A Study of the B-Z Transition of the AC-rich Region of the Repeat Unit of a Satellite DNA from Cebus by Means of Chemical Probes*

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The conformational changes induced by negative supercoiling in the AC-rich region of the repeat unit of a Cebus satellite DNA has been studied by chemical probes sensitive to alterations in DNA conformation. This region is constituted of a (GT/CA)$_n$ stretch (15 $\leq n \leq 18$) associated to a sequence rich in GT/CA. At high superhelical density, at least 100 base pairs in the AC-rich region adopt the Z conformation as judged by diethyl pyrocarbonate reactivity. This is confirmed by diethyl pyrocarbonate footprinting of the complex between antibodies to Z-DNA and the AC-rich region. Osmium tetroxide and hydroxylamine reveal some distortions of the Z double helix in the (GT/CA)$_n$ stretch. The terminal T residues of the stretch are hyperreactive with osmium tetroxide; the terminal left C residues but not the terminal right C residues are hyperreactive with hydroxylamine. Substitution of a few base pairs in the middle of the (GT/CA)$_n$ stretch also also some distortions of the Z double helix. In the GT/CA-rich sequence, distortion of the Z double helix is also supported by the hyperreactivity of osmium tetroxide with several T and C residues.

Natural and synthetic DNAs are flexible molecules which can exist in a variety of conformations (1–5). In plasmids, free energy of negative supercoiling is one of the most important factors to drive the transition from B form to unwound DNA (6) or to complex conformations, e.g. slippage (7) and cruciform structures (8, 9), left-handed Z double helix (10–12), or to still unknown structures (13–18).

In situ immunofluorescence techniques with antibodies to Z-DNA have shown that sequences able to adopt the Z conformation exist in the genome (19–24). In fixed metaphase chromosomes from Cebus, antibodies to Z-DNA interact strongly with segments corresponding to R band positive heterochromatin (23). These segments are constituted of a satellite DNA, the repeat unit of which contains an AC-rich region. The AC-rich region is constituted of a stretch of (GT/CA)$_n$, associated to a sequence rich in dinucleotides GT/CA. Antibody retention experiments have shown that, in vitro, the AC-rich region adopts the Z conformation (25). We were interested to know whether, under topological constraints, the whole GT/CA-rich sequence adopts a Z-like conformation in addition to the (GT/CA)$_n$ stretch.

Recently, several chemical reagents have been used to map the conformational changes at the nucleotide level. It has been found that their reactivity is strongly dependent upon the structure of DNA. Diethyl pyrocarbonate is hyperreactive with purines in Z-DNA or in single stranded DNA (26–31). Dimethyl sulfate has similar reactivity with the guanine residues in B-DNA or in Z-DNA (27). Osmium tetroxide and hydroxylamine react with denatured DNA. They do not react with B-DNA or Z-DNA but react with T (osmium tetroxide) and C (hydroxylamine) residues at the B-Z functions (27, 32, 33). Bromoacetaldehyde and chloroacetaldehyde react with A and C residues in denatured regions and at the B-Z interfaces (34–39).

In this work, using chemical probes we show that under topological constraints, about 100 base pairs of the AC-rich region adopt the Z conformation. The study has been performed in a large range of superhelical density, since recent results strongly suggest that in transient states DNA can be highly negatively supercoiled (40, 41).

MATERIALS AND METHODS

Plasmids and Antibodies—pCP1, pCP2, pCP3, and pCP4 contain a fragment of about 300 base pairs from four different repeat units of the Cebus satellite DNA (25). They were prepared after MluI degradation of the satellite DNA and cloned in PstI site of pBR322 (pCP1) or in Smal site of pUC18 (pCP2, pCP3, and pCP4). Closed circular plasmid DNAs were isolated according to standard procedures (42) and banded twice in a CsCl/ethidium bromide equilibrium density gradient. Plasmids of different negative superhelical density (s) were generated and characterized as described in Ref. 25. Topoisomerase I from chicken erythrocytes was prepared according to the method described in Ref. 43. Preparation and affinity chromatography purification of anti Z-DNA antibodies have been described previously (44).

