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Replication Forms of the Gene-Sized DNA Molecules of Hypotrichous Ciliates

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Using a method for obtaining DNA from 10 to 40 macronuclei for electron microscopy, we analyzed the structure of gene-sized, linear DNA molecules from S-phase macronuclei of two hypotrichous ciliates, *Euplotes eurystomus* and *Styx* sp. Three types of putative replicating intermediates were observed: (i) molecules with a bubble close to one end, (ii) molecules with single forks, and (iii) molecules with two forks. We conclude that: (i) each macronuclear DNA molecule replicates as an independent unit, (ii) the molecules contain an origin of replication close to one or both ends, and (iii) the mode of replication is bidirectional.

Hypotrichous ciliates (e.g., *Euplotes*, *Styx*, *Oxytricha*, and *Stylonychia* spp.), like other ciliated protozoans, contain two types of nuclei, micronuclei and macronuclei. The micronucleus is diploid, divides by mitosis, and makes little or no RNA during vegetative growth. It serves as the germ-line nucleus, undergoing meiosis and exchange during conjugation, and giving rise to a new macronucleus after conjugation. The micronucleus provides no indispensable role in vegetative cells since amicronucleated strains isolated from nature grow and divide vigorously. Micronuclear DNA has a high molecular weight and is organized into chromosomes, as in other eucaryotes.

The macronucleus divides by amitosis and functions as the somatic nucleus. It contains only gene-sized DNA molecules with a number-average size of 2,200 kilobase pairs and which, in aggregate, represent a few percent of the total sequence complexity of micronuclear DNA. The macronucleus of *Oxytricha nova* contains ~24,000 different DNA molecules, which are present in ~1,000 copies each. A review of the properties of macronuclear and micronuclear DNAs and steps in macronuclear development after conjugation has been published previously (12).

The mode of DNA replication in hypotrich macronuclei is unique. DNA systhesis occurs in a cytologically distant structure called a replication band (3, 14; M. T. Swanton and D. M. Prescott, J. Protozool., in press). Replication bands originate at one or two specific points on the macronuclear envelope and traverse the macronucleus, replicating all DNA molecules as they are encountered. In *Euplotes* spp. a band originates at each end of the rod-shaped macronucleus, and these travel toward one another. In *Styx*, *Oxytricha*, *Stylonychia*, *Holosticha*, and other genera with multiple macronuclei, a single band forms at one end of each ovoid macronucleus. The replication band mode of DNA synthesis is probably related to the fact that the macronucleus contains only gene-sized DNA molecules.

In this paper we present a method for preparing DNA from a small number of hand-isolated macronuclei and describe replicating forms of the gene-sized molecules in *Euplotes eurystomus* and *Styx* sp.

MATERIALS AND METHODS

E. eurystomus and *Styx* sp. were grown under nonsterile culture conditions with the alga *Chlorogonium* as the food. To isolate the macronuclei, the cells were lysed in a solution containing 1% Triton and 0.05% (wt/vol) spermidine phosphate (15). Macronuclei with replication bands were picked up with a micropipette under a dissecting microscope. We have usually used groups of 20 or 40 nuclei to prepare DNA, but the method works with as few as 10 nuclei. The nuclei were washed with distilled water three times to remove the lysing solution and cytoplasmic remnants. The nuclei were transferred in a small volume (~1 μl) of distilled water by a pipette to a 5-μl drop of pH 9 distilled water (pH adjusted with NaOH immediately before use) on a siliconized microscope slide. The lysis of the nuclei was monitored under the microscope.

The lysate (~6 μl) was picked up in the pipette and transferred to a plastic vial containing 15 μl of proteinase K (stock solution, 2 mg/ml; Beckman Instruments, Inc.) and kept at 21°C for 30 min. At the end of this period, 20 μl of formamide (Matheson, Coleman and Bell), 2.5 μl of a buffer containing 4 M Tris–0.4 M EDTA (pH 8.0), and 2.5 μl of cytochrome *c* stock

solution (5 mg/ml; Calbiochem) were added to the vial. The solution was mixed with a vortex mixer, and 10-μl portions of this solution were spread on the surface of distilled water as described by Kleinschmidt (7). When <20 nuclei were used, the spreading was done on drops of distilled water rather than in a trough (4). The protein monolayer was adsorbed to Parlodion-coated, 200-mesh copper grids, stained with ethanolic uranyl acetate, and rotary-shadowed with platinum-palladium alloy (80:20) (Ted Pella Co.). The grids were viewed in a Philips 301 electron microscope operated at 80 kV. Micrographs were taken at magnifications of 34,000 and 45,000. Magnifications in the microscope were calibrated with a carbon grating replica (2,160 lines per mm; E. Fullam Co.). The lengths of molecules in two- to threefold enlarged prints were measured with a Numonics graphic digitizer.

