Multiple Non-B-DNA Conformations of Polypurine·Polypyrimidine Sequences in Plasmids†

Mitsuhiro Shimizu,‡ Jeffery C. Hanvey,§ and Robert D. Wells*
Department of Biochemistry, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294

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ABSTRACT: A polypurine·polypyrimidine (Pur·Pyr) sequence with a central interruption in a plasmid can adopt multiple non-B-DNA conformations depending on the conditions as revealed by specific chemical probes (OsO_4, diethyl pyrocarbonate, and dimethyl sulfate) and two-dimensional electrophoresis. The relatively long mirror repeat Pur·Pyr sequences $(GAA)_9TTC(GAA)_8$ and $(GGA)_9TCC(GGA)_8$ form single canonical intramolecular triplexes at pH 7.0–6.0 in negatively supercoiled plasmids as isolated from *Escherichia coli*. With a lowering of the pH and/or an increase in the degree of negative supercoiling, these sequences undergo a novel conformational change as revealed by diethyl pyrocarbonate hypermodification of adenines in the middle of the polypurine strand and OsO_4 reaction with thymines in the center and the quarter points of the polypyrimidine strand. To evaluate this structure, a family of related Pur·Pyr sequences were cloned and studied. The non mirror repeat sequence $(GGA)_9TCC(GAA)_8$ forms a non-B conformation only under acidic pH conditions, but the structural properties are different from those of the mirror repeat sequences. Furthermore, when the central interruptions of a mirror repeat sequence were increased from 3 to 9 bp, two canonical triplexes formed independently at pH 5.0 [at the $(GAA)_9$ and $(GAA)_8$ regions in the sequence $(GAA)_9TTAATTCGC(GAA)_8$]. Thus, if an interruption is sufficiently long, the two halves of the Pur·Pyr sequence do not interact with each other. Novel types of folded DNA geometries which explain these results are described.

DNA has the capability to adopt several types of conformations as dictated by its sequence, and many factors can promote the interconversion from one form to another. This structural flexibility may play a role in key cellular processes. Alternative DNA conformations such as left-handed Z-DNA at alternating purine–pyrimidine sequences, cruciforms at inverted repeats, bent DNA, etc. are well documented [reviewed in Wells (1988), Wells and Harvey (1987), Lilley et al. (1987), and Rich et al. (1985)]. In addition to these unusual DNA structures, recent studies demonstrated that polypurine·polypyrimidine (Pur·Pyr)[1] sequences can form several types of non-B conformations [reviewed in Wells et al. (1988)]. Non-B conformations at Pur·Pyr sequences have attracted much attention because of the overrepresentation of the Pur·Pyr sequences in eucaryotic genomes and their proximity to regulatory regions and recombinational hot spots [reviewed in Wells et al. (1988) and references cited therein].

An intramolecular triplex (also called H-form DNA), which consists of a triple-stranded region containing $G{\cdot}G{\cdot}C^+$ and T·A·T triads (simultaneous Watson–Crick and Hoogsteen base pairs) and an unpaired purine strand, has been identified in a mirror repeat Pur·Pyr sequence containing G's and A's or poly(G) (Lyamichev et al., 1986, 1987; Christophe et al., 1985; Mirkin et al., 1987; Hanvey et al., 1988a,b, 1989; Collier et al., 1988; Voloshin et al., 1988; Kohwi & Kohwi-Shigematsu, 1988; Vojitskova et al., 1988; Htun & Dahlberg, 1988, 1989; Johnston et al., 1988; Shimizu et al., 1989). This triplex structure is stabilized by mildly acidic conditions and negative supercoiling. However, different types of triplexes may be formed depending on the sequences and environmental conditions. Htun and Dahlberg (1989) have reported that a $(GA)_{18}$ sequence forms one isomer of the triplex (5′-half of the pyrimidine strand as the third strand) at lower supercoil densities, whereas the other isomer (3′-half of the pyrimidine strand as the third strand) forms at higher supercoil densities. Parniewski et al. (1989) showed that an AG repeat with a several bp interruption in the center can adopt at least two different structures depending on the pH and negative supercoiling, which may be a mixture of both isomers of the triplex. Furthermore, some divalent metal ions may induce the structural changes from Pyr·Pur·Pyr triplexes (pyrimidine strand as the third strand) to Pyr·Pur·Pur triplexes (purine strand as the third strand) in G_{25-30} or $(AG)_{22}$ sequences in plasmids (Kohwi & Kohwi-Shigematsu, 1988; Kohwi, 1989; Bernues et al., 1989).

Longer Pur·Pyr sequences in plasmids have complex structural behaviors. The DR2 repeats of the segment inversion site of herpes simplex virus type 1, which contain a long Pur·Pyr strand bias with a high G+C content, adopt non-B conformations different from those of intramolecular triplexes in plasmids (Wohlrab et al., 1987; Wohlrab & Wells, 1989). Also, Htun and Dahlberg (1989) showed that a long GA repeating sequence forms metastable multiple conformers in a plasmid. Collier and Wells (1990) showed that $(GA)_{37}$ adopts an intramolecular triplex under moderate levels of supercoil stress at neutral pH. In addition, a recent circular

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* To whom correspondence should be addressed.
‡ Present address: Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Insitutes of Health, Bethesda, MD 20892.
§ Present address: Chemotherapy Division, Glaxo Inc., 5 Moore Dr., Research Triangle Park, NC 27709.

[1] Abbreviations: Pur·Pyr, polypurine·polypyrimidine; DEPC, diethyl pyrocarbonate; DMS, dimethyl sulfate; bp, base pair; 2D gel, two-dimensional gel; *E. coli*, *Escherichia coli*; $-\sigma$, negative supercoil density.

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dichroism study reveals six different conformational states of the polymer poly(AG)·poly(CT) at pH 8.5–2.5 (Antao et al., 1988). The different types of non-B structures adopted by Pur·Pyr sequences remain to be fully characterized.

Herein, we describe studies on the insert $(GAA)_9TTC(GAA)_8$, which has properties different from those of a canonical triplex. The structures and properties of this and three related sequences were investigated by chemical probing and 2D gel electrophoresis. These studies revealed that some of the Pur·Pyr sequences form a canonical triplex at or near neutral pH but adopt other non-B structures at lower pHs. Three-stranded models of the new structure(s) are proposed.

Materials and Methods

Plasmids. The vector plasmid pRW791 is a 1991-bp pBR322 derivative described previously (Hanvey et al., 1988b, 1989; Shimizu et al., 1989; McLean et al., 1986). pRW1707 was made as follows: two $(GAA)_8$ inserts, one with 5′ *Bam*HI and 3′ *Eco*RI sticky ends and the other with 5′ *Eco*RI and 3′ *Bam*HI sticky ends, were isolated from *Bam*HI plus *Eco*RI digests of pRW1704 and -1703, respectively (Hanvey et al., 1988b; Jaworski et al., 1989). These fragments were ligated, treated with *Bam*HI, and ligated into the *Bam*HI site of pRW791 according to standard procedures (Maniatis et al., 1982). The $(GGA)_8$ and $(GAA)_8$ inserts with 5′ *Bam*HI overhangs on each end were isolated from pRW1411 and -1408, respectively (Hanvey et al., 1988a, 1989). The $(GGA)_9TCC(GGA)_8$ insert containing 5′ *Bam*HI overhangs on each end was made by ligation of the $(GGA)_8$ insert with *Bam*HI sticky ends and, following partial digestion with *Bam*HI, was cloned into the *Bam*HI site of pRW791, forming pRW1724. The $(GGA)_9TCC(GAA)_8$ insert with 5′ *Bam*HI overhangs was made by ligation of the $(GGA)_8$ with $(GAA)_8$ and, following partial digestion with *Bam*HI, was cloned into the *Bam*HI site of pRW791, forming pRW1725. pRW1718 was made by treating pRW1707 with *Bst*BI, filling in the 5′-overhangs, ligating, digesting with *Eco*RI, filling in the 5′-overhangs, and ligating. The term "mirror repeat" was defined previously (Wells et al., 1988).

Plasmids were grown in *Escherichia coli* HB101 with chloramphenicol amplification and prepared by the alkaline lysis method and two cesium chloride gradient centrifugations (Maniatis et al., 1982).

