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Separation of Micronuclear DNA of *Stylonychia lemnae* by Pulsed-Field Electrophoresis and Identification of a DNA Molecule with a High Copy Number

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DNA from the hypotrichous ciliatae *Stylonychia lemnae* was separated by PFGE. In addition to the separation of the macronuclear DNA molecules with a size up to ~40 kb, we were able to separate the micronuclear DNA with a size between ~90 kb and 2 Mb. One very prominent 90-kb DNA band appeared on the pulsed-field gels. We propose that this 90-kb DNA fragment represents a linear plasmid residing in the micronucleus in a very high copy number. About 10% of the micronuclear DNA consists of the 90-kb DNA molecule. It appears in the micronucleus as well as in the macronuclear anlagen during macronuclear development but not in the mature macronucleus. Thus, the multicopy DNA is eliminated during fragmentation of the macronuclear anlagen DNA in the course of macronuclear development. Therefore, this 90-kb DNA molecule might serve as an excellent tool to study the recognition and elimination of DNA during nuclear differentiation of hypotrichous ciliates.

Hypotrichous ciliates are characterized by their nuclear dimorphism, containing diploid micronuclei and DNA-rich macronuclei. The macronucleus contains the transcriptionally active DNA of the vegetative cell, consisting of small DNA molecules with sizes between 500 bp and ~40 kb (for review, see Prescott 1994; Lipps and Eder 1996) and is divided by a mechanism called amitosis (Grell 1973). The typical macronuclear "genesized piece" contains an open reading frame of one gene and regulatory sequences (for review, see Maercker and Lipps 1993; Hoffman et al. 1995). The only similarity to chromosomes are telomeric sequences at the end of each macronuclear DNA molecule (Klobutcher et al. 1981). The copy number of these molecules varies between 10^3 and 10^6 . For this reason the DNA content in the macronucleus is much higher than in the micronucleus, although its complexity is much lower than that of micronuclear DNA (Prescott 1994). The micronucleus contains ~100–300 chromosomes (Ammermann et al. 1974) and undergoes normal mitosis during vegetative growth.

We decided to analyze size and structure of micronuclear DNA molecules by pulsed-field gel electrophoresis (PFGE). Several questions were addressed: (1) Is the size of micronuclear chromosomes exclusively in the range of several megabases, or does it also contain smaller chromosomes as, for example, *Saccharomyces cerevisiae* with the smallest chromosome only having a size of 225 kb (Carle and Olson 1985) or the unicellular fungus *Pneumocystis carinii* with 13–15 linear chromo-

somes with sizes between 300 and 700 kb (Stringer and Cushion 1998). (2) Do autonomous, extrachromosomal genetic elements exist in the micronucleus? A huge variety of linear or circular plasmids have been characterized in prokaryotes. They mostly encode genetic determinants which make the organism resistant to antibiotics or help in other ways to compete with other microorganisms occupying the same ecological niche (for review, see Actis et al. 1995). So far, there are only a few plasmids that have been identified in eukaryotic cells, for example, the circular 2 μ plasmid in yeast (Hollenberg 1982) or the mitochondrial giant linear plasmids in the ascomycete *Podospira anserina* (Hermanns et al. 1995). (3) What happens to specific micronuclear DNA molecules during macronuclear development? During sexual reproduction of hypotrichous ciliates, after two cells of different mating types have conjugated, a new macronucleus is formed from a zygote micronucleus, whereas the old macronucleus is resorbed. At the beginning of this process one micronucleus from each cell undergoes meiosis before fusion to a diploid synkaryon takes place. The zygote formed from the two haploid micronuclei is the precursor for new micronuclei and a new macronucleus (Ammermann 1971; Ammermann et al. 1974). Macronuclear differentiation is accompanied by extensive genome rearrangement processes: Micronucleus-specific DNA segments and also whole micronuclear chromosomes are eliminated. Polytene chromosomes are formed from some micronuclear chromosomes, and after fragmentation of these polytene chromosomes, most of the DNA sequences are degraded. Telomeres are added to the remaining macronuclear DNA molecules which,

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in a last step, are amplified by several rounds of replication (for review, see Klobutcher and Jahn 1991; Prescott 1994; Doak et al. 1997).

