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In vitro aggregation of the gene-sized DNA molecules of the ciliate *Stylonychia mytilus*

(multistranded DNA/macronucleus)

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ABSTRACT **Macronuclear DNA of hypotrichous ciliates exists in the form of gene-sized DNA molecules. It can be resolved on agarose gels into a continuum of sizes upon which is imposed a set of characteristic DNA bands. Most or all of the DNA molecules carry identical terminal inverted repeat sequences. By incubating macronuclear DNA under increasingly stronger ionic conditions, high molecular weight DNA aggregates and ring-like DNA structures are formed. Experimental evidence is presented that this aggregation is not due to the presence of identical single-stranded DNA ends on each macronuclear DNA fragment, and an alternative model for DNA aggregation is discussed.**

Macronuclear DNA of hypotrichous ciliates exists in the form of gene-sized DNA molecules (1–5) which are created during macronuclear development in a series of well-defined events (6–8). Macronuclear DNA can be separated by size on agarose gels, yielding a reproducible size distribution and banding pattern (2–5). The size of these DNA molecules varies between about 0.4 and 14 kilobases, with an average around 2–4 kilobases (2–5). Most or all of the DNA molecules contain the same terminal inverted repeat sequences of about 26 base pairs (refs. 9 and 10 and unpublished data), and evidence has been presented that macronuclear DNA fragments have flush duplex ends (11). A diagram of one macronuclear DNA fragment is shown in Fig. 5*a*.

In this communication I describe the *in vitro* aggregation of the gene-sized DNA molecules of *Stylonychia mytilus* which I interpret to be the result of multistranded DNA complexes formed by regions of sequence homology at the ends of molecules.

MATERIALS AND METHODS

S. mytilus were cultured, macronuclei were prepared, and macronuclear DNA was isolated as described (4, 12, 13).

Macronuclear DNA was dissolved in various buffers of different ionic strengths and incubated at room temperature for various time intervals (for details see legends to the figures). DNA was separated by size on 1% agarose gels (3, 4). For hybridization of cloned macronuclear DNA fragments to these agarose gels, DNA was transferred to nitrocellulose filters by the method of Southern (14). Cloned macronuclear DNA fragments were labeled with [^{32}P]dCTP by nick translation (15) and hybridized to the filter-bound DNA as described by Jeffreys and Flavell (16, 17).

To study the effect of formaldehyde on the ability to aggregate, I dissolved DNA at a concentration of 1 mg/ml in 10 mM Tris·HCl (pH 7.5) and added formaldehyde at concentrations between 0.1% and 1.5%. After incubation at room temperature for 30 min, 20× standard saline citrate (1× standard saline citrate is 0.15 M NaCl/0.015 M Na_3citrate) and 0.1–1.5% formaldehyde were added to make the samples 4× standard saline citrate. DNA was further incubated for 48 hr and then electrophoresed on a 1% agarose gel.

Density gradient centrifugation of macronuclear DNA was performed either on an ethidium bromide-containing cesium chloride gradient (18) or on a gradient containing ethidium bromide and 25% (wt/vol) metrizamide (19). After centrifugation, cesium chloride gradients were photographed under UV light. After fractionation, DNA fractions were used for either agarose gel electrophoresis or electron microscopy. Metrizamide gradients were fractionated, each fraction was precipitated with 2.5 vol of ethanol, and the precipitates were placed on agarose gels.

Electron microscopy of nucleic acids was performed as described (8).

RESULTS AND DISCUSSION

By incubating macronuclear DNA under increasingly stronger ionic conditions the typical DNA distribution and banding pattern on agarose gels (Fig. 1*A*, slots a, c, and e) disappears and only DNA of highly increased molecular weight is observed on these gels (Fig. 1*A*, slots b, d, and g). As shown in Fig. 1*B*, the formation of high molecular weight DNA aggregates is relatively slow. Aggregation can be detected on agarose gels after an incubation time of about 7 hr, and aggregation seems to be almost complete after 50–60 hr under the conditions used in this experiment (see legend of Fig. 1*B*). Increasing or decreasing the incubation temperature from room temperature to 45°C or 10°C did not significantly change the time course of aggregation. To see whether only identical DNA molecules form high molecular weight complexes, I hybridized cloned macronuclear DNA molecules to the gel shown in Fig. 1*B*. When aggregation proceeds, no multiples of these fragments can be detected, but a continuous background hybridization in the high molecular weight region of the gel is seen. It therefore has to be assumed that actually most or all DNA molecules can form aggregates with each other (Fig. 1*C*). The thermal stability of the complex is shown in Fig. 1*D*. The original banding pattern can be restored by heating the DNA sample to 65–70°C.

