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Conformational junctions between left-handed DNA in $(dA–dT)_{16}$ and contiguous B-DNA in a supercoiled plasmid contain chemically reactive bases

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Alternating adenine–thymine sequences in supercoiled DNA may undergo a transition to the left-handed Z-conformation in the presence of Ni^{2+} ions and high Na^+ concentrations [(1989) FEBS Lett. 243, 313–317]. In this work we have studied the junctions between B- and Z-conformations in a supercoiled plasmid containing a $(dA–dT)_{16}$ insert, by means of chemical probing. We observed enhanced reactivity of bases at both ends of the alternating tract to chloro- and bromoacetaldehyde. The degree of chemical reactivity was found to increase with the level of negative supercoiling. Only individual bases were observed to be reactive in the B–Z junctions, consistent with tightly localized interfacial regions.

Supercoiled DNA; Left-handed $(dA–dT)_{16}$; B–Z junction; Unpaired bases; Chemical probe; Bromoacetaldehyde; Chloroacetaldehyde

1. INTRODUCTION

The discovery of left-handed Z-DNA stimulated an enormous scientific effort, surveyed in numerous reviews (e.g. [2–4]). Regular alternation of *syn* and *anti* nucleoside conformations appeared to be one of the main characteristics of Z-DNA; such alternation occurs most easily in alternating purine–pyrimidine sequences. Z-DNA is readily formed under various conditions in $(dC–dG)_n$ and $(dA–dC)_n \cdot (dT–dG)_n$ but not normally in $(dA–dT)_n$ sequences. Several years ago left-handed DNA was observed in poly(dA–dT) · poly(dA–dT) under specific conditions including high NaCl concentration and presence of Ni^{2+} [5,6]. Recently we have shown [1] that left-handed DNA in the $(dA–dT)_{16}$ insert of pAT32 is stabilized by negative supercoiling, and occurs under conditions not sufficient to induce formation of left-handed DNA in poly(dA–dT) · (poly(dA–dT). We probed supercoiled DNA with diethyl pyrocarbonate (DEPC), a chemical probe that reacts preferentially with both left-handed and single-stranded DNAs [7–9]. This probe did not allow us to search for the conformational junctions between the left-handed segment and contiguous B-DNA (termed B–Z junctions).

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Abbreviations: BAA, bromoacetaldehyde; CAA, chloroacetaldehyde; DEPC, diethyl pyrocarbonate.

B–Z junctions have been studied in several laboratories (reviewed in [3,10]). It has been shown that these junctions contain distorted base pairs and/or unpaired bases [3,10]. So far no reports have been published about the existence and properties of the structural junctions between left-handed DNA formed in $(dA–dT)_n$ sequence and contiguous B-DNA. In this paper we used chloro- and bromoacetaldehyde (CAA and BAA, respectively), which react specifically with unpaired or rearranged base pairs, to probe the B–Z junctions in supercoiled pAT32 in the presence of 2.0 M NaCl and 0.2 M $NiCl_2$. We have found chemically reactive bases at both boundaries between B-DNA and the left-handed $(dA–dT)_{16}$ segment.

2. MATERIALS AND METHODS

2.1. *Plasmid DNA*

pAT32 (a recombinant 2,718 bp derivative of pUC19 that contains a $(dA–dT)_{16}$ insert cloned into the *Sma*I site [11]) was isolated from chloramphenicol amplified cells as described previously [12]. Samples of DNA of defined mean superhelical density ($\bar{\sigma}$) were generated according to [13].

2.2. *BAA, or CAA modification*

5 or 10 μg of plasmid DNA were modified in 100 μl total volumes. Reactions proceeded in 25 mM Tris-HCl (pH 7.8) plus 2.0 M NaCl and 0.2 M $NiCl_2$ unless otherwise stated, after 30 min preincubation at room temperature. Modification was performed by adding either BAA (prepared according to [14]) to concentration of 50 mM, or CAA (Fluka, doubly distilled) to concentration 0.2 M for 1 h at 37°C. After reaction, haloacetaldehydes were removed by double extraction with diethyl ether, and the DNA was ethanol precipitated and rinsed with 80% ethanol.