In this work we describe the separation of micronuclear DNA by PFGE. One DNA molecule with a size of ~90 kb appears in a very high copy number. By isolation of this DNA molecule and by hybridization analyses we show that the 90-kb band represents a DNA fraction that is present in the micronucleus and in intermediate nuclei during macronuclear processing (macronuclear anlagen) but not in the mature macronucleus, indicating that it is eliminated during macronuclear differentiation.

RESULTS

The size of condensed micronuclear metaphase chromosomes is ~1 μm (Ammermann et al. 1974; C. Maercker et al., unpubl.). Taking into consideration that (1) the condensation grade during mitosis is ~1:10,000 (Kitsberg and Cedar 1991), (2) the *Stylonychia* micronuclear DNA content is $\sim 1.5 \times 10^{10}$ bp (Prescott 1994), and (3) the chromosome number is ~100–300 per micronucleus (Ammermann et al. 1974), a molecular weight of the chromosomes in the megabase range would be expected. To determine the size of the micronuclear DNA molecules more exactly, total *Stylonychia* DNA was separated by PFGE (Fig. 1a–c). In addition to macronuclear DNA with the expected size of up to 40 kb (for review, see Kraut et al. 1986), a very prominent band with a size of ~90 kb was obvious (Fig. 1a–c). In addition, depending on the DNA preparation, the gel run, and the size of the slots in the gel, many bands with sizes between ~100 and 700 kb (especially in Fig. 1a) and two to three bands between 1 and 2 Mb (especially Fig. 1c, lanes 1,2) were visible on the gels. According to these results we propose that many of the micronuclear chromosomes have sizes between 100 and 700 kb and also between 1 and 2 Mb. However, it is also possible that the 1- to 2-Mb fraction in our experiments also contained micronuclear DNA that was not separated properly or remained in the slots, as a similar pattern was observed with other chromosomal DNAs, for example, from algae and bacteria, which were not separated properly under the applied conditions (not shown).

To determine which DNA molecules belong to the micronucleus and to the macronucleus, respectively, micro- and macronuclei were separated before preparing the plugs for PFGE. As expected, DNA molecules smaller than ~40 kb were of macronuclear origin (Fig. 1c, lane 3). However, all higher molecular weight DNA, including the 90-kb DNA molecule, were part of the micronucleus (Fig. 1c, lane 2).

Because the 90-kb band was most prominent on all pulsed-field gels we concentrated on the analysis of this band in more detail. To confirm again that the 90-kb DNA molecule is part of the micronucleus, total micronuclear DNA was hybridized with PFGE-separated DNA and, as shown in Figure 1d, gave a strong signal with the 90-kb band. In addition, the 90-kb band hybridized exclusively with micronuclei in situ hybridization experiments (Fig. 1f). Therefore, the 90-kb molecule most probably is part of micronuclear DNA.

To decide whether the 90-kb DNA is a linear molecule, a two-dimensional PFGE was performed (Fig. 2). By this method it is possible to discriminate among linear, relaxed circular, and supercoiled DNA molecules, because in the second dimension the interca-

