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Viscoelastic studies on *Tetrahymena* macronuclear DNA

(chromosome/DNA molecular weight/amplification/recombination/micronuclei)

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ABSTRACT **We have used viscoelastometry in an attempt to understand the physical organization of genetic material in *Tetrahymena* nuclei. The micronucleus or germ line nucleus is diploid. It divides mitotically during vegetative growth, and five pairs of chromosomes are seen in meiosis. The macronucleus, or somatic nucleus, is approximately 45-ploid, divides amitotically, and has no visible chromosomes at any stage. Viscoelastic analysis of *Tetrahymena* macronuclei reveals DNA molecules of 2–3 × 10^{10} daltons accounting for much, if not all, of the macronuclear DNA. Since the average chromosome in the micronucleus contains 2.4–2.7 × 10^{10} daltons of DNA, we deduce that the macronucleus of *Tetrahymena* contains chromosome-sized DNA molecules.**

The ciliated protozoa, *Tetrahymena*, are remarkable for their nuclear dimorphism; they possess a transcriptionally inactive, diploid, germinal micronucleus, and a transcriptionally active, polyploid, somatic macronucleus. During vegetative growth the micronucleus divides mitotically while the macronucleus divides amitotically. Sexual conjugation results in degeneration of the macronucleus while the micronucleus undergoes meiosis. Subsequent reciprocal exchange and fusion of two haploid gametic nuclei maintains the genetic continuity of the organism. From a daughter of the zygotic nucleus formed during conjugation a new macronucleus arises by amplification of the genetic material. Presumably, before macronuclear differentiation occurs the micronucleus and the macronuclear anlage contain identical genetic material. However, both go on to develop into quite different organelles with distinctly different functions. The mechanism by which *Tetrahymena* establish and maintain nuclear dimorphism remains an intriguing unsolved problem.

At the present time the genetics and molecular architecture of the nuclei of *Tetrahymena* are being intensively studied (for reviews see refs. 1–4). The diploid micronucleus contains five pairs of approximately equal-sized chromosomes clearly visible during conjugation. Genetic markers on this germinal nucleus are distributed in the normal mendelian fashion. The macronucleus, by comparison, does not contain visible chromosomes, but rather many "chromatin bodies," and genetic markers are apparently segregated randomly during vegetative fission. On the basis of cytological (5), genetic (6), and cytospectrophotometric (7, 8) evidence, the macronucleus appears to contain 45 haploid genomes after cell division, with each genome containing at least 90% of the sequences found in the micronuclear genome (9).

Of central importance to an adequate understanding of *Tetrahymena* nuclear dimorphism is an explanation of how DNA is organized within the two types of nuclei. Conceivably, micronuclear DNA resembles DNA from other organisms in which chromosomes are visible, such as *Drosophila* (10) and yeast (11, 12), in which chromosome-sized DNA molecules are observed, or dinoflagellates (13) and mice (14), in which large molecules somewhat smaller than chromosome size are observable. Macronuclear DNA, on the other hand, could fall into any of three possible size classes. First, the macronucleus could contain gene- or multigene-sized pieces of DNA similar to those observed in the protozoan *Oxytricha* (15). Second, the macronucleus could contain linear pieces of DNA corresponding in size to the chromosomes observed in the micronucleus. Third, all the DNA of one genome could be connected into a superchromosome (5, 16–18). We have attempted to determine which, if any, of these three alternative models applies to *Tetrahymena* macronuclear DNA using the recently developed technique of viscoelastometry (13, 19, 20).