One obvious explanation for this aggregation process would

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Abbreviation: 1× standard saline citrate, 0.15 M NaCl/0.015 M sodium citrate, pH 7.

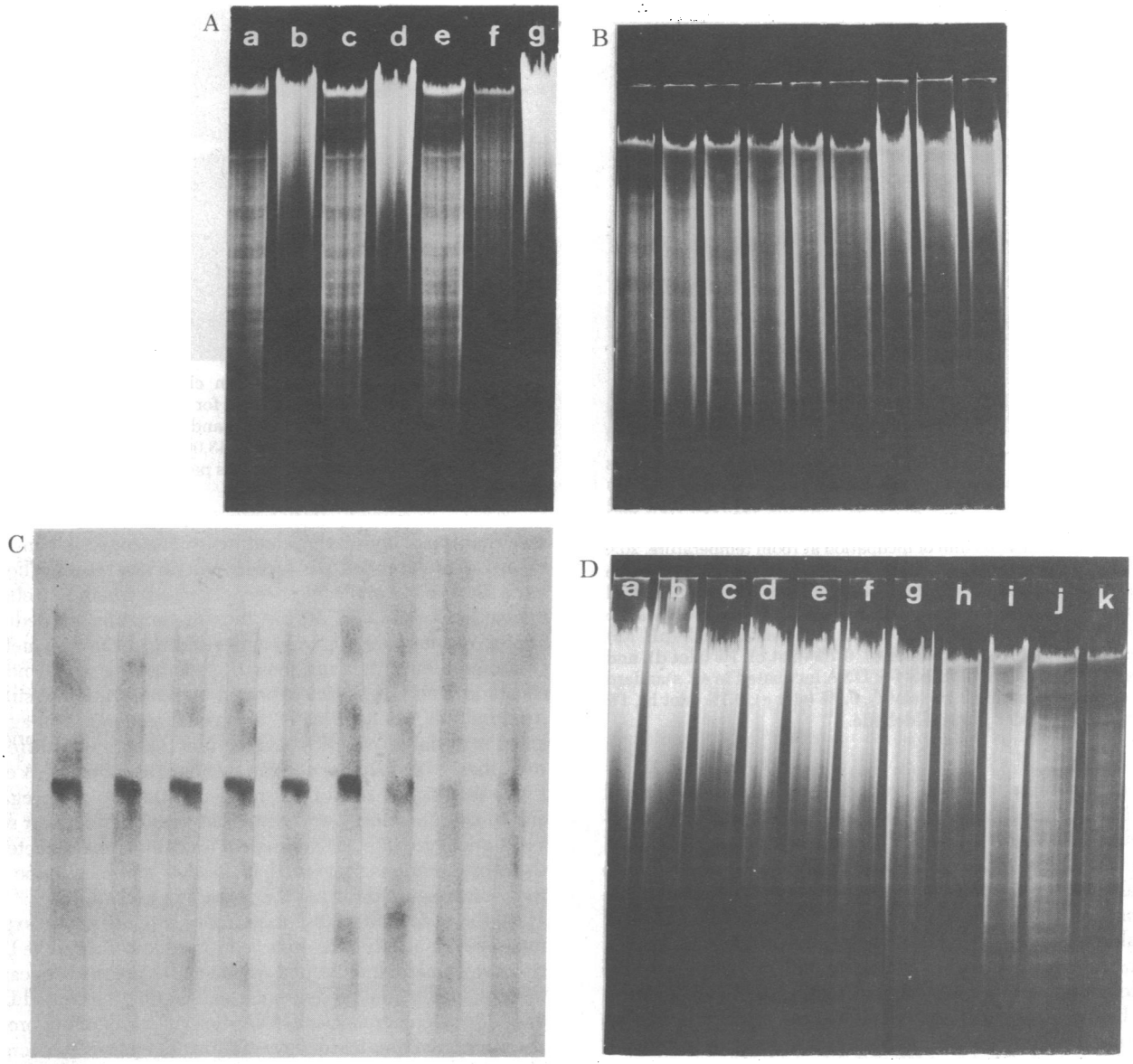

FIG. 1. (*A*) One percent agarose gel of macronuclear DNA incubated for 48 hr at room temperature under different ionic conditions. DNA was dissolved at a concentration of 1 mg/ml in the following buffers: slot a, 10 mM Tris·HCl, pH 7.5; slot b, 10 mM Tris·HCl/0.5 M NaCl, pH 7.5; slot c, 40 mM Na acetate, pH 7.8; slot d, 50 mM Na acetate/0.5 M NaCl, pH 5.0; slot e, 10 mM Tris·HCl, pH 8.8; slot f, standard saline citrate; slot g, 4× standard saline citrate. (*B*) Time course of DNA aggregation. DNA was dissolved at a concentration of 1 mg/ml in 4× standard saline citrate and incubated at room temperature. At various time intervals samples were taken and electrophoresed on a 1% agarose gel. Incubation time is (from left to right): 0, 1, 2, 3, 5, 7, 24, 30, and 40 hr. (*C*) The DNA of the gel of *B* was transferred to nitrocellulose filters and a ^{32}P-labeled, cloned macronuclear DNA fragment was hybridized to it. (*D*) Stability of the high molecular weight DNA aggregates against temperature. DNA was dissolved at a concentration of 1 mg/ml in 4× standard saline citrate and allowed to aggregate for 48 hr at room temperature. It was then heated in a water bath. At different temperatures samples were taken and run on a 1% agarose gel: slot a, 24°C; slot b, 30°C; slot c, 35°C; slot d, 40°C; slot e, 45°C; slot f, 50°C; slot g, 55°C; slot h, 60°C; slot i, 65°C; slot j, 70°C; slot k, 75°C.

