Environmental Influences on the *in Vivo* Level of Intramolecular Triplex DNA in *Escherichia coli*†

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ABSTRACT: We describe an assay for detecting intramolecular triple-stranded DNA in living cells. The assay involves quantitative analysis of the differential photobinding of 4,5′,8-trimethylpsoralen to 5′TA and 5′AT dinucleotides in a region of plasmid DNA containing the triplex-forming sequence $(GA)_7TA(GA)_7$. Psoralen photobinds to the central 5′TA within the $(GA)_7TA(GA)_7$ sequence in duplex DNA but not when the sequence exists as an intramolecular triplex structure. The reactivity of photobinding sites in regions flanking the intramolecular triplex-forming sequence, those that comprise the duplex–triplex junctions, either increase or decrease upon formation of the intramolecular triplex structure from duplex DNA. The pattern of trimethylpsoralen reactivity provides an indication of the conformation of the $(GA)_7TA(GA)_7$ sequence in plasmid DNA. The formation of the intramolecular triplex structure is dependent on both pH and negative superhelical density *in vitro*. The fraction of the $(GA)_7TA(GA)_7$ sequence that existed as an intramolecular triplex structure *in vivo* was dependent on the level of DNA supercoiling *in vivo* and changes in growth conditions which influence the intracellular pH. The Hy3 isomer was detected in living *Escherichia coli* cells.

Although a triple-stranded helix structure for synthetic homopolymers of nucleic acids was first described in 1957 (Felsenfeld et al., 1957), it was not until nearly three decades later that an intramolecular triple-stranded conformation was proposed to exist within the context of a double-stranded molecule (Lyamichev et al., 1986). The formation of intramolecular triple-stranded DNA requires a polypurine·polypyrimidine region at least 15 bp long (Lyamichev et al., 1989; Kohwi, 1989) with mirror repeat symmetry (Mirkin et al., 1987) and is favored under conditions of negative DNA supercoiling and either low pH (Lyamichev et al., 1985) or neutral pH in the presence of divalent cations (Kohwi & Kohwi-Shigematsu, 1988). To form an intramolecular triplex, the middle of the polypurine·polypyrimidine region unpairs, and then one strand folds back into the major groove, leaving the other half single-stranded (Htun & Dahlberg, 1989). The third strand pairs with Hoogsteen base pairing to the central purine strand. Detection of the unpaired single-stranded region has been the basis for several assays for triple-stranded DNA (Johnston, 1988; Kohwi & Kohwi-Shigematsu, 1988; Palecek, 1991).

Potential triple-strand-forming sequences are widespread throughout the genome of higher eukaryotes (Manor et al., 1988; Birnboim, 1978), and their location appears to be evolutionarily conserved (Wong et al., 1990). A biological role for intramolecular triplex DNA remains to be clearly established, although sequences capable of adopting this conformation have been mapped to origins of replication (Kinniburgh, 1989; Bianchi et al., 1990), suggesting they might be involved with initiation of DNA replication. In addition, potential triplex-forming regions have been shown to stop replication forks *in vitro* (Baran et al., 1991) and *in vivo* (Brinton et al., 1991). Triple strands have also been postulated to play a role in the regulation of gene expression (Postel et al., 1989), in recombination (Weinreb et al., 1990), and in the integration of human viruses into the human chromosome (Liu & Chan, 1990).

An intramolecular triplex can be formed in which the purine strand is donated (*pur*-pur-pyr) or in which the pyrimidine strand is donated (*pyr*-pur-pyr) as the third strand. In both cases, the acceptor (central) strand is the Watson–Crick paired purine strand. For both the *pur*-pur-pyr and the *pyr*-pur-pyr intramolecular triplex types two different isomers can exist. For example, Htun and Dahlberg (1989) and Glover and Pulleyblank (1990) have found that $(CT)_n$ stretches can adopt two different triplex isomers under acidic conditions, which they named Hy3 and Hy5. The Hy5 isomer, in which the 5′ end of the pyrimidine strand is donated, was found to exist in DNA with low levels of supercoiling (when incubated in pH 5 buffer *in vitro*). The Hy3 isomer, in which the 3′ end of the pyrimidine strand is donated, was shown to be the predominant isomer at higher ("natural" $\sigma \approx -0.06$) levels of supercoiling. Hr3 and Hr5 isomers, in which the 3′ and 5′ halves of the purine strand are donated, respectively, have been identified by several investigators (Kohwi & Kohwi-Shigematsu, 1988; Bernues et al., 1989, 1990; Kang & Wells, 1992). Many of the *pur*-pur-pyr intramolecular triplex structures form at neutral pH, often, but not always, in the presence of divalent cations (Kohwi & Kohwi-Shigematsu, 1988, 1993; Kang et al., 1992).

Several approaches have been applied to determine the existence of intramolecular triplexes *in vivo*. *Dam* methylation sites located within a potential triplex were found to be undermethylated *in vivo*, consistent with the presence of triple strands *in vivo* (Parniewski et al., 1990). Other methods used to address the existence of intramolecular triplex DNA *in vivo* include triplex antibody binding (Lee et al., 1989) and deletion analysis of potential triplex-forming sequences (Jaworski et al., 1989). The promoter for the *Drosophila* hsp26 gene has been shown to require $(CT)_n$ repeats (capable of forming H-DNA *in vitro*) for full activity (Glaser et al., 1990).

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However, no triplex structure was detected in $(CT)_n$ repeats in *Drosophila* nuclei, using diethylpyrocarbonate modification (Glaser et al., 1990). Two proteins were isolated which bound to the $(CT)_n$ repeats in this region (Gilmour et al., 1989). In contrast, a $(CT)_{16}$ stretch from the sea urchin histone gene was probed with OsO_4 2,2'-bipyridine *in situ* in *Escherichia coli*, and it was concluded that the Hy5 isomer is most likely to exist inside the cell (Karlovsky et al., 1990). This conformation was detected at levels of supercoiling likely to occur *in vivo*. In these experiments, cells were amplified with chloramphenicol and were then incubated in buffers at various pH levels (Karlovsky et al., 1990). The triplex conformation was detected when cells were incubated in pH 5.2 buffer (the *in vivo* pH was 5.7), but no triplex was detected at intracellular pH values greater than 6 (Karlovsky et al., 1990). Cholor-acetaldehyde modification detected an Hr3 intramolecular triplex in a $(G)_{44}$ or $(G)_{35}$ [but not $(G)_{25}$] insert in plasmids inside of *E. coli* cells (Kohwi et al., 1992). However, intramolecular triplex DNA was detected only at higher than native levels of supercoiling, following chloramphenicol amplification, which increases negative superhelicity. The sequence $(TTCCC)_{48}$ has been shown to adopt either the Hy3 or the Hr3 isomers *in vitro* (depending on ionic conditions), and the Hy3 structure formed even in linear DNA, as assayed by a single-strand-specific nuclease protection assay (Michel et al., 1992). In HeLa cells, this sequence was found to adopt predominately the Hr3 conformation *in vivo*, although small amounts of the Hy3 conformation were also detected (Michel et al., 1992).