RESULTS

Method of DNA preparation for electron microscopy. The procedure described here to obtain DNA from macronuclei has two major advantages. First, the method requires few nuclei, and these can be readily obtained by manual isolation techniques. This is especially advantageous in cases in which mass isolation of nuclei is difficult or large numbers of nuclei of a particular stage of cell cycle or development cannot be obtained. Second, the method involves minimal manipulations compared with routine nucleic acid extractions and is extremely rapid. The entire preparation, from cells to DNA on the grids, can be accomplished in less than 2 h. Although the method was devised for macronuclei of hypotrichous ciliates, it may also be useful for analysis of the DNA of other large eucaryotic nuclei.

Electron microscopy of replicating macronuclear DNA. The vast majority of macronuclear DNA is in the form of short, linear molecules, as described before (13). However, a careful search yielded a few molecules that are interpreted to be replicating structures. The frequency of these molecules is quite low (0.01 to 0.1% in *E. eurystomus*, depending on the preparation), but their distinctive structure enabled us to identify and photograph them readily. Among 51 such molecules from *E. eurystomus*, 41 had a fork at one end, 6 had forks at both ends, and 4 had a bubble close to one end. Single examples of these three types of molecules are shown in Fig. 1. Figures 2 and 3 illustrate additional molecules with one or two forks.

The frequency of replicating forms was higher in DNA from *Styx* sp. than in that from *E. eurystomus*, ranging from 0.1 to 0.5% depending on the preparation. This higher frequency probably results because macronuclei in *Styx* sp. are ovoid, with the consequence that the single replication band occupies a larger proportion of the macronuclear volume than do the two replication bands combined in the highly elongated

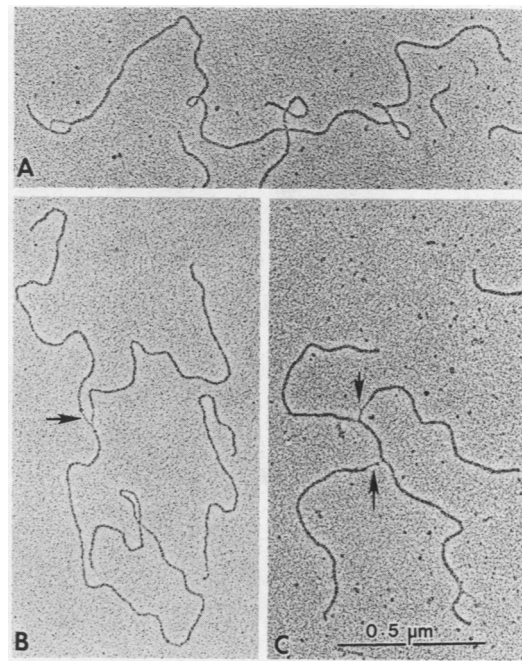

FIG. 1. Three types of replication forms in macronuclear DNA of *E. eurystomus*. (A) A molecule with a bubble near one end, (B) a molecule with a single fork, and (C) a molecule with two forks.

macronucleus of *E. eurystomus*. Among 60 replicating molecular forms from *Styx* sp., 57 had single forks, 1 had two forks, and 2 had a bubble at one end (Fig. 4).

In eucaryotes and procaryotes in general, the initial stage of replication is characterized by the formation of bubbles in DNA molecules of the type shown in Fig. 1 and 4. Replication then evidently proceeds bidirectionally (9). The low frequency of molecules with bubbles in our preparations would be expected if replication in a bubble is bidirectional, since one replication fork would soon run off the end of a molecule, creating a fork. In macronuclear DNA, the few bubbles we saw always occurred 200 to 300 base pairs from the end of a molecule, suggesting that origins of replication are located near ends, as in the DNA of bacteriophage T7 (2). Based on the nucleotide sequence of a macronuclear gene for actin, Kaine and Spear (6) suggested that a replication origin is ~150 base pairs from one end.

