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Nucleotide sequence of an heterochromatic segment recognized by the antibodies to Z-DNA in fixed metaphase chromosomes

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ABSTRACT

The purpose of this work was to analyse at the molecular level the DNA recognized by the antibodies to Z-DNA in in situ experiments. Antibodies to Z-DNA interact strongly with R-band positive heterochromatic segments of fixed metaphase chromosomes of Cebus (Viegas-Pequignot et al., 1983). These segments are constituted of a satellite DNA the repeat unit of which is about 1520 base pairs long. The base sequence of the repeat unit has been determined. It contains a $(AC)_n$ rich region which, in vitro, adopts the Z conformation under topological constraints. Experiments with nuclei suggest that this sequence is not predominantly in the Z conformation in vivo. The polymorphic structure of the $(AC)_n$ rich region argues for an active recombination sequence.

INTRODUCTION

It is now well recognized that conformational changes can be induced in synthetic and natural double-stranded polynucleotides by modification of the environmental conditions (2). Moreover natural closed circular DNA is generally negatively supercoiled. The negative supercoiling raises the DNA free energy which can be utilized in a variety of structural and dynamic processes (3-5). High salt conditions are necessary to induce the B form - Z form transition of poly(dG-dC).poly(dG-dC) while in low salt conditions the B-Z transition of $(dC-dG)_n.(dC-dG)_n$ sequences in a closed circular DNA is induced by an increase of the negative superhelical density (6). The discovery that the topological constraints can stabilize Z-DNA has strengthened the idea of a biological role for Z-DNA. The presence of Z-DNA has been revealed in fixed nuclei and in fixed metaphase chromosomes by means of antibodies to Z-DNA (6-8). In the genus Cebus, the antibodies to Z-DNA bind to euchromatic and heterochromatic segments of fixed metaphase chromosomes (1). A very heavy staining is detected in large segments which correspond to R-band positive heterochromatin whereas euchromatin shows a weak heterogeneous staining which consistently reproduces the R and T banding pattern. However, questions were raised about the possible role of fixatives (acetic acid, alcohol) used for

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the cytological preparations in the induction of these stainings since acetic acid may have dramatic effects at the molecular level. The removal of the proteins of the chromatin can change DNA accessibility and topological stress. Moreover, the B form - Z form transition is facilitated by protonation of the bases (9-12). In order to better characterize the DNA sequences recognized by the antibodies to Z-DNA and to give a molecular explanation for the cytological staining, we have undertaken a study of the DNA present in the R-positive heterochromatin regions of the chromosomes of Cebus appella which interacts strongly with the antibodies to Z-DNA. We show that these regions are constituted of a highly repeated DNA of about 1520 bp. This repeat unit contains a (AC) rich sequence which adopts the Z conformation in vitro. In isolated nuclei, it has not been possible to show that this sequence is in the Z conformation.

MATERIALS AND METHODS

DNA Purification

DNA was purified from the liver of a female Cebus appella after Maniatis et al. (13). The satellite was prepared as described in ref. 14. Briefly, the total DNA was fractionated by preparative centrifugation in BAMD/Cs_2SO_4 density gradient at pH 9.2 using a BAMD/nucleotide molar ratio of 0.12. The DNA concentration at the beginning of the centrifugation was 200 µg per ml. In these experimental conditions, the satellite was located at the foot of the main DNA peak. BAMD : 3,6-bis(acetomercurimethyl)dioxan was prepared as described in 15.

Construction and Characterization of Plasmids and Phages

Satellite DNA was digested by HaeIII (Boehringer). After electrophoretic separation on 1 % agarose gel, the three main bands were electroeluted and then purified on a NACS Prepac column (BRL).

The three fragments have been cloned in the Pst1 site of pBR322, by (dC-dG) homopolymer tailing (13). Three clones pCH11, pCH21 and pCH31 which carry respectively the satellite HaeIII fragments of about 1110, 250 and 160 base pairs (bp) have been studied. The insertion restored the restriction sites of HaeIII and Pst1. The (dG-dC) tails were 25-30 bp long. The sequence of the inserts has been determined by the Maxam and Gilbert method (16). In the case of pCH31 and pCH21, the inserts have been cut from the plasmids and the strands separated on polyacrylamide gels (13).

Some DNA fragments have been cloned in the Sma1 site of M13mp18. When necessary, the restriction sites of the fragments have been filled up with

Klenow DNA polymerase. TG1 cells have been transformed by ligated DNA after Hanahan (17). The sequence reactions were performed using the dideoxy chain termination procedure (18).

For preparation of radioactive labelled plasmids, HB101 cells carrying the plasmid were grown in minimal medium in the presence of 200 mM adenine and 1 µC per ml of [^{3}H] thymidine (Centre de l'Energie Atomique). Plasmids were prepared according to Birnboim and Doly (19). Specific activities ranged from 7000 to 15000 cpm per µg of DNA.

Degradation by restriction enzymes (Boehringer) has been performed as recommended by the supplier.

Nuclei Preparation and Nuclease Digestions

Nuclei were prepared from the liver as previously described (20). Acidic extraction of the nuclei in the presence of 0.3 NaCl was performed as described in 21. The reaction with formaldehyde was done according to the procedure of Solomon and Varshavsky (22). DNA extracted from the nuclei after S1 nuclease action were separated on horizontal agarose gels and transferred on a GeneScreen Plus membrane (New England Nuclear). Prehybridization and hybridization have been performed in 50 % formamide, 1 M NaCl, 1 % SDS. Hybridization was made in the presence of denatured sperm salmon DNA (100 µg/ml). The membranes were washed in 3 SSC, 0.1 % SDS (3 times) and then in 0.1 SSC, 0.1 SDS (2 times) at 42°C. The radioactive probe was a M13mp18 derivative carrying the full length repeat unit obtained by digestion of the satellite DNA by EcoRI.

