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Z-DNA immunoreactivity in fixed metaphase chromosomes of primates

(antibodies to Z-DNA/R- and T-banding)

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ABSTRACT **Antibodies against Z-DNA bind to fixed metaphase chromosomes of man and *Cebus albifrons* (Platyrrhini, Primate). By indirect immunofluorescence and indirect immunoperoxidase techniques, a heavy staining is detected in some segments of chromosomes of *C. albifrons*. These segments correspond to R-band-positive heterochromatin, which has a high G+C-base content. Euchromatin of human and *Cebus* chromosomes show a weak and heterogeneous staining that consistently reproduces an R- and T-banding pattern in both species. Because chromosome homologies previously were demonstrated between these distantly related species by chromosome banding, our results suggest that Z-DNA has been conserved during the course of primate evolution.**

Repeating sequences of alternating dC and dG deoxynucleotides can form the left-handed duplex DNA conformation, named Z-DNA, as demonstrated by x-ray diffraction studies of crystals of $(dC\text{-}dG)_n$ (1–3) and fibers of poly (dG-dC)·poly (dG-dC) (4). In solution, poly(dG-dC)·poly(dG-dC) can adopt the Z conformation (for general review, see ref. 5), and the B form–Z form transition is highly cooperative as first shown by Pohl and Jovin (6). The Z form is stabilized by high ionic strength, but several other factors can play an important role in the transition. For example, the midpoint of the B form–Z form transition of poly-(dG-m^5 dC)·poly(dG-m^5dC) occurs at 0.7 M NaCl instead of at 2.7 M NaCl as for poly(dG-dC)·poly(dG-dC) (7). Negative supercoiling is also efficient to stabilize the Z conformation. This was demonstrated by the study of intact plasmid DNAs and plasmid DNAs containing alternating d(pCpG) sequences (8–11). Sequences in Z conformation were also found in form V DNA (12–14), form V being prepared from plasmid DNA by hybridizing covalently closed complementary single strands (15). These results also show that sequences other than alternating $(dC\text{-}dG)_n$ can be in Z conformation.

By means of antibodies to Z-DNA, Nordheim *et al.* (16) revealed the presence of Z-DNA in chromosomes of *Drosophila melanogaster*. Other authors, using fixed or unfixed material from *Drosophila* and *Chironomus thummi thummi*, confirmed the binding of antibodies to polytene chromosomes, although there is no consensus concerning the band or interband localization of the fluorescent regions of medium intensity (16–19). Moreover, in chromosomes II and IV of *Chironomus*, a brilliant fluorescence was observed in heterochromatic regions near the centromere (17, 18). Recently, a specific staining of fixed metaphase chromosomes of *Gerbillus nigeriae* (Gerbillidae, Rodentia) (20) suggested to us that Z-DNA could exist in the chromosomes of other mammals.

In the present work, we report that antibodies to Z-DNA bind specifically to fixed metaphase chromosomes of man and *Cebus albifrons* (Platyrrhini, Primate) as visualized by fluorescent and peroxidase stainings. The karyotypes of the genus *Cebus* are known to have large heterochromatic segments whose richness in G+C bases suggested by cytogenetic methods (21) was confirmed by using a biochemical analysis (22). Moreover, because a clear analogy exists between the euchromatic material of the chromosomes of *Cebus* and man (23, 24), the comparison of the staining patterns produced by antibodies to Z-DNA in euchromatin may be considered a check of the stability of Z-DNA conformation during chromosome evolution.

In this work we show (*i*) that anti-Z-DNA antibodies bind to metaphase chromosomes of man and *Cebus* in a distinct and specific way and (*ii*) that an undisputable correspondence exists between the antibody binding and the chromosome banding patterns. In euchromatin, a weak but distinct and consistent staining is observed in the segments corresponding to the R- and T-bands, which are thought to be richer in G·C base pairs both in man and *Cebus*. Slight differences of intensity are observed from band to band: they are the same for all of the chromosomal segments of man and *Cebus*, which were found homologous by banding pattern and by gene mapping comparison (23–25). This fact underlines the stability of Z-DNA conformation during the evolution of mammalian chromosomes. Furthermore, the additional heterochromatic segments existing in *Cebus* chromosomes exhibit a consistent and very strong binding to anti-Z-DNA antibodies.

MATERIAL AND METHODS

Obtention of Metaphase Chromosomes. Metaphase cells were obtained from tissue cultures after skin biopsy of a female of *C. albifrons* held in captivity at the Museum National d'Histoire Naturelle, Paris.