be the presence of complementary single-stranded DNA at the ends of each DNA molecule. Depending on DNA concentration, either long aggregates or intramolecular DNA rings could be formed similar to the cohesion processes described for phage λ (20). For aggregation to occur one would have to assume complementary single-stranded DNA ends in addition to the previously identified identical inverted repeats on each DNA molecule (9–11). Such putative cohesive single-stranded DNA ends would have been lost during the procedure used to isolate the terminal inverted repeats (10). However, as discussed by Herrick and Wesley (11), this possible loss of single-stranded DNA ends is very unlikely.

There is further experimental evidence that the observed aggregation of DNA molecules is not due to cohesion processes via single-stranded DNA. Assuming a G+C content of about 30% for these hypothetical sequences, as it is for total macronuclear DNA (12, 21), the observed thermal stability of the aggregates suggests that they should be quite long, perhaps representing up to 0.05–0.1% of an average-sized DNA molecule (22). Because it would be necessary to assume that most or all of the DNA molecules carry identical single-stranded DNA ends (Fig. 1*C*), the total sequence complexity of ends should be very low; therefore, under the conditions used, renaturation should occur extremely fast (23). As shown in Fig. 1*B*, this is not the case. The effect of formaldehyde on the ability to aggregate is shown in Fig. 2. Formaldehyde reacts with the free amino