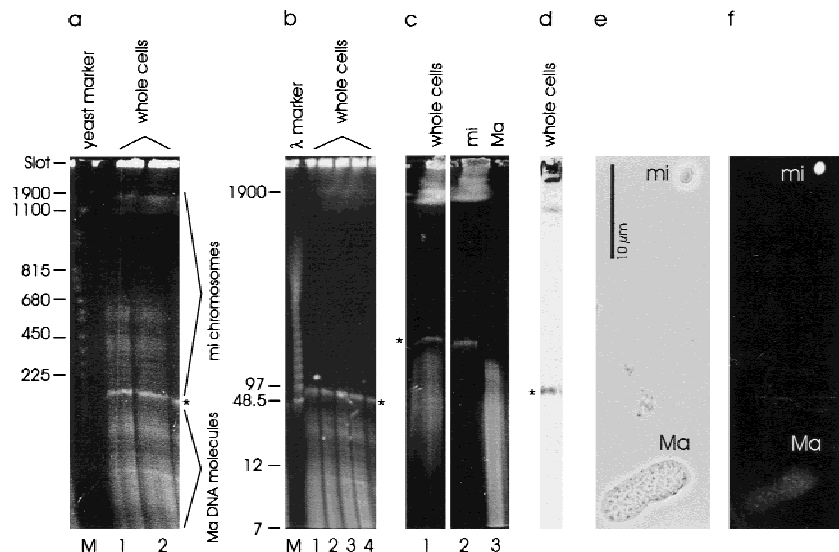


Figure 1 Visualization of *Stylonychia* DNA by PFGE and nuclear localization of the 90-kb DNA molecule by hybridization analyses. (a,b) DNA from whole *Stylonychia* cells was separated by PFGE and stained with ethidium bromide (a, lanes 1 and 2, and b, lanes 1–4). Two different DNA preparations were used in a and b, depending on the DNA preparation and the gel run, to show that the micronuclear chromosomes (mi) are more or less separated, whereas the 90-kb DNA molecule and the macronuclear DNA (Ma), with a size up to 40 kb, are visible on every gel. (c) DNA from whole cells (lane 1) and from isolated micronuclei (lane 2) or macronuclei (lane 3), respectively, was separated by PFGE and stained with ethidium bromide. (d) DNA from whole cells was separated by PFGE, transferred on a nylon filter, and hybridized with labeled micronuclear DNA. (e,f) In situ hybridization of micronuclear DNA with fixed *Stylonychia* cells. (e) Phase-contrast (bar, 10 μm); (f) in situ hybridization (FITC-labeled antibody against DIG-labeled micronuclear DNA). Sizes are given in kb. Molecular size markers were used: *Saccharomyces cerevisiae* chromosomes (lane M in a, Pharmacia), 1-kb ladder (7 and 12 kb in b, GIBCO BRL), λ concatemers (lane M in c, Bio-Rad). (*) 90-kb fraction.

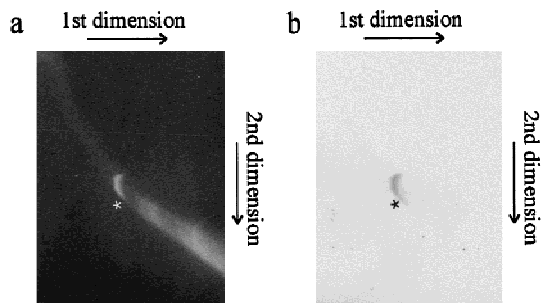


Figure 2 Two-dimensional PFGE of *Stylonychia* DNA. (a) Ethidium bromide-stained gel. (b) Southern hybridization of the gel shown in a, using the labeled 90-kb DNA molecule as a hybridization probe. (*) 90-kb DNA molecule.

lating agent ethidium bromide is present in the buffer, so that circular or supercoiled DNA molecules change their mobility in relation to linear molecules (Brewer and Fangman 1987). In our experiment after the second dimension it could easily be recognized on the gel that the 90-kb band is an integral part of the curve showing the linear DNA molecules of the micronucleus and the macronucleus (Fig. 2a). The 90-kb DNA molecule was also detectable by hybridization with itself, and the hybridization showed no other signals than the ethidium bromide-stained gel, indicating that a circular or supercoiled form of the DNA molecule does not exist (Fig. 2b).

To analyze whether the 90-kb band represents a homogenous DNA fraction, the isolated 90-kb DNA molecule was digested with *EcoRI*. Defined bands were obtained after this restriction digest (Fig. 3). The restriction fragments do not add up to 90 kb. This result may be due to the following reasons: Some restriction fragments may be of the same or similar sizes, which have not been separated properly, but still are different from each other. For example, this could be the case for the 4.9-kb *EcoRI* fragment, which gives a stronger signal than other restriction fragments on the gel. The largest band in the digest lane obviously represents the size of the undigested DNA. This could mean that it was only a partial digest, that is, that the DNA was not cut completely under the conditions applied in our experiment. The result suggests that the 90-kb DNA fraction is a homogenous DNA molecule or that at least it cannot be highly heterogenous.

To estimate the approximate copy number of the DNA molecule, micronuclear DNA was dot blotted. Both total micronuclear DNA and 90-kb DNA were labeled to comparable specific activity. Both probes were then used in excess for hybridization with the micronuclear DNA. The hybridization signal with whole micronuclear DNA was 10 times stronger than with the 90-kb DNA (data not shown). By assuming that the DNA content of the micronucleus is $\sim 1.5 \times 10^{10}$ bp

(Prescott 1994), the 90-kb fraction could be estimated to be $\sim 10,000$ copies per micronucleus.