2.3. *Mapping of BAA, or CAA modified nucleotide residues*

The DNA was cleaved with either *Eco*RI or *Hin*dIII, radioactively 3'-^{32}P-labeled and cleaved with *Bgl*I. The required fragment (approximately 1,450 bp, or 235 bp, respectively) was purified and sites of modification were determined at sequence resolution by treating the fragment with either hydrazine or formic acid (Maxam and Gilbert sequencing protocol [15] for pyrimidines and purines, respectively) and reacting with 1 M piperidine at 90°C for 30 min [16]. After extensive lyophilization the DNA was loaded on the sequencing gel next to the Maxam and Gilbert sequencing reactions of the unmodified fragment. Radioactive DNA fragments were observed by autoradiography of dried gels at −70°C with intensifier screens or with storage phosphor screens and a 400 phosphorimager (Molecular Dynamics).

2.4 *Diethyl pyrocarbonate (DEPC) modification and mapping of DEPC-modified nucleotide residues*

Performed as previously described [1] in 50 μl total volumes under specified ionic conditions after 30 min preequilibration at room temperature. Modification was performed by adding 3 μl DEPC (Sigma) at room temperature for 30 min.

3. RESULTS

3.1. *Choice of the single-stranded selective chemical probe*

Our previous data [1] have shown that treatment of supercoiled pAT32 (at native superhelical density) with DEPC in 2.0 M NaCl plus 0.2 M $NiCl_2$ generated modification patterns typical of left-handed DNA (modification of all adenines within the $(dA–dT)_{16}$ insert). To probe the conformational junctions it was necessary to use a chemical that reacts with perturbed base pairs and/or unpaired bases, but not with Z-DNA. A number of such probes are available [10] but most of them are not suitable for probing DNA structure stabilized by Ni^{2+} ions because of competing reactions (e.g. complexes of osmium tetroxide with nitrogenous ligands, hydroxylamine and potassium permanganate; K. Nejedlý, unpublished results); others do not generate modification patterns at single nucleotide resolution (e.g. glyoxal). Haloacetaldehydes (CAA and BAA), which react preferentially with unpaired adenines and cytosines [17], do not seem to react with $NiCl_2$ (K. Nejedlý, unpublished results) and a procedure for their application at nucleotide sequencing level has been recently elaborated [16]. This procedure is based on increased reactivity of modified bases to subsequent chemical modification prior to performing piperidine cleavage. The enhanced reactivities of unpaired bases may be detected by increased intensity of certain bands, or the appearence of new ones.

3.2. *CAA and BAA modification patterns*

We used CAA and BAA to probe putative structural junctions in both strands of supercoiled pAT32 at native superhelical density (σ) and at more negative σ. Supercoiled DNA was reacted with the probe in 0.2 M $NiCl_2$ plus 2.0 M NaCl (i.e. in the ionic conditions inducing the formation of left-handed DNA in $(dA–dT)_{16}$, [1]).

3.2.1. Bottom strand

Modification patterns of the bottom strand are shown in Fig. 1. Native ($\sigma = -0.06$) and more negatively supercoiled ($= -0.09$) DNA reacted with 0.2 M CAA exhibited modification of the second 5' cytosine (C448) outside the $(dA–dT)_{16}$ tract (Fig. 1, track 6; Fig. 4) with less intense modification of native DNA (not shown). This alteration of band intensity in the boundary could be detected by a hydrazine reaction (pyrimidines). No reaction was observed in this region with formic acid (purines) (Fig. 1, tracks 3, 4). This difference was not due to reduced CAA induced reactivity of bases to formic acid: native DNA modified with CAA in 2.0 M NaCl undergoes pronounced modification only in cruciform loop regions in DNA reacted with either hydrazine or formic acid (not shown), though the latter is less