MATERIALS AND METHODS

Solutions. The growth medium for all *Tetrahymena* cultures consisted of a sterile, filtered solution of 2% proteose peptone, 0.1% yeast extract, and inorganic salts (21, 22), hereafter referred to as PPY. The lysis solution contained 0.5 M Na_4EDTA (pH 9.5), and 6% (wt/vol) sodium decyl sulfate ($NaDecSO_4$). The resuspension solution was 0.5 M Na_4EDTA adjusted to pH 8.5. Nuclease-free Pronase was obtained from Calbiochem (San Diego) and dissolved in 0.5 M EDTA (pH 9.5) to a concentration of 1 mg/ml. Pronase solutions were prepared in advance and stored in 1-ml aliquots at −20°. Porcine spleen DNase was obtained from Sigma Chemical Corporation (St. Louis, MO); 20,000 units were dissolved in 1 ml of 0.5 M EDTA (pH 7.0) in advance and stored at −20°. Pronase substrate, hide powder azure (B grade), was obtained from Calbiochem.

Instruments. All viscoelastic measurements were performed with a Cartesian diver viscoelastometer as originally described by Klotz and Zimm (20) and modified by Uhlenhopp *et al.* (23).

Organisms. *Tetrahymena pyriformis* (GL), an amicronucleate strain, was obtained from M. A. Gorovosky. The micronucleate strain (formerly syngen 1 of the *T. pyriformis* complex and a derivative of strain B1868) has been recently designated *T. thermophila Chx-2/Chx-2* (cycl-S, II) and was obtained from P. Bruns via E. Orias (24, 25,‡).

Procedures. All work was conducted at room temperature, unless otherwise specified. Cell concentrations were determined with an electronic particle counter (Coulter counter model B).

Exponentially growing cultures of *Tetrahymena* were used throughout this study. To obtain cells in their exponential phase, we added 1 ml of *T. pyriformis* or *T. thermophila* stock culture in PPY (approximately 2 × 10^5 cells) to 100 ml of filtered, sterile

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Abbreviations: M_r, molecular weight; PPY, proteose peptone/yeast extract medium; $NaDecSO_4$, sodium decyl sulfate.

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‡ According to the genetic nomenclature recently proposed by *Tetrahymena* geneticists (E. Orias, personal communication).

PPY broth in a 250-ml erlenmeyer flask and placed it in a 30° incubator for 8–12 hr. The cell concentration at the end of this period averaged 1–2 × 10^4 cells/ml. Cultures were then divided into two 250-ml erlenmeyer flasks and incubated at 30° with shaking (approximately 150 cycles/min). Cells were harvested at a concentration of 2–8 × 10^4 cells/ml by centrifugation for 3 min at 1370 × *g* in a clinical centrifuge. The pellets obtained were compact, but easily dislodged, necessitating the careful removal of the supernatant. Cells were resuspended in an appropriate volume of resuspension solution and checked for sterility by visual examination and, on occasion, streaking on a nutrient agar plate to check for contaminating yeast or bacteria.

Lysates were prepared by a modification of the method used by Kavenoff and Zimm (10). Two milliliters of lysis solution were added to a clean viscoelastometer chamber maintained at 65°. One milliliter of resuspension solution containing the appropriate number of *Tetrahymena* cells was then pipetted rapidly into the viscoelastometer. Addition of resuspended cells to the lysing solution resulted in adequate mixing and rapid lysis, consistently yielding clear lysates. After incubation at 65° for 10 min, the temperature of the chamber was lowered rapidly to 50° with the simultaneous addition of 1 ml of Pronase solution, thawed immediately before inclusion. Upon addition of Pronase, no cloudy precipitate was observed. The lysate was then maintained at 50° for 4 hr, at which time the Cartesian diver was carefully placed in the chamber, stabilized in the center of the chamber for 30 min, and rotated to produce an elastic response.

Comparison studies of both *T. pyriformis* and *T. thermophila* in the absence of Pronase were conducted by the same procedure, except that 1 ml of 0.5 M EDTA (pH 9.5) was pipetted into the chamber in place of 1 ml of Pronase solution. Control experiments involved breaking the DNA enzymatically with DNase or hydrodynamically by repeated pipetting with a pasteur pipet.

Solvent viscosity was determined with an Ostwald viscometer submerged in a 50° water bath.