If the 90-kb DNA is a linear molecule of the micronucleus with a high copy number, it seems not to be a micronuclear chromosome but, rather, a linear plasmid. This led to another question: What is the structure of the ends of the DNA molecule, that is, does it contain terminal sequences homologous to *Stylonychia* telomeres? To address this issue, whole cell DNA, separated by agarose gels, was hybridized with *Stylonychia* telomere oligonucleotides. As expected, macronuclear DNA gave rise to strong hybridization signals (Fig. 4b,d, lanes 1). After separation by PFGE, the 90-kb DNA molecule also hybridized (Fig. 4d, lane 1). To assure that the hybridization signal obtained with the telomere oligonucleotides was specific, the ends of the linear DNA molecules were digested with exonuclease *Bal 31* for 5–30 min to remove the telomeric sequences before hybridization. After this treatment the hybridization signals disappeared (Fig. 4a,b, lanes 2–5). The same result was obtained after *Bal 31* digest of the isolated 90-kb DNA molecule (Fig. 4d, lanes 2–5). This result implied again that the DNA molecule is linear and that it contains telomeric sequences or at least similar G-rich sequences at its ends.

Because the described results strongly suggest that the 90-kb fraction is localized in the micronucleus and not in the macronucleus of vegetative cells, we determined how this DNA behaves during macronuclear

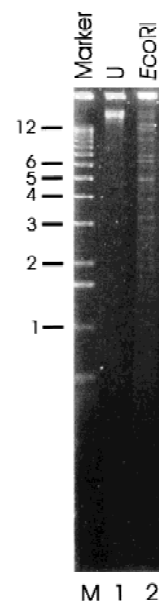


Figure 3 Restriction analysis of the 90-kb DNA molecule. The isolated 90-kb DNA molecule was digested with *EcoRI*. The DNA fragments were separated on an 0.7% agarose/TBE gel. (Lane M) Sizes in kb (1-kb ladder; GIBCO BRL); (lane 1) undigested 90-kb DNA; (lane 2) DNA digested with *EcoRI*.

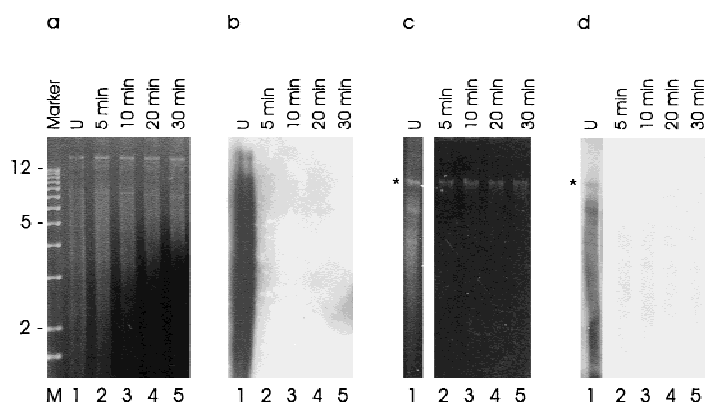


Figure 4 Analysis of the ends of the 90-kb DNA molecule. (a,b) As a control, *Styloynchia* DNA, isolated by classical methods (Ammermann et al. 1974), was separated by a 0.7% agarose/TBE gel before *Bal* 31 digestion (U, lane 1) and after *Bal* 31 digestion [(lane 2) 5 min; (lane 3) 10 min; (lane 4) 20 min; (lane 5) 30 min], stained with ethidium bromide (a), and hybridized with *Styloynchia* telomere oligonucleotides (b). (c,d) Whole cell *Styloynchia* DNA (lane 1) or the isolated 90-kb DNA molecule was separated before *Bal* 31 digestion by PFGE (U, lane 1) or at different time points after *Bal* 31 digestion on a 0.7% agarose/TBE gel (c, ethidium bromide stained) and hybridized with *Styloynchia* telomere oligonucleotides (d). (*) 90-kb DNA molecule.