We describe a novel assay for triple-stranded DNA that is based on differential photobinding of Me_3psoralen[1] to B-DNA, intramolecular triplex DNA, and the B-DNA–triplex junctions. This approach is applicable under a variety of conditions both in bacteria and in eukaryotes, and, unlike many of the assays described above, does not seriously compromise cell viability. The assay is based on the photobinding characteristics of 4,5',8-trimethylpsoralen. Me_3psoralen preferentially binds to 5'TA residues when they exit as duplex B-form DNA. Me_3psoralen does not appreciably bind to 5'TAs when single stranded (or in Z-DNA) (Sinden & Kochel, 1987; Kochel & Sinden, 1989). In the Z-DNA assay, certain 5'AT sequences were nonreactive in B-DNA but hyperreactive when they existed within B–Z junctions (Kochel & Sinden, 1989). Previously, we have used this approach to assay Z-DNA in living cells (Sinden & Kochel, 1987; Zheng et al., 1991). On the basis of these observations, we have designed the triplex-forming sequence $(GA)_7TA(GA)_7$, which contains a central 5'TA. This central 5'TA should photobind Me_3psoralen in B-DNA but not when it exists as a single-stranded loop at the tip of an intramolecular helix. In addition, the triplex region was engineered to contain flanking 5'TAs and minimally reactive 5'AT sequences. As shown below, the reactivity of these sites with Me_3psoralen changes in response to DNA conformation and is diagnostic for the duplex or intramolecular triplex forms of DNA.

EXPERIMENTAL PROCEDURES

Plasmids, Bacteria, and Growth Conditions. To construct plasmid pRDH4, the 36 bp insert shown in lowercase letters in Figure 1 was synthesized and cloned into the *Eco*RI site of plasmid pUC8. The insert, which can form intramolecular triplex structures, contains a 30 bp alternating (CT) repeat, in which the central C is replaced with an A. This was designed to provide a central 5'TA which is a preferred Me_3psoralen photobinding site in B-DNA (Esposito et al., 1988). The regions flanking the purine stretch were designed to contain additional Me_3psoralen photobinding sites (e.g., 5'TA and 5'AT). The Me_3psoralen photobinding sites are indicated in Figure 1 by TA_n or AT_n, where n is the location of the first nucleotide in the site.

Plasmid pRDH4 was initially transformed into DH5α strain of *E. coli*, and plasmid DNA was purified from 1 L of T-broth (Tartof & Hobbs, 1987) as described (Kochel & Sinden, 1988). pRDH4 was transformed into *recA56* derivatives of *E. coli* K-12 strains JTT1 (*topA*$^+$) and RS2 *topA10* (Sternglanz et al., 1981) for the *in vivo* experiments.

For the *in vivo* experiments, 500 mL of cells were grown in 2-L flasks with shaking for 24–36 h after inoculating the culture at A_{650} = 0.1 from an overnight culture. Cultures were grown in duplicate in either LB media (bactotryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; and 1.5 mL of 1 M NaOH per L of distilled water) or K media, which consisted of M9 buffer (1 g of NH_4Cl, 5.8 g of Na_2HPO_4, 3 g of KH_2PO_4, and distilled water to 1 L) plus (per liter) 0.03 g of $MgSO_4$, 0.07 g of $CaCl_2$, 10 g of glucose, 10 g of casamino acids, 1 μg of thiamine, and 0.05 g of uridine. Cells were photobound with Me_3psoralen as described below, and the DNA was purified as described previously (Kochel & Sinden, 1988, 1989).

Me_3psoralen Photobinding Procedure. To measure quantitatively the sites of Me_3psoralen photobinding *in vitro*, 20 μg of plasmid DNA was equilibrated in 1 mL of the appropriate buffer for 1 h at 37 °C to allow the formation of the triplex structure, and then 0.01 volumes of 4,5',8-trimethylpsoralen saturated in ethanol was added. Several different buffers were employed, including TEN buffer at pH 7.6 (TEN; 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 50 mM NaCl), pH 7 buffer (20 mM Tris-HCl, pH 7.0, 1 mM $MgCl_2$), and pH 5 buffer (20 mM Tris-acetate, pH 5.0, 1 mM magnesium acetate). After 2 min of equilibration at 4 °C, the DNA was irradiated with 360-nm light to introduce cross-links and monoadducts into the DNA. For the *in vitro* experiments, the DNA was irradiated with 0, 0.4, 0.8, and 1.2 kJ/m^2 360-nm light at an incident light intensity of 1.2 kJm^{-2} min^{-1} as described previously (Sinden & Kochel, 1987; Kochel & Sinden, 1988, 1989). Unincorporated Me_3psoralen was removed by ethanol precipitation of the DNA.

For the *in vivo* experiments, after 24–48 h of growth, cells were chilled to 4 °C, washed, and resuspended in 25 mL of ice-cold M9 buffer. Me_3psoralen (0.25 mL; saturated in ethanol) was added, and the cells were incubated 2 min at 4 °C. Aliquots of the cells were then irradiated for 2 and 5 min (2.4 and 6.0 kJ/m^2, respectively). These doses introduce a similar number of Me_3psoralen photoproducts as the conditions used for the *in vitro* analyses.

The ExoIII Me_3psoralen Mapping Assay. The level of Me_3psoralen photobinding to 5'TAs and 5'ATs within and flanking the intramolecular triplex-forming region was quantitated using the exoIII Me_3psoralen mapping procedure as described previously (Kochel & Sinden, 1988, 1989; Ussery et al., 1992). Briefly, after irradiation, the samples were cut with *Pvu*II, end-labeled with [γ-^{32}P]ATP, and digested with *Bam*HI to produce a 223 bp fragment containing the ^{32}P label at one end. This fragment was purified from a nondenaturing polyacrylamide gel and digested twice with 100 units of exoIII, photoreversed, and run on an 80-cm denaturing 5% polyacrylamide gel. The levels of photobinding to individual bases (exoIII stops) were quantitated using a Molecular Dynamics PhosphoImager.