Chemical Modifications—Plasmid DNAs were modified as described by Herr (26) for diethyl pyrocarbonate (DEPC) and by Johnston and Rich (27) for OsO$_4$ and hydroxylamine. Dimethyl sulfate reactions were performed according to Maxam and Gilbert (45).

Interaction with Antibodies—Preincubation for 1 h at 20 °C of 2 μg of plasmid with 20 μg of nonspecific IgG or antibodies to Z-DNA were carried out in 150 μl of 60 mM NaCl, 5 mM Tris-Cl, pH 7. Tubes were transferred in ice, following adjustment of the medium at 50 mM NaCl, 25 mM sodium cacodylate, 0.25 mM EDTA, pH 7. Samples were further processed according to Herr (26), with a phenol extraction step to remove antibodies prior to precipitation with ethanol.

Labeling—DNAs were digested with the appropriate restriction nucleases and labeled at either the 5' end (T4 polynucleotide kinase) or the 3' end (Klenow polymerase). Uniquely end-labeled DNA

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1 The abbreviation used is: DEPC, diethyl pyrocarbonate.
RESULTS

pCP1 contains the AC-rich region (about 100 base pairs) of a repeat unit of the Cebus satellite DNA (25). As shown in Fig. 1, between residues 30 and 59 there is a stretch of 15 GT/CA (written (GT)₃), rich in GT/CA (19 over 33 dinucleotides) in pCP1. The sequence does not show any apparent specificity.

We also took advantage of the variations in the sequence of the AC-rich region from one repeat unit to another (25) to study some sequences which are not in pCP1. Three clones have been studied, and their sequences are given in Fig. 7. They resemble that of pCP1 with a (GT)₃ stretch in which the following nucleotides are inserted: TT/AA for pCP2, GA/CT for pCP3, and TTGA/AACT for pCP4. Moreover, the base pair A/T in position 27 in pCP1 is replaced by a base pair C/G in the three clones. The sequence (ATCT/TAGA)⁹₀ is replaced by GGCC/CCGG in pCP4.

In order to determine the conformational changes of the AC-rich region as a function of the superhelical density of the plasmids, several chemical probes have been used in the presence or absence of the antibodies to Z-DNA.

Chemical Hyperreactivity of the AC-rich Region of pCP1

Diethyl Pyrocarbonate—The negative supercoil-dependent DEPC reactivity of the AC-rich region of pCP1 is shown in Fig. 2A for the AC strand and in Fig. 3A for the GT strand. With relaxed DNA, the purines are not hyperreactive. At a mean superhelical density σ = -0.05, in the (GT)₃ stretch, the A residues and to a less extent the G residues, are hyperreactive. A weak reactivity of some A residues and G residues is also observed on each side of the (GT)₃ stretch (residue A²³ on the left and up to residue 80 on the right). As −σ increases, more purines become hyperreactive (at σ = -0.085, up to residue 12 on the left side of the (GT)₃ stretch and up to residue 130 on the right side). At σ = -0.05 and -0.1, the patterns of reactivity are slightly different, but no new residues become hyperreactive. No difference of re-

Heterogeneity in Z-DNA

Fig. 1. Sequence of the AC-rich region of the fragment of the repeat unit of Cebus satellite DNA inserted in pCP1. Left and right refer to the orientation of the gels in Figs. 2, 3, and 5.

Fig. 2. Supercoil-induced hyperreactivity of the AC strand of pCP1. A, DEPC; B, OsO₄; C, hydroxylamine. G, G + A, C, T + C, Maxam and Gilbert specific reactions. The values of the negative superhelical density are indicated at the top of each lane. Left, right, and numbers refer to Fig. 1.
activity is observed between $\sigma = -0.1$ and $\sigma = -0.12$ (not shown).

At $\sigma = -0.1$, the reactivity of the GT/CA on the right side of the (GT)$_{15}$ stretch is similar to that of the GT/CA in the stretch with a strong hyperreactivity of the A residues and weaker hyperreactivity of the G residues. One exception is G residue 112 which is not hyperreactive.