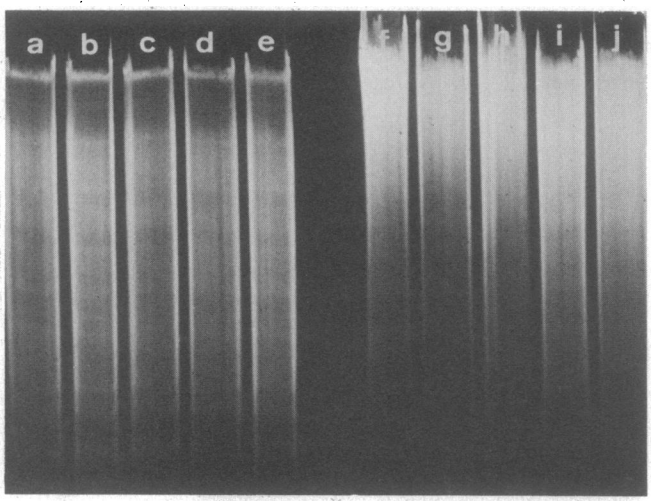

FIG. 2. Effect of formaldehyde on DNA aggregation. DNA was dissolved at a concentration of 1 mg/ml in 10 mM Tris·HCl (pH 7.5) and formaldehyde was added at concentrations between 0.1% and 1.5%. Samples were either incubated in 10 mM Tris·HCl and 0.1–1.5% formaldehyde or, after 30 min of incubation at room temperature, 20× standard saline citrate plus 0.1–1.5% formaldehyde was added to make the samples 4× standard saline citrate. DNA was further incubated at room temperature for 48 hr and then electrophoresed on a 1% agarose gel. Slots a–e: DNA incubated in 10 mM Tris·HCl (pH 7.5) containing 0.1% (slot a), 0.3% (slot b), 0.7% (slot c), 1% (slot d), and 1.5% (slot e) formaldehyde. Slots f–j: DNA incubated in 4× standard saline citrate containing 0.1% (slot f), 0.3% (slot g), 0.7% (slot h), 1% (slot i), and 1.5% (slot j) formaldehyde.

groups of cytosine, guanine, and adenine. At neutral pH and very low formaldehyde concentration, all bases in double-stranded DNA are protected from formaldehyde action. However, single-stranded DNA ends should be accessible to formaldehyde, and after reaction with it they should be unable to renature (24). As shown in Fig. 2, only a 10-fold excess of formaldehyde partially inhibits aggregation of DNA molecules. When DNA was allowed to aggregate under conditions where ring formation also occurs (incubating the DNA at concentrations below 50 μg/ml) and subsequently centrifuged on an ethidium bromide/cesium chloride gradient, an additional DNA fraction was observed (Fig. 3*b*, arrow). When this fraction

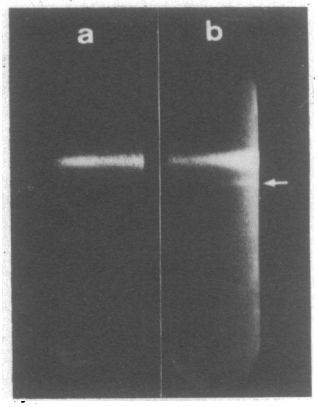

FIG. 3. Ethidium bromide/cesium chloride gradient of non-aggregated DNA (*a*) or DNA incubated for 48 hr at room temperature at a concentration of 10 μg/ml in 4× standard saline citrate (*b*). The gradient was centrifuged for 36 hr at 43,000 rpm in a Beckmann Ti 50 rotor at 25°C. (This experiment was performed at the suggestion of Max Birnstiel, Zürich.)

was examined under the electron microscope, it consisted mainly of supercoiled DNA circles of various sizes (although some linear DNA molecules were observed, which is probably due to contamination during the fractionation procedure). Similar results were obtained with ethidium bromide/metrizamide gradients (data not shown). Supercoiling would not be observed with DNA circles formed by renaturation of single-stranded DNA ends. Finally, electron micrographs of aggregated material revealed DNA structures that are very different from those expected for cohesion by single-stranded DNA ends. Fig. 4 shows gene-sized DNA molecules that have aggregated end to end and ring-like DNA structures. The fact that some short nonaggregated DNA molecules can still be detected in these preparations suggests that these structures may be partially disintegrated during the spreading procedure.

All the experiments discussed above provide strong experimental evidence that the observed formation of ring-like DNA structures and high molecular weight aggregates *in vitro* cannot be explained by the presence of identical single-stranded DNA ends on each macronuclear DNA molecule. I therefore propose the alternative model for aggregation of gene-sized macronuclear DNA molecules shown in Fig. 5. DNA molecules may be