[1] Abbreviations: Me_3psoralen, 4,5',8-trimethylpsoralen; exoIII, exonuclease III; CAA, chloroacetaldehyde.

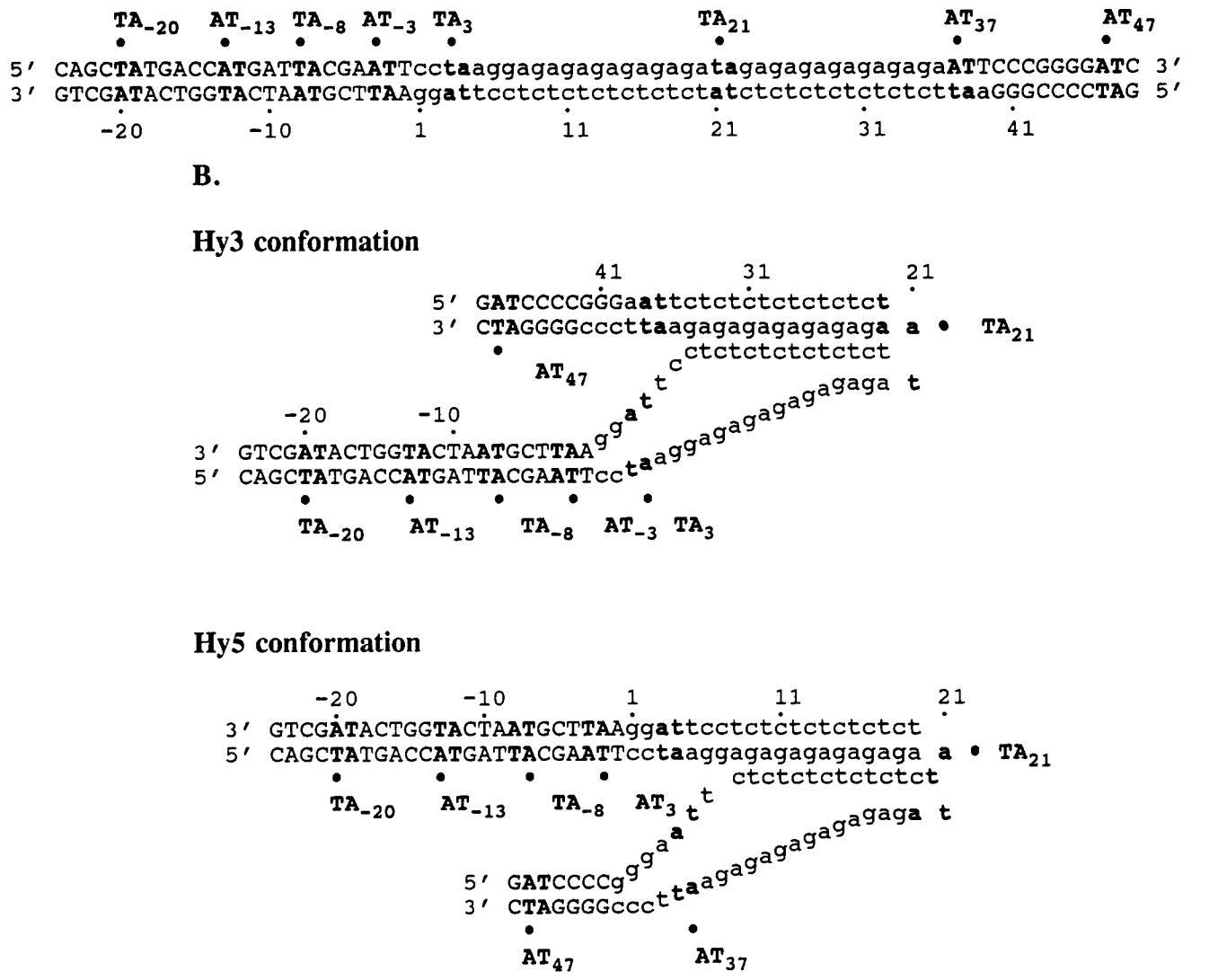

FIGURE 1: (A) Sequence of the intramolecular triplex-forming region of pRDH4. The first lowercase c in the purine-rich (top) strand of the insert was arbitrarily chosen as base number 1. The 5′TA's and 5′AT's, which are preferentially photoreactive with Me_3psoralen, are shown in bold and are numbered above the top strand. (B) The intramolecular Hy5, Hy3, and triplex isomers of pRDH4. The labeling is the same as in part A. In Hy3 and Hy5, the 3′ and 5′ ends of the polypyrimidine strand are donated as the third strand, respectively.

Chemical Modification in Vitro and in Situ. For *in vitro* chemical modification experiments, 1 μg of pRDH4 was incubated in 100 μL of the appropriate buffer for 1 h at 37 °C and then modified with either chloroacetaldehyde or OsO_4 bipyridine. Plasmid was modified with chloroacetaldehyde *in vitro* by adding 4 μL of chloroacetaldehyde (50% v/v) followed by incubation for 30 min at 37 °C. The chloroacetaldehyde was then extracted twice with 50 μL of ether and precipitated by the addition of 10 μL of 3 M potassium acetate and 300 μL of ethanol. pRDH4 was modified with OsO_4 by the addition of 2,2′-bipyridine and OsO_4 to a final concentration of 2 mM each to the 100-μL sample. Modification was allowed to proceed for 10 min at 37 °C, and then the sample was diluted with 100 μL of TEN buffer, extracted twice with ether, and then ethanol-precipitated. Modified bases were detected by a primer extension assay (Ussery et al., 1992). Briefly, 100 ng of DNA was extended with the Stoffel fragment of *Taq* polymerase (Cetus) for 10 cycles. Each cycle consisted of melting at 96 °C for 2 min, hybridization at 60 °C for 30 s, and extension for 5 min at 80 °C.

For *in situ* chemical modification experiments, cells were grown as described above, pelleted, and resuspended in 50 mL of M9 buffer containing 4 mL of chloroacetaldehyde. The cells were then shaken at 37 °C for 20 min and then pelleted and rinsed twice with M9 buffer, as described by Kohwi-Shigematsu and Kohwi (1992). Cells were modified with OsO_4 bipyridine as described by Karlovsky et al. (1990). Plasmid DNA was purified and analyzed using the primer extension assay.

