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SHORT COMMUNICATION

The Size of DNA Molecules and Chromatin Organization in the Macronucleus of the Ciliate *Didinium nasutum* (Ciliophora)

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ABSTRACT

Pulsed-field gel electrophoresis (PFGE) was applied to analyze the molecular karyotype of the ciliate *Didinium nasutum*. The data obtained indicate that *D. nasutum* belongs to the ciliate species with subchromosomal macronuclear genome organization. No short "gene-sized" DNA molecules were detected. Macronuclear DNAs formed a continuous spectrum from 50 kbp to approximately 1,000 kbp in size with a peak plateau between 250 and 400 kbp. The macronuclear DNA molecules were packed into chromatin bodies of 80–265 nm in size. Comparison of the PFGE and electron microscopic data shows that most if not all chromatin bodies contain more than one DNA molecule.

CILIATED protists contain two morphologically and functionally different types of nuclei in each cell: a germinal micronucleus, which is usually diploid and transcriptionally inactive, and a somatic macronucleus, which is DNA-rich and transcriptionally active during vegetative growth. In the course of the sexual process (conjugation or autogamy), the micronucleus undergoes meiotic divisions leading to the formation of gamete nuclei that exchange between cells and fuse to produce the zygotic nucleus (synkaryon). The mitotic products of the synkaryon finally become the new micronuclei and macronuclei of the following vegetative cells. During the macronuclear development, the primordial chromosomal DNA is amplified, fragmented, and in part eliminated. Thus, contrary to the nuclear genome organization in Metazoa, the macronuclear genome of ciliates is represented by a set of relatively small DNA molecules (for reviews see, Jahn and Klobutcher 2002; Raikov 1995).

All ciliate species can be divided into two groups: the ciliates with macronuclear DNA molecules of subchromosomal size (from several tens up to several hundred kbp), and those with gene-sized (0.05–66 kbp) macronuclear DNAs. Each of these DNA molecules is terminated by telomeres at both ends, lacks a centromere and is usually considered as a nano-, mini- or micro-chromosome (Jung et al. 2011; Ricard et al. 2008; Zagulski et al. 2004).

The size of macronuclear DNAs is an important characteristic of a species, which shapes to a great extent both the higher order chromatin structures in the nucleus and, presumably, the spatial organization of chromatin in the nucleoplasm. The latter assumption is supported by data on the distribution of replication foci in the nuclear space detected by fluorescence microscopy after incorporation of halogenated nucleotides during S phase (Postberg et al. 2005, 2010). Such spatial and temporal replication patterns are widely used for comparative analysis of chromatin arrangement in the nuclei of various eukaryotes. The experiments carried out on many evolutionarily distant metazoan species and different cell types demonstrated that in all cells studied, the special replication patterns

observed in successive stages of S phase were quite similar (Alexandrova et al. 2003; Habermann et al. 2001; O'Keefe et al. 1992; Sadoni et al. 1999). It evidences an extremely conserved higher order chromatin arrangement in the metazoan nucleus. On the contrary, the spatial and temporal replication patterns in the macronuclei of ciliates with both gene-sized macronuclear DNA, e.g. in *Stylonychia lemnae* (Postberg et al. 2005), and subchromosomal DNA, e.g. *Paramecium caudatum* (Tanaka and Watanabe 2003), differed from each other and from those observed in the nuclei of all metazoans studied so far. Moreover, it was assumed by Postberg et al. (2006) that the absence of typical chromosomes could determine an alternative spatial organization of vectorial synthesis and processing of rRNA. It was shown, that in the ciliate *S. lemnae*, which contains gene-sized macronuclear DNAs, rRNA is processed in a bipartite and inverse manner compared to a typical metazoan nucleolar organization (Postberg et al. 2006). The nucleoli of another ciliate, *Didinium nasutum*, also display the bipartite structure, with an inverted disposition of its main components (Leonova et al. 2006, 2012, 2013). Our electron microscopic observations provide an indirect evidence of subchromosomal sizes of *D. nasutum* macronuclear DNA (Karajan et al. 1995). However, no direct pulsed-field gel electrophoresis (PFGE) data have yet been published.

In this work, we used PFGE to estimate the size of *D. nasutum* macronuclear DNAs. The PFGE data were compared with those obtained by electron microscopy.

MATERIALS AND METHODS

Cultures

The laboratory strain of *D. nasutum* was grown at room temperature in lettuce medium and fed *P. caudatum* cultivated separately. *Paramecium* cultures were maintained at the Centre of Core Facilities "Culture Collection of Microorganims", St. Petersburg State University.