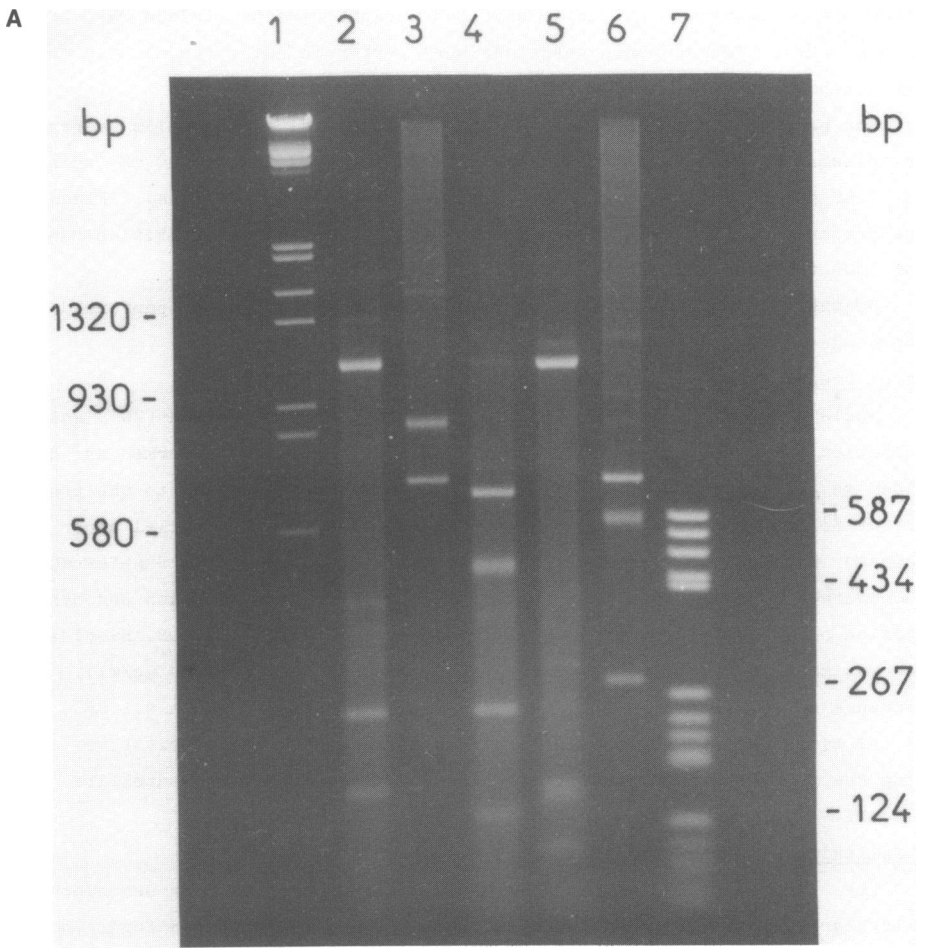
Crosslinking of Anti-Z DNA Antibodies to Plasmids

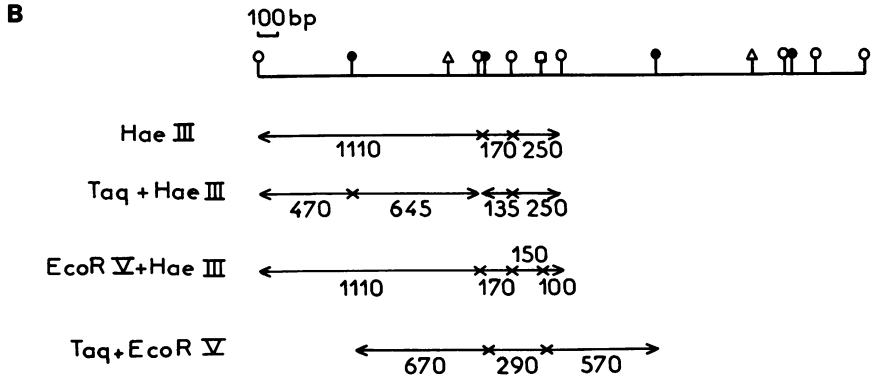
The binding of the antibodies to plasmids was performed as described by Pulleyblank et al. (23). DNA samples (100 µg/ml) were incubated for 2 hours at room temperature in the presence of antibodies (0.6 mg/ml) in 50 mM NaCl, 10 mM Tris pH 7.4, 10 mM $MgSO_4$, 1 mM DTT, then fixed overnight by glutaraldehyde (0.1 %). The fixation reaction was terminated with 0.1 % $NaBH_4$ and the samples were digested by MnL1. The fragments were analysed on a 10 % polyacrylamide gel. Restriction fragment crooslinked to antibodies molecules do not enter in the gel.

In Vitro S1 Nuclease Degradation of the Plasmids

DNA at 60 µg per ml in 30 mM sodium acetate, 70 mM NaCl, 1 mM $ZnSO_4$, pH 4.6 was incubated with 2000 units per ml of S1 nuclease (Boehringer) at 37°C for 1 hour. The reaction was stopped by ethanol precipitation after neutralization with 50 mM Tris pH 10.