RESULTS

Design and Characterization of pRDH4. Plasmid pRDH4 was designed to contain an intramolecular triplex-forming $(GA)_n$ region with a central 5′TA dinucleotide that should photobind Me_3psoralen in the B conformation. Me_3psoralen photobinding to the central TA (TA_{21}) should be ablated upon formation of an intramolecular triplex since this dinucleotide will exist within the single-stranded loop of the triplex structure. The reactivity of Me_3psoralen within the B-DNA–triplex junction region is expected to be characteristically different from that for duplex DNA since there will be a single-stranded

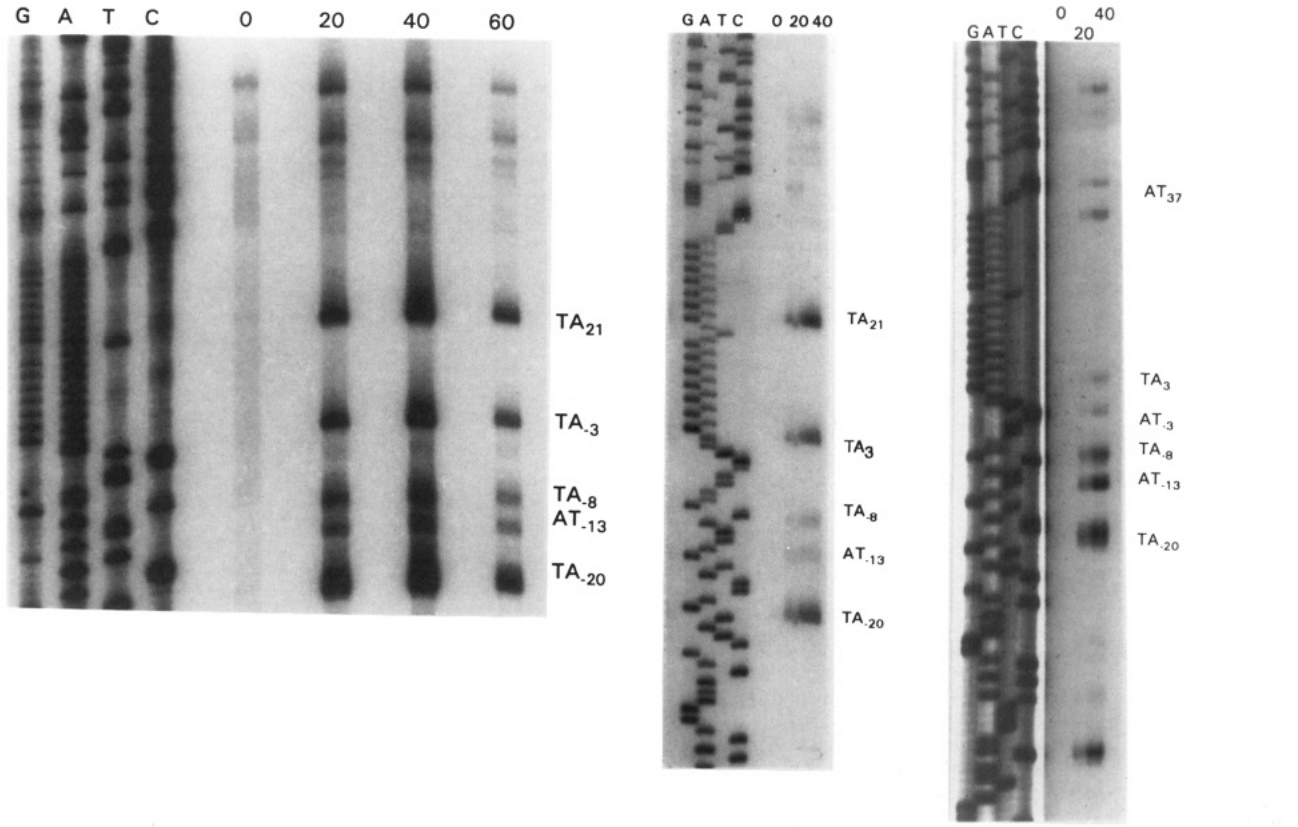

FIGURE 2: The Me_3psoralen exoIII assay of pRDH4. Panel A (left) shows an exoIII assay on samples treated with Me_3psoralen and 0, 20, 40, and 60 s of 360-nm light. Linear plasmid DNA was incubated in pH 5 buffer (without Mg^{2+}; 20 mM Tris-acetate, pH 5, 1 mM EDTA) for 1 h at 37 °C before Me_3psoralen photobinding, as described in the text. The positions of the major exoIII stops are indicated to the right. DNA sequence markers are shown on the left. Panel B (middle) shows results of an exoIII assay of naturally supercoiled DNA ($\sigma \approx -0.06$) pRDH4 treated with Me_3psoralen and light for 0, 20, and 40 s in TEN. Panel C (right) shows the pattern of exoIII stops for the same DNA shown in panel B, but which was photobound in pH5 buffer containing Mg^{2+}. The origin of the band below AT_{37} is uncertain. It could represent a psoralen photobinding site in the purine tract present in the Hy5 conformation. Alternatively, it could represent a contaminant or an incomplete digestion product present in these samples, although there are no other signs of incomplete exoIII digestion in these lanes. This band was not observed in other experiment with DNA of similar superhelical density incubated and photobound under these conditions.

region on one side of the junction. The reactivity of the intramolecular triplex DNA forming region to Me_3psoralen photobinding should be dependent on the particular structural isomer formed (either Hy5 or Hy3 as shown in Figure 1). Certain sites, especially 5′TA residues, may not photobind Me_3psoralen as well when in an unwound duplex–triplex junction as in B-form DNA. On the other hand, other 5′AT sites, which show weak reactivity to Me_3psoralen in B-DNA, may show enhanced reactivity in the junction. An increased reactivity was observed previously for B–Z junctions (Kochel & Sinden, 1988, 1989).

Analysis of pRDH4 on two-dimensional agarose gels (using pH 5 buffer in the first dimension) revealed a transition to the triplex form occurring at the eighth topoisomer. This corresponds to a superhelical density for the triplex transition of $\sigma = -0.031$. *In vitro* characterization with OsO_4, bipyridine, and chloroacetaldehyde showed that the Hy5 conformation (Figure 1B) existed at this superhelical density in the pH 5 buffer (data not shown). At higher levels of negative superhelical density ($\sigma \approx -0.06$) the Hy3 conformation (Figure 1B) existed. This is in agreement with the findings of Htun and Dahlberg (1989) and Glover and Pulleyblank (1990).