Topoisomer populations were prepared as described (Singleton & Wells, 1982; Wohlrab et al., 1987).

Chemical Probes. The modifications of the plasmids by OsO_4 (Sigma), diethyl pyrocarbonate (Sigma), and dimethyl sulfate (Aldrich) were performed as described previously (Hanvey et al., 1988a,b, 1989; Shimizu et al., 1989).

Two-Dimensional (2D) Gel Electrophoresis. 2D gel electrophoresis was performed as described previously (Wang et al., 1983; Collier et al., 1988; Zacharias et al., 1988; Shimizu et al., 1989; Wohlrab & Wells, 1989).

Results

We demonstrated recently that simple repeating Pur·Pyr sequences about 30 bp in length form intramolecular triplexes in supercoiled plasmids (Hanvey et al., 1988a,b, 1989; Collier et al., 1988; Shimizu et al., 1989). To understand this unorthodox structure, relatively longer Pur·Pyr sequences were characterized. Figure 1 shows the Pur·Pyr sequences of the recombinant plasmids used in this study. pRW1707 and -1724 contain $(GAA)_9TTC(GAA)_8$ and $(GGA)_9TCC(GGA)_8$, respectively, which are mirror repeat sequences, but with different G+C content. We found that these Pur·Pyr sequences have structural properties different from those of shorter

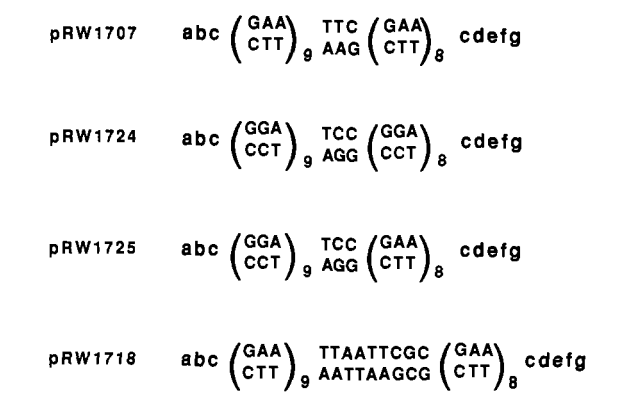

FIGURE 1: Inserts in plasmids. The multiple cloning site is in lower-case letters: (a) *Sst*I; (b) *Xma*I; (c) *Bam*HI; (d) *Xba*I; (e) *Hin*cII; (f) *Pst*I; (g) *Hin*dIII. The sequences of the inserts are in upper-case letters. The *Bam*HI site, GGATCC, adds three homopurines or homopyrimidines to the end of each Pur·Pyr insert. Thus, for example, pRW1724 is actually $(GGA)_9TCC(GGA)_9$ when the *Bam*HI site is included.

Pur·Pyr sequences such as $(GAA)_4TTC(GAA)_4$. To understand this behavior, we constructed other plasmids with related sequences. pRW1725 contains a $(GGA)_9TCC(GAA)_8$ insert, which is a non mirror repeat. pRW1718 contains the $(GAA)_9TTAATTCGC(GAA)_8$ insert, which is similar to pRW1707 except that the central interruption is 9 bp. All the inserts were cloned into the *Bam*HI site of pRW791 and were characterized by DNA sequencing of both strands using the chemical degradation method (Maxam & Gilbert, 1980).

Chemical Probing of the $(GAA)_9TTC(GAA)_8$ Insert in pRW1707. OsO_4, DEPC, and DMS are widely used to study non-B-DNA structures such as intramolecular triplexes, left-handed Z-DNA, and cruciforms [reviewed in Wells and Harvey (1987) and Wells et al. (1988)]. OsO_4 is substantially more reactive to thymines in single-stranded than in double-stranded DNA. DEPC shows greater reactivity toward purines (A > G) in single-stranded DNA and in a syn conformation as in Z-DNA. Also, it was shown that guanines involved in triplexes are protected from dimethyl sulfate modification.

Figure 2 shows the OsO_4, DEPC, and DMS modification sites of pRW1707 at pH 7.0 and 5.0 for the plasmid with a supercoil density as isolated from *E. coli* ($-\sigma \sim 0.06$). At pH 7.0, OsO_4 modified thymines in the center of the purine strand (positions 28 and 29). Also, the 5′-half of the purine strand (positions 2–27) and the adenines in the center of the pyrimidine strand (positions 28 and 29) were hyperreactive to DEPC. This hyperreactive pattern of the insert is the same as that reported previously for relatively short Pur·Pyr sequences (about 25–35 bp in length) at around pH 5.0 (Hanvey et al., 1988a,b, 1989; Collier et al., 1988; Voloshin et al., 1988; Vojitskova et al., 1988; Htun & Dahlberg, 1988; Johnston, 1988; Shimizu et al., 1989). This indicates that the $(GAA)_9TTC(GAA)_8$ sequence forms a canonical intramolecular triplex structure at pH 7.0 in supercoiled plasmids isolated from *E. coli*. It has been shown that longer Pur·Pyr sequences in supercoiled plasmids show S1 hypersensitivity at neutral pH, although the S1 hypersensitivity was enhanced by acidic pH conditions (Htun et al., 1984, 1985; Evans & Efstratiadis, 1986; McCarthy & Heywood, 1987; Collier et al., 1988). Also, Collier and Wells (1990) recently showed that a long Pur·Pyr sequence $[(GA)_{37}]$ forms an intramolecular triplex at neutral pH as revealed by chemical probes and 2D gel electrophoresis.

As seen in Figure 2, at pH 5.0 the hyperreactive pattern of the insert to specific chemical probes changed dramatically.