**Osmium Tetroxide**—No reaction is detected between OsO$_4$ and relaxed DNA (Figs. 2B and 3B). At $\sigma = -0.05$, several residues on both strands become hyperreactive. All the T residues on the AC strand (even T$^{129}$) and some C residues (C$^{69}$, C$^{79}$, and to a lesser extent C$^{89}$, C$^{79}$, C$^{79}$, and C$^{99}$) are hyperreactive. On the GT strand, three T residues on the left of the (GT)$_{15}$ stretch and also some T residues at the ends of the (GT)$_{15}$ stretch are hyperreactive. The T residues on the right of the (GT)$_{15}$ stretch are not or poorly reactive.

As $-\sigma$ increases, an extension of the reactive region is observed. At $\sigma = -0.1$, on the GT strand, some T residues are reactive up to T residue 125, and on the AC strand C residue 101 is hyperreactive. On the other hand, the pattern of reactivity of the already reactive bases is modified. Some residues become less reactive (T$^{29}$, C$^{59}$, C$^{69}$, C$^{79}$, C$^{79}$, C$^{99}$, and C$^{99}$) and the T residues in the (GT)$_{15}$ stretch). Some residues become more reactive (T$^{12}$, T$^{79}$, C$^{69}$, T$^{129}$). Finally, some residues present a complex behavior; the reactivity of the T residue at position 60 increases up to $\sigma = -0.07$ and then decreases (a similar behavior is observed but to a less extent for T residues 23, 27, 75, 77, 81, and 83).

**Hydroxylamine**—Hydroxylamine has been reacted with relaxed and highly supercoiled pCP1 ($\sigma = -0.1$). Only the results relative to the AC strand are shown in Fig. 2C. With the relaxed DNA, a slight reactivity with all the residues is observed. At $\sigma = -0.1$, the same background of reactivity is observed but a few residues are hyperreactive: C$^{23}$, C$^{39}$, C$^{79}$, and C$^{85}$. In the (GT)$_{15}$ stretch, C residue 30 is a strong hydroxylamine target. C residues 32 and 34 are less modified. In the GT strand, only C residues 26 and 62 are hyperreactive (not shown).

**Dimethyl Sulfate**—In relaxed DNA, all the G residues react equally well with dimethyl sulfate (Fig. 3C). On the GT strand, at $\sigma = -0.1$, G residues 69, 79, 85, 101, 111, 115, and 121 are hyperreactive. A small increase of the reactivity of the adjacent G residues is observed (except G residue 86). The reactivity of the other G residues is similar to that observed with relaxed DNA. On the AC strand, G residues 26, 62, and 110 are hyperreactive at $\sigma = -0.1$ (not shown).

A summary of the reactivity of pCP1 at $\sigma = -0.1$ with the chemical probes is given in Fig. 4.

**DEPC Footprint of Anti-Z-DNA Antibodies over the AC-rich Region of pCP1**

The reactivity of DEPC with the complexes formed between pCP1 and the antibodies to Z-DNA has been studied (Fig. 5). At $\sigma = -0.05$, in the absence of antibodies, the (GT)$_{15}$ stretch and the GT/CA dinucleotides up to about residue 80 are reactive with DEPC. The presence of the antibodies induces an extension of the purine reactivity which can be detected on the AC strand from residues 22 up to 120 and on the GT strand up to residue 5. At $\sigma = -0.1$, in the presence of the antibodies, the hyperreactivity of the G residues is reduced, while that of most of the A residues is not modified. There are several exceptions: the hyperreactivity of A residues 59, 117, and 123 is enhanced, and that of 130 is inhibited. In addition, A residues 133, 135, 137, and 139 become hyperreactive. The hyperreactivity of C and T residues with OsO$_4$ (not shown) and of G residues with dimethyl sulfate (Fig. 3C) is

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**Fig. 3. Supercoil-induced hyperreactivity of the GT strand of pCP1.**

A, DEPC. B, OsO$_4$. C, dimethyl sulfate. G, G + A, C, T + C, Maxam and Gilbert specific reactions. The values of the negative superhelical density are indicated at the top of each lane. In C, lane $0.1+Z-Ab$ corresponds to the dimethyl sulfate footprinting of the anti Z-DNA antibodies on the GT strand of pCP1 at a superhelical density of $-0.1$. 