In Vitro Analysis of Me_3psoralen Photobinding to the Intramolecular Triplex-Forming Region. ExoIII digestion patterns for linear (relaxed) DNA treated with psoralen and 0, 20, 40, and 60 s of 360-nm light at pH 5.0 are shown in Figure 2, panel A. Patterns of exoIII stops are also shown for supercoiled DNA photobound at pH 7.6 in Figure 2, panel B. When no Me_3psoralen photoproducts are introduced into the DNA, exoIII digested through the region showing no stops. Upon the introduction of photoproducts in either buffer, a number of prominent stops were observed that corresponded to 5′TA and 5′AT dinucleotides. As shown in gels of Figure 2 and representative tracings in Figure 3, there are strong stops at TA_{-20} and TA_{21} and weaker stops at AT_{-13}, TA_{-8}, and TA_3. The strong stop at TA_{21} indicates the presence of duplex DNA at this center position within the poly(pu)·poly(py) region.

When supercoiled DNA samples were incubated at pH 5.0 in the presence of Mg^{2+}, conditions that support the formation of intramolecular triplex structure, the pattern of Me_3psoralen photobinding was quite different. As shown in Figure 2, panel C, the strong stop at the central TA_{21} was absent while the stop at TA_{-20} remained. In addition, the intensity of the stops at AT_{-13} and TA_{-8} increased while the intensity of the stop at TA_3 decreased relative to their reactivity in duplex DNA. Moreover, a new stop at AT_{-3} was present. There was also a new band that corresponded to Me_3psoralen photobinding to a position at the end of the poly(Pu) strand (AT_{37}). A tracing of the data in Figure 2C is shown in Figure 3, panel A.

From the patterns shown for relaxed or supercoiled DNA in Figure 2, it is evident that the relative intensity of the bands did not change significantly as a function of the photobinding treatment. Consequently, subsequent experiments in Figures 3 and 4 compare samples at a single dose of photobinding.

Densitometric quantitation of these exoIII stops is shown in Table I. In linear DNA in TEN buffer at pH 7.6 the

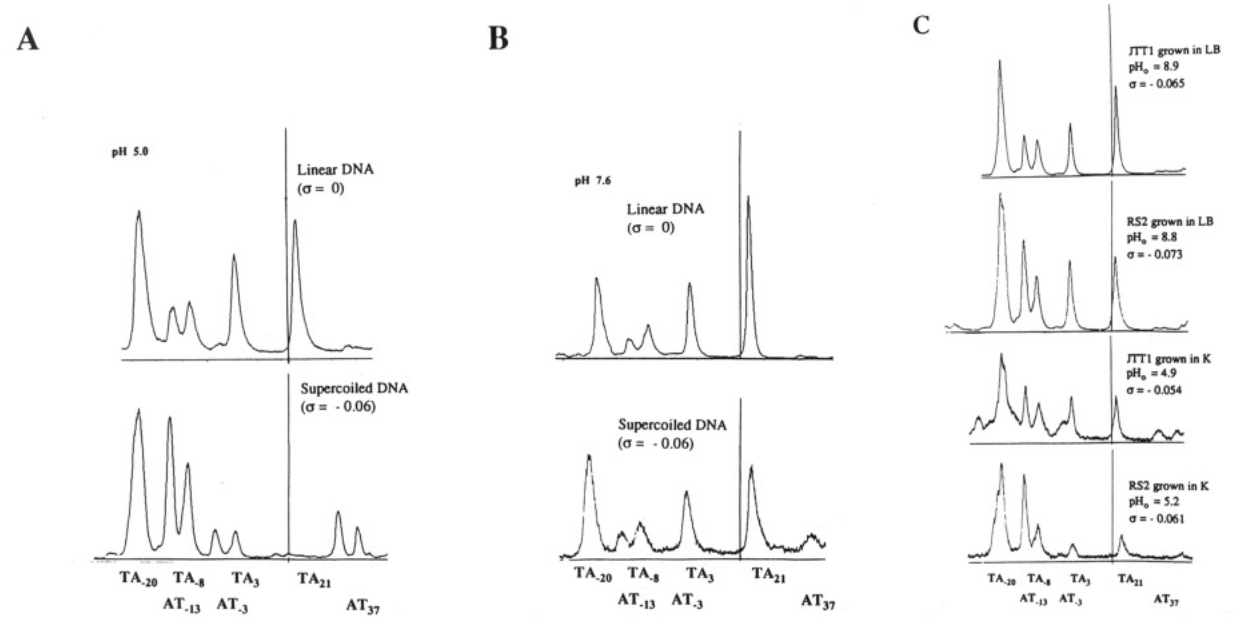

FIGURE 3: Densitometric tracings of exoIII assays of psoralen photobinding. Panel A shows densitometric tracings of the exoIII stops linear DNA photobound with Me_3psoralen in pH 5 buffer and of supercoiled DNA ($\sigma = -0.06$) in pH 5 buffer with Mg^{2+}. The tracing for supercoiled DNA is from the 60 s irradiation time point shown in Figure 2C. Panel B shows tracings of exoIII digestions of linear and supercoiled DNA samples photobound for 60 s with Me_3psoralen in TEN buffer at pH 7.6. Panel C shows four tracings from plasmid DNA photobound with Me_3psoralen in living *E. coli* strains JTT1 or RS2. The top two tracings represent plasmid from cells grown 24 h in Luria broth (LB). The bottom two represent plasmid from cells grown 24 h in K media. The superhelical densities were determined on one-dimensional agarose gels containing chloroquine. pH_0 represent the pH of the media at the time the cells were harvested for the photobinding analysis, which, for all four samples, was done under identical conditions in M9 buffer.