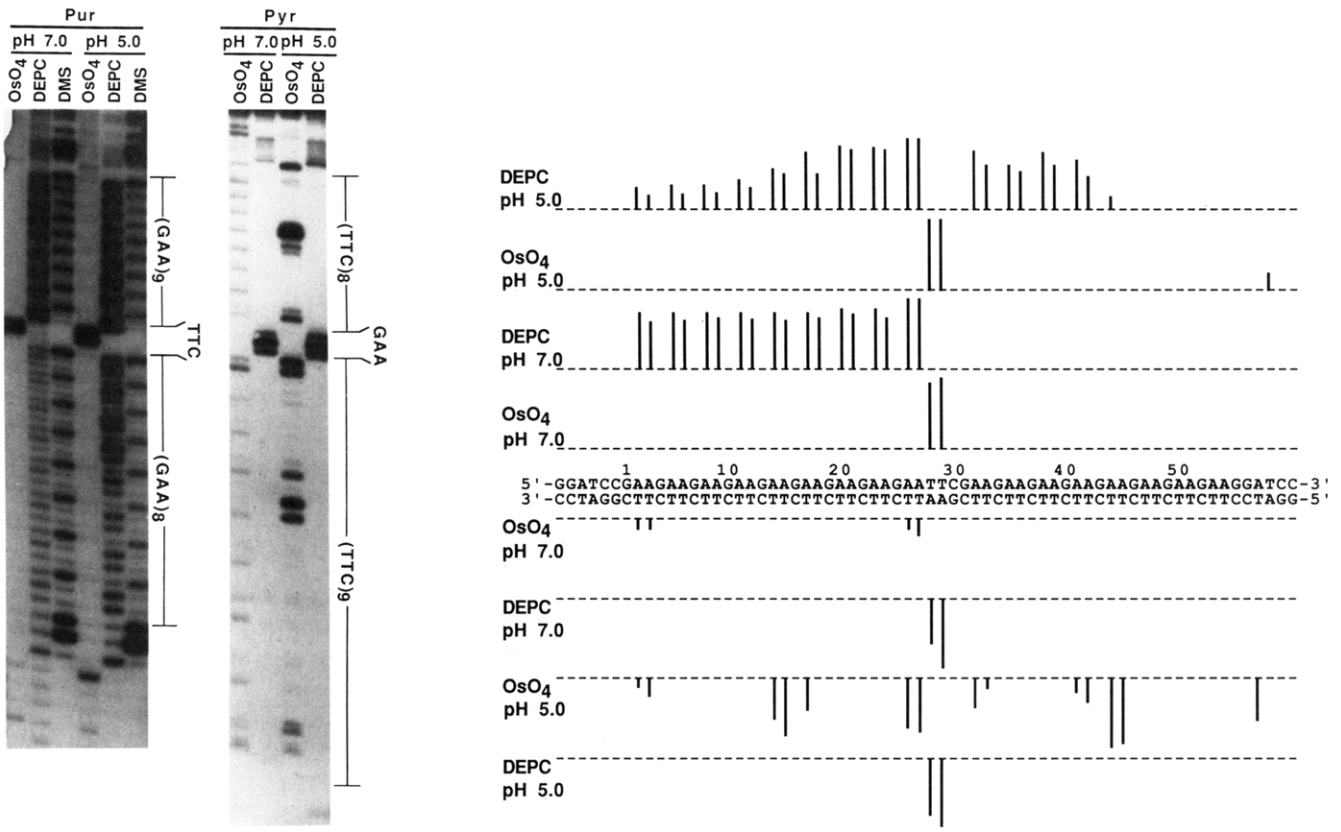

FIGURE 2: Fine mapping of OsO_4, DEPC, and DMS sites in supercoiled pRW1707 at pH 7.0 and 5.0. (Left panel) OsO_4, DEPC, and DMS modification sites of pRW1707 at the nucleotide level. For the OsO_4 modifications, 3 μg of plasmids was preincubated in 25 μL of 0.1 M Tris–acetate buffer containing 2% pyridine (pH 7.0 or 5.0) for 30 min at 22 °C, and then, the reactions were performed for 30 min at 22 °C with 2 mM OsO_4. The reactions were terminated by extraction with ether and precipitation of the DNA with ethanol. For the DEPC reactions, 3 μg of plasmids was preincubated in 100 μL of 0.3 M Tris–acetate buffer (pH 7.0) or 0.3 M sodium acetate buffer (pH 5.0) for 30 min at 22 °C. Then, the reactions were initiated by the addition of 10 μL of DEPC and vortexing and after 30 min were stopped by precipitation of the DNA with ethanol. For the DMS modifications, 3 μg of plasmids was preincubated in 100 μL of 0.3 M Tris–acetate buffer (pH 7.0) or 0.3 M sodium acetate buffer (pH 5.0) for 30 min at 22 °C. The reactions were performed for 1 min at 22 °C with 1% DMS and then terminated by the addition of 5 μL of 2-mercaptoethanol and precipitation of the DNA with ethanol. The subsequent workup was as described (Hanvey et al., 1988a; Shimizu et al., 1989). The purine strand was radioactively labeled at the *Hin*dIII site, whereas the pyrimidine strand was labeled at the *Xma*I site by filling in the 5′-overhangs with the Klenow fragment of DNA polymerase. The sequence of the insert is shown on the right side of the gel. The gel is 5′ to 3′ from top to bottom. Pur, purine strand; Pyr, pyrimidine strand. (Right panel) Summary of the modification patterns for pRW1707. The length of each line corresponds to the relative intensity of a modification site based on densitometric analysis of the autoradiograms using a Bio-Rad 620 video densitometer. The reproducibility of chemical probe studies is estimated, in general, to be within ±20%. The first G after the upper *Bam*HI site (GGATCC) is designated as position 1.

DEPC predominantly modified the middle of the purine strand at positions 14–44, although adenines at positions 2–12 are somewhat hyperreactive to DEPC. Also, in addition to the hyperreactive thymines in the central region of the insert observed at pH 7.0, new OsO_4-reactive sites appeared at the center of the $(TTC)_9$ region (positions 14–17) and of the $(TTC)_8$ region (positions 41–45). Guanines in the 5′-half of the $(GAA)_9$ region (positions 4, 7, and 10) and in the 3′-half of the $(GAA)_8$ region (positions 46, 49, and 52) were protected from DMS modification. Thus, the $(GAA)_9TTC(GAA)_8$ sequence gives a novel hyperreactive pattern to these specific chemical probes, which is quite different from that of any other Pur·Pyr sequence reported previously (Evans & Efstratiadis, 1986; Hanvey et al., 1988a,b, 1989; Collier et al., 1988; Kohwi & Kohwi-Shigematsu, 1988; Voloshin et al., 1988; Vojitskova et al., 1988; Htun & Dahlberg, 1988, 1989; Johnston, 1988; Parniewski et al., 1989; Shimizu et al., 1989; Bernues et al., 1989). These results indicate that the $(GAA)_9TTC(GAA)_8$ sequence undergoes a novel conformational change with lowering of the pH.

Structural Polymorphism of pRW1707 as a Function of pH and Negative Supercoiling. To examine the structural changes of the $(GAA)_9TTC(GAA)_8$ insert, OsO_4 modifications were performed as a function of pH with supercoiled plasmid as isolated from *E. coli* ($-\sigma \sim 0.06$). Figure 3 shows the OsO_4 modification sites in the pyrimidine strand of pRW1707 in the pH range of 7.5–4.5. At pH 7.5, no OsO_4 hyperreactive sites were observed. Between pH 7.0 and pH 6.0, OsO_4 strongly modified thymines in the center and 3′-junction of the pyrimidine strand, indicating that this sequence forms an intramolecular triplex between pH 7.0 and pH 6.0. The additional hyperreactive sites appeared at pH 5.5 or below, showing that there is a transition to another or other non-B-DNA structures at or below pH 5.5 at this supercoil density.

To further characterize this behavior, OsO_4 and DEPC reactions were also performed as a function of negative supercoil density. Figure 4 shows DEPC and OsO_4 modification patterns of the insert in the range of supercoil density from −0.026 to −0.09 at pH 6.0 and 5.0. At pH 6.0, OsO_4 reacted with thymines in the center and 3′-junction of the pyrimidine strand, whereas DEPC modified the 5′-half of the purine strand in the range −0.03 to −0.062, indicating that this insert forms only one isomer of an intramolecular triplex (3′-half of the pyrimidine strand as the third strand) under these conditions. However, even at pH 6.0, as the negative supercoil density increased to greater than −0.077, additional OsO_4 reactive sites appeared at the quarter points (positions 14–17 and 41–45), and DEPC hypermodified the 5′-half of the

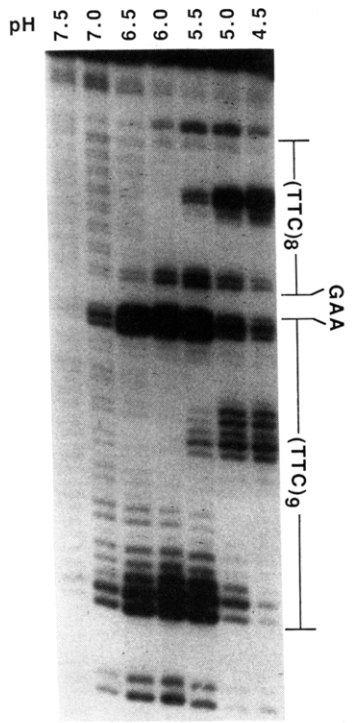

FIGURE 3: Fine mapping of OsO_4 sites of the pyrimidine strand of supercoiled pRW1707 as a function of pH. OsO_4 reactions were carried out in 0.1 M Tris–acetate buffer containing 2% pyridine at the indicated pHs as described in the legend of Figure 2. The pyrimidine strand was radioactively labeled at the *Eco*O109 site, which is located 31 bp upstream from the *Sst*I site (Shimizu et al., 1989). The gel is 5′ to 3′ from top to bottom. The sequence of the insert is shown on the right side of the gel.

$(GAA)_8$ region (positions 31–42).

At pH 5.0 and lower supercoil density (~−0.03), the hyperreactive pattern shows the formation of a single intramolecular triplex in pRW1707. As the supercoil density increases, the modification pattern changes, which reveals the formation of the novel conformation. It should be noted that at high supercoil density (−0.077 to −0.09) the 5′-half of the $(GAA)_9$ region (positions 1–12) becomes less reactive to DEPC (Figure 4A). This suggests that the hyperreactive patterns observed under certain conditions (between supercoil density −0.077 and −0.09 at pH 6.0 and between supercoil density −0.046 and −0.077 at pH 5.0) were probably from mixtures of multiple non-B conformations (intramolecular triplex and other).