The activity of Pronase under the lysis conditions used in this study was ascertained with a general proteolytic substrate, hide powder azure. Lysate incubation mixtures, with or without $NaDecSO_4$, were prepared as indicated above except that hide powder azure was added to a final concentration of 0.25 mg/ml instead of cells. The time course of Pronase-catalyzed release of the azure dye from the collagen at 50° was monitored at 595 nm (26).

Determination of Molecular Weights and Populations. Viscoelastic retardation times are dependent on molecular size, temperature, solvent viscosity, cation concentration, and DNA concentration. Correction factors for the last four variables must be obtained before molecular size can be calculated from retardation data. The dependence on DNA concentration can be resolved graphically by plotting experimentally determined retardation times (τ values) against cell (i.e., DNA) concentration, and extrapolating to zero concentration. τ^0 is then defined as the value of τ at infinite dilution. τ^0 may be corrected for solvent viscosity and temperature effects using the following relationship to obtain $\tau^0_{50,w}$, the value of τ at infinite dilution and 50°, if the solvent is H_2O (10):

$$\tau^0_{50,w} = \frac{0.005468\ T}{323\ \eta_{sol}} \cdot \tau^0. \qquad [1]$$

[The viscosity of water at 323 K is 0.005468 poise (0.0005468 Pa·sec).] Finally, the value of $\tau^0_{50,w}$ must be corrected for the cation concentration in the lysate. Three formulas for this final correction have been derived, and each was used to calculate the molecular weight (M_r) of the macronuclear DNA in *Tetrahymena*.

$$M_r = 2.2 \times 10^8\ (\tau^0_{50} \cdot 1.67)^{0.6} \quad \text{(ref. 10)} \quad [2]$$

$$M_r = 2.45 \times 10^8 (\tau^0_{50,w})^{0.63} \quad \text{(ref. 12)} \quad [3]$$

$$M_r = 2.75 \times 10^8 (\tau^0_{50,w})^{0.623} \quad \text{(ref. 12)} \quad [4]$$

[The first equation, derived by Kavenoff and Zimm (10), actually corrects for both solvent viscosity and salt concentration in the final equation, necessitating the use of τ^0_{50}, rather than $\tau^0_{50,w}$.]

The absolute number (L_1), as well as the size, of largest molecules in a solution can also be calculated from viscoelastic data from the following equation (10):

$$L_1 = \frac{\eta_0 \Gamma_1}{kT \cdot \tau_1 \cdot \Delta\theta}, \qquad [5]$$

where k is Boltzmann's constant in ergs/K, T is temperature in K, η_0 is the solvent viscosity in poise, τ_1 is the longest retardation time in seconds, Γ_1 is the total diver recoil due to the largest molecules alone, and $\Delta\theta$ is the total rotation of the diver initiating the experiment.

RESULTS

In this study, preliminary work had shown that $NaDecSO_4$ concentrations higher than those used by Kavenoff and Zimm (10) were necessary to lyse *Tetrahymena* completely. Questions arose as to the activity of Pronase at these higher $NaDecSO_4$ concentrations. Fig. 1 shows the time course, with and without $NaDecSO_4$, of Pronase-catalyzed digestion of hide powder azure, a general proteolytic substrate, under conditions identical to the viscoelastic lysis conditions. These results show that a $NaDecSO_4$ concentration of 3% does not appreciably affect Pronase activity.