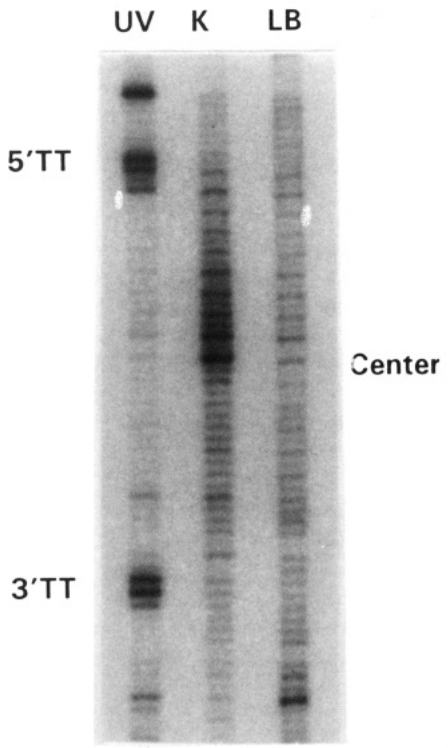

FIGURE 4: Chloroacetaldehyde modification of pRDH4 *in situ*. pRDH4 was treated with CAA *in situ* as described under Materials and Methods. The sites of covalent modification were identified by primer extension. The primer used the purine-rich strand as the template. As markers, the primer extension products of a pRDH4 treated with UV light was included in the gel. The positions of primer extension stops corresponding to the 3′ and 5′ TT dinucleotides (within the *Eco*RI sites) flanking the $(GA)_7TA(GA)_7$ sequence are indicated. The position of the center of the triplex-forming sequence is also shown. K and LB refer to modified DNA from cells grown in K media or Luria broth, respectively.

sequence $(GA)_7TA(GA)_7$ exists only as duplex DNA and the center TA (TA_{21}) photobound Me_3psoralen at a ratio 1.55 times that of the reference TA at position –20 (i.e., the TA_{21}/TA_{-20} ratio = 1.55). The relative rate of Me_3psoralen photobinding to this position was dependent on the ionic conditions. The ratio was 0.64 in linear DNA in pH 7.0 buffer (20 mM Tris, pH 7.0, 1 mM $MgCl_2$) (data not shown) and nearly identical (a TA_{21}/TA_{-20} ratio = 0.69) for linear DNA incubated in pH 5 buffer with no $MgCl_2$. We found that in general the reactivity of the TA_{21} was reduced in the presence of Mg^{2+}. Significant reactivity was present at pH 5.0 in the presence of Mg^{2+} indicative of the presence of duplex DNA at this position in linear DNA under these conditions (data not shown). The relative intensities of the other exoIII stops was not as sensitive to ionic conditions as the central TA_{21}.

In supercoiled DNA at neutral pH, little triplex would be expected to exist and the ratio of photobinding to the central TA was quite high. In DNA with $\sigma = -0.03$ and –0.06, the TA_{21}/TA_{-20} ratios were 1.21 and 0.75, respectively, which, although high, were lower than the value of 1.55 for relaxed DNA in TEN buffer. However, when these DNAs were incubated at pH 5.0, the TA_{21}/TA_{-20} ratios equalled 0.06 and 0.04 for DNAs with $\sigma = -0.03$ and –0.06, respectively. Under these conditions the TA_{21} in all of the plasmid topoisomers should exist as a single-stranded loop at the tip of a triplex. In supercoiled DNA at pH 5.0 the poly(Pu)·poly(Py) sequence existed in the triplex conformation as determined from analysis of two-dimensional agarose gels and from OsO_4 modification in agreement with the results of Htun and Dahlberg (1989) (data not shown).

There were characteristic changes in the Me_3psoralen reactivity of bases flanking the purine stretch that were diagnostic for the presence of the intramolecular triplex. In the B-form, there was strong photobinding to TA_3 and weak binding to TA_{-8} and AT_{-13}. Photobinding to TA_3 decreased at low pH in supercoiled DNA samples where the Hy3 intramolecular triplex should predominate (Figures 2 and 3; Table I). Figures 2 and 3 show that AT_{-13} becomes much more reactive to Me_3psoralen photobinding in supercoiled

Table I: Relative Intensity of ExoIII Stops[a]

	in vitro						in vivo			
	pH 7.6			pH 5.0	pH 5.0 + Mg^{2+}		LB media		K media	
exoIII stop	0	–0.03	–0.06	linear	–0.03	–0.06	JTT1	RS2	JTT1	RS2
AT_{37}	0.03	0.01	0.19	0.03	0.04	0.09	0.03	0.06	0.14	0.08
TA_{21}	1.55	1.21	0.75	0.69	0.06	0.04	0.58	0.40	0.35	0.12
TA_3	0.85	0.82	0.68	0.52	0.09	0.11	0.32	0.33	0.28	0.14
AT_{-3}	0.04	0.03	0.05	0.07	0.07	0.11	0.06	0.02	0.16	0.06
TA_{-8}	0.49	0.36	0.33	0.34	0.36	0.49	0.29	0.33	0.35	0.30
AT_{-13}	0.22	0.20	0.21	0.28	0.50	0.61	0.28	0.42	0.31	0.67
TA_{-20}	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

[a] Relative intensities of the exoIII stop values were calculated as (intensity of a band)/(intensity of TA_{-20}) for the tracings shown in Figure 3. The intensity represents the radioactivity in the bands which was quantitated using a Molecular Dynamics Phosphoimager system. Data were analyzed in two ways: by a densitometer-like scan down each lane and then comparing the relative area under each peak; alternatively, bands were individually quantitated. The relative ratios determined by both methods agreed within 5%.

DNA at pH 5.0 (see Table I). In addition, 5′AT_{-3} does not react with Me_3psoralen when in duplex DNA. However, it does react when the intramolecular triplex is formed at pH 5. These changes may be consistent with the presence of an unwound duplex/triplex junction at the 3′ end of the pyrimidine strand (see Figure 1B) and suggest the existence of the Hy3 conformation. In some experiments with supercoiled DNA samples with $\sigma = -0.03$ to -0.06 at pH 5.0, reactivity of AT_{37} was also observed as shown in Figure 2, panel C, and Figure 3, panel A. There was no reactivity at this position in linear DNA where no triplex exists. This band may be consistent with the presence of the Hy5 conformation. Under many conditions a mixture of both isomeric forms will exist (Htun & Dahlberg, 1989; Glover & Pulleyblank 1990; Glover et al., 1990), and patterns of exoIII stops consistent with the existence of both isomers should be present.