At pH 7.0, above negative supercoil density ~−0.05, this insert forms one isomer of an intramolecular triplex (3′-half of the pyrimidine strand as the third strand) as shown by both OsO_4 and DEPC modification and does not form the other isomer of the triplex (5′-half of the pyrimidine strand as the third strand) (data not shown). Recently, Htun and Dahlberg (1989) showed that a Pur·Pyr sequence $[(AG)_{18}]$ forms both isomers of the triplex; one isomer (3′-half of the pyrimidine strand as the third strand) was favored at higher supercoil densities, but the other isomer (5′-half of pyrimidine as the third strand) formed at lower supercoil densities. Apparently, this reaction does not occur for the $(GAA)_9TTC(GAA)_8$ sequences under the conditions employed. These results demonstrate that a new non-B-DNA structure, different from an intramolecular triplex, forms as a consequence of a lowering of the pH, as well as increasing supercoil tension.

Supercoil-induced changes in the primary helix were studied by 2D agarose gel electrophoresis. This method has been widely used to detect the supercoil-induced structural transitions from B-DNA to unusual structures adopted by Pur·Pyr sequences (Lyamichev et al., 1985, 1987, 1989; Mirkin et al., 1987; Pulleyblank et al., 1985; Collier et al., 1988; Htun & Dahlberg, 1989; Shimizu et al., 1989; Wohlrab et al., 1987; Wohlrab & Wells, 1989; Wells et al., 1988). Figure 5 shows typical 2D agarose gel electrophoretic patterns of pRW1707 at pH 6.0 and 4.6. At pH 6.0, the structural transition occurred at topoisomer −8, and the amount of relaxation was about six superhelical turns, which is consistent with the entire insert (54 bp) being unwound by triplex formation. On the other hand, at pH 4.6, the structural transition occurred at topoisomer −5. No such transition was observed for the vector pRW791 (data not shown). This is consistent with previous reports (Lyamichev et al., 1985; Pulleyblank et al., 1985; Collier & Wells, 1990) which showed that a decrease of the pH reduces the requirements of negative supercoiling for formation of non-B structures at Pur·Pyr sequences.

Other differences in the 2D gel profile between pH 6.0 and pH 4.6 were observed. After the transition, each topoisomer consisted of two primary spots and one minor spot at pH 4.6, whereas only one spot with a relaxation of about six supercoils was observed at pH 6.0. At pH 4.6, one major spot had a relaxation of about six superhelical turns, whereas the other spot showed about five supercoils relaxed. Between topoisomers −6 and −9, the spot that was relaxed by six supercoils was dominant, whereas after topoisomer −9 the spot that was relaxed by five supercoils was dominant. Similar 2D gel patterns were observed when the first dimension was run at pH 5.0; that is, after the transition, two major spots were observed in the same linking difference topoisomer, although the transition required more supercoil energy than at pH 4.6 and less than at pH 6.0 (data not shown). This indicates that multiple non-B conformations probably coexist at pH 5–4.6, which are the intramolecular triplex and the other novel conformation(s), whereas a single intramolecular triplex is dominantly formed at pH 6.0. These 2D gel data are consistent with the chemical probe results.

Non-B-DNA Structures at the $(GGA)_9TCC(GGA)_8$ *and* $(GGA)_9TCC(GAA)_8$ *Inserts in pRW1724 and pRW1725.* To evaluate the sequence requirements for the structural polymorphisms in Pur·Pyr sequences, we constructed pRW1724 and -1725, which have $(GGA)_9TCC(GGA)_8$ (mirror repeat) and $(GGA)_9TCC(GAA)_8$ (non mirror repeat) inserts, respectively.

The pattern of DEPC and OsO_4 hyperreactivities at pH 5.0 for supercoiled pRW1724 as isolated from *E. coli* ($-\sigma$ ~0.06) is summarized in Figure 6, bottom panel. At pH 5.0, DEPC predominantly reacted with adenines in the middle of the purine strand (positions 15–45). Guanines in this region were also somewhat reactive. OsO_4 strongly reacted with thymines at the center of each $(GGA)_9$ region (positions 15, 18, 42, and 45) of the pyrimidine strand and at the central interrupted region (positions 27 and 28). At pH 7.0, adenines in the 5′-half of the purine strand were slightly modified by DEPC, and thymines in the central interrupted region (positions 27 and 28) were reactive to OsO_4 (data not shown). These modification patterns of pRW1724 at pH 5.0 and 7.0 are similar to those found with pRW1707, indicating that the $(GGA)_9TCC(GGA)_8$ sequence also has the same structural properties as the insert in pRW1707.

Furthermore, topoisomer populations of pRW1724 were subjected to 2D gel electrophoresis. At pH 4.6, the structural transition occurred at topoisomer −4. After the transition, each topoisomer showed mainly two spots (although the −9 and −10 topoisomers have two major spots and one minor spot), one with a relaxation of six supercoils and the other with five

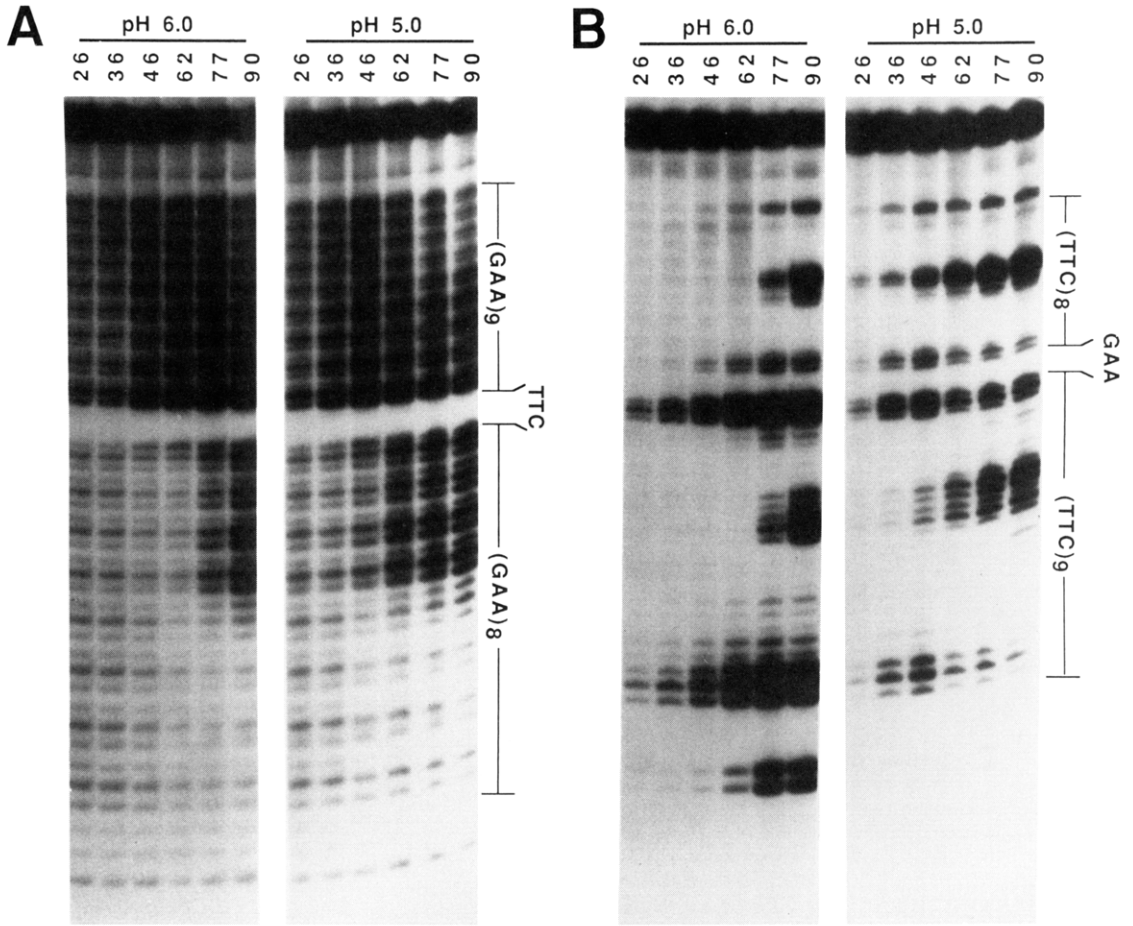

FIGURE 4: Fine mapping of DEPC and OsO_4 sites in pRW1707 as a function of negative supercoil density at pH 6.0 and 5.0. (Panel A) DEPC modification sites of the purine strand at pH 6.0 and 5.0 at the indicated average supercoil density ($-\bar{\sigma} \times 1000$). DEPC modification was performed in 0.1 M Tris–acetate buffer (pH 6.0 or 5.0) as described in the legend of Figure 2. The purine strand was radioactively labeled at the *Hin*dIII site. (Panel B) OsO_4 modification sites of the pyrimidine strand at pH 6.0 and 5.0 at the indicated average supercoil density ($-\bar{\sigma} \times 1000$). OsO_4 reactions were performed in 0.1 M Tris–acetate buffer containing 2% pyridine (pH 6.0 or 5.0) as described in the legend of Figure 2. The pyrimidine strand was radioactively labeled at the *Eco*O109 site. The gel is 5′ to 3′ from top to bottom. The sequence of the insert is shown on the right side of the gel.

