Large Scale Synchronous Mating and the Study of Macronuclear Development in *Euplotes crassus*

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ABSTRACT After conjugation in hypotrichous ciliates, a new macronucleus is produced from a copy of the micronucleus. This transformation involves large-scale reorganization of DNA, with conversion of the chromosomal micronuclear genome into short, gene-sized DNA molecules in the macronucleus. To study directly the changes that occur during this process, we have developed techniques for synchronous mating of large populations of the hypotrichous ciliate *Euplotes crassus*. Electron microscope studies show that the micronuclear chromosomes are polytenized during the first 20 h of macronuclear development. The polytene chromosomes lack the band-interband organization observed in other hypotrichs and in the Diptera. Polytenization is followed by transectioning of the chromosomes. We isolated DNA at various times of macronuclear development and found that the average molecular weight of the DNA decreases at the time of chromosome transectioning. In addition, we have shown that a small size group of macronuclear DNA molecules (450–550 base pairs) is excised from the chromosomal DNA ~10 h later in macronuclear development.

An extreme reorganization of the genomic DNA occurs during the life cycle of hypotrichous ciliated protozoans. Hypotrichs, like other ciliates, have two types of nuclei—micronuclei and macronuclei. The DNA in the micronucleus is organized into typical eucaryotic chromosomes that segregate by mitosis during vegetative growth and undergo meiosis in cell mating. However, the micronucleus is unusual in that it has little or no RNA synthesis. In contrast, the macronucleus does not contain chromosomes, but instead the DNA is organized into small, gene-sized molecules that range in size from ~400 base pairs (bp)^1^ to ~15,000 bp. This nucleus divides amitotically and synthesizes all of the nuclear RNA needed for vegetative existence of the cell.

During conjugation, two cells pair, the micronucleus in each undergoes meiosis, a cytoplasmic bridge is formed, and haploid micronuclei are exchanged. The migratory micronucleus fuses with one of the stationary haploid micronuclei, and the cells separate. The unused haploid micronuclei and the old macronucleus then degenerate. The new diploid micronucleus divides by mitosis without cell division, and one of the daughter micronuclei develops into a new macronucleus. The change in DNA from chromosomal organization in the micronucleus to gene-sized molecules is a main event of macronuclear development. In addition, development of the macronucleus includes large scale elimination of sequences in the genome. Development of the macronucleus in *Oxytricha nova* is accompanied by elimination of ~95% of the genomic DNA sequences. A similar reduction occurs in *Stylonychia lemnae* (2). The amount of reduction has not been measured in *Euplotes crassus*. A third event in hypotrich macronuclear development is the addition of inverted terminal repeats, which apparently serve as telomeres on all macronuclear gene-sized DNA molecules after they are excised from the chromosomes (3).

This genomic reorganization occurs during a 3-d period after conjugation. It includes polytenization of the chromosomes (1), transection of the chromosomes into short segments by lamina-like material, and enclosure of the chromosome segments into vesicles (7, 10). The DNA content of the maturing macronucleus decreases during the vesicle stage. Macronuclear development ends with disappearance of the vesicle organization, multiple rounds of replication of the remaining DNA, and elongation of the macronucleus into its typical crescent shape.

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^1^ Abbreviation used in this paper: bp, base pair(s).
Macronuclear development has been difficult to study biochemically because of insufficient material. We present here a system for synchronous macronuclear development in large cultures of the hypotrich E. crassus and some initial results on DNA changes.

MATERIALS AND METHODS

Methods for Obtaining and Handling Mating Populations of E. crassus: E. crassus was first isolated from nature by Heckmann (4). The cells used here were a gift from Dr. P. Luporini, who obtained them on the coast of Chisimaio, Somalia.