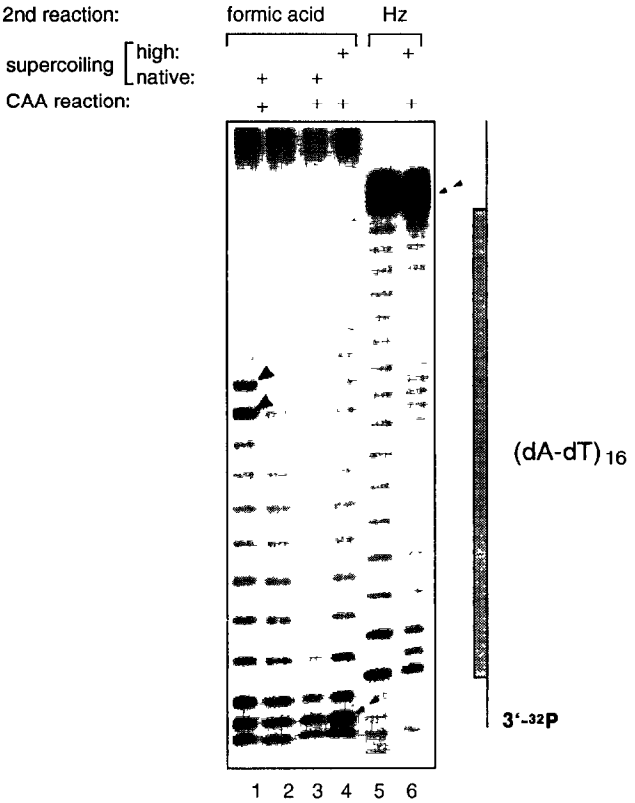

Fig. 1. CAA modification of pAT32 under Z-forming conditions: lower strand. Mapping of CAA modification of the lower strand of the *Eco*RI–*Bgl*I fragment (containing the $(dA–dT)_{16}$ insert) of native (tracks 1,3) and more negatively supercoiled ($\bar{\sigma} = -0.09$, tracks 4,6) plasmid. Supercoiled DNA was modified with 0.2 M CAA (1 h, 37°C) in 25 mM Tris-HCl (pH 7.8), 2.0 M NaCl (track 1), plus 0.2 M $NiCl_2$ (tracks 3,4,6). Purified DNA was cleaved by *Eco*RI and consequently treated as described in section 2. DNA was subsequently reacted with either formic acid (tracks 1–4) or with hydrazine (tracks 5,6). Samples in tracks 2 and 5 were not reacted with CAA, and may be used for reference. Simple arrows denote modification of the cruciform loop, double arrows show the modified bases within presumed B–Z junctions. The vertical line indicates the extent of the $(dA–dT)_{16}$ segment

intense. Similarly, there is modification of the second 3′ guanine (G413) outside the tract (Fig. 1, track 4) of formic acid reacted sample, but not of hydrazine one.

Similar results were obtained with 50 mM BAA modified plasmid. Both native and more negatively supercoiled ($\bar{\sigma}$ = −0.07) DNAs showed modification of the second 5′ cytosine (C448) outside the $(dT–dA)_{16}$ tract (not shown). Quantitative analysis by phosphorimaging revealed increased intensity of this band by 45% (native) or 65% ($\bar{\sigma}$ = −0.07), respectively, in comparison to control (BAA-unmodified) sample. Preliminary experiments with another $(dA–dT)_{16}$ bearing plasmid (pTA16; [18]) indicated enhanced BAA reactivity of the first 3′ adenine outside the insert in the bottom strand (not shown).

3.2.2. Upper strand

Fig. 2 presents the results of CAA modification of the upper strand of both native and more negatively supercoiled ($\bar{\sigma}$ = −0.09) pAT32 DNA. We detected modification of the second 5′ cytosine (C413) outside the $(dA–dT)_{16}$ tract (Fig. 2, tracks 1, 3, 4; Fig. 4); reactivity of native DNA being lower than that of the more highly supercoiled DNA (Fig. 2, track 3). This C413 modification band was detected both by hydrazine (Fig. 2, tracks 3, 4) and formic acid (Fig. 2, track 1) reactions. In addition, there was enhanced modification of the adjoining cytosine (C412) in native DNA (Fig. 2, track 3). At the opposite junction enhanced modification of the second 3′ guanine (G448) was observed, again more intense with increased negative supercoiling (not shown).