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the presence of nonspecific IgG. DNA, a function of the superhelical density. This AC-rich region is nonspecific of kc.

Maxam and Gilbert specific reactions. This AC-rich region of pCP1.

Fig. 4. Summary of the changes in chemical reactivity in the AC-rich region of pCP1. σ = -0.1. ○, DEPC; ▲, OsO₄; □, hydroxylamine; ●, dimethyl sulfate. Filled, open, and dotted symbols indicate strong, intermediate, and low hyperreactivity, respectively.

Chemical Reactivity of pCP2, pCP3, and pCP4

The main difference between pCP1 and pCP2, pCP3, and pCP4 is the presence of a dinucleotide or a tetranucleotide embedded in the (GT), stretch (Fig. 7). We focus our attention on these inserted bases, the chemical reactivity of the other parts of the AC-rich region being essentially similar to that observed with pCP1.

At high superhelical density, the 5’ T and the 5’ A residues in TT/AA (pCP2) are hyperreactive with DEPC and OsO₄, respectively (Fig. 6). In pCP3, G and T residues in GA/CT are hyperreactive with DEPC and OsO₄, respectively. When the two dinucleotides are associated in a tetranucleotide (pCP4), the reactivity of the bases is not changed as compared to that of the dinucleotides.

The TT/AA dinucleotide in the (GT), stretch (pCP2) modifies the reactivity of the bases on its left side but not on its right side (Fig. 6). The gradient of reactivity of the T residues with OsO₄ observed at low superhelical density is no longer observed at high superhelical density. At σ = -0.1, only the last GT/CA presents an unusual chemical reactivity: G and T residues are hyperreactive, while the A residue reacts as the other A residues in the (GT), stretch.

GA/CT introduces small changes in the reactivity of the bases in the (GT), stretch (pCP3); only the last T residue on the right is weakly reactive with OsO₄ (Fig. 6).

In pCP4, at σ = -0.05, a gradient of reactivity of the T residues with OsO₄ is observed on the right side of the TTGA/AATC insert, whereas a poorly resolved reactivity is observed on the left side.

Hydroxylamine does not react with the inserted bases (not shown).

A summary of the reactivity of pCP2, pCP3, and pCP4 inserted nucleotides is given in Fig. 7.

DISCUSSION

We have used several chemical probes and antibodies to Z-DNA to study the conformational changes of the AC-rich region present in the repeat unit of a Cebus satellite DNA as a function of the superhelical density. This AC-rich region is...
The heterogeneity of Z-DNA is illustrated in the figure below, showing the supercoil-induced hyperreactivity of the GT strand of pCP2, pCP3, and pCP4 with: A, DEPC; and B, OsO₄. The values of the negative superhelical density are indicated at the top of each lane. Sequences of the inserted nucleotides in the (GT)ₙ stretch are indicated. Left and right refer to Fig. 7.

**Fig. 6.** Supercoll induced hyperreactivity of the GT strand of pCP2, pCP3, and pCP4 with: A, DEPC; and B, OsO₄. The values of the negative superhelical density are indicated at the top of each lane. Sequences of the inserted nucleotides in the (GT)ₙ stretch are indicated. Left and right refer to Fig. 7.

The DEPC reactivity pattern observed in the presence of the antibodies to Z-DNA strengthens the suggestion that all the GT/CA of the sequence adopt the Z conformation since at \( \sigma = -0.1 \), the presence of the antibodies abolishes the hyperreactivity of the G residues (Fig. 5B).

We conclude that at high superhelical density, the GT/CA in the AC-rich region, from residues 30 to 127, are in the Z form. Nevertheless, the hyperreactivity of some bases, such as G₁₂₂ and G₁₂₄ residues, leads us to suggest some local structural distortions of the canonical Z double helix. This is confirmed by the reactivity of the other chemical probes with the whole sequence and even with the (GT)₁₅ stretch.