Growth of Dunaliella salina (Food Source): Like all other hypotrichous ciliates, E. crassus requires live food. The free-swimming, flagellated algae D. salina was used as the food organism. The algae were grown in nonsterile artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH) supplemented with vitamin B12 (0.2 μg/ml), vitamin B1 (1 μg/ml), FeSO4 (10 μg/ml), and 1 ml/liter of a mixture of 0.5 M NaNO3, 0.12 M Na2EDTA, 0.5 M H2BO3, 0.15 M NaH2PO4, H2O, 2 mM MnCl2, 4 H2O, 15 μM ZnCl2, 8 nM CoCl2·6 H2O, 8 nM CoSO4·5 H2O, and 1 μM (NH4)6Mo7O24·4 H2O. This solution was made in 5-gal Pyrex glass carboys and stirred vigorously. Approximately 1 liter of dense culture (5 x 10^5 cells/ml) was added to the carboy as an inoculum, and the algae was grown at room temperature under constant lighting using fluorescent grow lamps (Growlux, Inc.) and aeration with the building air supply. The algae grew to a density of ~5 x 10^6 cells/ml within 5 days and could be used for ~2 wk.

Vegetative Cultivation of E. crassus: The generation time of E. crassus was ~10 h, although slight variability was seen between different cell lines. Cells were cultured only in glass containers; organisms became immobilized in plastic containers. When Euplotes were grown with 5 x 10^5 algae/ml, they reached a maximum density of 3 x 10^7 cells/ml. Euplotes stocks were grown in test tubes (10 ml) filled with algae culture. Euplotes could be starved for several weeks without detectable loss of viability. Stocks were renewed weekly by removing an aliquot with at least 100 cells from a tube and placing them into another tube containing a fresh alga culture. Stocks were inoculated into 5-gal Pyrex glass carboys and stirred vigorously. Approximately 1 liter of dense culture (5 x 10^6 cells/ml) was added to the carboy as an inoculum, and the algae was grown at room temperature under constant lighting using fluorescent grow lamps (Growlux, Inc.) and aeration with the building air supply. The algae grew to a density of ~5 x 10^9 cells/ml within 5 days and could be used for ~2 wk.

Establishment of Rejuvenated Mating Stocks: Recent exconjugants were found to be incompetent to mate as was observed by Heckmann (4). After 30-40 fissions, competence was acquired. Approximately 500 fissions after the establishment of competence, the cells began to senesce as noted by a decreased efficacy of mating, appearance of occasional intranuclear mating, and an increased generation time. To rejuvenate new mating stocks of Euplotes, complementary mating stocks were mixed. 2 d after conjugation, individual exconjugants were transferred to separate culture tubes. Exconjugants were distinguished from cells that did not pair by having matured macronuclei that appear as optical holes in the cells (Figure 1 C). Of 100 tubes started in this way, generally >80 clones were obtained. These clones were maintained with a constant food supply for 4 wk and then starved for 2 d. Approximately 100 cells from each tube were mixed in a glass petri dish (10 cm) and left undisturbed on an inverted microscope for 1.5 h. After this time, individual pairs of cells that were just beginning to fuse were removed from the dish and separated by repeated pipetting. Once separated, the two cells were transferred to separate tubes containing algae. Fifteen pairs were separated in this way and cultured separately. To test for mating efficiency, the latter clones were starved for 2 d, and several hundred cells of each of the clones were mixed pairwise in glass petri dishes and observed for frequency of mating pairs. The two clones that gave the highest number of mating pairs in the shortest time were used for experiments.

Cultivation of Cells for Mass Mating: Several modifications of the growth conditions were used to maximize the efficiency of mating of large cultures. Typically, a large mating involved 60 liters of algae culture. Four 5-gal carboys were inoculated with algae (1 liter) 6 d before the start of cultivation of the ciliates. The algae were then poured into 40 glass baking dishes (1.5 liter/dish), and the two mating stocks were cultured separately by adding ~3 x 10^6 Euplotes to each dish. After inoculation, the dishes were covered with plastic wrap to prevent evaporation. 2 d after the cells had exhausted their food supply, they were mixed. This was done by pouring two dishes of opposite mating cells into a 4-liter beaker, and then returning the cells to the same dishes. All of the dishes were mixed over a 15-min period.