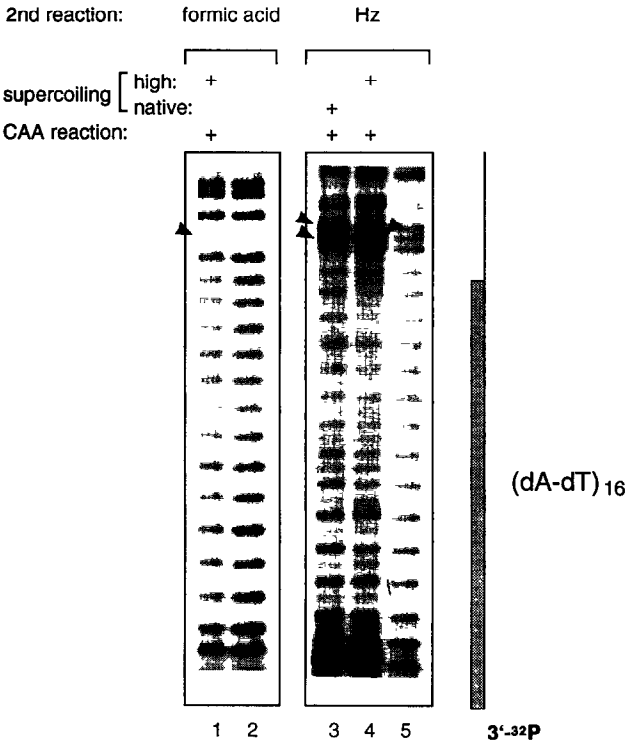

Fig. 2. CAA modification of pAT32 under Z-forming conditions: upper strand. Mapping of CAA modification of the upper strand of the *Hin*dIII–*Bgl*I fragment (containing the $(dA–dT)_{16}$ insert) of native (track 3) and more negatively supercoiled ($\bar{\sigma}$ = −0.09, tracks 1,4) plasmid. Reaction conditions were identical to those given in Fig. 1. DNA was cleaved by *Hin*dIII and consequently treated as described in section 2. Samples in tracks 1 and 2 were secondarily reacted with formic acid, in tracks 3–5 with hydrazine. DNA in tracks 2 and 5 was unmodified with CAA.

The dependence of both BAA, and CAA modification of boundary(ies) upon superhelical density reflects the effect of superhelical stress on the B to Z transition within $(dA–dT)_n$ insert. More negative supercoiling increases the proportion of $(dA–dT)_{16}$ inserts present in the Z conformation, but does not affect either the width or location of boundary(ies).

Probably both C–G and G–C base pairs flanking the insert which are 'in phase' with alternating adenines and thymines adopt Z conformation, because within both boundaries there was modification of the next but one base pair to the insert (Fig. 4). Thus the Z-segment is expanded to include these extra bases. With plasmid pTA16 we observed BAA modification of the first 3′ A in the bottom strand adjoining the insert, which is 'out of phase' with the insert, limiting the extent of left-handed region.

In addition to reactivity of unpaired cytosines and adenines we have observed the reactivity of unpaired guanines with CAA, in agreement with the results of others [19]. Although less reactive, guanine residues react at the exocyclic amino group and the ring nitrogen in N-1 or N-3 position [20,21].

3.3. *Cleavage at sites of CAA-modified bases with nuclease S_1*

Chemically modified bases might be susceptible to cleavage by the single-strand-selective nuclease S1. However, S1 nuclease cleavage of 3′-labeled fragments reacted with CAA under Z-forming conditions, followed by resolution at single nucleotide level, showed no site-specific cleavage at the junction regions (not shown), in agreement with known inability of this enzyme to recognize and cleave an isolated modified single bases [14].

Modification of only one base pair within both B–Z junctions suggests that the junctions are quite narrow. We previously obtained similar results with osmium tetroxide modified B–Z junctions in $(dC–dG)_n$ bearing plasmid cleaved by S1 nuclease, whereas hot piperidine cleavage showed modification of both boundaries [12].