The pellet was dissolved in 50 mM NaCl, 10 mM Tris-HCl pH 7.2, 10 mM


A
1 2 3 4 5 6 7
bp
bp
1320 -
930 -
580 -
- 587
- 434
- 267
- 124


B
100 bp
Hae III
1110
170 250
Taq + Hae III
470
645
135 250
EcoR V+Hae III
150
1110
170
100
Taq+EcoR V
670
290
570

$MgSO_4$, 1 mM dithiothreitol and digested by the restriction enzymes at 4 units par µg of DNA for 4 hours at 37°C. The DNA fragments were separated on a 2 % agarose gel in Tris-borate buffer. The size of the fragments were determined using known molecular weight markers (Boehringer).

In Situ Hybridization

For in situ hybridization, the 1112 bp fragment of the Cebus satellite inserted in pCH11 recut by HaeIII has been transferred in the SmaI site of the M13mp18. The single-stranded form of the phage has been modified on 5 % of the bases by N-acetylaminofluorene (AAF). Protocole of modification by AAF (24), purification of the antibodies to guanosine-AAF (25), R-banding, anti Z-DNA immunostaining (1) and in situ hybridization (26) techniques have been previously described.

Other Methods

The samples at various superhelical densities were generated and characterized as described in (27). Topoisomerase I from chicken erythrocytes was prepared after (28).

Preparation and affinity chromatographic purification of anti Z-DNA antibodies have been previously described (29).

Nitrocellulose filter assays were performed as described in (27). The experimental conditions were : medium : 70 mM NaCl, 30 mM EDTA, 5 mM Tris pH 7.2, antibodies concentration 100 nM, DNA concentration 20 pM in molecules of plasmid.

Densitometry was done on the photographic negatives of the gels with a Joyce-Loebl microdensitometer.

RESULTS

The very large R-positive heterochromatic segments which bind the antibodies to Z-DNA are constituted of a repetitive DNA which has been isolated as a satellite (14). This satellite represents about 10 % of the total DNA. It is cleaved by HaeIII in three bands of about 1110, 250 and 160 bp (Fig. 1A). Densitometric determination of the amounts of DNA present in each band

Fig. 1 - A) Degradation of the satellite DNA by restriction enzymes. Lane 2 : Hae III ; lane 3 : Taq I ; lane 4 : Taq I + HaeIII ; lane 5 : EcoRV + HaeIII ; lane 6 : Taq I + EcoRV. Lane 1 : DNA digested by EcoRI and HindIII ; lane 7 : pBR322 digested by haeIII. 1 % agarose gel electrophoresis in TBE. B) Partial restriction map of the repeat unit (after A). Two repeat units are presented. For each enzymatic degradation, the lengths of the fragments measured in A are given. In the case of TaqI + HaeIII, a 26 bp fragment is not visible on the gel. The experimental results for EcoRI restriction site mapping are not shown. ○ HaeIII ; ● TaqI ; □ EcoRV ; △ EcoRI.

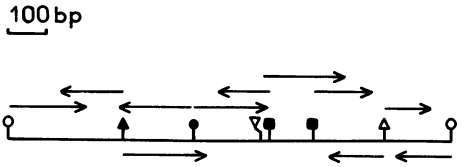


Fig. 2 - Sequencing strategy for the insert of pCH11.

```
        10         20         30         40         50         60
CCTCAGTGGT ACATGCGAAA TGAGCTGGTT TCCATCACCA CGCTCTCGCC AATCATCAAC

        70         80         90        100        110        120
ACCAATGTCT TCAAACCTAC ATGGATTGCG TTTTCAGGAG GGTTGGGGGT TATCCAAGGT

       130        140        150        160        170        180
TCTCAGTAAC ATGCTACCAG AGGAAAAAGC GTCTGCTGCT CAGGTTCTGG AGACACAAAC

       190        200        210        220        230        240
AATGACACAC CCACACCCAC CGACACACAC CCACACACAC AAAATCCCAC ACCCACACAC

       250        260        270        280        290        300
ACCCACACAG ATACACACAC ACACACACAC ACACACACAC ACACTCGGGC ATCCCACACA

       310        320        330        340        350        360
ACGGGTCGTG TCCAGCCAAA TACTTAGTAG TATGAGGTCG GCTTTGAACC CATCCATGCT

       370        380        390        400        410        420
GCAAAGGAAG AGACACCTTT CCCTAATTCA CACGTCCATT CTCCCTCTTC TAATCCAATG

       430        440        450        460        470        480
CTGTCTCCTC CTCAAGAACA TGCTTGCATA TGAAATATGA TTCATTCGAT AAGACACGGC

       490        500        510        520        530        540
TCATGACCAC AAGGAGCACA CCCCTAGGGT GAGTTCTGCT TCCTAGCAAG CCTACCTGTG

       550        560        570        580        590        600
GGTCTGGACA CCTCCCAAAG TGCTTCACGC CTTCATTGCA CCCTCAGACG CAGACAAGGG

       610        620        630        640        650        660
GACTCCAAAG GACTCATGTG TCTCCCATGC TGTCTGAAAA CGACTCCAAT TTCTCCTGGG

       670        680        690        700        710        720
GACCAAAAGT CACAAGAAAA GTAGCTTTTT ATGGGGAGAG CACACAGCTT TTTGAAAATA

       730        740        750        760        770        780
CACAGCTAAA ACGGTCCCTG CTTTAACTGC AAAAGCACTT AAAGCAGGGA CCGTTTTAGC

       790        800        810        820        830        840
TGTGTATTCT GAAAAACTGG TCTAGCACTT AAAGCTCGGA CAGCTTGAGC TGTGTATTTT

       850        860        870        880        890        900
CAAAAAGTTA TCTCTCGGTG CCATAGCTAA TGAAGTCAAA ATGAGGGATC TGTGTCCACT

       910        920        930        940        950        960
TGAGTTGAGC ATTCTTTTGT TCATAAAGGA TTCCGCTCTG TTGGAATTCT TTAGAACGGA

       970        980        990       1000       1010       1020
TTCTCATTCT TGTCAATCCA CTGTCAATTT TCTCTGCTAA TTTCTAGGAG AGGTAACAGA

      1030       1040       1050       1060       1070       1080
GTTCATCCAA AAGCACAGGT GTATGAAAGT CCAGATTGAC AAGTAGACTC CACAAACAGG

      1090       1100       1110       1120       1130       1140
CACCCAGCTG GAAAAGGGAT CGTTCCCGTG GG
```