The results of several viscoelastic experiments on *Tetrahymena* cells both possessing and lacking micronuclei, in the presence and absence of Pronase, are given in Fig. 2. In all cases, retardation times were equivalent within experimental error, indicating that neither the digestion with Pronase nor the presence of micronuclear DNA alters the viscoelastic species. A computer-generated least squares line extrapolating τ_1 values for *T. pyriformis* in the presence of Pronase to infinite dilution indicates little concentration dependence in 2 M salt, a result

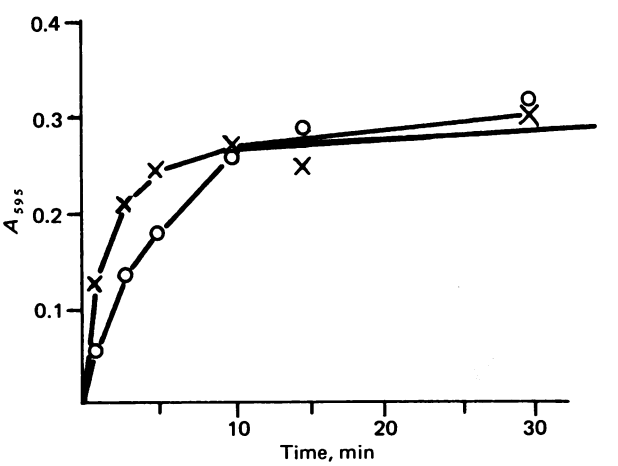

FIG. 1. Activity of Pronase under cell lysis conditions. The Pronase assay contained 1 ml of Pronase (1 mg/ml), 1 ml of resuspension solution, 1 mg of hide powder azure, and 2 ml of lysis solution with (O) or without (×) $NaDecSO_4$. The mixture was incubated at 50° and the release of the dye from the collagen was measured by recording the increase in absorbance at 595 nm.