3.4. *The effect of decreased pH on the B–Z transition*

The transition of $(dA–dT)_{16}$ inserts into the left-handed form was observed (just as for the polynucleotide $(dA–dT)_n$) at acidic pH (5.3–5.5) due to the presence of high concentration of $NiCl_2$. It is difficult to approach neutral pH because of $NiCl_2$ hydrolysis. As we

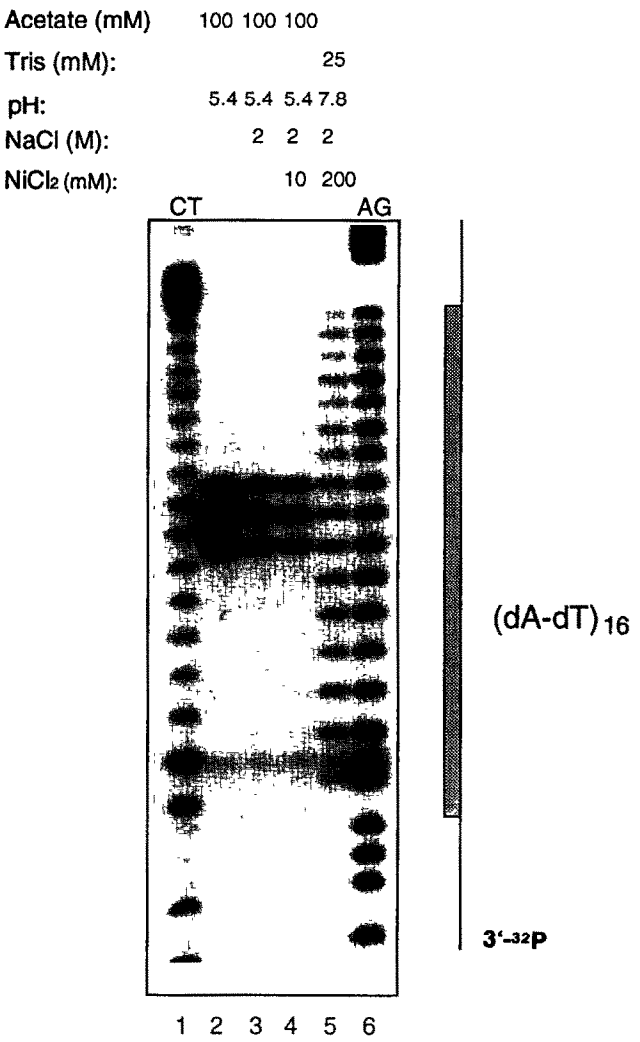

Fig. 3. The effect of ionic conditions on the pattern of DEPC modification of the $(dA–dT)_{16}$ sequence in native supercoiled pAT32. DNA was modified with DEPC (30 min, room temperature) in 0.1 M sodium acetate buffer, pH 5.4 (track 2) plus 2.0 M NaCl (track 3), or 2.0 M NaCl and 10 mM $NiCl_2$ (track 4). Track 5 contains pAT32 reacted with DEPC in 25 mM Tris-HCl buffer, pH 7.8 plus 2.0 M NaCl and 0.2 M $NiCl_2$. Tracks 1 and 6 contain sequencing reactions of C+T and A+G, respectively. The vertical line indicates the extent of the $(dA–dT)_{16}$ segment.

have suggested previously [1], the B to Z transition is probably not induced by final pH values, which were nearly the same in 1.5 M NaCl plus 0.2 M $NiCl_2$, when the insert adopted cruciform structure. Cruciform was extruded either in acetate buffer (pH 5.4), or in buffered high salt (2.0 M NaCl) in the presence or absence of 10 mM $NiCl_2$ (Fig. 3, tracks 2–4), i.e. no sign of denaturation bubble occurrence due to acidic pH. These results confirm the idea that the lower pH is not the cause of DEPC reactivity 'mimicking' the B–Z transition under the conditions used.

4. DISCUSSION

In this paper we have shown that the boundaries between left-handed DNA in $(dA–dT)_{16}$ and contiguous B-DNA contain chemically reactive bases. These are located outside the $(dA–dT)_{16}$ segment suggesting that all the segment plus flanking C–G and G–C base pairs adopt left-handed form under the given conditions.