Metaphase cells from human lymphocytes were cultured according to usual methods (26). Cell cultures were treated by colchicine for 1 or 2 hr and then for 10 min with a hypotonic solution of diluted human serum (1:6, vol/vol). Cells were fixed in most of the experiments for at least 40 min with two different solutions (solution I, ethanol/chloroform/acetic acid, 6:3:1, vol/vol; solution II, ethanol/acetic acid, 3:1, vol/vol). A prolonged fixation (45 min to 48 hr), with only the last fixative solution was used for some human cultures.

The following banding methods were used to characterize the chromosomes of the specimen of *C. albifrons*: R-banding

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(RHA, RTBA; see ref. 27), T-banding, and C-banding (27, 28).

Anti-Z-DNA Antisera and Purified Antibodies. Antisera against Z-DNA were elicited in rabbits immunized with poly(dG-dC)·poly (dG-dC) having 12% of the bases modified with chlorodiethylenetriamineplatinum(II) chloride (29, 30). It has been shown that this modified polynucleotide exists in the Z form under physiological conditions (31). The antibodies to Z-DNA were purified by affinity chromatography. The specificity of the antisera and of the purified antibodies has been described (29, 30).

Immunological Stainings. All the experiments were performed the day after the slide preparation.

Anti-Z-DNA antisera and antibodies were used at a 1:35 to 1:50 dilution. The incubation time of the antibodies or antisera varied according to the staining method used in order to provide an amplification of the binding of anti-Z-DNA antibodies. When a fluorescent staining was used, slides were incubated at 37°C for 30–90 min. Slides were washed with 100 mM phosphate buffer (pH 6.7) prior to incubation, which lasted 30 min with a fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin (IgG) serum. This fluorescent serum (Nordic-Tubu) was used at a 1:50 dilution. Stained preparations were mounted in the same phosphate buffer, and cells were photographed under a UV light microscope (HBO 200 mercury vapor lamp).

When peroxidase staining was used, the incubation time was 15–30 min at 37°C. Slides were washed with phosphate buffer prior to incubation (15 min) with peroxidase-labeled anti-rabbit IgG goat serum (Nordic-Tebu) at a 1:50 dilution. Cells were photographed under an ordinary light microscope.

RESULTS

C. albifrons. The karyotype of *C. albifrons* (Primate, Platyrrhini), comprised of 54 chromosomes (32), is identical to that of *Cebus capucinus*. A detailed analysis of the karyotype of *C. capucinus* has been carried out with different banding methods and *in situ* hybridization. This karyotype is characterized by an abundant heterochromatin strongly stained after R-, C-, and T-bandings (Fig. 1 *a* and *b*; see also ref. 21).

These segments strongly incorporate 5-azacytidine (33), they are late replicating, and they possess a repetitive G+C-rich DNA (22).

After treatment with anti-Z-DNA antibodies followed by immunofluorescent staining, two levels of fluorescence appeared on the fixed metaphase chromosomes (Figs. 1*c* and 2*b*). The first one, very intense, was located in R-band-positive heterochromatic segments. The second one, weaker, was distributed in all of the euchromatic segments, and it could be subdivided into three levels of intensity. The weakest one corresponded to the localization of the G-bands, the more intense one corresponded to the localization of the R-bands, and the brightest one, located in some telomeric regions, corresponded to the T-bands. These different levels of fluorescence permitted the identification of all the chromosomes of the karyotype. The same patterns were obtained when the immunoperoxidase method was used (Fig. 3).

Man. In contrast to the karyotype of *C. albifrons*, the human karyotype did not present large R-band-positive heterochromatic segments. Thus, no intense fluorescence comparable to that of heterochromatic segments of *C. albifrons* resulted after incubation with Z-DNA antibodies and fluorescent labeling. Nevertheless, in euchromatin a nonhomogeneous staining was observed: on both chromatids there was a weak but consistent banding pattern. As in the case of *C. albifrons*, it corresponded to a R- or T-banding with three levels of fluorescence: T-bands showing the brightest fluorescence, R-bands showing a less intense one, and G-bands showing the weakest one (Figs. 2*a* and 3). The same chromosomal staining pattern was observed when the peroxidase method was used (Fig. 3).

