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DNAs with unusual properties revealed by field inversion gel electrophoresis of agarose-encapsulated DNA from mammalian cells

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Abstract Distinct DNA fractions (fr-DNAs), moving separately from bulk DNA, were revealed by field inversion gel electrophoresis of DNA from intact cells lysed and deproteinized in agarose plugs. These fr-DNAs (~2% of the total DNA) were ubiquitously present in nuclei of all mammalian cells studied, including human normal and tumor tissues, and showed a typical electrophoretic pattern (three bands with constant mobilities termed *a*-, *b*-, and *c*-DNA). Characteristic mobility shifts induced by gamma irradiation of *a*- and *b*-DNAs suggest their non-linear conformation. In fact, electron microscopy of *a*- and *b*-DNAs from Namalwa cells revealed rosette-like structures stabilized by a central protease-resistant knob. Comparative PCR analysis revealed qualitative differences between genomic fingerprints of *a*- and *b*-DNAs on the one hand and chromosomal DNA on the other. The results obtained suggest that fr-DNAs originate either from some specific chromatin regions due to non-random cleavages or from an autonomous intranuclear structure, not identified as yet.

Key words: Chromosomal structure; DNA domain; Extrachromosomal DNA; Mammalian cell; Pulsed field gel electrophoresis

1. Introduction

Pulsed field electrophoresis of DNA from higher eukaryotes shows that apart from giant chromosomal DNA (ch-DNA) remaining at the start of the gel some minor DNA fractions can move into the gel [1–6]. This phenomenon is usually attributed to the action of endogenous nucleases [4,5], amplification phenomena [2,6], topoisomerase II activity or the presence of a small subpopulation of apoptotic cells [1,7,8], since DNA fragments of large size (50–700 kbp) were seen at early stages of apoptosis [1,7–10]. Native DNA prepared by cell lysis in agarose with proteinase K and detergent treatment contained a DNA fraction (denoted forum-DNA), mainly in a range between 50 and 150 kbp, and it was suggested that this DNA appeared as a result of non-random chromosomal DNA cleavages and may correspond to some regular higher-order structures in the eukaryotic chromosomes [5]. This conclusion agrees with a more recent findings that long-range fragmentation of the eukaryotic genome by exogenous or endogenous nucleases proceeds in a specific fashion via preferential DNA cleavage at matrix attachment sites [11] and that large-scale fragmentation of mammalian DNA in the course of apoptosis proceeds via excision of chromosomal DNA loops and their oligomers [12].

Using pulsed field gel electrophoresis of agarose-embedded DNA we had observed earlier [13–15] separately moving DNA fractions, which differed in properties from those mentioned above. Here, we have characterized in more detail these ubiquitous fractions (operationally termed fr-DNAs), found in all mammalian cells studied so far.

2. Materials and methods

2.1. Cells

Human fibrosarcoma HT 1080 cells were maintained in a monolayer culture in Eagle's MEM supplemented with 10% fetal bovine serum, penicillin and streptomycin. Namalwa, a non-EBV producer cell line [16], was propagated as a suspension culture in Eagle's basal medium with 20% fetal calf serum.

2.2. Tissue specimens

Primary gastric cancer tissues (adenocarcinoma) and corresponding normal mucosa samples were obtained from the Cancer Research Centre (Moscow). The samples were frozen in liquid nitrogen immediately after surgery and stored at −70°C until use.

2.3. Field inversion gel electrophoresis (FIGE)

Agarose plugs containing 1.5–6.0 μg of undigested DNA were prepared by lysis of intact cells or isolated nuclei in agarose with proteinase K detergent treatment (1 mg/ml proteinase K and 1% SDS at 50°C for 48 h) [17]. The plugs (10–40 μl) were used for FIGE in a vertical 1% agarose gel in the presence of 0.5×TPE buffer (89 mM Tris-phosphate and 2 mM EDTA, pH 8.0) for 3 h at 200 V (8 V/cm) and 17–20°C with PC 750 pulse controller (Hoefer Scientific Instruments). A ramp factor of 6.6 was applied. For quantitative determinations and PCR analysis, DNA samples were recovered by dissolution of gel slices with a chaotropic agent ($NaClO_4$) and adsorption on Whatman GF/C filters [18]. DNA samples were stained with Hoechst 33258 and measured with a Perkin-Elmer 3000 spectrofluorometer [19].