supercoil turns. Between topoisomers −5 and −8, the spot that was relaxed by six supercoils was dominant, whereas after topoisomer −9 the spot that was relaxed by five supercoils was dominant (data not shown). This behavior of pRW1724 in the 2D gel at pH 4.6 was similar to that of pRW1707, although the structural transition required less energy than that for pRW1707. The differences in the transition points of pRW1707 and -1724 were probably due to the different G+C contents of the inserts, since the G+C contents of Pur·Pur sequences affect the thermostability and supercoil energy needed for triplex formation (Hanvey et al., 1989). Therefore, the results of chemical probing and 2D gel electrophoresis indicate that both pRW1707 and -1724 show the same structural polymorphisms as a function of pH and supercoiling.

Figure 6 (top) shows the nucleotide-level mapping at pH 5.0 of OsO_4- and DEPC-modified sites of supercoiled pRW1725 isolated from *E. coli* ($-\sigma \sim 0.06$). Figure 6 (right, bottom) shows the summary of the modification patterns. DEPC hypermodified the 5′-half of the $(GAA)_8$ region (positions 31–45) and the 3′-end of the $(GGA)_9$ region and the interrupted region (positions 24–27). The 5′-half of the $(GGA)_9$ region was slightly modified by DEPC, which was enhanced by a lowering of the pH to 4.5. OsO_4 strongly reacted with thymines in the center of the $(GAA)_8$ region (positions 41, 42, 44, and 45) and at the interrupted region (positions 27 and 28). Also, thymines at positions 15, 18, and 24 were reactive to OsO_4. This hyperreactive pattern is quite different from that of pRW1707 and -1724, indicating that the non-mirror repeat sequence, $(GGA)_9TCC(GAA)_8$, forms a non-B-DNA structure(s) different from the mirror repeat sequences in pRW1707 and -1724.

At pH 7.0, no OsO_4 and DEPC hyperreactive sites were observed for pRW1725 (data not shown). This is also different from pRW1707 and -1724, whose inserts form intramolecular triplexes around pH 7.0. This can be easily explained by the fact that intramolecular triplex formation requires or strongly prefers a mirror repeat Pur·Pyr sequence (Mirkin et al., 1987; Hanvey et al., 1988b, 1989).

The topoisomer populations of pRW1725 were also subjected to 2D gel electrophoresis as shown in Figure 7. The gel pattern for pRW1725 shows a smooth progression until topoisomer −4, and then, a sharp break in mobility was observed such that topoisomer −8 migrates the same as topoisomer −3 in the first dimension. The amount of total relaxation was about five supercoils, indicating that approximately 50–55 bp of primary helix is unwound if one supercoil turn is relaxed per turn of primary helix of Pur·Pyr sequences (10–11 bp) (Htun & Dahlberg, 1989; Collier & Wells, 1990). Also, the 2D gel profile of pRW1725 is different from those of pRW1707 and -1724. That is, after the transition, pRW1707 and -1724 show two main spots (plus one minor spot) of the same linking difference topoisomer, whereas pRW1725 shows a single spot for each topoisomer. A similar 2D gel pattern was observed for pRW1725 at pH 5.0, although the transition

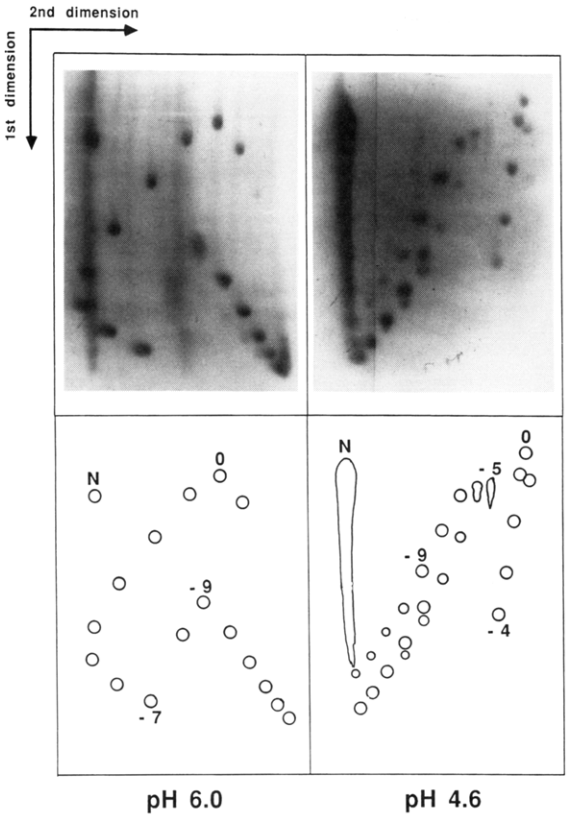

FIGURE 5: 2D agarose gel electrophoretic analysis of pRW1707. Mixtures of topoisomer populations were prepared as described previously (Singleton & Wells, 1982; Wohlrab et al., 1987) and subjected to the first-dimension gel electrophoresis in 2% agarose at 3 V/cm in 20 mM Tris–acetate (pH 6.0 or 4.6) containing 1 mM $MgCl_2$ at 27 °C. After the gel had been soaked in the buffer used in the second dimension, electrophoresis in the second dimension was carried out at 3 V/cm at a 90° angle to the first dimension in 40 mM Tris–acetate (pH 8.3)/1 mM EDTA containing 1.5 or 20 μM chloroquine phosphate (Sigma) for the first-dimension gel at pH 6.0 or at 4.6, respectively. (Upper panels) Gel photographs; (lower panels) schematic representations of gel photographs.

requires more supercoil energy than at pH 4.6 (data not shown).

In summary, at acidic pH (~5) both mirror repeat and non mirror repeat Pur·Pyr sequences form a non-B-DNA structure(s), and the energy required for the transition was nearly the same. However, the patterns of chemical modification, and thus the structure(s) formed, by mirror and non mirror repeat Pur·Pyr sequences are different. Also, near neutral pH, the mirror repeat Pur·Pyr sequences (about 55 bp in length) form intramolecular triplexes, whereas the non mirror repeat Pur·Pyr sequence of the same length does not form a non-B-DNA structure.

Two Intramolecular Triplexes Form in the $(GAA)_9TTAATTCGC(GAA)_8$ *Insert in pRW1718.* To investigate the complex structural properties of pRW1707, the central interrupted region was increased from 3 to 5, 7, or 9 bp by filling in unique *Bst*BI (TTCGAA) or *Eco*RI (GAATTC) sites or both in the insert of pRW1707. pRW1716, -1717, and -1718 contain $(GAA)_9TTCGC(GAA)_8$, $(GAA)_9TTAATTC(GAA)_8$, and $(GAA)_9TTAATTCGC(GAA)_8$ inserts, respectively. The DEPC hypermodification pattern of the purine strand of pRW1716 and -1717 at pH 5.0 was the same as that of pRW1707 (data not shown), indicating that increasing the interruption (up to 7 bp) does not affect the formation of the non-B-DNA structures. Recently, we showed (Shimizu et al., 1989) that an interruption of a similar length in the center of shorter Pur·Pyr sequences