4.1. *Conformation of $(dA–dT)_n$ sequences*

It has been shown that $(dA–dT)_n$ sequences inserted in negatively supercoiled plasmids may adopt two different structures in addition to left-handed DNA: one of them is cruciform [11,22,23], while the other exhibits uniform thymine reactivity to osmium tetroxide along the tract (possibly a consequence of poor TpA stacking) and torsional deformability. The latter has been observed in linearized plasmids as well as negatively supercoiled DNA [18,24]. A related structure has been recently observed as a uniform chemical reactivity of $(dA–dT)_n$ sequences contained in positively supercoiled DNA [25]. Conformational variability of $(dA–dT)_n$ sequences may be of biological significance if the above mentioned conformations are also formed in vivo. Cruciform formation by $(dA–dT)_n$ sequences as a function of the level of negative superhelicity has been recently demonstrated in *E. coli* [26]. The conditions under which we demonstrated left-handed DNA in $(dA–dT)_{16}$ are far from physiological. Left-handed DNA can be formed, however, in $(dA–dT)_n$ at substantially lower Ni^{2+} concentration under conditions closer to physiological if the $(dA–dT)_n$ segment is surrounded with other alternating purine–pyridimine sequences [27].

4.2. *Conformational junctions between left-handed and right-handed DNAs*

At present the exact structure of the B–Z junction is

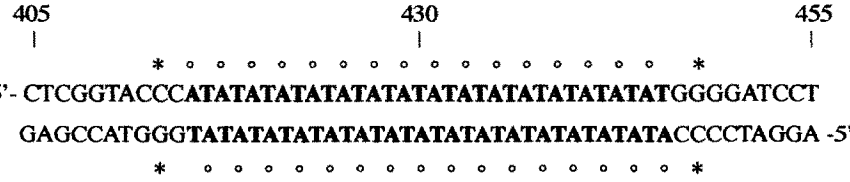

Fig. 4. Location of the CAA (asterisk) or DEPC (circle) modified bases found within the pAT32 fragment containing $(dA–dT)_{16}$ insert and its flanking sequences under Z-forming conditions used in this study. The alternating $(dA–dT)_n$ sequence is highlighted in bold.

not known. The results so far obtained suggest that the B–Z junction may not be characterized by a single structure. More probably different structures can be expected depending on nucleotide sequence, environmental conditions and other factors. Experimental data obtained by enzymatic and chemical probes, NMR and other techniques (reviewed in [10]) suggest the presence of distorted base pairs or unpaired bases in the junctions, in agreement with the results of structural modelling. Recent studies have demonstrated other peculiar features of the B–Z junctions which include enhanced intercalation of some drugs [28], and probably also DNA bending at the site of the junction [29]; not all data concerning the DNA bending are in complete agreement [30] and further work will be necessary.

The pronounced reactivity of particular bases in the B–Z junction regions to haloacetaldehyde suggests that these have a distinct local conformation. BAA or CAA reaction at adenine for example generates a cyclic etheno adduct at the N^1 and N^6 positions [17], which are not available when the base is conventionally basepaired with a thymine. However, these positions become accessible to covalent modification if the basepair becomes broken, or perhaps rearranged (to a Hoogsteen pair for example). Thus enhanced chemical reactivity of bases in the interfacial region might reflect a significant fraction of basepair breakage, or rearrangement to an alternative pairing scheme.

Haloacetaldehydes have been used to probe the conformations of bases in various B–Z junctions (reviewed in [10]), but with one exception [16] DNA cleavage at the site of modified bases was revealed with S1 nuclease; this enzyme may not, however, recognize and cleave DNA at a single modified base [14]. The single reactive bases that we observe in each strand at the boundaries of the left-handed segment adopted by $(dA–dT)_n$ sequences (Fig. 4) suggest that the conformational junction is narrow. This is similar to B–Z junctions observed in supercoiled plasmids containing left-handed DNA in $(dC–dG)_n$ segments [10,12] and differs from the much wider junctions observed with $(dA–dC)_n \cdot (dT–dG)_n$ sequences [10,31]. It is not clear whether the narrowness of the junction in pAT32 is connected with the $(dA–dT)_{16}$ sequence or whether it is due to the high salt environment containing $NiCl_2$.

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