Detection of Intramolecular Triplex DNA in Vivo. To detect intramolecular triplex DNA *in vivo*, *E. coli* cells containing pRDH4 were treated with Me_3psoralen and light as described under Materials and Methods. Several features of the pattern of Me_3psoralen photobinding *in vivo* for plasmids from RS2 grown in K media are diagnostic for the existence of the intramolecular triplex *in vivo*. The tracings of the exoIII stops are shown in Figure 3, panel C, and quantitation of these stops is shown in Table I. First, the intensity of the central TA stop, with a (TA_{21}/TA_{-20}) ratio of 0.12, was substantially lower than that observed for the duplex form of the sequence, which in various buffers had ratios of 0.69–1.55 (see Table I). Second, photobinding to the TA_3, flanking the purine region (e.g., the TA_3/TA_{-20} ratio), was significantly reduced to a ratio of 0.14 compared to 0.85 for the B-form (in TEN). Third, the reactivity of the AT_{-13} was increased, to 0.67, compared to a value of 0.22 (in TEN) in duplex DNA.

The formation of triplex DNA is favored by higher levels of negative supercoiling. To examine the influence of supercoiling *in vivo* on triplex formation, we have analyzed the amount of the intramolecular triplex in wild-type *topA*$^+$ strain (JTT1) and the corresponding topA10 strain of *E. coli* (RS2). DNA in the RS2 strain has about 25% more negative supercoiling *in vivo* than the *topA*$^+$ strain (Pruss et al., 1982; Bliska & Cozzarelli, 1987; Zheng et al., 1991). In either Luria broth or K medium, there was more triplex DNA in the *topA10* strain than the topA$^+$ strain (Figure 3, panel C; Table I). This is evident from a change in the ratio of the central TA_{21} as well as a change in the reactivity flanking the poly(Pu)·poly(Py) region. In either media the TA_{21}/TA_{-20} ratio was lower (and the AT_{-13}/TA_{-20} ratio higher) for DNA purified from RS2 cells than from JTT1 cells (Table I).

To examine the influence of pH on triplex DNA *in vivo*, cells were grown to stationary phase in either Luria broth or

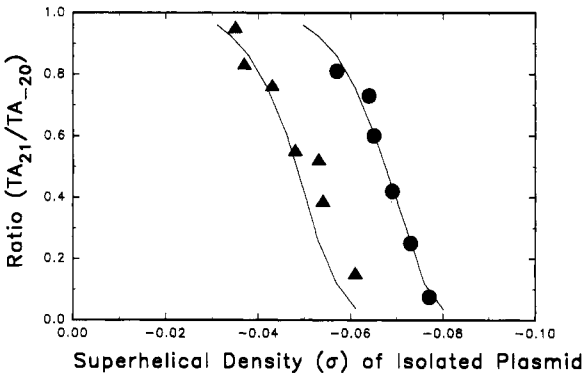

FIGURE 5: Dependence of the TA_{21}/TA_{-20} ratio on superhelical density and growth conditions. The superhelical density of the plasmids purified from cells varied with the strain used, as expected, but also with time of growth. In general, plasmids purified from cells grown for 36 h were less negatively supercoiled than plasmids grown for a shorter period of time. The first three data points for each set of data are from DNA purified from JTT1, the latter three from RS2. The data points were determined by densitometric analysis of purified DNA on an agarose–chloroquine gel. (▲) Plasmids from cells grown in K media; (•) plasmids from cells grown in LB media. The solid line represents the cumulative normal distribution curve generated assuming a Gaussian distribution of eight topoisomers, as described by Zheng et al. (1991), with the distribution centered at $\sigma = -0.045$ for cells grown in K media and $\sigma = -0.065$ for cells grown in Luria broth. The cumulative normal distribution is standardized at a value = 1 to the TA_{21}/TA_{-20} ratio = 1.

K medium. The intracellular pH is known to vary in relation to the external pH (Booth, 1985). After 24 h, the pH of K media decreased to near 5, resulting in an estimated *in vivo* pH of 7.1 (Dibrov, 1991). In Luria broth the extracellular pH was 8.7, resulting in an estimated intracellular pH of about 7.8. Plasmid DNA from both *topA*$^+$ and *topA10* cells grown in K media had Me_3psoralen photobinding patterns that were much more like those for the intramolecular triplex than DNA from the plasmids isolated from the same cells grown in Luria broth (Figure 3, panel C). In plasmids from *topA*$^+$ cells grown in LB, the Me_3psoralen pattern was very similar to that for the B-DNA conformation (pH 7.0, linear DNA). For DNAs of similar superhelical density, the TA_{21}/TA_{-20} ratios *in vivo* were consistently lower in cells grown in K media than Luria broth. The TA_{21}/TA_{-20} ratios for multiple repeats of these experiments ranged from 0.08 to 0.95 and are shown in Figure 5, where they are plotted as a function of superhelical density of the purified DNA. The significance of this relationship is discussed under Discussion.

CAA and OsO_4 Bipyridine Modification in Vitro and in Vivo. Chemical modification with OsO_4 and CAA was used to confirm the existence of triplex DNA and to determine which isomer existed *in vivo*. Plasmid pRDH4 was modified

with OsO_4 bipyridine as described (Karlovsky et al., 1990). Consistent with the results of Htun and Dahlberg (1989) and Glover et al. (1990), at lower superhelical densities at pH 5.0 *in vitro*, the 3′ junction (of the Pu strand) was hypersensitive to OsO_4 modification (suggestive of the Hy5 conformation), while at higher superhelical densities the 5′ junctions (of the Pu strand) became hypersensitive (data not shown). Modification with chloroacetaldehyde also revealed a pattern consistent with the existence of the Hy5 conformation at low levels of supercoiling and the Hy3 conformation at higher levels of supercoiling. At lower superhelical densities at pH 5.0 there was reactivity over the 3′ half of the purine-rich strand and little reactivity of the pyrimidine strand (data not shown).

To determine which isomer existed inside *E. coli*, cells were modified with chloroacetaldehyde *in situ*. The pyrimidine strand was modified only at the center, while the 5′ half of the purine strand was modified in cells grown in K media (Figure 4). Cells grown in Luria broth showed very slight modification in the 5′ half of the purine strand. These results are consistent with the existence of Hy3 *in vivo* and consistent with more of the triplex structure in cells grown in K media than in Luria broth.