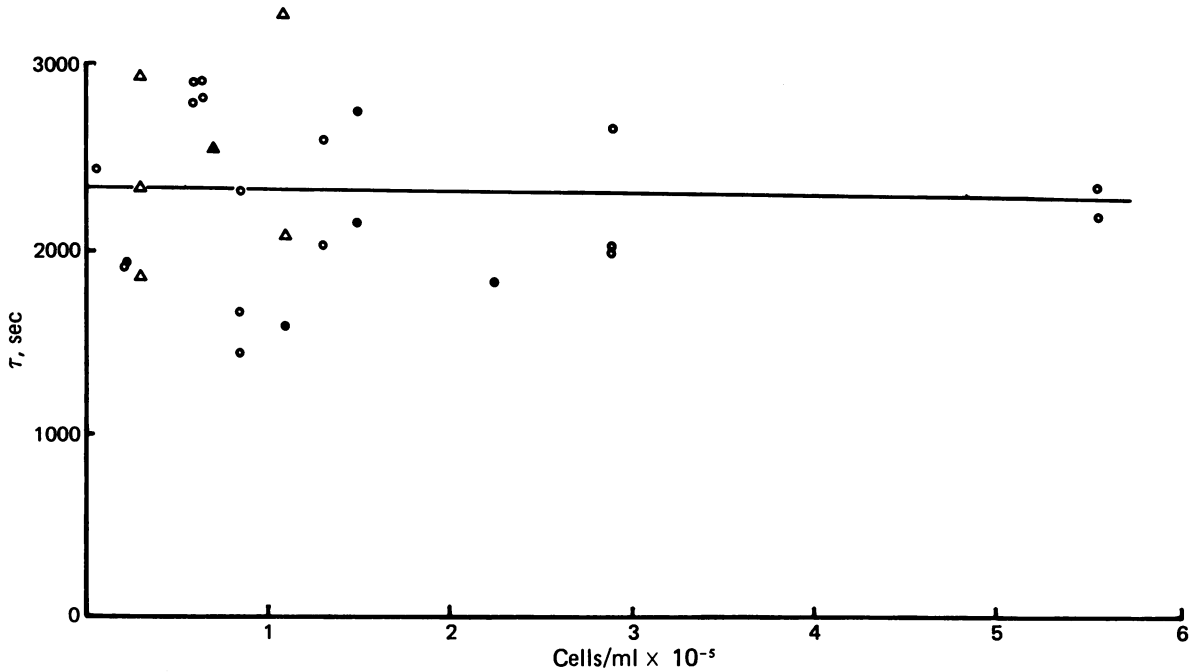

FIG. 2. Retardation times of various *Tetrahymena* lysates as a function of cell concentration. *Tetrahymena* cells were harvested, lysed, and examined by viscoelastometry. *T. pyriformis* lysates prepared (○) with Pronase, (●) without Pronase; *T. thermophila* (△) with Pronase, (▲) without Pronase. More than one point at a given cell concentration indicates that multiple viscoelastic relaxations were conducted on a single lysate. The computer-generated least squares line was constructed using only the data for the experiments with *T. pyriformis* in the presence of Pronase. τ^0, the *Y* intercept of this line, is 2340 ± 108 sec (SD).

in agreement with other viscoelastic work on eukaryotic DNA in 2 M sodium (10, 12). Table 1 lists molecular weights calculated from $\tau^0 = 2340$ sec by each of the three methods cited previously (Eqs. 2–4).

A single diploid *T. thermophila* micronucleus contains 0.8–0.9 pg of DNA during its G_2 phase (1). Ray's microscopic work with micronuclei during meiosis and postmeiotic haploid mitosis showed that the haploid micronucleus contains five approximately equal-sized chromosomes (27). Dividing the haploid micronuclear DNA equally among five chromosomes requires that the average chromosome contain from 2.4 to 2.7 $\times 10^{10}$ daltons of DNA. Our results indicate that the macronuclei of *T. pyriformis* and probably of *T. thermophila* contain DNA corresponding in size to that calculated to be contained within a *T. thermophila* micronuclear chromosome.

Viscoelastic data obtained from organisms having DNA of $M_r \geq 10^{10}$ suffer from a relatively high degree of experimental uncertainty. Retardation times obtained from the same lysate can deviate from one another by 30–50%. Correction of τ values for cation concentration are also uncertain. Kavenoff and Zimm (10) considered 30–40% a reasonable estimate of the precision of their results with *Drosophila*. We feel that a similar degree of accuracy is present in our results with *Tetrahymena*.

L_1, which can be converted easily to the number of largest DNA molecules present per *Tetrahymena* cell, is subject to even greater uncertainty, as originally noted by Kavenoff and Zimm (10). Calculations for various lysates yielded values from 200 to 400 DNA molecules of $M_r = 2\text{–}3 \times 10^{10}$ per cell. As indicated by Eq. 5, L_1 is subject to the cumulative errors of several experimental parameters. Nevertheless, the general range of 200–400 molecules is in good agreement with the predicted value of 350 chromosome-sized molecules in logarithmically growing *Tetrahymena*, indicating that much of the macronuclear DNA is present as chromosome-sized molecules. However, L_1 only measures the number of largest molecules in a given lysate. Smaller molecules do not contribute appreciably to the viscoelastic response and could easily escape detection.

Table 1. Calculation of DNA molecular weight from τ^0

Method	M_r
Kavenoff & Zimm (10)	$3.14 \pm 0.75 \times 10^{10}$
Lauer & Klotz (12)	$2.03 \pm 0.46 \times 10^{10}$
Ross & Scruggs, as cited by Lauer & Klotz (12)	$2.18 \pm 0.50 \times 10^{10}$

Controls. A high shear control to break high M_r DNA molecules was performed on an individual lysate after removal of the Cartesian diver by drawing the chamber contents rapidly up into a pasteur pipet three times (10, 12, 17). Upon replacement of the rotor, no recoil was observable, indicating that shear stress destroyed the viscoelastic species. In other experiments, in which the rotor was removed carefully and replaced but the solution was left intact, no loss of recoil was noted.