2.4. DNA topological form conversion assay

FIGE gels were stained with 0.1 μg/ml ethidium bromide. Agarose slices, containing *a*- and *b*-DNA, were excised and gamma-irradiated (0.1–10 krad) from the ^{137}Cs source (flux, 524 rad/min) at 20°C and analyzed by the second FIGE one day later [20–23].

2.5. Electron microscopy

DNA electroeluted from agarose slices was spread on a hypophase of distilled water by the protein monolayer technique in the presence of 50% formamide [24]. DNA was picked up onto electron microscopic grids with freshly prepared collodion film. The grids were rotary shadowed with Pt-Pd alloy (4:1, w/w) and examined in a JEM-100CX (Jeol, Japan) electron microscope at 80 kV.

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Abbreviations: ch-DNA, chromosomal DNA; fr-DNA, DNA fractions revealed by pulsed field electrophoresis; FIGE, field inversion gel electrophoresis

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2.6. PCR analysis

Thirty-five cycles of PCR were performed on an automated thermocycler PT 3.2/1 (Biolux, Russia) in 50 μl PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, at 80 μM of each deoxynucleotide, 4% formamide, 0.01% gelatin, 0.1% Triton X-100) containing 30–50 ng of DNA fractions, 5 units of Tth polymerase (Biolux, Russia), and 20 pmol of each primer. Analysis of multiple polymorphic loci using *Alu* oligonucleotide-directed PCR was as described [25].

3. Results

3.1. Detection of fr-DNA

Apart from ch-DNA on the start of the gel, FIGE of agarose-embedded native DNA constantly reveals characteristic DNA fractions (~2% of the total DNA) entering the gel (Fig. 1). They consist of two rather clear-cut bands with apparent sizes of roughly 400 kbp and 200–250 kbp (*a*- and *b*-bands, respectively), and a third one (*c*-band of ~25–30 kbp), which is observed only occasionally presumably due to its gradual diffusion out of agarose plugs during prolonged storage. The *a* and *b* bands, sometimes blurred with a smear differently expressed in various preparations (Fig. 1B,C), were always found in all cell types studied so far.

We assume a nuclear origin of fr-DNAs, since virtually the same patterns were obtained both for intact cells and isolated nuclei (Fig. 1B, lanes 3 and 4), while no such bands were seen in the cytoplasmic fraction (not shown). Detection of fr-DNAs not only in cultured cells (Fig. 1A) but also in animal tissues (Fig. 1B) makes it unlikely that they result from some DNA contamination (for example, with mycoplasmas). On the other hand, detection of fr-DNAs in both normal and tumor human tissues (Fig. 1C) testifies against their relation to tumor growth.

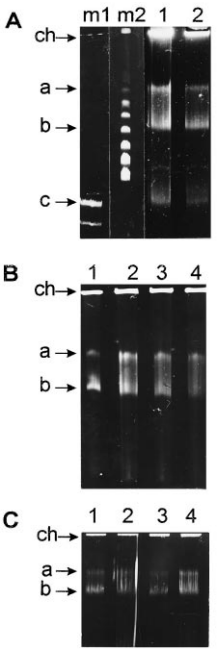

Fig. 1. FIGE analysis of fr-DNAs in various mammalian cells and isolated nuclei. A: Human cultured cells (lane 1, HT1080; lane 2, Namalwa). B: Animal tissues (lane 1, rat thymus; lane 2, rat liver; lane 3, rat spleen; lane 4, nuclei isolated from rat spleen). C: Human normal and tumor tissues (primary gastric cancer tissues: lanes 1 and 3; corresponding normal mucosa: lanes 2 and 4). Ch-DNA and *a*-, *b*-, and *c*-DNA are indicated by arrows. m1, λDNA/*Hin*dIII digest; m2, λDNA ladder.