DISCUSSION

We have examined the effect of changes in intracellular levels of supercoiling and pH on the level of intramolecular triplex *in vivo*. The results of the Me_3psoralen and CAA experiments demonstrate the existence of the protonated, Hy3 isomer of the $(GA)_7TA(GA_7)$ intramolecular triplex in living *E. coli* cells. In addition, they show a superhelical density dependence for the formation of intramolecular triplex DNA and show that the growth conditions and the extracellular environment can affect the level of the triplex formation in living cells.

pRDH4 was designed with a central 5′TA to report the Me_3psoralen photobinding properties of the center of the triplex DNA. The reactivity at this central TA ($5'TA_{21}$) compared to the control TA_{-20} should be inversely proportional to the fraction of the region that exists as an intramolecular triplex. This assumes the Me_3psoralen photobinding is "all or none", in which Me_3psoralen binds to B-DNA but not to DNA in the intramolecular triplex conformation. If this linear relationship exists *in vivo*, then the center TA_{21}/TA_{20} ratio should reflect the fraction of the pu·py region that exists as an intramolecular triplex *in vivo*. The reactivity of several Me_3psoralen junction binding sites is also indicative of the presence of intramolecular triplex formation *in vivo*. Me_3psoralen photobinds weakly to the $5'AT_{-13}$ in the B-DNA form but showed an enhanced affinity when it existed as a junction at the end of the triplex. The increase in the photobinding at AT_{-13} and the decrease in Me_3psoralen photobinding at TA_3 are suggestive of intramolecular triplex DNA. Moreover, the cumulative evidence of the chemical probes, in addition to the Me_3psoralen photobinding, argues strongly in favor of an intramolecular triple helix. The chloroacetaldehyde reactivity is consistent with the existence of the Hy3 isomer *in vivo*. The Hy5 isomer was detected *in situ* from OsO_4 bipyridine studies in a similar sized $(GT)_n$ sequence (Karlovsky et al., 1990).

The level of intramolecular triplex formation *in vivo* was dependent on the pH of the media and the level of supercoiling, consistent with the effects of these parameters *in vitro* (Htun & Dahlberg, 1989; Lyamichev et al., 1985; Mirkin et al., 1987). Figure 5 shows TA_{21}/TA_{-20} ratios determined for DNA from cells grown in either Luria broth or K media, plotted as a function of the level of supercoiling of the purified plasmid. The ratios decreased with increasing negative supercoiling for plasmids from cells grown in both media, and, in both cases at the higher levels of negative supercoiling, a low TA_{21}/TA_{-20} ratio (consistent with the presence of triplex DNA) was observed. The TA_{21}/TA_{-20} ratios from the different media are offset by a difference in supercoiling that corresponds to a change in linking number of about $\Delta L = 2$. The excellent correspondence between the TA_{21}/TA_{-20} ratios and the purified superhelical density suggest that the formation of intramolecular triplex DNA *in vivo* is superhelical density dependent. In addition, the results suggest that the "effective level of unrestrained supercoiling", with respect to the formation of intramolecular triplex DNA, is different in cells grown under different conditions. Even though the linking number of DNA purified from cells grown in K media or Luria broth may be the same, a higher effective level of unrestrained supercoils (in terms of intramolecular triplex formation) existed in cells grown in K media.

There may be at least two factors that explain why more triplex was found in K media compared to LB. First, the intracellular pH of K media-grown cells may be lower. By 24 h the external pH of the media dropped from 6.8 to 5.0, resulting in an estimated internal pH drop from near 7.6 to about 7.1. In cells grown in LB media, the estimated intracellular pH increased slightly from 7.6 to about 7.8 (Dibrov, 1991). It is possible that subtle pH changes *in vivo* may have a significant influence on the formation of H-DNA. Previous results have shown that highly supercoiled DNA would form an intramolecular triplex *in vitro* at pH 7.50, but not at pH 7.84 (Htun & Dahlberg, 1988); thus a difference between $pH_i \approx 7.1$ and $pH_i \approx 7.8$ might result in a lower superhelical requirement for triplex formation. Second, the level of restraint of supercoils and/or the ionic environment surrounding the DNA may be different under these two growth conditions. It is known for example that the level of supercoiling can change under different growth conditions (Sinden & Pettijohn, 1981; Balke & Gralla, 1987).

It is difficult, with the present intramolecular triplex-forming sequence, to quantitate the amount of triplex DNA *in vivo*. This is due to the variation in reactivity of the central TA_{21}, the reactivity of which would ideally provide an excellent quantitative probe of triplex DNA. The TA_{21}/TA_{-20} ratio varied in different buffers that should contain the poly(pu)·poly(py) region in the B conformation. Although it is difficult to make a conclusive estimation of the level of triplex DNA *in vivo*, the level may be similar to that found for Z-DNA and cruciforms (as high as 50–80%). The ratio *in vivo* changed from a low value of about 0.1 to a higher value of 0.95. If the ratio of 0.95 represented that obtained for the duplex form *in vivo*, then, to produce a ratio of 0.1, as much as 90% of plasmids containing the poly(pu)·poly(py) sequence would have contained this sequence in the intramolecular triplex form.

The level of intramolecular triplex DNA detected *in vivo* appears to be dependent on the stage of growth of the bacteria. Plasmids purified from either *E. coli* strain treated with Me_3psoralen and light while in log phase contained little of the intramolecular triplex DNA conformation (data not shown) while plasmids from the *topA10* cells in stationary phase contained a significant amount of intramolecular triplex DNA. In our experiments, the cells were grown for 24–36 h and resuspended in M9 buffer (at physiological pH) for the photobinding protocol. Other researchers have not detected intramolecular triplex DNA in *E. coli* under physiological

conditions (Karlovsky et al., 1990; Kohwi et al., 1992) although these researchers did not look at cells grown to stationary phase. Karlovsky et al. (1990) found intramolecular triplex DNA in cells that were chloramphenicol treated (which increases the level of negative DNA supercoiling) and then incubated in 0.1 M buffer at pH 5.2. Intramolecular triplex DNA was not detected in cells incubated in buffers with pH $\geq$ 5.4. Kohwi et al. (1992) also only detected intramolecular triplex DNA in chloramphenicol treated cells in which the level of negative supercoiling in plasmid DNA increases above that naturally found in wild-type cells. Growth into stationary phase may be required to allow the accumulation of high levels of intramolecular triplex DNA, since the processes of DNA replication and transcription may drive intramolecular triplex DNA back into the linear form. Replication and transcription are apparently responsible for a low level of cruciforms in DNA found in cells grown in log phase (Zheng et al., 1991; Morales et al., 1990), whereas high levels of cruciforms can be detected in cells in stationary phase (Zheng et al., 1991). This is in contrast to Z-DNA, which exists at the same level in log phase and stationary cells, whether located inside or outside of a transcription unit (Zheng et al., 1991; Rahmouni & Wells, 1989).

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