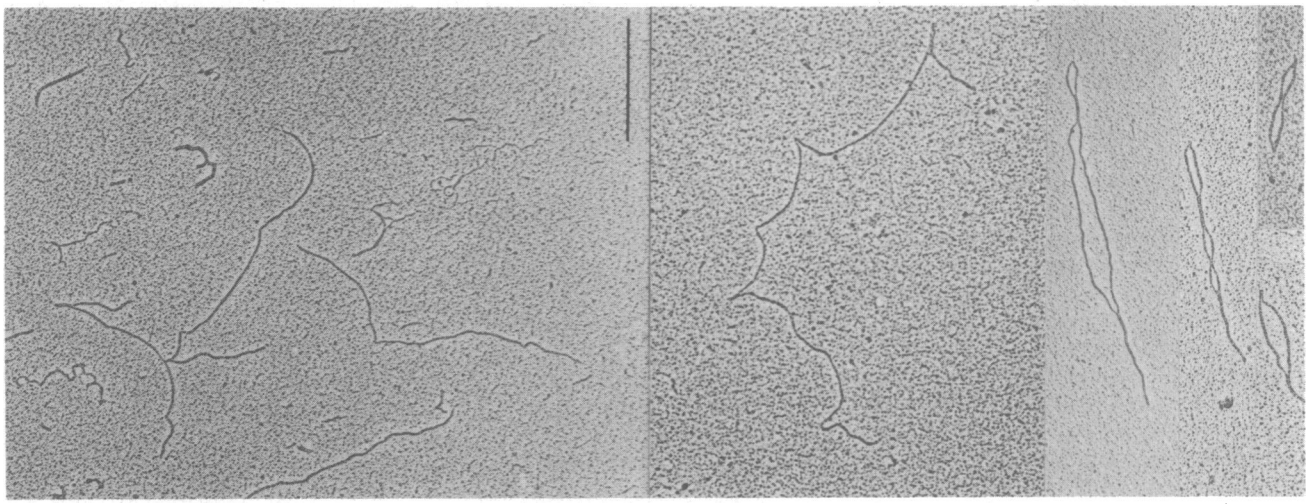

FIG. 4. Electron micrographs of end-to-end aggregated DNA fragments and ring-like DNA structures. DNA was allowed to aggregate for 24 hr at concentrations between 1 μg/ml and 1 mg/ml in 4× standard saline citrate. Mostly end-to-end aggregation of DNA fragments was observed; only at low DNA concentrations were up to about 5% ring-like DNA structures detected. (Bar = 0.5 μm.)

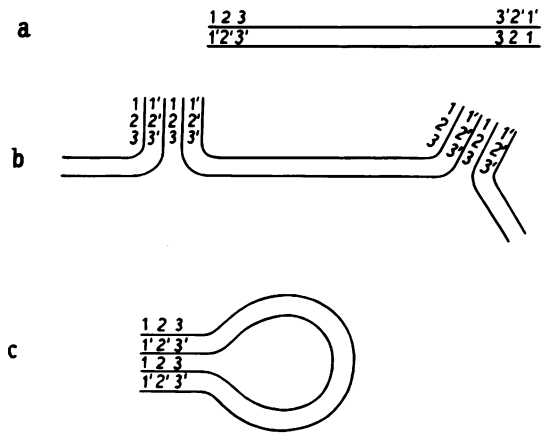

FIG. 5. (*a*) Model of one gene-sized macronuclear DNA fragment showing the terminal inverted DNA repeats. 1–3 and 1′–3′ represent homologous DNA bases. Slightly modified after Lawn (10). (*b* and *c*) Model for DNA aggregation by the formation of tetrastranded DNA complexes at regions of sequence homology. The formation of such structures could lead to either high molecular weight DNA aggregates (*b*) or ring-like DNA molecules (*c*).

able to form multistranded DNA complexes at regions of sequence homology (i.e., the terminal inverted repeats present on each DNA molecule). In its simplest form this could be tetrastranded DNA complexes similar to the structures proposed by Wilson (25). Formation of such tetrastranded DNA could lead either to the formation of intramolecular ring-like DNA or to end-to-end association, resulting in high molecular weight DNA aggregates. Formation of tetrastranded DNA from DNA duplexes at regions of sequence homology has been postulated in some recombination models (25, 26). Although such structures have not been isolated and characterized, molecular models have been described demonstrating their plausibility and specificity (25, 26). The model presented above is consistent in several important respects with the observations discussed in this communication. Because additional hydrogen bonds have to be formed (25), the reaction should depend very much on ionic conditions but should occur relatively slowly. The complex should be less stable than the original double helices (25), and it might be expected to result in structures similar to those observed in the electron microscope. Because the terminal repeat of the gene-sized DNA molecules is inverted, multistranded DNA complexes should form "necks." Due to the stiffness of the DNA duplexes, these necks may appear in the electron microscope to be considerably longer than they really are. Although it has not been described previously, it seems likely that DNA rings closed by tetrameric DNA should become supercoiled, behaving like covalently closed DNA circles. If this interpretation proves to be correct, the proposed structures would have a major impact not only on our view of macronuclear structure in ciliates (27) but also on recombination processes (25, 26, 28) and on eukaryotic chromosome structure in general.

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