Molecules with single forks formed the most abundant class. Of the three arms present in such forked molecules, two were always of the same length (Table 1) and presumably represent the replicated daughter portions of a molecule. The third arm is believed to be the unreplicated parental DNA. In a few molecules, one of the putative daughter branches contained a short,

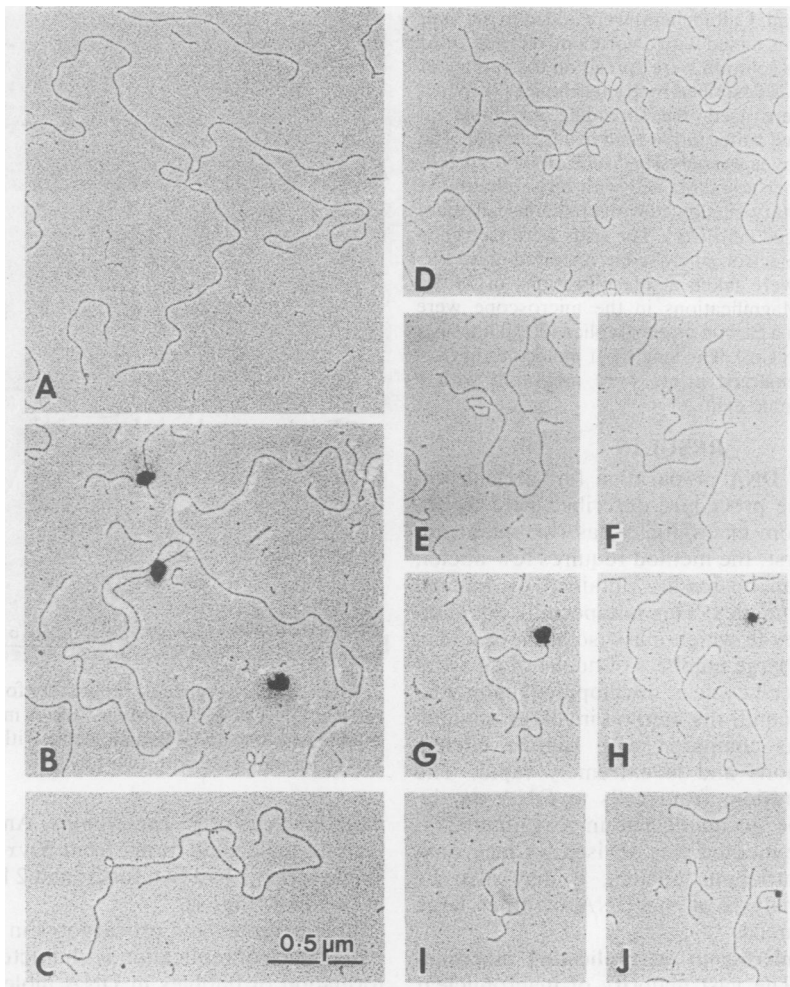

FIG. 2. Replication forms of macronuclear DNA of *E. eurystomus* with single forks. The three arms of these 10 molecules were measured; the measurements are recorded in Table 1. The "whisker" at the replication fork in (A) may be due to displacement of the end of a new daughter strand by the rejoining of the two parental strands.

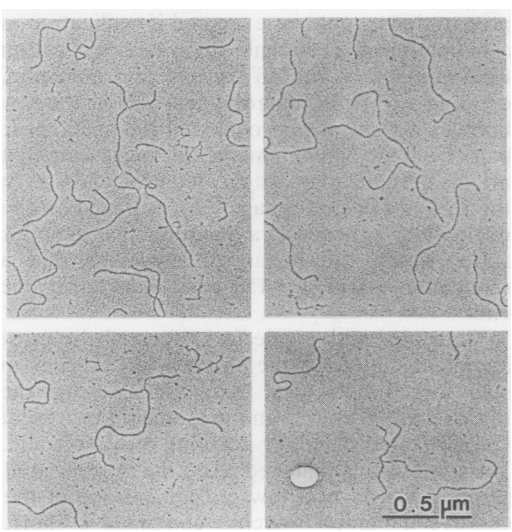

FIG. 3. Two-forked replication forms of macronuclear DNA from *E. eurystomus*. In some molecules, single-stranded regions are present at replication forks.

identifiable region of single-stranded DNA (Fig. 1C). Such single-stranded regions have been observed before (5, 10) in replicating DNA and are believed to reflect discontinuous replication on a lagging strand.

Some molecules have two forks apparently proceeding toward each other from the two ends of the molecule (Fig. 1, 2, 3, and 4). We think that these molecules have originated replication near both ends, probably by bubbles that have expanded and run off the ends. In these two-forked molecules the lengths of the arms belonging to one fork are the same, but the lengths of the arms of the two forks are different; this may mean that the two replication forks have trav-