Isolation of Maturing Macronuclei and Old Macronuclei: 40 liters of exconjugants were concentrated on 10-μm nitex (Tetko) and washed through cotton to separate them from algae debris. The cells were pelleted at 140 g for 1 min. They were removed from the dish and separated by a temperature lysis solution (0.5% Triton X-100, 10 mM Tris pH 8.0, and 0.5% spermidine hexahydrate) was added to the 2-ml pellet. The cells were resuspended and transferred to a glass Dounce homogenizer on ice and lysed. The cell lysate was layered onto a percoll step gradient. The gradient was constructed in a 30-ml test tube (Corex) with 3.5-ml steps of 30, 40, 45, 30, and 25% percoll in lysis buffer. The sample was made up to 20% percoll, layered onto the gradient, and spun in a HB-4 rotor in a Sorvall centrifuge (Beckman Instruments, Inc., Fullerton, CA) at 7,000 g for 5 min. The maturing macronuclei were collected from the 30, 35, and 40% steps, and the old macronuclei formed a pellet at the bottom of the tube. The developing macronuclei and old macronuclei are easily and unequivocally identifiable by light microscopy. Both types of nuclei were resuspended in 5% sucrose, 10 mM Tris pH 8.0, and pelleted by centrifugation at 200 g for 10 min. The procedure was used to separate old macronuclei from maturing macronuclei at all stages of development.

DNA Extractions: DNA from whole cells was isolated according to published procedures (9). DNA was isolated from nuclei by resuspending a pellet of nuclei in 6 vol of solution containing 0.5% SDS, 10 mM Tris pH 8.0, 100 mM EDTA, 250 mM NaCl, adding protease K to a final concentration of 500 μg/ml, and incubating the mixture at 37°C for 6 h. This solution was brought to 6 ml with saturated CaCl2 and ethidiurn bromide (final Rf = 1.4) and spun at 45 K rpm for 20 h in a Beckman L2 (Beckman Instruments, Inc.) with a type 65 rotor. The DNA was removed from the gradient, extracted with butanol, dialyzed against 10 mM Tris pH 8.0, 0.1 mM EDTA and ethanol precipitated.

FIGURE 1 Light micrographs of vegetative, mating, and exconjugant cells. Pictures were taken of cells (A) just before mixing of opposite mating types; (B) pairs of cells 2 h after mixing competent cells; and (C) 30 h after mixing of competent cells. The clear, circular area is the developing macronucleus. Bar, 100 μm.
Preparation of Exconjugant Cells for Electron Microscopy: Exconjugants were collected into pellets by centrifugation and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 30 min at room temperature. They were washed with 0.1 M cacodylate buffer and then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 min and finally washed with cacodylate buffer. The exconjugants were dehydrated with ethanol, transferred to 100% propylene oxide, and embedded in an Epon-Araldite mixture. Cells were identified in thin Epon-Araldite wafers with the aid of a light microscope and were remounted for sectioning. Sections were stained with lead and uranyl acetate and examined with a JEM 100c electron microscope.

Agarose Gel Electrophoresis, Southern Transfer, Hybridization, and Nick Translation: DNA samples were separated on 1% agarose gels at 100 V (9) and transferred to nitrocellulose for hybridizations (12). DNA probes were labeled by nick translation (11). Hybridization and washing conditions were those of Boswell et al. (3).

RESULTS

Electron Microscopy of Developing Macronuclei

The fine structure of macronuclear development in E. crassus was compared with other previously studied hypotrichs (7, 10). In brief, polytene chromosomes were observed between 20 and 40 h after cell mixing. (Macronuclear development begins ~20 h after cell mixing [Fig. 2]). Polytenization was accompanied by a large increase in DNA content (data not shown). Unexpectedly, the polytene chromosomes of E. crassus did not show the band–interband structure typical of polytene chromosomes in other hypotrich species (7, 10), but have the appearance of thick, homogeneously staining cords (Fig. 3A). Band–interband organization is easily observed in the polytene chromosome of E. eurystomus, Stylonychia lemnae, Stylonychia pustulata, Oxytricha nova, O. fallax, and

![Figure 3A](image)

![Figure 3B](image)

FIGURE 3. Electron micrographs of maturing macronuclei. Exconjugant cells undergoing macronuclear development were fixed at (A) 38 h after cell mixing during the polytene stage, and (B) 65 h during the vesicle stage, and prepared for electron microscopy as described in Materials and Methods. × 20,000.
Holosticha sp. by phase microscopy of fixed cells stained with aceto-orcein, and by electron microscopy of sectioned cells fixed with osmium and/or glutaraldehyde (1, 7, 10; unpublished results). Band–interband organization could not be detected in the polytene chromosomes of E. crassus with any of these microscope techniques. About 40 h after cell mixing, the polytene chromosomes were transected by a lamina-like material that first appeared as a coating on the surface of the chromosome (Fig. 3A) and after transection, formed vesicles around short chromosome segments (Fig. 3B). The vesicle disappeared ~74 h after cell mixing. There were no further notable changes in fine structure until the macronucleus acquired the typical organization of the vegetative macronucleus by ~95 h after mixing.