Pulsed-field gel electrophoresis

For preparation of DNA samples, 20 ml of *D. nasutum* culture containing 10^3–10^4 cells per ml was used. The ciliates were well-fed and then starved for 24–30 h; prior to making a sample, cells were washed out of the "Paramecium" medium in Peter's buffer (Sonneborn 1970). Then, the cells were gently concentrated by centrifugation at 500 *g* for 5 min. The resulting cell suspension was mixed in a 1:1 ratio with molten 1.5% SeaKem agarose (FMC Corp., Philadelphia, PA) in 0.125 M EDTA, pH 8.0, at 50 °C. This suspension was poured into templates to prepare agarose blocks as described earlier (Nekrasova et al. 2010). The blocks were placed into a lysing buffer (0.5 M EDTA, pH 9.5; 10% sodium lauroylsarcosine; 100 μg/ml proteinase K; Sigma Chem. Co., St. Louis, MO) and incubated for 2 d at 55 °C.

Samples of *Paramecium quadecaurelia* DNA were prepared as described earlier (Nekrasova et al. 2010). *Saccharomyces cerevisiae* chromosomes (CHEF DNA Size Marker #170-3605; Bio-Rad Laboratories, München, Germany) were used as electrophoretic markers. The PFGE was carried out using an original apparatus (Mezhevaya et al. 1990), the reorientation angle was 120°. The different pulse/duration PFGE conditions were used as described by Nekrasova et al. (2010) and Rautian and Potekhin (2002). The 4-mm-thick 1% SeaKem agarose (FMC Corp.) gels were prepared with 0.5X TBE buffer. Electrophoresis was carried out in the same buffer at 10 V (14 °C). After PFGE, the gels were stained with 0.5 μg/ml ethidium bromide solution and photographed on a UV transilluminator. For analysis of PFGE patterns, computerized densitometry was performed as described earlier (Rautian and Potekhin 2002).

Electron microscopy

The interphase macronuclei were isolated from groups of individuals in logarithmic growth phase which were synchronized by selecting and transferring the dividing cells into a vial with a fresh culture medium (Karajan et al. 1995). The macronuclei were manually isolated with needles in the solution containing 0.5% Triton X-100 (Serva GMBH, Heidelberg, Germany) in 0.1 mM borate buffer, pH 8.7–9.0, washed in 0.1 mM borate buffer and incubated for 10–120 min in a drop of the same buffer for lysis and spreading of chromatin. The spread chromatin was layered on top of a solution containing 4% paraformaldehyde, 0.1 M sucrose, 0.1 mM borate buffer, pH 8.7, and centrifuged for 10 min at 3,000 *g* to deposit the chromatin onto a freshly ionized parlodion-carbon supporting film as described by Miller and Bakken (1972). Six to eight macronuclei per electron microscopic grid were used. Then, the grids were washed in 0.4% Kodak Photoflo solution, pH 7.8, air-dried and rotary shadowed with palladium–platinum alloy (1:4) at 6° angle. For ultrathin sectioning, the cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) for 1 h at room temperature, dehydrated in a graded series of alcohol and embedded in Epon-Araldite.

The specimens were examined in a JEM-100CX electron microscope (JEOL Ltd., Tokyo, Japan).

The size of the chromatin bodies was measured on scanned negatives with ImageJ software (http://rsbweb.nih.gov/ij).

RESULTS AND DISCUSSION

Pulsed-field gel electrophoresis pattern of *D. nasutum* DNA is shown in Fig. 1a (lanes 1 and 2). No short DNA molecules corresponding to "gene-sized" DNAs were detected. The distribution of most DNA molecules looked like a continuous spectrum ranging from 50 kbp to over 1,000 kbp. DNA density along the lanes increased gradually from about 50 kbp and reached a plateau at 250 kbp. The densest region was from 250 to 400 kbp after which the density rapidly decreased. No pronounced bands were observed in these regions (Fig. 1a). The absence of any