Fig. 3 - Sequence of the insert of pCH11. Heavy line : $(AC)_n$ cluster.
Arrows : direct and inversed repeat sequences. ▲ MnL1 ; ● Taq1 ; △ EcoRI.

```
        10         20         30         40         50         60
CCCACAGCAA TCCCTGTGCA GAAATCGAGA AACAGAGACA GAAAGTCACC CGCCTAAGGC

        70         80         90        100        110        120
TTGTCACCTG AAAGTGAGTG GCTGACTGAC TGGCACAGGG CTGGAAATCA AACCCAAATC

       130        140        150        160        170        180
TTCGGACCTC TAATTGCACA GGGTGCACTG CCTCCCCCAG G
```

Fig. 4 - Sequence of the insert of pCH31. ● TaqI.

indicates that the three fragments are in equal number and thus the size of the repeat unit is about 1520 bp. The relative position of the three fragments can be deduced from double cleavage with restriction enzymes. Fig. 1A shows the cleavage of the satellite DNA by HaeIII, TaqI, TaqI + HaeIII, EcoRV + HaeIII and TaqI + EcoRV. From the length of each fragments (Fig. 1B) one gets an unambiguous positioning of the fragments. The cleavage of the satellite by several restriction enzymes suggests a low polymorphism in the sequence of the repeat unit.

Sequence Analysis

The three fragments obtained by cleavage with HaeIII were cloned in pBR322. The cloned pCH11, pCH21 and pCH31 carrying respectively the fragments of 1112, 250 and 161 bp have been sequenced. The strategy used to sequence the pCH11 insert is shown in figure 2. In figures 3 - 5, the sequences of the three inserts are given. It has to be pointed out that the restriction sites found by gel electrophoresis (Fig. 1A) are also found in the sequences of these randomly selected fragments of the repeat unit. The frequency of the bases in the repeat unit is T : 23.6 ; C : 27.2 ; A : 28.9 ; G : 20.2 mole %. If the (AC) rich sequence (see below) is deleted, the frequencies are more similar (T : 24.8 ; C : 25.5 ; A : 27.3 ; G : 22.2 mole %) but the guanines are still under represented. A low representation of the CpG doublet (2.17 %) is also observed. The (G+C) and (A+T) contents calculated for individual 50

```
        10         20         30         40         50         60
CCCAATGTGA CAGTTCAATG GCTTCACAAG AGCTTTCACG GATTGAAGTC AACATCAAAA

        70         80         90        100        110        120
GACATTAGAT TCCATCGGCA CAGTCTTTGG TCCCTTGTCT CTAATGAGAA TTTGCCCGGT

       130        140        150        160        170        180
TCCAATTTGA ACATTGGGTC TAACCCGAGA TATCTTGCCT TTCTACCTGA AACTCTTGAA

       190        200        210        220        230        240
AAGGCTTGAT TCACTTAGGA TCTCCTGCTA CAAAGTCAGA GTCAAGAGCC CTCTTGAGCA

       250        260        270        280        290        300
AAGCTCGCGG
```

Fig. 5 - Sequence of the insert of pCH21. □ EcoRV.

1	A C A C A C C C A C A C A G A T	$(A\,C)_{11}$	G C A G G C A T C C C A C A C A A C G
2	. . . . .	$(A\,C)_{13}$	. . . . .
3	. . . . .	$(A\,C)_{16}$	. . . A T . . . . .
4	. . . . .	$(A\,C)_{16}$	. . . . .
5	. . . . .	$(A\,C)_{16}$	. . . . .
6	. . . . .	$(A\,C)_{17}$	. . . . .
7	. . . . . A	$(A\,C)_{20}$	. . . . .
8	. . . . .	$(A\,C)_{20}$	. . . . .
9	. . . . .	$(A\,C)_{24}$	. . . . .
10	. . . . . A	$(A\,C)_{24}$	. . . . .
11	. . . . .	$(A\,C)_{25}$	. . . . .
12	. . . . .	$(A\,C)_{27}$	. . . . .
13	. . . . .	$(A\,C)_1\,T\,C\,(A\,C)_{15}$	. . . . .
14	. . . . .	$(A\,C)_7\,T\,C\,(A\,C)_7$	. . . . .
15	. . . . .	$(A\,C)_{17}\,T\,C\,(A\,C)_3$	. . . . .
16	. . . . .	$(A\,C)_8\,G\,C\,(A\,C)_6$	. . . T . . . . .
17	. . . . .	$(A\,C)_6\,A\,A\,(A\,C)_{11}$	. . . . .
18	. . . . .	$(A\,C)_{16}\,A\,A\,(A\,C)_5$	. . . . .
19	. . . . . C . C A C C C	$(A\,C)_{14}\,T\,C\,A\,A\,(A\,C)_4$	. . . . .
20	. . . . T . . . . . C . C C C G C	$(A\,C)_{18}$	. . . . .