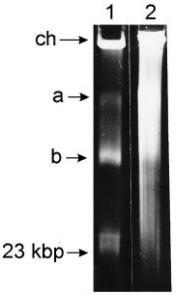

Fig. 2. Comparison of fr-DNAs and apoptotic fragments. Agarose-embedded Namalwa cells, intact or treated with 5 μM etoposide for 16 h (lanes 1 and 2, respectively), were processed as usual and subjected to FIGE.

3.2. Discrimination of fr-DNAs from apoptotic fragments

To exclude an apoptotic origin of fr-DNAs, Namalwa cells treated with etoposide, a topoisomerase II inhibitor capable of inducing apoptosis in lymphoid cells [26,27], were used as a model system. Control cells or cells treated with 5 μM etoposide for 16 h were used for FIGE of agarose-embedded DNA (Fig. 2). Evidently, *a* and *b* bands clearly seen in control cells become superimposed in etoposide-treated cells with a smear of heterogeneously sized fragments spreading from the start.

3.3. Topological properties of fr-DNA

In our preliminary experiments, it was found that relative mobilities of fr-DNAs with respect to linear DNA markers depended considerably on the FIGE conditions indicating a possibility of non-linear conformation of fr-DNAs. This prompted us to study the topological properties of fr-DNAs by the method previously described [20–23]. Creation of single-strand break(s) in supercoiled DNA converts it into the open circular form and further, upon their accumulation and appearance of a solitary double-strand break, into a linear molecule. These transitions are reflected by characteristic electrophoretic shifts. In contrast, linear DNA retains its electrophoretic mobility upon accumulation of single-strand breaks, while the appearance of a random double-strand break leads to a heterogeneously sized smear [20–23].

In the following experiments, appropriate agarose slices were excised from FIGE gels and DNA molecules were gamma-irradiated (0.1–10 krad) from a ^{137}Cs source directly in the gel to avoid mechanical shearing. The effect of such treatment was assessed by the second FIGE. *Saccharomyces cerevisiae* chromosome I DNA (225 kbp) was used as a control linear molecule. Accumulation of break(s) in this DNA brought about the appearance of heterogeneously sized fragments, which is characteristic of linear molecules (Fig. 3A). Irradiation of *a*-DNA at doses of 5–10 krad caused a mobility shift to a distinct position of approx. 25 kbp (Fig. 3B). Low doses (0.1–1 krad), capable of inducing rare single-strand breaks, brought about an upward mobility shift of *b*-DNA (Fig. 3C), i.e. it moved slower than the control DNA and comigrated with band *a* (lanes 3 and 4). Possibly, this shift reflects some decompactization of the structure. After a 5–10 krad exposure, a distinct band of ~25 kbp size was observed (lanes 5 and 6). The data suggest that a torsionally stressed *b*-DNA