differentiation. Cells of two different mating types were mixed and the different nuclei from exconjugants were separated according to their sizes at different time points and prepared for PFGE. At day 3 the macronuclear anlagen contained fully developed polytene chromosomes, so that the main macronuclear anlagen fraction had diameters between 20 and 40 μm ; some of them were smaller and copurified with the macronuclei (10–20 μm). The diameter of the micronuclei was <10 μm . At day 4 DNA fragmentation and degradation had started. Therefore, most of the macronuclear anlagen were already smaller, with diameters ranging between 10 and 20 μm , so that they were in the same fraction as the macronuclei. From the micronuclear fractions (Fig. 5a, lanes 1,4) and from whole vegetative cells (Fig. 5a, lane 7) the 90-kb DNA fraction clearly could be isolated. The DNA concentrations in the other lanes, however, were too low to see the 90-kb band. Therefore, the gel was blotted again and the DNA was hybridized with the 90-kb DNA. This hybridization revealed that the 90-kb DNA appeared both in the polytene chromosome nuclei (20- to 40- μm fraction, day 3) and in the late anlagen stage during fragmentation of the polytene chromosomes (10- to 20- μm fraction, day 4). The 10- to 20- μm fraction contained macronuclei and small macronuclear anlagen. However, the hybridization signal most probably is exclusively due to anlagen nuclei, because, as is shown in Figure 1c, the macronuclei do not contain the 90-kb molecule. Therefore, we propose that the 90-kb DNA fraction remains in the macronuclear anlagen and is only eliminated after fragmentation of the polytene chromo-

somes, before the replication of the macronuclear DNA molecules.

DISCUSSION

In this work the micronuclear DNA of the hypotrichous ciliate *S. lemnae* was characterized by PFGE for the first time. The sizes of the DNA molecules have been estimated to be approximately between 90 kb and 2 Mb. A very prominent 90-kb DNA fraction was the object of further analysis. It is suggested that the 90-kb DNA is a linear molecule and that it contains telomere-like sequences at its ends. In the micronucleus it was found in a high copy number, and it is also present in precursor nuclei (macronuclear anlagen). On the contrary, it could not be detected in the mature macronucleus, indicating that it is eliminated during nuclear differentiation.

Two-dimensional PFGE of *Styloynchia* DNA showed that the 90-kb band appeared as a single, linear fraction on the ethidium bromide-stained gel (Fig. 2a). Even the hybridization of the DNA separated in two dimensions with the 90-kb DNA revealed only one signal emanating from the linear molecule. No other signals from spots, which would have been due to different mobilities of nicked circle or supercoiled 90-kb molecules (Brewer and

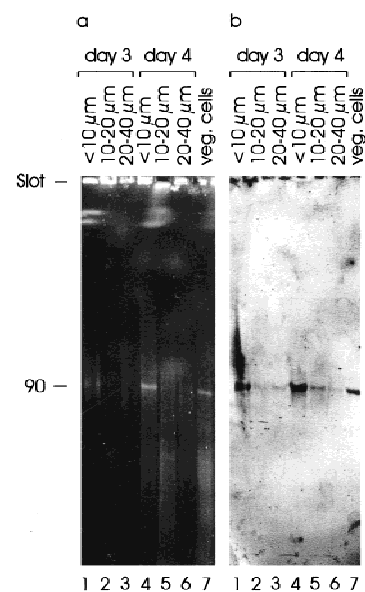


Figure 5 The 90-kb DNA molecule in exconjugants. (a) Nuclei of exconjugant cells (lanes 1–3, 3 days after the mixing of two mating types; lanes 4–6, day 4 after the mixing of two mating types) were separated by size (lanes 1,4, <10 μm ; lanes 2,5, 10–20 μm ; lanes 3,6, 20–40 μm) and the DNA was run on a pulsed-field gel and stained with ethidium bromide. Whole vegetative cells were taken as a control (lane 7). (90) The 90-kb DNA fraction. (b) The gel shown in a was blotted and the DNA was hybridized with the 90-kb DNA fraction.

Fangman 1987) could be detected (Fig. 2b). Therefore, the 90-kb fraction most likely is linear DNA.

The hybridization of the 90-kb DNA molecule with oligonucleotides containing *Stylonychia* telomeric sequences, which disappeared after exonucleolytic digest by *Bal* 31, suggested that it is a linear molecule with *Stylonychia* telomeres at its ends. However, telomere hybridization with the 90-kb band is not as strong as we would expect, that is, the high copy number of the 90-kb molecule compared with hybridization with macronuclear DNA (Fig. 4d, lane 1). This effect is even more prominent when high hybridization temperatures are used for this experiment (58°C; data not shown). Therefore, we cannot exclude that the 90-kb DNA molecule does not contain telomeric sequences that are completely homologous to the *Stylonychia* telomeres but are only similar to those sequences.