Fig. 6 - Sequence of the $(AC)_n$ rich region in 20 randomly selected clones. Fragments of about 470 bp obtained after digestion of the satellite DNA by TaqI plus HaeIII (Fig. 1) are cloned in the SmaI site of M13mp18 and the sequence determined by the Sanger procedure (18).

bp segments do not allow to distinguish any particular rich region or any asymmetry between the strands. On the other hand, the DNA satellite does not present any significant homology with the α satellite of primates (30) or with known repetitive DNAs (31).

In pCH11 insert, two sequences located near base 230 and 750 respectively, are of interest. A (AC) rich region is present around the base 230 (Fig.

```
A  750 C G T C A A T T T C  G T C C C T G G C A  A A A T C G A C A C  A T A A A A G T T T  T T 709
                |                                          |                 | |
B  755 G C A C T T A A A G  C A G G G A C C G T  T T T A G G T G T G  T A T T C T G A A A  A A 796
C  805 . . . . . .  . T C . . . . A . C  . . G . . C . . . .  . . . . T . C . . .  . . 846
                |              | |     | |     |
A  750 C G T C A A T T T C  G T C C C T G G C A  A A A T C G A C A C  A T A A A A G T T T  T T 709
```

Fig. 7 - B and C : direct repeat units of pCH11 (fig. 3). In B only the bases different from A are written. A : Complementary strand of B and C in the reverse orientation. Bars indicate the mispairings.

3). Between the bases 180 and 284, 38 doublets (AC) are in phase and among them, a cluster of 16 exists between bases 252 and 285. The polymorphism of this region in the repeat unit has been studied. The fragment between HaeIII (base 1) and TaqI (base 466) restriction sites (Fig. 3) prepared from the satellite DNA has been cloned in M13 phage. Figure 6 shows the sequences of 20 randomly selected clones. The range of length of the $(AC)_n$ clusters is 10 to 25 doublets. In 7 out of the 20 clones, this alternation is interrupted by one or two not related doublets. Some divergences are observed in the boundary regions. In two clones, the 5' sequence AGAT is not present. About 100 bases in each side of the (AC) rich region have been sequenced. A random divergence of about 0.5 % is observed.

The segments 755-796 and 805-846 are two direct repeats with 20 % divergence while the segments 755-796 or 805-846 and 709-750 are inverted repeats (Fig. 7). In a hairpin loop or cruciform conformation, the percentages of mismatch are respectively 10 and 15 %.

Interaction With Anti Z-DNA Antibodies

Several results have shown that $(AC)_n$ sequences can adopt the Z conformation in supertwisted DNA (6-7). We have studied the affinity of the anti Z-DNA antibodies toward pBR322 and pBR322 with the three inserts. Relaxed pBR322 is not recognized by the antibodies to Z-DNA. As the DNA is negatively supercoiled, it binds to the antibodies as shown by the retention of the complexes antibodies-pBR322 on nitrocellulose filters (6). We have compared the amount of pCH11, pCH21 and pCH31 retained on the filters in the presence of the antibodies to Z-DNA as a function of the superhelical density (σ) (Fig. 8). In our experimental conditions (70 mM NaCl, 30 mM EDTA, 5 mM Tris pH 7.2), 50 % of pBR322 are retained at $\sigma = -0.066$, while 50 % of pCH11 are retained at $\sigma = -0.053$. pCH21 and pCH31 behave as pBR322. These experiments show that pCH11 contains a sequence which preferentially adopts the Z-conformation as compared to pBR322. The position of the sequences recognized by the anti Z-DNA antibodies can be more precisely positioned after the crosslink of antibodies and DNA by glutaraldehyde. Figure 9 shows the densitometric traces from a photograph of an ethidium stained electrophoretic gel of the antibodies-DNA complexes digested by MnlI. The antibodies bind to two restriction fragments. One fragment of 194 bp is only present in pCH11. It carries the (AC) rich region (segment between bases 140 and 332 of the insert, Fig. 3). The other is a 165 bp fragment which is present in pBR322 and pCH11. It corresponds to the segment between bases 1906 and 2071 (in the numeration of pBR322 after Sutcliffe, 32). It has to be noted that the only $(GC)_3$ sequence

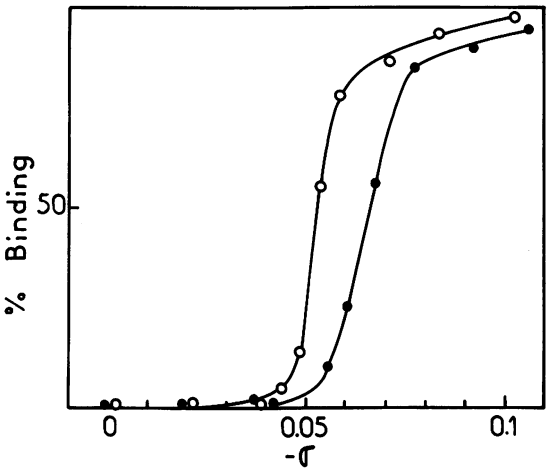


Fig. 8 - Percentage of binding of negatively supercoiled plasmids to anti Z-DNA antibodies as a function of superhelical density (σ) o-o pCH11 ; ●-● pBR322, pCH21, pCH31.