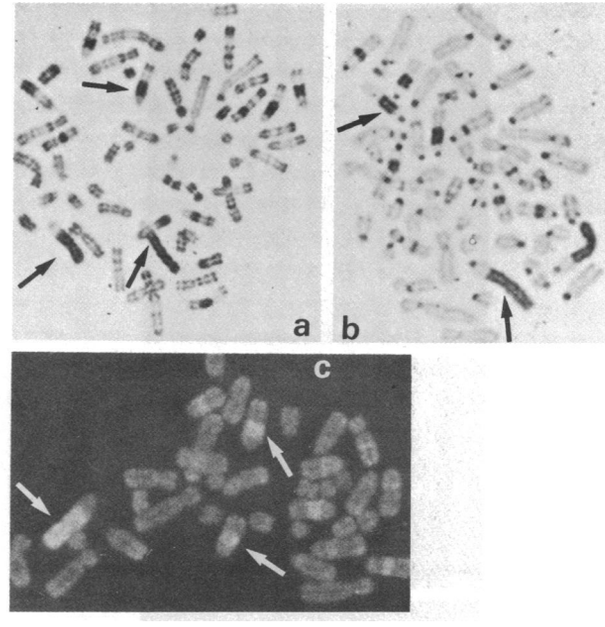

FIG. 1. Chromosomes from metaphase cells of *C. albifrons*. (*a*) After R-banding treatment and Giemsa staining; arrows indicate some chromosomes with large R-band-positive heterochromatic segments. (*b*) After C-banding treatment and Giemsa staining; some of the large heterochromatic segments are indicated by arrows. (*c*) After staining with antibodies to Z-DNA in the indirect immunofluorescence method.

Several control experiments were performed to test whether the immunoreactivity was due to the presence of Z-DNA and to the specificity of the antibodies. The same staining pattern of chromosomes from man or *Cebus* was produced by the whole antiserum or the purified antibodies to Z-DNA; we used several antisera to Z-DNA elicited in different rabbits immunized with different samples of poly(dG-dC)·poly(dG-dC) modified by chlorodiethylenetriamineplatinum(II) chloride. The staining pattern remained unaltered when the fixation time of the metaphase cells was prolonged from 45 min to 48 hr. The staining was abolished when the antiserum to Z-DNA was preincubated with a large excess of poly(dG-br^{5}dC)·poly(dG-br^{5}dC) polymer (the molar ratio of nucleotide residues to anti-Z-DNA antibodies was ≈200:1), which adopts the Z conformation under physiological conditions (30). No staining resulted when the antibodies were replaced by a serum obtained before immunization ("preimmune serum").

DISCUSSION

The karyotypes of the species belonging to the genus *Cebus* are characterized by the presence of large nonjuxtacentromeric segments of heterochromatin. After cytogenetic methods of banding, these segments react as follows: (*i*) they are R-, T-, and C-band positive and G- and Q-band negative and (*ii*) they present a poor incorporation of 5-bromodeoxyuridine (21) and a heavy incorporation of 5-azacytidine (33). By *in situ* hybridization, it has been shown that these heterochromatic segments possess a very repetitive G+C-rich satellite DNA (22). These segments clearly differ from the juxtacentromeric heterochromatin, which is R-, T-, G-, and Q-band negative (21) and for which no information exists concerning G+C richness.

The anti-Z-DNA antisera produce a distinctive pattern of

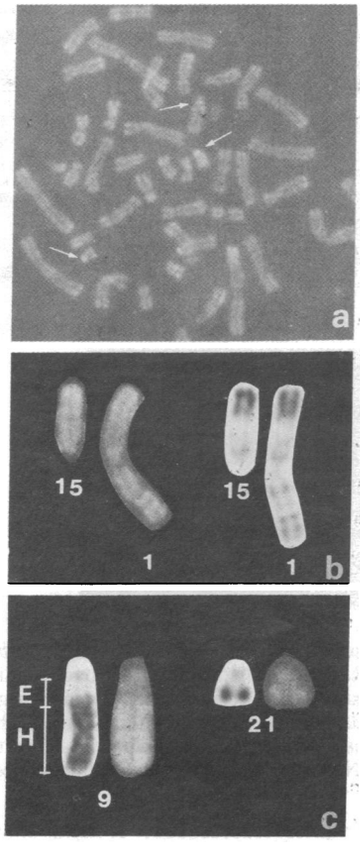

FIG. 2. (*a*) Human metaphase chromosomes from lymphocytes in culture, stained by antibodies to Z-DNA in the immunofluorescence method. The pale but distinctive staining pattern corresponds to R- and T-banding. The juxtacentromeric segments of the short arms of the acrocentric chromosomes (arrows indicate some examples) may present in some subjects a more intense fluorescent region. (*b*) Metaphase chromosome 15 of *C. albifrons*, homologous to the short arm of chromosome 1 in man. On the left are chromosomes stained by antibodies to Z-DNA in the immunofluorescence method. On the right are chromosomes after R-banding and Giemsa staining. (*c*) Metaphase chromosome 9 of *C. albifrons* having euchromatic segments homologous to chromosome 21 of man. For each pair of chromosomes, the element on the left was submitted to an R-banding treatment and a Giemsa staining; the element on the right was stained by antibodies to Z-DNA in the immunofluorescence method. Chromosome 9 of *Cebus* possesses an additional large R-band-positive heterochromatic segment rich in G-C base pairs, which strongly fixes the antibodies. E, Euchromatin; H, heterochromatin.