As judged by DEPC (Figs. 2 and 3), at a superhelical density equal to or higher than \(-0.05\), the (GT)₁₅ stretch in pCP1 adopts the Z conformation. At a superhelical density greater than \(-0.05\), most of the A and G residues on the right of the (GT)₁₅ stretch become hyperreactive with DEPC (at \( \sigma = -0.1 \), up to A₁₁₂ and G₁₁₆ residues on the AC and GT strands, respectively). The hyperreactivity of all the GT/CA (except the G residue in the GT/CA₁₂₂ and 124) is characteristic of Z-DNA (26–31). It has been shown that DEPC is also hyperreactive with unpaired purines present in loops resulting from the slippage of the two strands of a double helix or from the formation of cruciform structures (7–9). These two structures seem rather unlikely in our case since the sequence does not contain large inverted repeats, and it is not expected that all the purines are reactive after slippage of the two strands.

The DEPC reactivity pattern observed in the presence of the antibodies to Z-DNA strengthens the suggestion that all the GT/CA of the sequence adopt the Z conformation since at \( \sigma = -0.1 \), the presence of the antibodies abolishes the hyperreactivity of the G residues (Fig. 5B).

We conclude that at high superhelical density, the GT/CA in the AC-rich region, from residues 30 to 127, are in the Z form. Nevertheless, the hyperreactivity of some bases, such as G₁₁₂ and G₁₁₄ residues, leads us to suggest some local structural distortions of the canonical Z double helix. This is confirmed by the reactivity of the other chemical probes with the whole sequence and even with the (GT)₁₅ stretch.

At \( \sigma = -0.05 \), the terminal 4 T residues (left side) and the terminal 3 T residues (right side) of the (GT)₁₅ stretch are hyperreactive with OsO₄, while the central T residues are not reactive. This hyperreactivity decreases as the superhelical density increases, but even at \( \sigma = -0.1 \) the terminal 2–3 T residues are OsO₄-sensitive, indicating the distortion of the Z double helix. However, the distortion is not the same on each side of the stretch as shown by hydroxylamine which reacts with the terminal left C residues of the stretch and does not react with the terminal right ones.

For consideration of the reactivity of the dinucleotides embedded in a (GT/CA)ₙ sequence in Z conformation, we...
discuss first the (GT), stretch within pCP2, pCP3, and pCP4 (Figs. 6 and 7). As judged by DEPC, at $\sigma = -0.1$, all the GT/CA are in Z conformation. It is known that in negatively supercoiled DNA, the dinucleotides TT/AA and GA/CT adopt a left-handed conformation when embedded in a (CG), sequence in the Z conformation (47, 48). The calculated free energy values are 3.9, 3.4, and 1.3 kcal/mol for TT/AA, GA/CT, and GT/CA, respectively (49, 50). All together, these results suggest that at high superhelical density, the inserted dinucleotides TT/AA and GA/CT or the inserted tetranucleotide TTGA/AACT (pCP2, pCP3, and pCP4, respectively) are in the Z form. For all these inserted nucleotides, those in syn conformation show an increase of reactivity while those in anti conformation are not reactive (Figs. 6 and 7). The association of the two dinucleotides in a tetranucleotide (pCP4) does not modify the pattern of reactivity. It has been assumed that the weak hyperreactivity of G residues in a (GT)$_n$ stretch results from protection by the 3'-flanking thymine-C$_3$ methyl group (27). Within pCP2, the G residue in the first GT/CA on the left of the TT/AA insert is more reactive with DEPC than the other G residues of the stretch, and the T residue is hyperreactive with OsO$_4$ (Figs. 6 and 7). Since in this case the G residue is no longer protected from DEPC by the T residue as in the other GT/CA, it can be concluded that the conformation of the dinucleotide has been modified by the presence of the insert. A larger accessibility related to the presence of a T residue in 3' in place of a G residue as found in a (GT)$_n$ stretch, and/or a distortion of the Z double helix, can also explain the hyperreactivity of the T residue with OsO$_4$. Such differences in reactivity are not observed when GA/CT is inserted (pCP3).