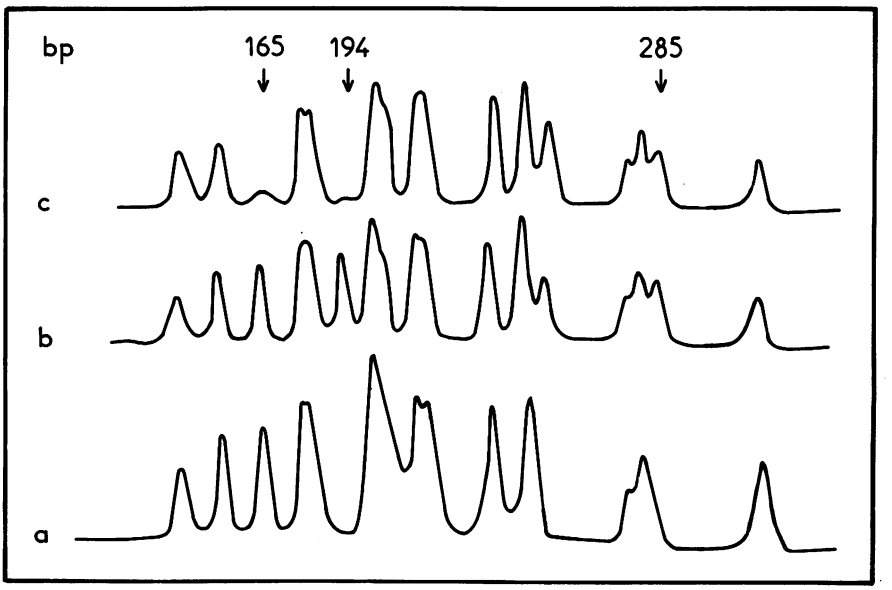


Fig. 9 - Densitometer traces of gel electrophoretically separated MnL1 digest of plasmid-antibodies complexes cross-linked with glutaraldehyde. a) pBR322-non specific antibodies. b) pCH11-non specific antibodies. c) pCH11 anti Z-DNA antibodies.

in pBR322 is in this segment. On the other hand, no crossreaction has been detected between the antibodies and the 285 bp fragment of pCH11 which carries the direct and inverted repeat units (between MnL1 sites 582 and 883, Fig. 3).

Sensitivity of pCH11 to S1 Nuclease

It is known that the junctions between B and Z conformations are sensitive to S1 nuclease (6-7). We have performed a study of the S1 sensitivity of pCH11 in order to confirm that the (CA) rich sequence adopts the Z conformation. Negatively supercoiled pCH11 ($\sigma = -0.1$) has been first cleaved by S1 nuclease and then by the restriction endonuclease HaeIII (HaeIII releases the intact insert from pCH11). S1 specific fragments can be observed on the gel in addition to the restriction fragments. In figure 10, one can see an intense band (890 bp) with in both sides less intense bands distant of 40-50 bp and two other bands (750 and 370 bp). The sum of these two latter bands corresponds to the total insert. On the other hand, the complementary part of the triplet (about 230 bp) is not seen on the electrophoresis. However, this band can be revealed by autoradiography. After incubation of pCH11 with S1 nuclease, the fragments have been labeled in 5' with ^{32}P and then cleaved with HaeIII. The presence of the 230 bp fragment is revealed by autoradiography of the gel electrophoresis (not shown). In the same experimental conditions, the relaxed pCH11 is not cleaved by S1 nuclease.

In order to establish the location of the S1 nuclease sensitive structures, S1 treated supercoiled pCH11 has been also cleaved with HhaI. This restriction enzyme does not cleave the insert. A set of S1 sensitive fragments of 800 and 700 bp are found (Fig. 10) which correspond to a fragment of 1500 bp including the insert of 1120 bp (Fig. 10). A broad band appears at about 1200 bp but the resolution of the gel does not allow to distinguish whether several bands are present. These experiments with HaeIII and HhaI give an unambiguous mapping of the S1 sensitive structures as shown in figure 10. From these results and from the knowledge of the sequence of the insert one can correlate the triplet with the (AC) rich region and the other S1 sensitive structure with the inverted repeats.

In Situ Hybridization

In situ hybridization have been performed in order to confirm the correspondence between the insert of pCH11 and the heterochromatic regions of the metaphase chromosomes. The insert of pCH11 has been transferred in M13mp18 and the single-stranded form of the phage (mCH11) modified by AAF has been used as a non radioactive probe. This probe hybridizes specifically on