Synchrony of Mating

To study directly the changes in DNA that occur in exconjugants, large numbers of synchronously developing macronuclei are needed. These were obtained by using Euplotes mating types that mate with a high degree of synchrony in mass cultures. The degree of synchrony was measured by counting the number of paired and exconjugant cells at various times after mixing of opposite mating types (Figs. 1 and 2). Pairing (Fig. 1B) occurred ~2 h after complementary mating stocks were mixed. Approximately 15 h after mixing of opposite mating types, the paired cells separated. The synchrony of cells entering the process of macronuclear development was determined by counting the number of cells with maturing macronuclei (Fig. 1C). Maturing macronuclei appear as transparent round structures near the center of the exconjugant cells at 35 h postmixing of opposite mating types and gradually increase in size to ~20-μm diam by 50 h. They remain until the end of development (95 h postmixing). At this time, the maturing macronucleus elongates and assumes a crescent shape on the left side of the organism typical of a vegetative macronucleus. These observations show that macronuclear development in mass cultures is sufficiently synchronous for analysis of DNA changes.

Fidelity of Excision of Macronuclear Genes

When mature macronuclear DNA is distributed by size on an electrophoretic gel, a pattern is obtained that is typical for a given species of hypotrich. For example, E. aediculatus, O. nova, and S. pustulata can be readily distinguished from one another by their DNA patterns (13). The pattern is characterized by the size distribution of all of the DNA and by the presence of bands representing differentially amplified molecules of a given length. For example, a band ~7,400 bp represents the differentially amplified rDNA (14).

The constancy of the DNA pattern in a species is evidence that excision of genes from chromosomes and DNA processing overall always occur in the same way during macronuclear development, i.e., a given gene always occurs in the same sized DNA molecule in the mature macronucleus.

We carried this kind of analysis one step further in E. crassus by comparing the DNA patterns of two complementary mating stocks and of new exconjugant cells derived from mating of these stocks. The patterns, illustrated in Fig. 4, were always the same.

A more precise measure of the fidelity of processing is provided by identification of the location of particular genes within the DNA pattern by Southern blot hybridization. To do this, total macronuclear DNA was cloned into the Eco R1 site of pBR325 by the procedure described by Klobutcher et al. (6). A 730-bp full length, cloned macronuclear molecule (designated as pEc4) taken from the macronuclear library showed that the sequence in this molecule is present in the same sized DNA molecule (730 bp) and in approximately the same copy number in DNA from both parental stocks and in a mixture of new exconjugant cell DNAs and in three exconjugant clones (Fig. 5). Seven additional exconjugant clones gave the same result. In all 13 cases, the pEc4 molecule hybridized to only one size class of molecules, which was 730-bp long. These experiments were repeated using two other full length cloned macronuclear molecules, pEc13 (650 bp) and pEc31 (600 bp). For all DNA samples, pEc13 hybridized only to 650-bp molecules and pEc31 only to 600-bp molecules.

These results add further evidence that DNA processing is always the same, within the limits of the resolving power of Southern blot hybridizations.

Degradation of Old Macronuclei

During macronuclear development, the old macronucleus
FIGURE 5 Hybridization of pEc4 to macronuclear DNA. DNAs isolated from mating stocks (mt5 and mt5'), 90 h exconjugants, and three exconjugant clones (F1 stocks) were electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and hybridized with 32p-labeled pEc4. The size of the molecules to which pEc4 hybridized (730 bp) is indicated.

is destroyed. Old macronuclei in exconjugant cells stained with acetocarmine appear pynotic but are retained until the end of development of the new macronucleus. DNA isolated from old macronuclei at various times during development was electrophoresed in a 1% agarose gel and stained with ethidium bromide. The distribution and relative intensity of the different size classes of old macronuclear DNA are indistinguishable from vegetative macronuclear DNA, indicating that the old macronuclear DNA remains intact at least until very near the end of macronuclear development (74 h in Fig. 2). About the time that the new macronucleus achieves its crescent shape, the old macronucleus is no longer visible in acetocarmine-stained cells.