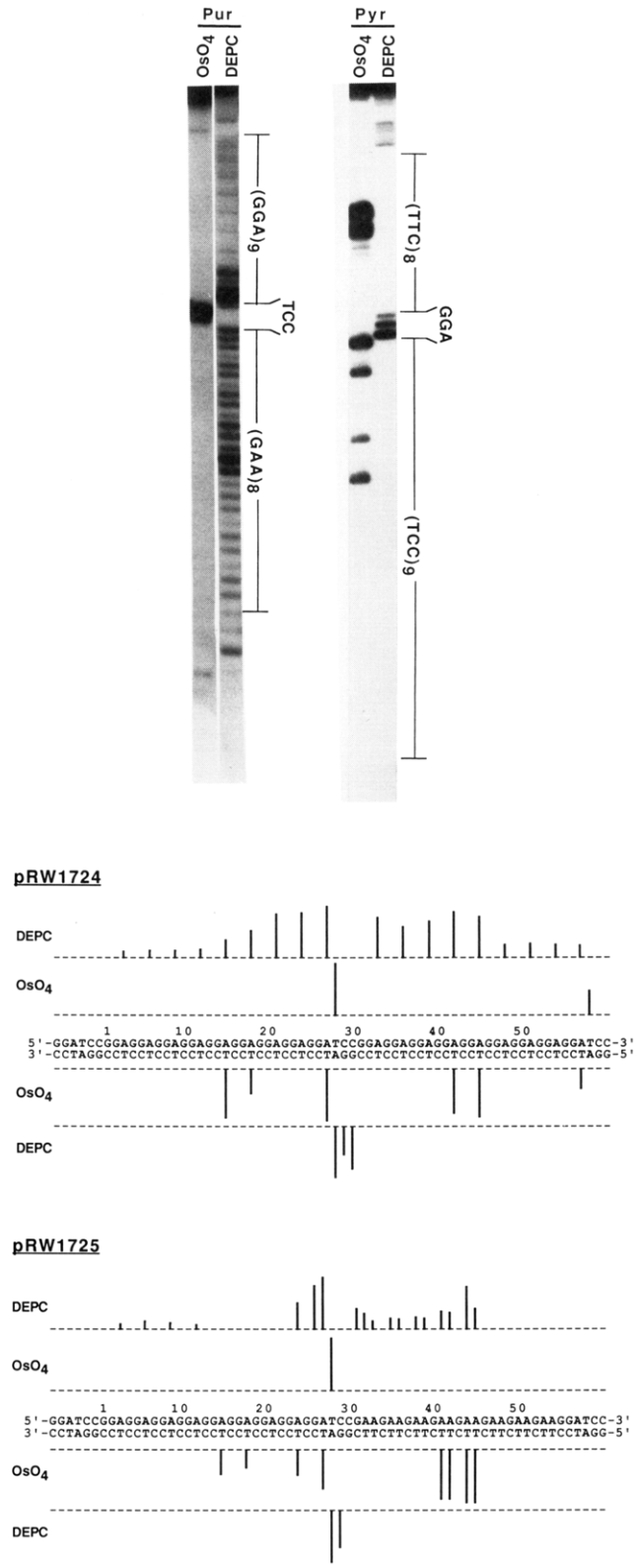

FIGURE 6: Fine mapping of OsO_4 and DEPC sites in supercoiled pRW1724 and -1725 at pH 5.0. (Top panel) OsO_4, DEPC, and DMS modification sites of pRW1725 at the nucleotide level at pH 5.0. The chemical modifications were performed as described in the legend of Figure 2. The gel is 5′ to 3′ from top to bottom. Pur, purine strand; Pyr, pyrimidine strand. The sequence of the insert is shown on the right side of the gel. (Bottom panel) Summary of the modification patterns for pRW1724 and -1725 at pH 5.0. The length of each line corresonds to the relative intensity of a modification site based on densitometric analysis of the autoradiograms using a Bio-Rad 620 video densitometer. The reproducibility of chemical probe studies is estimated, in general, to be within ±20%. The first G after the upper *Bam*HI site (GGATCC) is designated as position 1.

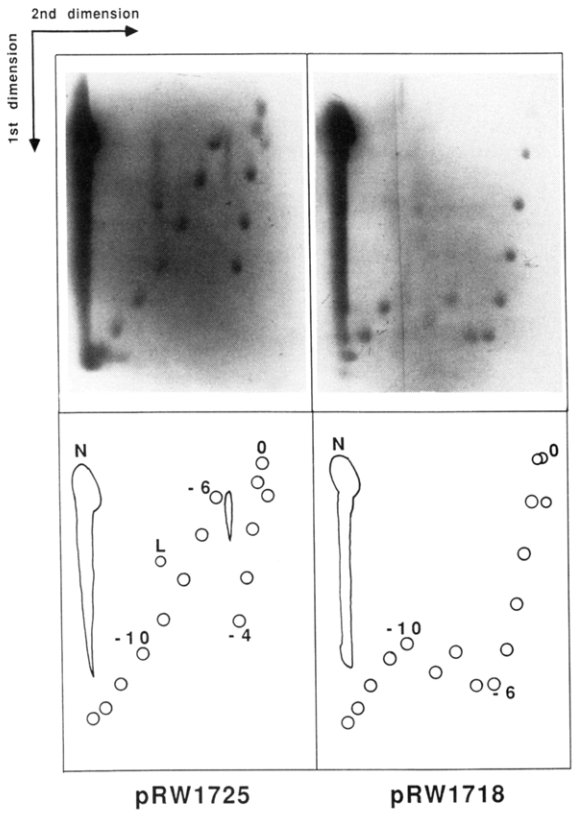

FIGURE 7: 2D agarose gel electrophoretic analysis of pRW1725 and -1718 at pH 4.6. The experimental conditions are described in the legend of Figure 5. (Upper panels) Gel photographs; (lower panels) schematic representations of gel photographs.

[$(GAA)_4(N)_n(GAA)_4G$, n = 3, 5, 7, and 9] does not eliminate the formation of intramolecular triplexes in supercoiled plasmids at pH 5.0.

However, with an increase in the interruption to 9 bp, the hyperreactive pattern of the insert changed dramatically. Figure 8 (top) shows the nucleotide-level mapping of OsO_4- and DEPC-modified sites of supercoiled pRW1718 ($-\sigma \sim 0.06$) at pH 5.0. Figure 8 (bottom) shows the summary of the modification patterns. Under this condition, the adenines in the 5′-half of both the $(GAA)_9$ and $(GAA)_8$ regions (positions 2–15 and 38–51, respectively) and in the central interrupted region are hyperreactive to DEPC, which is different from that of pRW1707. OsO_4 also modified thymines in the center of the $(TTC)_9$ and $(TTC)_8$ regions (positions 12–17 and 47–51) and in the interrupted region (positions 28–33). The hyperreactive patterns of the $(GAA)_9$ and $(GAA)_8$ regions in the insert are very similar to those of $(GAA)_9$ in pRW1704 (Hanvey et al., 1988b) and $(GAA)_8$ in pRW1408 (Hanvey et al., 1988a), suggesting that two separate intramolecular triplexes form at the $(GAA)_9$ and $(GAA)_8$ sequences in the $(GAA)_9TTAATTCGC(GAA)_8$ insert of pRW1718.

To clarify this point, 2D gel electrophoresis of pRW1718 was performed at pH 4.6. As seen in Figure 7, the gel pattern shows a smooth progression until about topoisomer −6, and then, a biphasic transition was observed between topoisomers −7 and −11. The supercoil energy required for the transition of pRW1718 is higher than that of pRW1707, -1724, and -1725 and is nearly the same as that of pRW1701, which contains the shorter $(GAA)_4TTC(GAA)_4$ insert as reported previously (Shimizu et al., 1989). The total amount of relaxation was about six superhelical turns. This demonstrates that as the negative superhelicity increases the $(GAA)_9$ [or $(GAA)_8$] region forma a triplex first and that, after a further