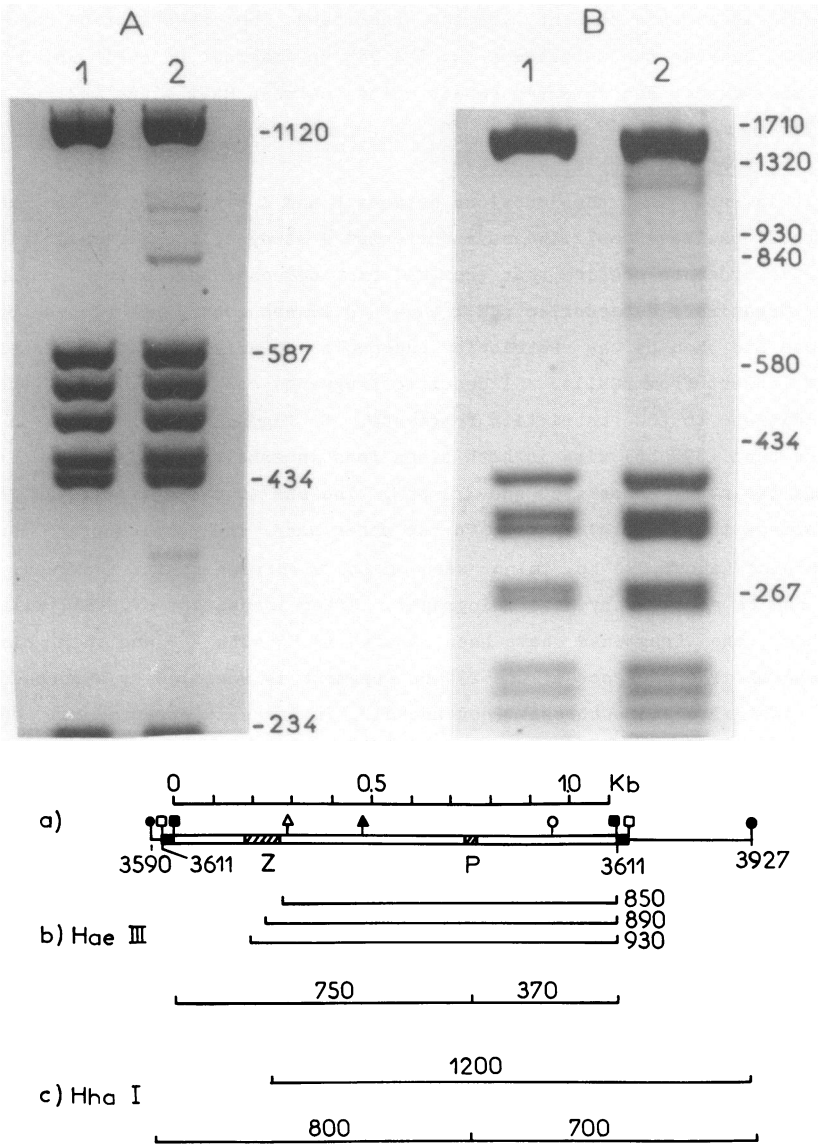


Fig. 10 - Cleavage of pCH11 with S1 nuclease followed by restriction enzyme digestion. A) HaeIII, B) Hha1. Negative superhelical density (σ) of the sample, 1) σ = 0 ; 2) σ = - 0.1.
a) Partial restriction map of the insert of pCH11. Box : insert, line : pBR322 ; filled boxes : homopolymer tail (25-30 bp). Lower values correspond to the coordinates of pBR322, upper scale corresponds to the insert of Cebus appella. Z : Z region ; P : palindromic region. ● Hha1 ; □ Pst1 ; ■ HaeIII ; △ SFaNI ; ▲ Taq1 ; ○ EcoRI.
b) Size of S1 specific bands derived from the HaeIII 1120 bp band.
c) Size of S1 specific bands derived from the Hha1 1500 bp band.

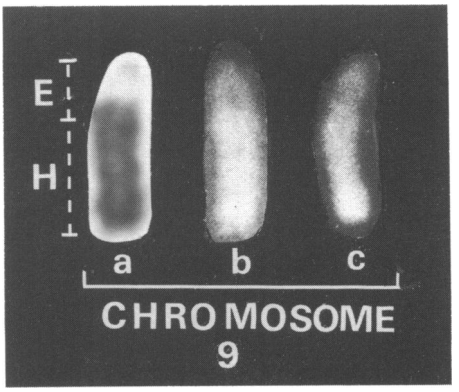


Fig. 11 - Chromosome 9 from Cebus appella. a) R-banding. b) Anti Z-DNA immunostaining. c) In situ hybridization with single-stranded form of mCH11 modified by AAF. Antiguanosine-AAF antibodies fixation is revealed by fluorescein conjugate second antibodies. E : euchromatic regions ; H : heterochromatic region.

the heterochromatic R-positive regions which are also reactive with the antibodies to Z-DNA (Fig. 11).

Nuclease Degradation of the Nuclei

We have tested the presence of B-Z junctions in the nuclei. Nuclei were first digested by S1 nuclease, the DNA was extracted and then was digested by EcoR1 nuclease which releases the complete repeat unit of the satellite. If B-Z junctions were present in the chromatin, S1 specific bands are expected to be revealed by hybridization with a satellite probe after electrophoresis. Incubation with S1 nuclease were carried out over a large range of time and concentration but no specific bands were observed (not shown). Similar results were obtained when the nuclei were treated in acidic medium in the presence of 0.3 M NaCl (conditions in which some chromatin proteins are released but the nucleosomal structure is preserved (21)) or when the nuclei were treated with formaldehyde (with or without acidic extraction). It has to be noticed that the cruciform structure observed in vitro was not observed in the treated nuclei.

We have also used micrococcal nuclease and DNase 1 in order to study the nucleosomal organization and the pitch of the helix in the (AC) rich region after the procedures described by Gross et al. (33). Our conclusion is that the conformation of the (AC) rich sequence is, in vivo, very close to the B conformation (not shown).

DISCUSSION

We had shown that the antibodies to Z-DNA bind strongly to some segments of fixed metaphase chromosomes of Cebus. These segments are heterochromatic and are positively stained after R-banding techniques. We now show that these segments correspond to a satellite DNA with a repeat unit of about 1520 bp. This repeat unit contains a (AC) rich sequence which, in vitro, adopts the Z conformation. By digestion of the chromatin in the nuclei by various nucleases, we are not able to demonstrate that this sequence adopts the Z conformation in the liver cells.

The satellite DNA purified by cesium gradient centrifugation is cleaved in a limited number of fragments by several restriction enzymes. A very low percentage of multimers of the repeat unit is observed with all these enzymes. This low polymorphism in the repeat unit allows to establish a partial restriction map (Fig. 1). The nucleotide sequence of the repeat unit has been determined by the study of three fragments obtained after HaeIII degradation of the satellite (Fig. 3-5). The comparison of the nucleotide sequence and of the restriction map obtained by digestion of the total satellite shows that the sequenced fragments are representative for the average sequence. In the repeat unit, no particular global structure or symmetry have been observed and no significative homology with other repetitive DNAs from primates has been found. In particular, the 250 bp fragment obtained after HaeIII degradation of the satellite is not related to the α satellite DNA as previously reported (34).