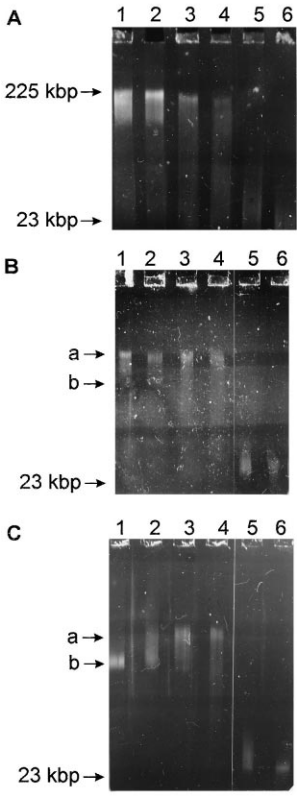

Fig. 3. Topological properties of fr-DNAs. Agarose-embedded Namalwa DNA was subjected to FIGE, slices containing appropriate fr-DNA were excised, gamma-irradiated with 0, 0.1, 0.5, 1, 5, and 10 kilorad (lanes 1, 2, 3, 4, 5, and 6, respectively), and subjected to second FIGE a day later. A: Control: *S. cerevisiae* chromosome I DNA (225 kbp). B: *a*-band DNA. C: *b*-band DNA. Original positions of 225 kbp DNA, *a*-DNA, *b*-DNA and 23 kbp marker are indicated by arrows.

converts initially into a relaxed form (*a* band) and then into a linear form (*c* band) as a result of consecutive generation of single- and double-strand breaks.

3.4. Electron microscopy of fr-DNA

Electron microscopy of electroeluted fr-DNAs spread by the protein monolayer technique showed that they have a rosette-like structure (Fig. 4A). Contour lengths of *a*-DNA and *b*-DNA appeared to be about 18.3 ± 4.7 μm and 49 ± 5.5 μm ($\sim$55 kbp and 147 kbp, respectively). Each rosette appears to consist of a single central knob, from which loop-shaped DNA comes out. The interconnected rosettes form large aggregates. The fact that the larger *b*-DNA moves faster than the smaller *a*-DNA can be explained by a higher degree of its compactization in accordance with topological assay data.

The rosette structure is not an artifact of specimen preparation, since in a control experiment with lambda phage DNA fragments of 23.1 kbp size processed in the same way, only linear molecules (8.5 ± 1.1 μm) were observed (Fig. 4B). Neither can it be explained by the large size of *a*-DNA and *b*-DNA, since high molecular weight calf thymus and human DNA appeared in our preparations as long-stretched molecules up to 60–80 μm in length with no rosettes seen (not shown).

3.5. PCR analysis of fr-DNAs

The question of sequence diversity was addressed by comparative PCR of fr-DNAs and ch-DNA. We compared these fractions using *Alu* oligonucleotide-directed PCR [25]. With about half a million *Alu* elements distributed randomly in the human genome, every region of several kilobase pairs is expected to contain a single-copy DNA segment flanked by these repeats. The intervening DNA fragment can be amplified by PCR with a single *Alu*-specific oligonucleotide primer when two flanking *Alu* elements are in the opposite orientation. Simultaneous amplification from many genomic loci results in multiple bands, which served in this study as a genomic fingerprint.

Using this approach, we observed qualitative differences between ch-DNA and *a*- and *b*-DNAs (Fig. 5). Some major bands of ch-DNA, especially in the lower part of the gel, were not detected in fr-DNAs, and vice versa in the upper part. Taken together, the data obtained imply that ch-DNA and fr-DNAs may constitute different entities and that fr-DNA sequences are heterogeneous. The latter conclusion is substantiated by the finding of some unique gene sequences (*bcl-2*, *hsp70*, *c-Ha-ras1*, and *p53*) present in fr-DNAs from Namalwa cells (not shown).