The result of the *Eco*RI digest revealing distinct restriction fragments (Fig. 3) clearly makes it possible that the 90-kb band might be a homogenous DNA fraction. The sum of the restriction fragments obviously is <90 kb, which is also true for other restriction digests (not shown). Despite the possibility that this might be due to similar restriction fragments that cannot be separated properly or due to an incomplete digest (see Results), it also can be speculated that the structure of the 90-kb band might represent a linear dimer of a 45-kb molecule or even a multimer of a smaller sequence. If that is the case, we would expect several restriction fragments of the same sizes that cannot be discriminated on the gel. The completion of subcloning and sequencing of the DNA molecule will give more detailed information about its structure.

The most surprising result was the very high copy number of this DNA molecule in the micronucleus. In addition, it also was detectable in the late macronuclear Anlagen but not in the mature macronucleus (Fig. 5). Presently, it is not known whether the 90-kb DNA molecule becomes amplified during polytenization of the micronuclear chromosomes, but it seems to be stable until fragmentation of the polytene chromosomes takes place. Even if 10,000 copies per micronucleus is a rough estimation—moreover, the copy number does not seem to be the same in every *Stylonychia* strain (data not shown)—the copy number is definitely higher than would be expected from any micronuclear chromosome.

Taking our experimental results into consideration, the origin of the 90-kb DNA molecule, if it is not a micronuclear chromosome, remains to be determined. From the following experimental results, it was concluded that the molecule does not represent mitochondrial DNA. (1) After isolating micronuclei from *Stylonychia* and separating its DNA by PFGE, the 90-kb DNA molecule was a very prominent fraction (Fig. 1c,

lane 2). The result was the same when micronuclei where isolated by a saccharose cushion using low speed centrifugation (Ammermann et al. 1974) to exclude contamination with organelles other than nuclei (not shown). (2) By hybridization of pulsed-field separated DNA with micronuclear DNA, the 90-kb band showed a very strong signal (Fig. 1d). (3) The micronuclei showed a very strong hybridization signal after in situ hybridization of *Stylonychia* nuclei with the 90-kb molecule (Fig. 1f). (4) After cloning and sequencing of ~1 kb of the DNA molecule we did not find a remarkable homology to a mitochondrial gene or any other known gene (C. Maercker, unpub.). (5) By using primers binding to a conserved region of the mitochondrial cytochrome *b* gene, we were not able to amplify a PCR fragment homologous to mitochondrial DNA with the 90-kb DNA molecule as a template (data not shown). (6) The 90-kb fraction also was present in the 20- to 40- μ m nuclear fraction, where small organelles such as mitochondria would not appear normally (Fig. 5). Therefore, although linear mitochondrial genomes have been identified in ciliates and other organisms (for review, see Nosek et al. 1998), it is very unlikely that the 90-kb DNA molecule is a mitochondrial genome, or another organellar genome, as was found in *Plasmodium* and related species (for review, see Wilson and Williamson 1997).

Despite the fact that in hypotrichous ciliates no endosymbionts have been found in the micronucleus so far, the high copy number is a strong argument against the 90-kb DNA molecule being the genome of a nuclear endosymbiont. In addition, the DNA molecule is much smaller than the genomes of, for example, the bacteria *Holospora elegans* or *Holospora obtusa*, which are micronucleus- or macronucleus-specific endosymbionts, respectively, of the ciliate *Paramecium caudatum* (Fujishima and Görtz 1983; Görtz and Fujishima 1983). Also *H. elegans*-bearing paramecia are genetically dead, as most of the exconjugants die (Görtz and Fujishima 1983), whereas *Stylonychia* cells bearing the 90-kb molecule, even with this high copy number, are able to undergo the sexual life cycle. Also, virus particles in such large number would be lethal for the cell, making it very unlikely that the 90-kb DNA molecule is a viral genome.

Instead, the 90-kb DNA molecule seems to have similarities to large linear plasmids that have been identified in *Streptomyces* and other *Actinomycetales*. These plasmids have sizes of up to several hundred kilobases, which can replicate autonomously with a mechanism proposed to be similar to the autonomous replication of parvoviruses (Picardeau and Vincent 1998; Qin and Cohen 1998; Redenbach et al. 1998). To our knowledge, no plasmids of this size have been found in nuclei of eukaryotic cells, but it would be worth noting, after completion of sequencing, if there

are any similarities between the 90-kb DNA molecule and eukaryotic or even prokaryotic DNAs.