A DNase control, using the method of Kavenoff and Zimm (10), was attempted, but no enzymatic degradation of the chamber contents could be induced. Neither the DNase solution prepared according to Kavenoff and Zimm, nor our own preparation, added to the chamber at 37°, incubated overnight, warmed to 50°, and tested for recoil, caused any DNA degradation. Instead, a dense, cloudy precipitate was generated when DNase solutions were pipetted into the chamber. Presumably, the DNase was being denatured and precipitated by the high detergent concentration in the lysate. Similar results were obtained by Lauer and Klotz (12) when attempting a DNase control on yeast DNA. It was necessary to use a $NaDecSO_4$ concentration higher than that used by Kavenoff and Zimm in order to lyse *Tetrahymena* cells.

DISCUSSION

In spite of the fact that macronuclear DNA never condenses to form visible chromosomes, our results indicate that the genetic material in the macronucleus of *T. pyriformis* and *T. thermophila* consists primarily, if not entirely, of DNA molecules having the same size as that calculated for the chromosomes of *T. thermophila* micronuclei. By our analytical procedures we cannot detect molecules corresponding in size to an entire haploid equivalent of DNA (superchromosome), as has been suggested for macronuclei of *Tetrahymena* (5, 8, 16–18) and *Paramecium* (18). Moreover, the molecular weight of the macronuclear DNA of the two species of *Tetrahymena* does not change appreciably when Pronase is deleted from the lysis mixture, suggesting that protein linkers do not connect the chromosome-sized DNA duplexes into superchromosomes. If superchromosomes do exist, the DNA contained within them must be held together by bonds sensitive to disruption by high salt and detergent alone. One example of such a haploid superchromosomal-segregating unit not excluded by our data would be a collection of five chromosome-sized DNA molecules held together by a detergent-sensitive membrane attachment site.

In addition, our data indicate that micronuclear DNA is not of superchromosome length. If this were true, the micronucleate species, *T. thermophila*, would show a viscoelastic response for a DNA molecule of one haploid equivalent (1.25×10^{11} daltons) even in the presence of approximately 26 times more macronuclear DNA of chromosome length ($2–3 \times 10^{10}$ daltons). We observe no such viscoelastic species in *T. thermophila*, leading us to believe that the micronucleus contains DNA of chromosome length or smaller.

Since a preponderance of the nuclear material in both species of *Tetrahymena* can be accounted for by molecules in the size range of $2–3 \times 10^{10}$ daltons, smaller size molecules, such as gene-sized pieces, are probably not present in great abundance. However, we cannot rule out the possibility that a small amount of gene-sized DNA does exist in the micro- and/or macronucleus since it would not be detected in the presence of the larger, chromosome-sized DNA molecules by our technique. Furthermore, the possibility still exists that in fact the chromosome-sized DNA molecules are made up of smaller units joined together by linkers that are resistant to detergent, high salt concentrations, and Pronase.

The importance of establishing the presence or absence of gene-sized pieces of macronuclear DNA in *Tetrahymena* is related to the phenomenon of genetic dislinkage during macronuclear assortment (2). Markers that are observed to segregate together in micronuclear meiosis appear unlinked during random assortment of the macronuclear genetic material (28, 29). The results presented here do not support previous explanations of this phenomenon in terms of fragmented macronuclear chromosomes (30). If the macronucleus does in fact contain only chromosome-sized DNA molecules, then an alternative explanation must be sought for genetic dislinkage, such as the high rate of recombination postulated by Orias (31). Such a mechanism would presumably allow rapid relocation of genetic material, making all markers appear unlinked while preserving the structural integrity of the chromosome-sized DNA molecules. The results presented here make such an explanation increasingly attractive.

In summary, we find that macronuclei of logarithmically growing *T. pyriformis* or *T. thermophila* contain 200–400 DNA molecules of M_r $2–3 \times 10^{10}$, supporting the contention that the mature *Tetrahymena* G_1 macronucleus contains 45 haploid genomes, each genome composed of five entire chromosome-sized DNA molecules. Our results also lend support to the generalization that the chromosome-sized DNA molecule is the fundamental organizational unit in the eukaryotic nucleus. The macronucleus of *Tetrahymena* now stands with the nuclei of *Drosophila* and yeast in support of this concept.

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