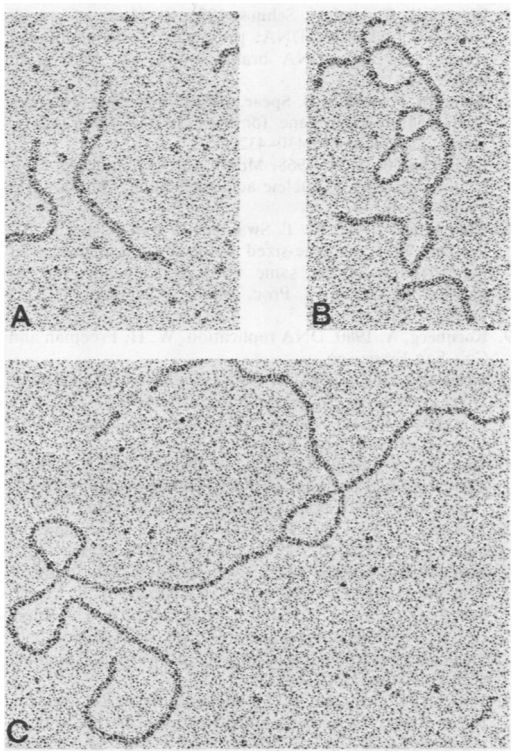

FIG. 4. Replication forms of macronuclear DNA from *Styx* sp. (A) A molecule with a terminal bubble, (B) a molecule with two forks, and (C) a molecule with a single fork.

elled at different rates or that initiation occurred at one end before it occurred at the other.

It is possible that some molecules have a single replication origin and others have an origin near each end. Alternatively, all molecules may have an origin near each end, but one end enters the replication band well before the other so that most molecules appear with a single fork. Those with two forks may represent molecules in which both ends have entered the replication band, but asynchronously.

TABLE 1. Lengths of the three arms in single-forked molecules from the macronuclei of *E. eurystomus* shown in Fig. 2

Molecule	Length (μm)		
	X	Y	Z
A	1.63	1.61	1.49
B	3.00	3.07	1.52
C	1.49	1.48	0.88
D	1.10	1.09	1.22
E	0.38	0.37	1.21
F	0.62	0.63	0.59
G	0.13	0.14	0.58
H	0.27	0.27	0.29
I	0.35	0.36	0.18
J	0.13	0.15	0.23

The terminal location of origins of replication in hypotrichs contrasts with the nearly central origin(s) reported for the extrachromosomal rDNA of *Tetrahymena thermophila* (1, 16). However, the rDNA of *T. thermophila* is a palindrome with two coding regions, and the molecules in hypotrichs contain only single coding sequences.

Our interpretation of the observations is represented by the diagram in Fig. 5. A replication origin may be present near each end of the gene-sized molecules internal to the inverted terminal repeats (ITRs), or telomeres, that terminate all molecules (8, 11). The ITRs terminate in a 16-base single-stranded region, forming a 5′ gap (8). Neither the ITRs nor 16-base 5′ gaps are thought to be involved in initiation, partly because the

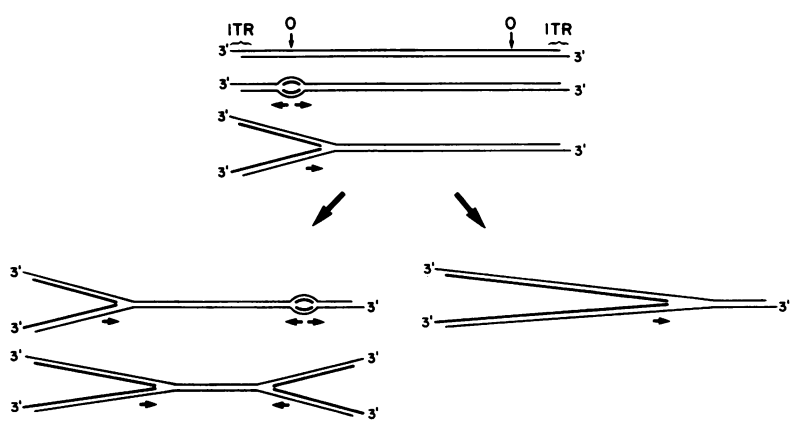

FIG. 5. Suggested replication patterns for macronuclear DNA molecules of hypotrichs. O, Origin of replication; ITR, inverted terminal repeat sequence or telomere. Some molecules may replicate starting from a single origin (right); others may replicate with asynchronous initiations at two origins (left).

presence of bubbles suggests that origins are some distance from ends. In *E. eurystomus* all molecules have in common a 5-base-pair sequence located 17 bases in from the inner end of the ITRs on both ends. This 5-base-pair sequence is too close to the ends of the molecules (53 bases from 3′ ends) to fit with our estimate from electron microscopy that origins are 200 to 300 base pairs from ends. The ITRs must replicate as a replication fork passes off the end of the molecule. How the single-stranded tails replicate remains unknown. Current work on sequencing a number of gene-size molecules in hypotrichs may give information about the natural precise location of replication origins.

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