4. Discussion

Fr-DNAs content is estimated to be about 2% of total

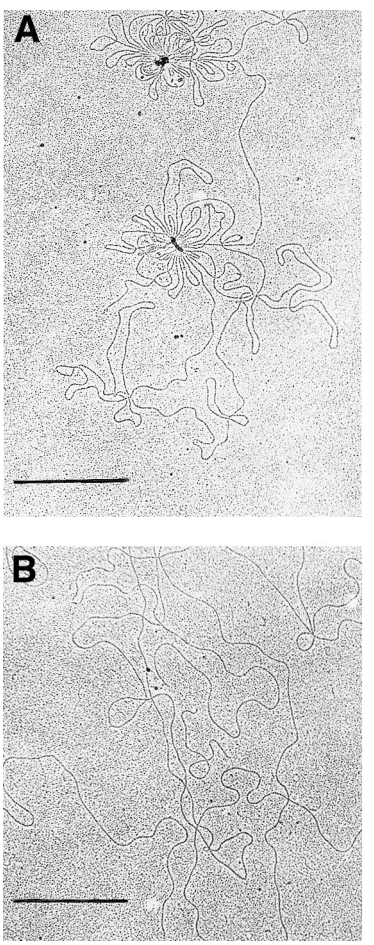

Fig. 4. Electron micrograph of electroeluted fr-DNA. A: *b*-DNA. B: Control (23.1 kbp fragment of lambda phage DNA). Bar: 1 μm.

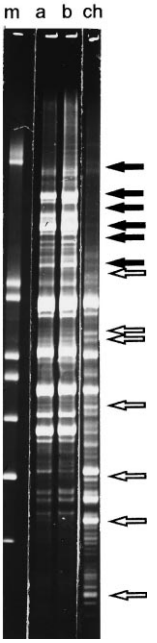

Fig. 5. *Alu*-oligonucleotide-directed PCR of ch-DNA and fr-DNAs. Similar amounts (~50 ng) of DNA fractions from Namalwa cells separated by FIGE were tested by PCR with a single *Alu*-specific primer [25]. a: *a*-DNA; b: *b*-DNA; ch: chromosomal DNA; m: pBR322/*Hinf*I digest. Empty arrows indicate bands specific for ch-DNA, black arrows indicate bands specific for fr-DNAs.

DNA. Some features of fr-DNAs seem to be rather unusual: (i) presence in all cells studied so far (cultured cells and tissues of humans and animals, both normal and malignant), whoch is at odds with extrachromosomal amplified sequences present only in transformed cells [28,29]; (ii) distinct electrophoretic pattern constant in all cell types (Fig. 1); (iii) specific rosette-like electron microscopic appearance of *a*- and *b*-DNAs; (iv) large size and sequence heterogeneity. By these criteria, they differ from polydispersed small circular DNA [30,31] as well as from heterogeneously sized apoptotic fragments and mtDNA.

The origin of fr-DNAs is not clear yet. The simplest explanation would be that this DNA appears as a result of non-random chromosomal DNA cleavages within sensitive regions and its presence in the gel reflects the existence of regular higher-order looped structures in eukaryotic chromosomes [1,3–5,11,12]. In addition, a rosette-enriched DNA fraction was found electron microscopically in DNA isolated from mouse L-M cell nuclei [32].

However, some features of fr-DNAs contradict this assumption. First, chromatin domains are heterogeneous in size (20–200 kbp), so their cleavage would presumably lead to the appearance of heterogeneously sized fragments like those seen upon etoposide-induced chromatin fragmentation (Fig. 2) or like forum-DNA [5], rather than to distinct bands. Second, it is expected that because of cell variability in endogenous degradative enzyme activities and peculiarities of chromatin structure, certain cell or tissue specificity would have to manifest itself in an extent and character of such chromatin fragmentation, which is obviously not the case for fr-DNAs. Third, the fact that *a*- and *b*-DNAs preserve rosette-like conformation after hard proteinase K-detergent treatment (1 mg/ml proteinase K, 1% SDS at 50°C for 48 h) may indicate that it is stabilized in an unusual way. Finally, PCR analysis argues in favor of qualitative differences between ch-DNA and fr-DNAs. For these reasons, it is not excluded that fr-DNAs can occur in vivo as autonomous structures. In this connection, it is worth mentioning the so called polymorphic interphase karyosomal associations (PIKAs), which were revealed by an immunocytochemical approach in nuclei of all human cell lines tested, both transformed and primary cultures [33]. PIKAs represent previously uncharacterized, large and highly polymorphic structural domains, which contain less compact DNA than other areas of the nucleus. One may suggest that fr-DNAs are somehow related to these nuclear domains.

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