DNA Isolated from Maturing Macronuclei

DNA isolated from maturing macronuclei at various times after mating showed the pattern of decrease in the average molecular weight of the DNA from chromosomal size (micronuclear) to gene-size (macronuclear) DNA. Electrophoretic gels of this DNA and DNA from vegetative cells in a 1% agarose gel are shown in Fig. 6. Macronuclear DNA, which is randomly broken during isolation, migrates predominantly at about the same position as does the largest fragment of Hind III-digested λ DNA (lane mt5). Macronuclear DNA size ranges from ~15 kbp to ~400 bp. Polytene DNA (39 h), which has probably been sheared during preparation, remains near the top of the gel in about the same position as micronuclear DNA, showing that little or no cleavage of chromosomal DNA has occurred by this time. Subsequently, about the time that the lamina-like material transects the polytene chromosomes (47 h after cell mixing), the average molecular weight of the DNA has decreased. At 56 and 65 h, the distribution of DNA sizes is similar to that in mature macronuclear DNA, although the banding pattern typical of vegetative macronuclear DNA is not yet present. The banding pattern, as illustrated by mt5 DNA in Fig. 6, appears sometime between 65 h and the completion of macronuclear development at 74 h.

Timing of Excision of Genes

The timing of excision of particular genes from the polytene chromosomes was determined as follows. Macronuclear DNA was separated in a 1.4% agarose gel, the DNA molecules in the 450–550-bp range were removed from the agarose, nick translated, and used as a heterogeneous probe for Southern hybridizations to DNAs isolated from various stages of development (Fig. 7). This allowed us to follow the excision of multiple macronuclear genes simultaneously. This probe hybridized to one broad sized class of molecules (450–550 bp) in macronuclear DNA isolated from the mating stocks (e.g., lane mt5 in Fig. 7) and from other vegetative cells (data not shown). Macronuclear DNA of 450–550 bp hybridized only to high molecular weight DNA prepared from macronuclei before 50 h after cell mixing, indicating that the molecules in the 450–550 bp range had not yet been excised from chromosomal DNA. The small amount of hybridization in the 450–550 size range at 47, 50, and 70 h is due to the presence of a small amount of contaminating DNA from old macronuclei. At 50 and 70 h (vesicle stages), the composite probe

FIGURE 6 DNAs were isolated from maturing macronuclei at different stages of development, electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light. The number above each lane indicates hours after mixing of mating types. Lane mt5 contains DNA from one of the two mating types used to generate the exconjugant cells that were used to obtain the maturing macronuclear DNA.
intermediate forms that are subsequently resolved to mature genes (550-650 bp), suggesting the presence of slightly larger molecules than the final mature genes (550-650 bp vs 450-550 bp), indicating that excision is precise within the limits of resolution of Southern blot hybridization.

We present initial evidence that genes excised from chromosomal DNA appear first in somewhat larger molecular weight form than that found in the mature macronucleus. Genes represented by molecules in the 450-550-bp range in the mature macronucleus first appear in molecules ranging from ~550-650 bp in the vesicle stage of macronuclear development. A report of a more extended analysis of such intermediates will be reported elsewhere (11a).

Polytene chromosomes have been observed during macronuclear development in the dozen or so hypotrich species examined thus far (e.g., references 1, 7, and 8). In all previous cases, the polynete chromosomes were found to have a distinct band–interband organization, similar to that seen in polynete chromosomes of Dipterans. Because the transsectioning of the polynete chromosomes in species of *Stylonychia* and *Oxytricha* and in *Euplotes eurystomus* occurs by the development of lamina through all interbands, it has been inferred that the band–interband organization is important in guiding the cutting up of the chromosomes. This idea is contradicted in *E. crassus*, in which the usual band–interband organization is not present in the polynete chromosomes. Obviously, whatever guides transsection of polynete chromosomes does not require the visible organization of the DNA into bands and interbands.

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