staining in these two types of heterochromatic segments of *C. albifrons*. The large R-band-positive heterochromatic segments (nonjuxtacentromeric heterochromatin) are strongly stained, and the R-band-negative heterochromatin (juxtacentromeric heterochromatic segments) is weak.

In human karyotype, there are usually no large segments of R-band-positive heterochromatin, and the heterochromatic segments, restricted to juxtacentromeric regions, are not G+C rich as judged by cytogenetic methods. Indeed, anti-Z-DNA antibodies do not produce strong staining comparable to that of R-band-positive heterochromatin of *C. albifrons*. At present, a heavy staining is found on heterochromatic segments of polytene chromosome of *C. thummi thummi* (17), on some heterochromatic segments of fixed metaphase chromosomes of a rodent (20), and here in *Cebus* chromosomes. Nevertheless, it is clear that a strong antibody binding is not related to heterochromatin in general but to specific types of it. For instance, the results obtained in *Cebus* clearly indicate that only the G+C-rich heterochromatic segments bind intensively anti-Z-DNA antibodies. Although the sequences of these fragments are not known, it has been shown that these segments correspond to a G+C-rich satellite. The buoyant density of this satellite is 1.705 g/cm^3 in CsCl (22). It can be estimated that the G+C content is about 50%.

Concerning euchromatin, the interpretation of the staining pattern is less clear-cut because the euchromatic segments are weakly stained. However, anti-Z-DNA antibodies always give a consistent and distinctive pattern. Moreover, this staining coincides in man and *Cebus* with the banding pattern observed on metaphase chromosomes when cytogenetic methods are used. The chromosomes show R- and T-banding; the G-bands are always weaker than the R-bands. The fraction called T-bands, which is the most resistant to heat denaturation and therefore possibly the richer in G+C content (34–37), is consistently the brightest.

At this point, some comments have to be made on the antibody specificity and the fixation of chromosomes. The antibodies were elicited in rabbits immunized with poly(dG-dC)·poly(dG-dC) chemically modified with chlorodiethylenetriamineplatinum(II) chloride. The antibodies were found to bind to a number of synthetic polynucleotides in Z form and to form V DNA, but no crossreaction was detected with native or denatured DNA (calf thymus or *Micrococcus luteus* DNA), single-stranded or double-stranded RNA, deoxyguanosine, or deoxycytidine (14, 29, 30). The chromosome staining is abolished when the antibodies are first incubated with poly(dG-br^{5}dC)·poly(dG-br^{5}dC) polymer, which has the Z conformation irrespective of the salt concentration (30). On the other hand, there is no staining when the antibodies to Z-DNA are replaced by a preimmune serum. These control experiments strongly suggest that the binding of the anti-Z-DNA antibodies to man and *Cebus* chromosomes reveals the presence of DNA segments having the Z conformation. Nevertheless, the chromosomes have been subjected to acidic treatment. This can induce formation of Z-DNA by removal of some proteins or by another process. In this case, the stained segments are only potential Z-DNA. Recent observations do not exclude an effect of fixative but indicate that the staining is qualitatively independent of the fixation methods. In fact, in a recent work, the same antibodies to Z-DNA as those used here were found to bind to most of the nuclei in a fixed tissue section of rat (38). Four unrelated fixatives (methanol, acetone, acetic acid, and picric acid/$HgCl_2$) were used, and the same qualitative pattern of Z-DNA immunoreactivity was detected irrespective of the method of fixation. Another experiment carried out on the ciliate *Stylonychia mytilus* showed the same fluorescence pattern after fixation with 95% ethanol or with 45% acetic acid (39).