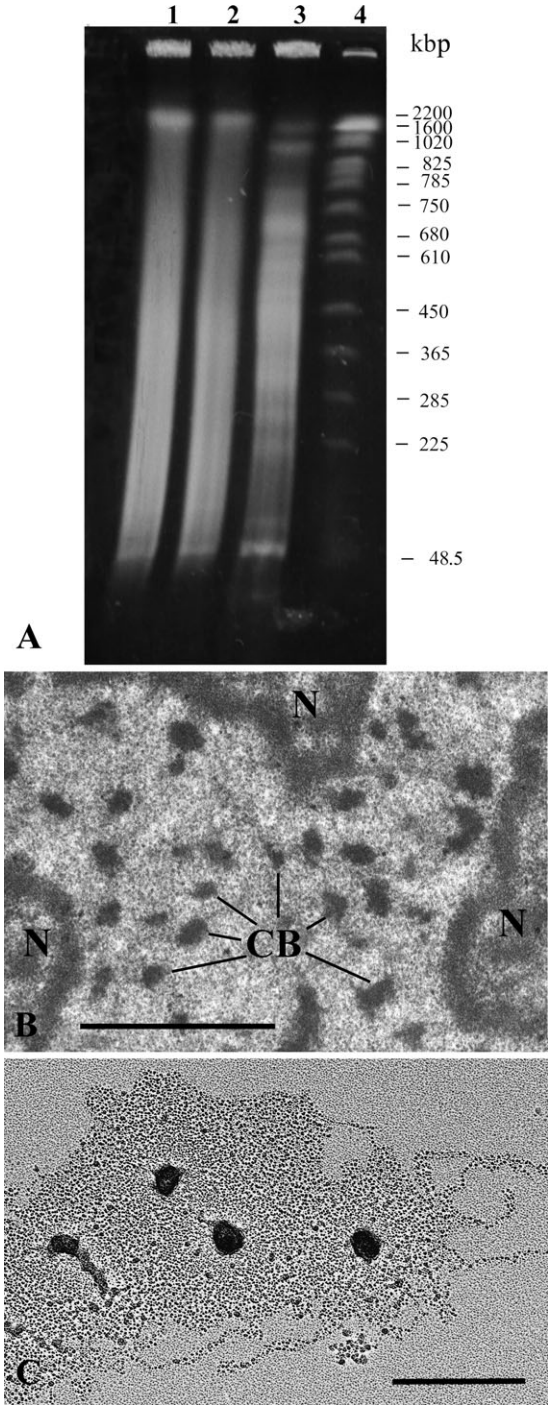

Figure 1 Pulsed-field gel electrophoresis of macronuclear DNA and electron microscopy of chromatin from *Didinium nasutum*. **A**. Molecular karyotype of *D. nasutum* (lanes 1 and 2); macronuclear DNA from *Paramecium quadecaurelia* (lane 3); size markers (chromosomes of *Saccharomyces cerevisia,* lane 4). The separation conditions (pulse time – duration): 20 s – 6 h, 60 s – 8 h, 110 s – 18 h, 160 s – 6 h. **B.** A fragment of an ultrathin section of *D. nasutum* macronucleus. CB = chromatin bodies, N = nucleoli. Scale bar = 1 μm. **C.** Partially decompacted macronuclear chromatin after incubation in 0.1 mM borate buffer for 30 min. Halos of nucleosomal fibers appear around electron dense chromatin bodies. Scale bar = 0.5 μm.

bands in *D. nasutum* karyotype indicates that there are no hyperamplified molecules or sets of molecules with similar length.

A somewhat similar distribution of DNA molecules (i.e. appearing as a single asymmetric peak lacking pronounced bands) was observed in *P. caudatum*, for example (Rautian and Potekhin 2002). In several other ciliates, e.g. in *P. quadecaurelia* (Fig. 1a, lane 3) and in all other species of the *P. aurelia* complex, the whole spectrum of macronuclear DNA molecules can be subdivided in the PFGE gels into several bands "embedded" in a smooth back-cloth of less amplified molecules, which may be grouped in specific regions (Nekrasova et al. 2010).

In the *D. nasutum* molecular karyotype, the only prominent band co-migrated with the ~2,000-kbp chromosome-sized DNA molecules of *S. cerevisiae*. Our PFGE samples were prepared from the intact *D. nasutum* cells and contained both macronuclear and micronuclear DNA. As micronuclear chromosomal DNAs are known to be of much higher molecular weight than the macronuclear chromosomal fragments (Prescott 1999), this high molecular weight band most probably corresponded to micronuclear DNA. This conclusion is also supported by data obtained by Rautian and Potekhin (2002). These authors studied the molecular karyotypes of *Paramecium* species and found that such a high molecular weight band is more prominent in the species with a higher percentage of micronuclear DNA with the total genomic DNA of the cell. Each cell of *D. nasutum* contains 2–4 micronuclei (4–6 μm in diam.) and one oblong macronucleus 50–70 μm in length and 8–13-μm thick (Leonova et al. 2013; Raikov 1982). Thus, the volume of each micronucleus is about 1.5% of the volume of the macronucleus, and the amount of micronuclear DNA in our PFGE preparations can be estimated as about 6% of macronuclear DNA. This value is close to that in *P. bursaria*, where the high molecular weight DNA band at about 2,200 kbp corresponds to micronuclear DNA (Rautian and Potekhin 2002).