It has been verified by the use of a non radioactive probe that the cloned fragments correspond to the R-positive heterochromatic segments of the metaphase chromosomes which bind strongly the antibodies to Z-DNA (Fig. 11).

Two regions of the repeat unit deserve some comments : i) Around the base 750 of the pCH11 insert (Fig. 3) there are two long direct repeats preceded by one inverted repeat ; ii) Around the base 230 of the pCH11 insert a (AC) rich region is present (Fig. 3).

i) The two direct repeats and the inverted repeat present a low divergency. Their homology and their length argue for the formation of a cruciform and/or slipping structure. The presence of such structures, in vitro, is supported by the sensitivity of this region to S1 nuclease when pCH11 is supertwisted (a detailed analysis of the behavior of this region as a function of the superhelical density will be published elsewhere).

In the nuclei, we have not been able to demonstrate a cruciform structure for this region.

ii) The (AC) rich region near the base 230 (Fig. 3) can be divided in a (AC) cluster preceded by a sequence rich in (AC) and (ACCCA). A rather large polymorphism is found in this region. The length of the $(AC)_n$ cluster as deduced from the study of 20 randomly selected clones varies between 10 and 25 doublets. These large variations are characteristic of DNA sequences which undergo fast unequal crossing-over. A similar $(AC)_n$ cluster has been found in the bovine 1.709 satellite (35).

In the 5' side of the $(AC)_n$ cluster, there is a series of ACCCA sequences. Unequal crossing-over of such sequences with $(AC)_n$ sequences could participate to the extension of $(AC)_n$ cluster according to the mechanism proposed by Smith (36). Multiple direct repeats of structure $(AC)_nCCC(AC)_m$ present in this region are in favor of such a mechanism. An exemple could be seen in the clone 10 (Fig. 6) in which an (ACCCA) sequence present in all the other clones has been transformed by the substitution of the central C.

In all the clones but 2, the $(AC)_n$ cluster is bordered in 5' by an AGAT sequence. In the case of the two clones in which this sequence is absent one supplementary tetranucleotide is present (Fig. 6). In 7 clones, the $(AC)_n$ cluster is interrupted by one or two nonrelated dinucleotides which might correspond to recombination events in which the dinucleotides flanking the (AC) sequences have been removed.

The region of the $(AC)_n$ cluster presents a very complex structure which might reflect the mechanism of recombination. In the case of the repeat unit of bovine 1.709 satellite, the boundary regions of the $(AC)_n$ cluster are not related to the ones presented here. In particular, the 5' region is constituted by a $(ACGC)_3$ sequence preceded by a nearly perfect series of cytosines (35). If the boundary regions are related to the mechanisms of crossing-over (36), different mechanisms could be at the origin of AC clusters.

On the other hand, sequences similar to that between bases 180 and 250 (Fig. 3) have been found at the tips of the yeast chromosomes and are assumed to be involved in the mechanism of the telomers growth (37-38).

It is well established that, *in vitro*, under topological constraints, $(AC)_n$ sequences can adopt the Z-conformation (6-7). Interaction with antibodies to Z-DNA show that the (AC) rich sequence of the repeat unit adopt the Z conformation *in vitro* when inserted in a supertwisted plasmid (Fig. 8-9). S1 nuclease degradation evidences the complexity of the behavior of this sequence (Fig. 10). In a general manner, after incubation of a DNA with S1 nuclease and then with the appropriate restriction enzyme, a Z segment gives rise to two doublets corresponding to the cleavage of the two B-Z junctions. The

cleavage of the supertwisted plasmid pCH11 by S1 nuclease in the (AC) rich region gives rise to three bands, i.e. an intense band with in both sides two less intense bands distant of 40-50 bp. Up to now, a triplet has not yet been reported for S1 nuclease sensitive structures. By comparison with the doublet obtained in the case of a unique Z segment and from the knowledge of the sequence, it is suggested that in the region 180-280, two segments in the Z-conformation are separated by a segment being not in the Z-conformation. These two segments could be the 180-220 and 233-280 segments. However, because of the complexity of the region between 180 and 250 in which there are many direct or indirect repeat sequences, other S1 sensitive structures could exist. Work is in progress to establish the exact behavior of this region as a function of the topological conditions.

Degradation of the chromatin in the nucleus have been performed by means of three nucleases in order to establish the presence of the Z-conformation. It has not been possible to prove the presence of Z-DNA in the (AC) rich sequence of the repetitive unit which constitutes the R positive, anti Z-DNA positive heterochromatic segments of the Cebus appella chromosomes. This conclusion is in agreement with the experiments of Gross et al. on the dispersed (AC) sequences (24). They conclude that these sequences do not exist predominently in the Z form. In our more favorable system (high content of repetitive DNA (about 10 % of the genome), knowledge of the sequence), even after formaldehyde fixation or extraction of some non-histone proteins, no Z-DNA is detected.

Thus, the in situ immunochemical staining indicate the presence of DNA sequences with potential for Z-DNA formation but the experiments with nuclei suggest that these sequences are not predominantly in the Z-form in vivo. However, these experiments do not mean that Z-DNA does not exist in vivo. It has been reported that Z-DNA is involved in the mechanism of recombination (39-40). In the Cebus satellite, the sequence recognized by the antibodies to Z-DNA seems to be an active recombination region. It is tempting to speculate that this sequence could play a role in the mechanism of amplification of the segment which carries it.

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