An alternative explanation would be that the strong and weak stainings reflect different accessibilities of DNA segments in Z conformation to the antibodies. This seems unlikely. The results presented here for both euchromatin and heterochromatin are in agreement: the segments showing the stronger staining are considered to have a higher G+C-base content than those which are not stained. This is true for euchromatic regions of man and *Cebus* and also for *Gerbillus nigeriae* as previously reported (20). Preliminary results with other species belonging to several orders of mammals (Rodentra, Insectivora, and Artiodactyla) confirm these first observations. Thus, these results on the antibodies binding to metaphase chromosomes support the hypothesis that the staining patterns reveal an intrinsic property of the chromosome structure, the particular conformation of the DNA double helix.

A last point to consider concerns the chromosomal analogies and evolutionary relationships between the karyotypes of man and *Cebus*. It was proposed that the euchromatic segments of

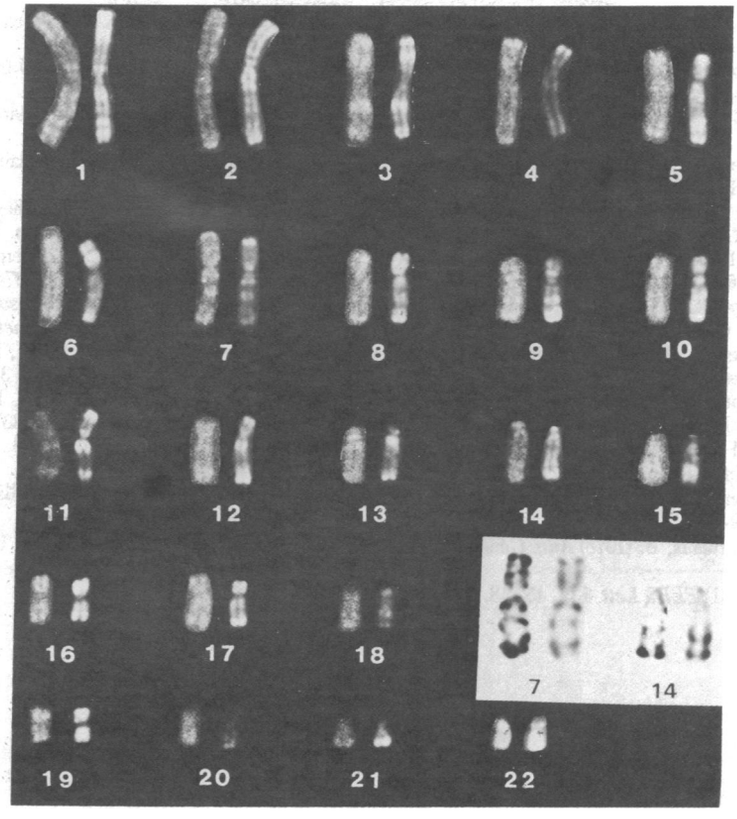

FIG. 3. Human autosomes 1–22. The left chromosome of each pair was stained by antibodies to Z-DNA in the immunofluorescence method. The right chromosome of each pair was submitted to a prolonged R-banding treatment and stained by acridine orange. (*Inset*) Human chromosomes 7 and 14 after anti-Z-DNA staining and immunoperoxidase labeling (left chromosome of each pair) and after R-banding and Giemsa staining (right chromosome of each pair).

the chromosomes of man and *Cebus* are very similar, and banding analogies were given for almost all chromosomes of these species (23, 24). This result was further confirmed by gene mapping (25) and by the chronology of DNA replication of the bands (40). If one takes into account the results concerning the fixation of anti-Z-DNA antibodies at the level of euchromatin, a good analogy exists between the staining patterns of those two species: the brighter euchromatic bands of the human karyotype are also the brighter ones in the *Cebus* karyotype.

For instance, if one considers the chromosome 15 of *Cebus* and the short arm of the human chromosome 1, the same staining pattern of euchromatin is observed after R-banding and after applying anti-Z-DNA antibodies (Fig. 2*b*). The same is true for the chromosome 9 of *Cebus* and the chromosome 21 of man (Fig. 2*c*), although at first glance *Cebus* looks different. It possesses a large additional R-band-positive heterochromatic segment, strongly stained by the antibodies to Z-DNA.

According to our results, the R-banding pattern produced by anti-Z-DNA antibodies would correspond to the presence of Z-DNA in euchromatin; thus, we conclude that these structures are present in two distantly related species, man and *Cebus*. Furthermore, these R-bands are considered as the chromosomal segments that possess the higher concentration of structural genes in human karyotype (41). This would support the assumption that a relationship exists between the presence of Z-DNA and the control of gene expression (16). It would further agree with the recent results claiming that potential Z-DNA-forming sequences are dispersed in the human genome (42).

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