In interphase, *D. nasutum* macronucleus DNA molecules were packed into chromatin bodies uniformly distributed in the macronucleus (Fig. 1b). Chromatin bodies were sometimes aggregated into short fibers of two to four bodies. The size of a single chromatin body varied from 80 to 265 nm (mean size ± SD = 183 ± 38 nm, $N = 426$). Upon incubation of isolated macronuclear chromatin in 0.1 mM borate buffer within 10–120 min, chromatin clumps gradually decondensed. A "halo" of long chromatin nucleosomal fibers appeared around them (Fig. 1c). No short chromatin fibers, corresponding to "gene-sized" DNAs (0.15–25 kbp) were detected. The process of chromatin body decondensation in low salt buffer in *D. nasutum* was quite similar to that described for other "subchromosomal" ciliate species, such as *Bursaria truncatella* and *P. caudatum* (Leonova et al. 2004; Tikhonenko et al. 1984). Thus, electron microscopic and PFGE data are in good agreement and indicate that *D. nasutum* belongs to the species with "subchromosomal" macronuclear genome.

To our knowledge, *D. nasutum* is the only representative of the class Litostomatea to which the sizes of macronuclear DNA molecules has been documented thus far. In spite of the rather broad distribution of macronuclei with "gene-sized chromosomes" across species belonging to distantly related lineages of ciliates, e.g. Spirotrichea, Phyllopharyngea, Armophorida, Clevelandellida (Riley and Katz 2001), the "subchromosomal-type" macronucleus is probably a more typical feature of many other groups within Ciliophora, including Litostomatea (Jahn and Klobutcher 2002; Raikov 1995).

Electron microscopic and PFGE data allow us to estimate the number of DNA molecules per a chromatin body. If we approximate the chromatin bodies and nucleosomes by spheres, the size of DNA molecules in a body *L* will be given by equation:

$$L = k\ L\mathrm{nucl}\ (V\mathrm{chrb}/V\mathrm{nucl}) = k\ Lnucl\ (D\mathrm{chrb}/D\mathrm{nucl})^3,$$

where *L*nucl is the size of DNA per a nucleosome (bp), *k* the packing coefficient, *D*chrb and *D*nucl the diameters of a chromatin body and nucleosome, respectively, *V*chrb and *V*nucl the volumes of a chromatin body and nucleosome, respectively. As nucleosomes are tightly packed in chromatin bodies (Tikhonenko et al. 1984), we can take $k = 0.74$ (from the well-known task about packing of oranges in a box, Sloane 1984). Taking the values *L*nucl = 200 bp (Kornberg 1977), *D*nucl = 10 nm (Olins and Olins 1974), we find that the smallest single chromatin body 80 nm in size contains at least 76 kbp DNA, which is significantly larger than the smallest 50 kbp DNAs detected by PFGE. Analogously, the chromatin bodies 100, 183, 200, and 250 nm in diam. are estimated to contain at least 148, 907, 1,184, and 2,313 kbp DNA, respectively.

It should be noted that the calculated values only give a lower estimates of DNA size, because (i) the DNA repeat unit is longer in macronuclear nucleosomes than 200 bp (Prescott 1999); (ii) the volume of a nucleosome, consisting of the disk-shaped histone octamer (11 nm in diam. and 5.5 nm in height), DNA repeat unit, and one linker histone, is significantly smaller than the volume of 10 nm sphere assumed in our calculations. Comparison of the calculated values with PFGE data allows us to conclude that many (if not all) macronuclear chromatin bodies in *D. nasutum* contain more than one DNA molecule. The same situation has been observed in the ciliate *B. truncatella*. It was shown on spread chromatin preparations that each *B. truncatella* chromatin body can contain several long DNA molecules attached by their ends to the organizing center of the body (Novikova and Popenko 1998). As it was shown by Murti and Prescott (2002), in such structures macronuclear DNAs are connected to each other at their telomeric ends by protein–DNA interactions.

In conclusion, the data obtained show that *D. nasutum* belongs to the ciliate species with subchromosomal macronuclear genome and its structural organization is similar to that observed in *B. truncatella*.

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