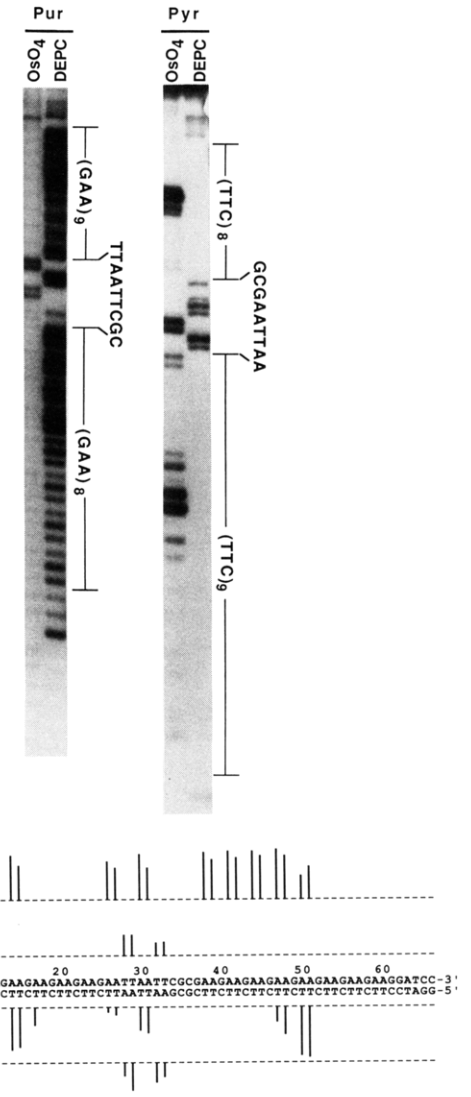

FIGURE 8: Fine mapping of OsO_4 and DEPC sites in supercoiled pRW1718 at pH 5.0. (Top panel) OsO_4, DEPC, and DMS modification sites of pRW1718 at the nucleotide level at pH 5.0. The chemical modifications were performed as described in the legend of Figure 2. The gel is 5′ to 3′ from top to bottom. Pur, purine strand; Pyr, pyrimidine strand. The sequence of the insert is shown on the right side of the gel. (Bottom panel) Summary of the modification patterns for pRW1718 at pH 5.0. The length of each line corresponds to the relative intensity of a modification site based on densitometric analysis of the autoradiograms using a Bio-Rad 620 video densitometer. The reproducibility of chemical probe studies is estimated, in general, to be within ±20%. The first G after the upper *Bam*HI site (GGATCC) is designated as position 1.

increase in the supercoiling, the other region independently forms a different triplex. Previously, a biphasic transition was observed in a 2D gel for a plasmid containing two separate segments with the potential to form Z-DNA (Zacharias et al., 1984, 1988; Jaworski et al., 1987; Kelleher et al., 1986). Hence, the structure of the $(GAA)_9TTAATTCGC(GAA)_8$ insert in supercoiled pRW1718 is not certain but may be a pair of triplexes (Discussion).

Discussion

Herein, we present evidence that a single Pur·Pyr sequence can adopt multiple non-B-DNA conformations, including a canonical intramolecular triplex and a novel structure, in plasmids depending on the pH and negative supercoiling. At or near neutral pH (7.0–6.0), relatively long mirror repeats [$(GAA)_9TTC(GAA)_8$ and $(GGA)_9TCC(GGA)_8$], but not a

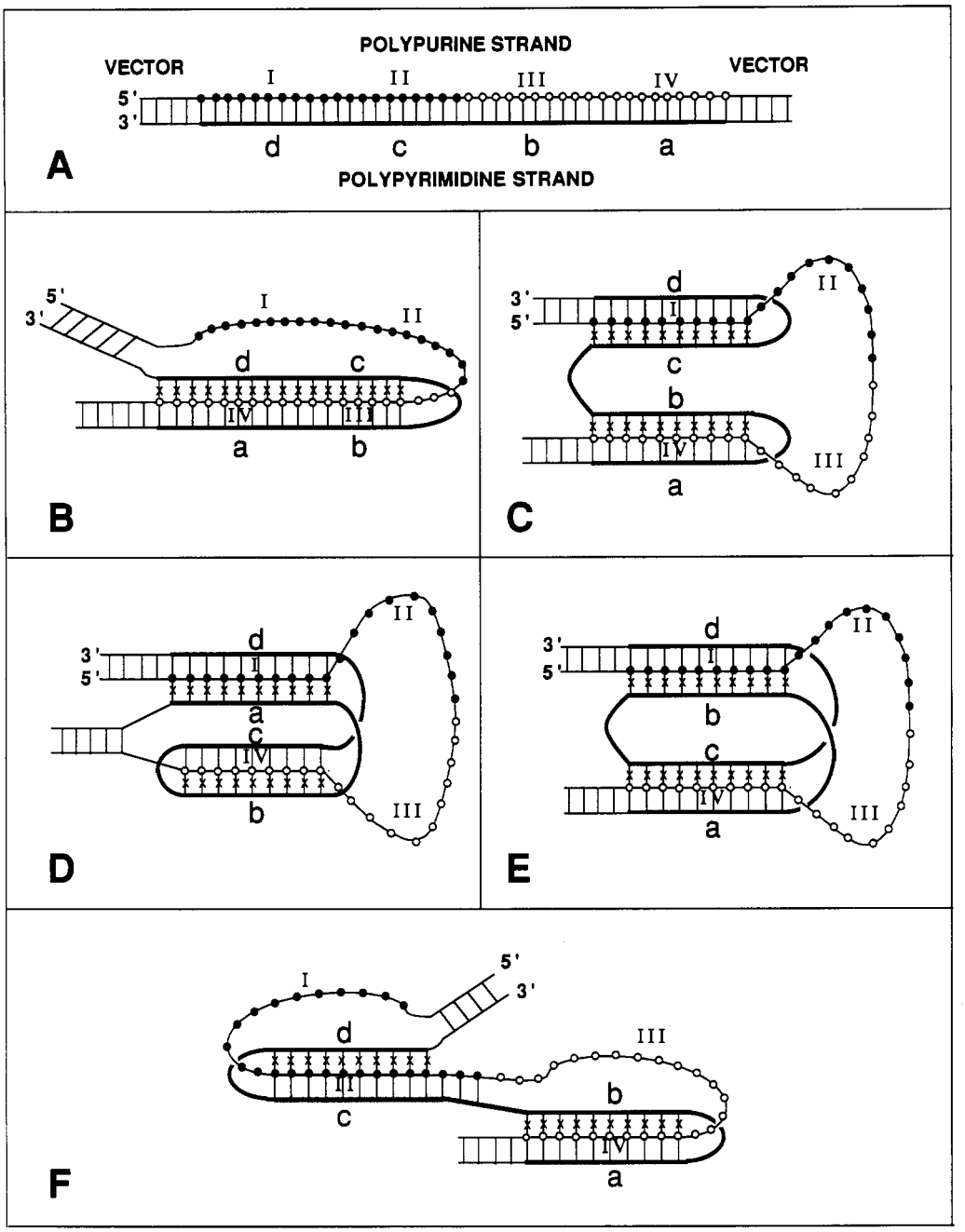

FIGURE 9: Possible triple-stranded DNA structures for pRW1707, -1724, -1725, and -1718. (A) Duplex of the Pur·Pyr sequence. The lines indicated as (●) and (○) are the 5′-half and 3′-half, respectively, of the polypurine strand. The thick lines are the polypyrimidine strand. For convenience, the polypurine strand is divided into four regions designated as I–IV from 5′ to 3′, whereas the polypyrimidine strand is designated a–d. (B) An intramolecular triplex (H-form) model. Regions I and II of the polypurine strand are unpaired. Regions III and IV of the polypurine strand associate with regions b and a of the polypyrimidine strand through Watson–Crick base pairs and with regions c and d through Hoogsteen base pairs, respectively. (C–E) Possible non-B conformations for pRW1707 and -1724 at low pH. Regions II and III of the polypurine strand and the center and quarter points of the polypyrimidine strand are unpaired. The guanines and adenines in regions I and IV of the polypurine strand are involved in Hoogsteen base pairs. (C) Regions I and IV of the polypurine strand associate with regions d and a of the polypyrimidine strand, respectively, through Watson–Crick base pairs and also with regions c and b, respectively, through Hoogsteen base pairs. (D) Regions I and IV of the polypurine strand associate with regions d and c of the polypyrimidine strand, respectively, through Watson–Crick base pairs and also with a and b through Hoogsteen base pairs. (E) Regions I and IV of the polypurine strand associate with regions d and a of the polypyrimidine strand, respectively, through Watson–Crick base pairs and also with b and c through Hoogsteen base pairs. (F) The two halves of the Pur·Pyr sequence separately form two canonical intramolecular triplexes (5′-half of the pyrimidine strand as the third strand). Regions I and III of the polypurine strand are unpaired. Regions II and IV of the polypurine strand associate with regions c and a of the polypyrimidine strand, respectively, through Watson–Crick base pairs and also with d and b through Hoogsteen base pairs. (Ladder symbols) Watson–Crick base pairs; (crossed ladder symbols) Hoogsteen base pairs. All models are schematic drawings and do not accurately represent the numbers of bp in the inserts (Figure 1).

non mirror repeat [$(GGA)_9TCC(GAA)_8$], form single intramolecular triplexes (Figure 9B) in supercoiled plasmids. In addition, the mirror repeat Pur·Pyr sequences in pRW1707 and -1724 can adopt other or another non-B conformations at or below pH 5.5, which is different from the canonical triplex.