It will be crucial to sequence the ends of the molecule to see clearly whether it contains *Stylonychia* telomeres at its ends or longer inverted terminal repeats like, for example, yeast plasmids (McNeel and Tamanoi 1991), *Tetrahymena* mitochondrial telomeres (Morin and Cech 1988), retrotransposons at the ends of *Drosophila* chromosomes (Danilevskaya et al. 1999), or other genetic elements (for review, see Sakaguchi 1990; Rohe et al. 1992). The complete sequence of the DNA molecule will reveal if it contains any insertions of, for example, micronuclear DNA, similar to giant linear plasmids of the ascomycete *P. anserina* in which mtDNA sequences are integrated (Hermanns et al. 1995).

On the other hand, it is possible that the DNA molecule represents a palindrome like the macronuclear rDNA in *Tetrahymena* (Kapler et al. 1994) or a concatemer of several shorter DNA fragments that can be added to 90 kb, as is known from *Neurospora* plasmids (Yang and Griffiths 1993). With DNAs from some *Stylonychia* strains strong bands with a much larger size than 90 kb were detected on pulsed-field gels, some of which also hybridized with the 90-kb fragment, possibly representing multimers of the DNA molecule (C. Maercker, unpubl.).

Whether or not the 90-kb DNA molecule has any function—it might contain genes important for vegetative growth or for macronuclear differentiation of *Stylonychia*—we only can speculate at the moment. Although we did not find hybridization of the 90-kb band with a gene-sized DNA molecule (data not shown), it still might code for a gene product that is expressed only as specific time points during the life cycle of *Stylonychia*. Further characterization of the DNA of different *Stylonychia* and other ciliate strains, as well as cloning, sequencing, and expression studies, for example, by Northern analysis of different nuclei and macronuclear precursors, respectively, will elucidate the meaning of the extrachromosomal DNA molecules. At least the DNA degradation machinery seems to eliminate the 90-kb DNA before the processing of the mature macronucleus. Therefore, the DNA molecule might be a nice vehicle to study the recognition and programmed elimination of DNA during macronuclear differentiation of hypotrichous ciliates.

METHODS

Preparation of DNA and PFGE

Stylonychia cells were cultivated as described by Ammermann et al. (1974). Conjugation was achieved by mixing two different mating types (Ammermann et al. 1974), and exconjugants were incubated for 3–4 days at room temperature until macronuclear anlagen with fully developed polytene chromo-

somes and later macronuclear anlagen stages were visible by phase contrast microscopy.

Stylonychia DNA was isolated as described by Ammermann et al. (1974), or it was prepared especially for PFGE, modified after Birren and Lai (1993): 6×10^4 – 8×10^4 cells were collected on 30- μ m gauze (Heidland) in a volume of ~1 ml. Five hundred microliters of cell suspension was mixed with the same volume of 50°C prewarmed 1% low melting point (LMP) agarose (GIBCO BRL) in 0.5 M EDTA. The mixture was poured into disposable plug molds (~80 μ l for one plug; Bio-Rad). To prepare DNA for PFGE from different nuclei, cells were lysed and the nuclei were separated according to size using 40-, 20-, and 10- μ m gauze (Ammermann et al. 1974), before mixing with the prewarmed agarose. After 30 min at 4°C the agarose blocs containing the *Stylonychia* cells or nuclei, respectively, were incubated in 400 μ l of 0.5 M EDTA, 1% laurylsaracoline, and 10 μ l of proteinase K (10 mg/ml, Sigma) overnight at 55°C. The blocs can be stored at 4°C for ~1 week before application on a pulsed-field gel. For PFGE the Gene Navigator System with the hexagonal electrode was used (Pharmacia). About half of a bloc (25-well comb) or a whole bloc (14-well comb) was loaded in one slot of a 1.1% agarose gel (GIBCO BRL) and filled up with 1% LMP agarose in $0.5 \times$ TBE. To retain the DNA agarose bloc directly in front of the slot, the slots were filled with LMP agarose. The gels were run in $0.5 \times$ TBE at 200 V, 70-sec pulse time, for 22–23 hr at 8°C (temperature of the buffer in the chamber). Afterward the gel was stained with ethidium bromide in the same buffer.

