alternating purine/pyrimidine sequence (Johnston & Rich, 1985). Although B–Z junctions are sensitive to S1 nuclease in DNA of physiological superhelical densities (Singleton et al., 1982), it has recently been shown that the B–Z junctions contain few, if any, unpaired bases (Kang & Wells, 1985). However, in highly supercoiled DNA, $\sigma < -0.1$, there are unpaired bases near the B–Z junctions, and the junctions extend into sequences flanking the alternating purine/pyrimidine tract (Kang & Wells, 1985; Johnston & Rich, 1985). If the binding of intercalating drugs is favored by an unwound helical region as suggested by Banerjee and Sobell (1983) [see Walker et al., (1985a) for a discussion], then the photobinding of $Me_3$-psoralen in higher than physiologically supercoiled DNA may be preferential for the B–Z junction regions. This may explain our observations that in highly supercoiled DNA we see an increase in the loss of restriction at the central *Eco*RI site in pUR222HZ3 (Figure 1A). Although the inhibition of *Eco*RI restriction was much lower for plasmid pCGTA-C (<10% at $\sigma = -0.087$), we observed that the inhibition of cutting increased as a function of superhelical density for samples containing similar numbers of psoralens per kilobase (T. J. Kochel and R. R. Sinden, unpublished results). Preliminary results using the method of Becker and Wang (1984) to identify for the base pair the site of psoralen photoaddition also suggest preferential binding of $Me_3$-psoralen adjacent to the ends of the Z-DNA fragment in highly supercoiled DNAs (T. J. Kochel and R. R. Sinden, unpublished observations).

Numerous chemical and enzymatic probes for Z-DNA have been developed [see Rich et al. (1984), Johnston & Rich (1985), and Herr, (1985)]. Z-DNA assays involving antibody binding (Lafer et al., 1981) or binding of chiral cobalt phenanthroline compounds (Barton & Raphael, 1985) rely on binding to the Z conformation. It has recently been shown that antibody binding actually drives the equilibrium toward the Z-helical form (Lafer et al., 1985). Methods of selective chemical modification of Z-DNA such as carbethoxylation of the N-7 position of purines (Herr, 1985) may, following modification, favor a Z equilibrium by stabilizing the Z-DNA conformation. In many of these assays, the probes are reacting with Z-DNA. Consequently, the assays may stabilize Z-DNA or drive a B–Z equilibrium toward the Z form, resulting in an overestimation of the extent of Z-DNA. This has recently shown to be the case for Z-DNA antibody binding (Lafer et al., 1986). $Me_3$-psoralen as an intercalating drug does not preferentially react with the Z conformation. The effects of intercalation and photobinding of $Me_3$-psoralen are such that the B–Z conformation will be driven toward the B form. The reduced rate of cross-link formation to DNA in the Z form may actually reflect trapping and reaction with B-form DNA during equilibrium transitions between the B and Z conformations. Consequently, this assay may result in an underestimation of Z-DNA. It should also be pointed out that reduced binding of $Me_3$-psoralen to DNA may reflect a number of non-B-DNA helical forms, not exclusively Z-DNA. However, an important point with regards to the $Me_3$-psoralen cross-linking assay is that detection of a Z conformation (or a non-B-form helix) could *not* be due to its formation by the assay procedure. With the applicability of $Me_3$-psoralen photobinding in vivo, the differential rate of cross-linking to $(CG)_6TA(CG)_6$ should provide an assay for Z-DNA and a sensitive measure of unrestrained torsional tension in living cells.

*Initial Application of the Cross-Linking Assay for Z-DNA in Vivo.* The results shown demonstrate the applicability of the $Me_3$-psoralen cross-linking assay for Z-DNA in vivo. The results, however, are not yet conclusive as to the existence of Z-DNA in wild-type *Escherichia coli* under normal growth conditions. The results suggest a superhelical density in vivo of $\sigma = -0.035$ at which some fraction of $(CG)_6TA(CG)_6$ may exist as Z-DNA in chloramphenicol-amplified *E. coli*. However, it is possible that the relative rates of cross-linking in vivo show a slightly different relationship than observed in vitro, such that data in Figure 5 for DNA in JTT1 may actually reflect a relaxed or entirely B-DNA conformation. A more rigorous demonstration of Z-DNA would be indicated by an increase in the initial relative rates of cross-linking (to that for relaxed DNA) following relaxation of supercoils in vivo, and experiments to test this are under way. Additionally, we are analyzing the initial relative rate of cross-linking in a strain mutant in topoisomerase I from which DNA is more negatively supercoiled. Results of these experiments will be published separately.

A significant conclusion, however, from the in vivo analysis shown here is that the effective level of unrestrained torsional tension in vivo in JTT1 appears lower than the purified superhelical density $\sigma = -0.06$ at which Z-DNA clearly existed and was assayable in vitro. Although one cannot completely exclude the possibility that changes in linking number occur during purification protocols, presumably this superhelical density reflects the linking number in vivo. However, chloramphenicol-amplified cells probably do not represent a truly natural in vivo situation, and it has been suggested that am-

plification may result in an increase in superhelical density (Haniford & Pulleyblank, 1983b, 1985). We have shown previously that DNA in *E. coli* is equilibrated with unrestrained torsional tension (Sinden et al., 1980). It seems clear that the *E. coli* intracellular environment in which DNA is packaged with counterions and histone-like HU protein, which has been shown to restrain supercoils (Broyles & Pettijohn, 1986), is not as conducive to Z-DNA formation as an in vitro environment. The results presented here are in agreement with previous observations that palindromes that form cruciforms rapidly in vitro at "physiological levels of supercoiling" exist in the linear conformation in vivo (Sinden et al., 1983; Haniford & Pulleyblank, 1985; Greaves, et al., 1985). Experiments are in progress to more clearly define the effective level of unrestrained torsional tension in vivo using this assay for left-handed Z-DNA.

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