We now consider the GG/CC embedded in the AC-rich sequence of pCP1 (Figs. 2-4). Thermodynamic and chemical studies have shown that GG/CC can easily adopt the Z conformation when embedded in Z-DNA (54). The pattern of reactivity here observed at high superhelical density, for all the GG/CC (positions 68, 78, 84, 100, 114, and 120), is in agreement with a previous finding, i.e. hyperreactivity of the G residue in syn conformation with DEPC and of the G residue in anti conformation with dimethyl sulfate (27). Moreover, we note that the G residues in syn conformation are more hyperreactive with DEPC than the G residues in the dinucleotide GT/CA. The C residues in anti conformation are not reactive with OsO$_4$, while the C residues in syn conformation are hyperreactive. However, the hyperreactivity of all the C residues is not the same, and even some C residues ($C^3$, $C^5$) are reactive with hydroxylamine. This probably reflects some distortion all along the Z double helix.

From all the results relative to the GA/CT and GG/CC, we conclude that in the dinucleotide Gpur/Cpyr embedded in a (GT/CA)$_n$ stretch in the Z conformation, the G residue is not protected from reaction with DEPC, and the pyrimidine is hyperreactive with OsO$_4$. Up to now, the reactivity of OsO$_4$ with the C residues was only observed with single stranded structures.

According to the thermodynamic studies a CG/GC out of phase with a purine/pyrimidine alternation does not easily adopt the Z conformation (47). In pCP1, the CG/GC at position 110 is out of phase (Fig. 1). At $\sigma = -0.1$, this dinucleotide does not seem to largely distort the double helix: C residues 111 and 112 are not hyperreactive with OsO$_4$, while T residues 109 and 113 are slightly hyperreactive. Thus we assume that this dinucleotide adopts the Z conformation with the G residue in the anti conformation. As in GG/CC, the G residue in anti conformation (with a purine on the 3' side) is strongly reactive with dimethyl sulfate. The C residue in syn conformation is not reactive with OsO$_4$. At $\sigma = -0.1$, the conformation of the oligonucleotides ATCT/TAGA at position 60 and GGAGTTT/TCCCTAAAA at position 84 cannot be determined from our experiments. As judged by the chemical probes, it can be stated that the bases are not unpaired. In the tetranucleotide, T residue 60 is hyperreactive with OsO$_4$, but C residues 62 and 63 are not. In the octanucleotide, T residue 88 and C residue 85 are hyperreactive with OsO$_4$, but C residues 82, 84, and 92 are not. Moreover, in presence of the antibodies to Z-DNA, the G residues are protected from DEPC. Thus we suggest that these oligonucleotides can adopt a Z-like conformation. We are, however, aware that the sequence of the octanucleotide is not favorable to form Z-DNA, and thus the presence of two separate blocks (residues 30–83 and 92–127) in Z conformation separated by a distorted structure cannot be excluded.

At high superhelical density, the chemical reactivity of the boundaries between the sequence in Z conformation and the remaining part of the DNA are very different. On the left side, the sequence from residues 12 to 29 presents a complex reactivity which does not allow us to determine the exact position of the B-Z junction. On the right side, the reactivity of the GT/CA suggests a Z conformation up to residue 127. Since the tetranucleotide CATT/GTAA at position 128 is hardly modified by the chemical probes, the B-Z junction spreads over only four base pairs, a value in agreement with various studies (10, 55-57). A last comment is relative to the binding of the antibodies to Z-DNA. At $\sigma = -0.1$, in the absence of antibodies, A residue 130 belongs to the B-Z junction. In the presence of the antibodies, A residue 130 is no longer reactive, but several other A residues (135, 137, 139, 141) become hyperreactive with DEPC. It can be concluded that the antibodies stabilize the Z form of a sequence which in the absence of the antibodies does not adopt the Z conformation even at $\sigma = -0.12$. This supports previous works showing the shift of the B $\leftrightarrow$ Z equilibrium toward the right by the antibodies to Z-DNA (28, 29, 55–57).

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