Two-dimensional PFGE was performed according to Brewer and Fangman (1987), with modifications: After the run in the first dimension, the gel was equilibrated in 0.2 μ g/ml ethidium bromide in $0.5 \times$ TBE for 2 hr. The position of the rack with the gel was then changed to 90°, and the run in the second dimension followed the same conditions as in the first dimension, but in 0.2 μ g/ml ethidium bromide in $0.5 \times$ TBE.

Isolation of the 90-kb DNA Molecule

The 90-kb DNA band was eluted from pulsed-field gels. The ethidium bromide-stained band was cut out from about four to five gels. The agarose blocs containing the 90-kb DNA molecule were collected in a Biotrap elution chamber (Schleicher & Schuell). The DNA was eluted from the gel pieces overnight at 100 V in $0.5 \times$ TAE or $1 \times$ TAE according to the supplier's instructions. The eluate (~500 μ l) was applied on a Microcon 30 column (Amicon) and centrifuged for 12 min at 13,000 rpm in a Heraeus-Minifuge. Alternatively, the DNA fragment was isolated by centrifugation of the agarose piece containing the DNA through filter wool (Rivalon; Heraeus-Minifuge at 8000 rpm for 10 min) before application on a Microcon 30 column. The desalted DNA solution in a volume of about 20 μ l was used for digoxigenin (DIG) labeling (Feinberg and Vogelstein 1983; Boehringer Mannheim) or for digestion with restriction endonucleases according to the supplier's instructions (GIBCO BRL).

DNA Transfer and Hybridizations

The DNA from agarose gels was transferred on a Nylon plus membrane (Qiagen or Schleicher & Schuell) in $10 \times$ SSC according to Southern (1975) and the supplier's instructions. The genomic DNA probes were labeled by the random oligo-labeling method with DIG-dUTP (Feinberg and Vogelstein 1983; Boehringer Mannheim) The oligonucleotides containing *Stylonychia* telomeric sequences (Ptel1, 5'-CCCCAAAA-

CCCCAAAACCCCGTCGACGGCG-3'; Pte12, 5'-CGCCGTCGACGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGG-3') were labeled by DIG-dUTP tailing with terminal transferase according to the supplier's instructions (Boehringer Mannheim) or by a T4 polynucleotide kinase labeling reaction using [γ - 32 P]ATP (Sambrook et al. 1989). Hybridization was done in $4\times$ SSC, $10\times$ Denhardt's medium, 0.1% SDS at 65°C overnight with genomic probes or at 42°C (Fig. 4d, lane 1), or 58°C (Fig. 4, b, lanes 1–5, and d, lanes 2–5) with the oligonucleotides. Following hybridization, the filters were washed twice for 5 min in $2\times$ SSC, 0.1% SDS, at room temperature and twice for 15 min in $0.1\times$ SSC, 0.1% SDS, at hybridization temperature. The hybridization signals were detected by the DIG detection method using nitro blue tetrazolium and X-phosphate for the color reaction (Boehringer Mannheim) or by autoradiography, respectively.

In situ hybridization with whole nuclei was performed as described earlier (Maercker et al. 1997): The hybridization was done overnight in a humid chamber in $2\times$ SSC at 65°C. Following hybridization the slide was washed in $2\times$ SSC at room temperature and in $0.1\times$ SSC at 65°C. The following steps were incubation with anti-DIG-fluorescein Fab fragments (2 mg/ml) in TS buffer (100 mM Tris/HCl at pH 7.5, 150 mM NaCl), washing in TS buffer, and analysis with a Leitz DMRB microscope by phase-contrast or fluorescence microscopy with blue light (470 nm) to visualize the anti-DIG-fluorescein Fab fragments.

Dot Blot Analysis of the 90-kb DNA fraction

Micronuclear DNA was isolated and sonicated with a Branson 250 sonifier and transferred on a filter in different dilutions. Whole micronuclear DNA and the 90-kb DNA fraction, respectively, were labeled to the same specific activity, and the same number of counts of each labeling reaction were taken for hybridization, using the probes in excess concentrations. The signals on the autoradiographs were measured by densitometry (Bio-Rad) to set the strength of the hybridization signals of whole micronuclear DNA and the 90-kb DNA fraction in relation to each other.

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