The novel conformational state was observed by chemical probing and 2D gel electrophoresis. DEPC hypermodifies adenines in the middle of the polypurine strand of pRW1707 and -1724, and OsO_4 reacts with thymines at the center and quarter points of the polypyrimidine strand. One possible explanation for this hyperreactive pattern of the

$(GAA)_9TTC(GAA)_8$ and $(GGA)_9TCC(GGA)_8$ inserts is a mixture of small triplexes in the inserts in different plasmids; i.e., nucleation events occur at several points in the insert. Recently, we demonstrated that the $(GAA)_9TTC(GAA)_4$ sequence forms a mixture of two intramolecular triplexes with perfect stems in different plasmids; one was formed by the $(GAA)_9$ region and the other by the $(GAA)_4TTC(GAA)_4$ region (Hanvey et al., 1989). Thus, if the mixture of small triplexes forms in the insert of pRW1707, three primary regions are possible: the $(GAA)_9$, $(GAA)_4TTC(GAA)_4$, and $(GAA)_8$ regions. These triplexes have about the same stability (Hanvey et al., 1989) because their length and G+C content are almost identical. Hence, if these triplexes form in different plasmid molecules, DEPC modification sites should be observed for adenines at positions 2–44 of the polypurine strand with approximately equal intensity. However, the DEPC hyperreactive sites observed for pRW1707 and -1724 do not support this idea. Also, 2D gel electrophoresis analyses indicate that this novel conformation gives a relaxation of approximately five superocils, which indicates that almost the entire Pur·Pyr sequence is unwound. Thus, the 2D gel data also are not consistent with the formation of a mixture of small triplexes.

On the basis of the results of chemical probing and 2D gel electrophoresis, possible models for the structure of the $(GAA)_9TTC(GAA)_8$ and $(GGA)_9TCC(GGA)_8$ inserts of pRW1707 and -1724 at low pH were constructed (Figure 9). We assumed that the hyperreactive adenines and thymines are located in the single-stranded regions; i.e., the middle of the polypurine strand and the quarter points and center of the polypyrimidine strand would be unpaired. Also, guanines in the 5′-half of $(GAA)_9$ and the 3′-half of $(GAA)_8$ regions in pRW1707 are somewhat protected, suggesting that the guanines in these regions may be involved in Hoogsteen base pairs. Thus, the model of Figure 9C is a structure that simultaneously contains two triplexes in the insert; one isomer (5′-half of the pyrimidine strand as the third strand) forms in the $(GAA)_9$ region and the other isomer (3′-half of the pyrimidine strand as the third strand) in the $(GAA)_8$ region, which is different from the canonical triplex model (Figure 9B). Alternatively, other three-stranded models are possible for these sequences (Figure 9D,E). In these models, the two halves of the Pur·Pyr insert interact with each other. In Figure 9D regions a and b of the polypyrimidine strand associate as the third strand with regions I and IV of the polypurine strand, respectively, whereas in Figure 9E regions b and c of the polypyrimidine strand associate with regions I and IV, respectively (see legend to Figure 9).

To study the interaction between the two halves of the Pur·Pyr inserts, the interruption in pRW1707 was increased from 3 to 5, 7, or 9 bp. Although the 5- and 7-bp interruptions do not affect the formation of the novel conformation, the pattern of chemical modification changed dramatically with an increase in the interruption to 9 bp (pRW1718). Also, 2D gel electrophoresis analysis of pRW1718 revealed a biphasic structural transition, indicating that the $(GAA)_9$ and $(GAA)_8$ regions formed unwound structures independently. These results imply that the two canonical triplexes (3′-half of the pyrimidine strand as the third strand) form separately in the insert of pRW1718 (Figure 9F); i.e., if the interrupted region is long enough, no interactions will occur between the segments. Therefore, this suggests that in the $(GAA)_9TTC(GAA)_8$ and $(GGA)_9TCC(GGA)_8$ sequences the two halves of the inserts interact with each other.

To test the possible models in Figure 9C–E, the non mirror repeat sequence, $(GGA)_9TCC(GAA)_8$ was constructed (pRW1725). Interestingly, pRW1725 had structural properties different from those of the mirror repeat sequences. At acidic pH, although almost all of the entire Pur·Pyr insert of pRW1725 was unwound (as shown by 2D gel electrophoresis), no multiple spots were observed. Also, the DEPC and OsO_4 hyperreactive patterns were different from those of pRW1707 and -1724 and any Pur·Pyr sequence reported previously. If the model of Figure 9C is universal, the non mirror repeat $(GGA)_9TCC(GAA)_8$ sequence also should form this structure. However, the results with pRW1725 are not easily explained by the model in Figure 9C. In any case, this model cannot be ruled out completely, as it is possible that the disruption of the mirror motif causes changes in the free energy of nucleation and formation of the non-B structure and/or alters the melting behavior of the Pur·Pyr insert. As a result, a different non-B structure may form in pRW1725.

Alternatively, if the two halves of the Pur·Pyr insert are interacting, as shown in Figure 9D,E, to form a three-stranded region, the insert of pRW1725 cannot form these structures because of the nonmirror motif. Thus, the models of Figure 9D,E are consistent with the results of pRW1707, -1724, and -1725. At present, however, it is difficult to identify unambiguously the non-B conformation of pRW1707 and -1724 at low pH. Other models might also be possible. The molecular details of this conformation remain to be fully clarified and may require the development of new conformational probes.

Recently, Htun and Dahlberg (1989) proposed a mechanism for triplex formation. According to their model, the two isomers are not topologically equivalent: one isomer (3′-half of the pyrimidine strand as the third strand), which relaxed more supercoils than the other isomer, forms at higher supercoil densities, and the other isomer (5′-half of the pyrimidine strand as the third strand) occurs at lower supercoil densities. However, at pH 7.0 and 6.0, the $(GAA)_9TTC(GAA)_8$ sequence forms only one isomer (Figure 9B) of the triplex (3′-half of the pyrimidine strand as the third strand); no formation of the other isomer was observed in the range of supercoil density −0.01 to −0.09. Previously, we also observed no formation of the other isomer (5′-half of the pyrimidine strand as the third strand) for shorter Pur·Pyr sequences (about 25–30 bp) in the range $-0.01 < -\sigma < -0.07$ at pH 5.0 as revealed by OsO_4 modification (Hanvey et al., 1989; Shimizu et al., 1989). Due to differences in the Pur·Pyr sequences, flanking regions, and experimental conditions, their model may not be applicable for these Pur·Pyr sequences. Perhaps other mechanisms for triplex formation are possible under certain circumstances, since two pathways were proposed for cruciform extrusion depending on the environment [reviewed in Lilley et al. (1987)].

In summary, a given Pur·Pyr sequence can adopt more than one non-B conformation including a novel triplex structure. Since other workers have reported that other Pur·Pyr sequences can adopt more than one type of non-B conformation [reviewed in Wells et al. (1988), Antao et al. (1988), Parniwski et al. (1989), Htun and Dahlberg (1989), Wohlrab and Wells (1989), Lyamichev et al. (1989), Bernues et al. (1989), Kohwi (1989), Usdin and Furano (1989), and Collier and Wells (1990)], we conclude that Pur·Pyr sequences are highly polymorphic depending on the conditions. This behavior suggests that these structural changes may be induced in vivo by protein binding or alterations in supercoiling during cellular processes. As an example, the *E. coli* single-stranded DNA binding protein preferentially binds to and alters the conformation of

a triplex (Klysik, Shimizu, and Wells, submitted for publication).

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