The Vegetative Micronucleus has a Critical Role in Maintenance of Cortical Structure in *Tetrahymena thermophila*

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**ABSTRACT.** The vegetative role of the germinal micronucleus of *Tetrahymena thermophila* was studied. Amicronucleate cells that had spontaneously arisen in star strains (very aged clones with defective micronuclei) were observed in B* as well as in A* and in C* and were found to lack the oral apparatus and to have disordered ciliary rows. To reduce uncertainty given the very small micronucleus and possible effects of aging factors, we induced amicronucleate cells in a young clone by treatment with the antitubulin drug, nocodazole, and observed their cortical structure and nuclei. Amicronucleate cells gradually lost their oral apparatus and then their ciliary rows became disordered, even without cell division, and they became crinkled cells. It can be concluded that the vegetative micronucleus appears to play a critical role in the maintenance of the cortical structure, especially of the oral apparatus.

*Tetrahymena thermophila*, a ciliated protozoan, has two nuclei which differ in structure and function: the diploid (2N=10) (19) micronucleus, which is germinal, and the polygenomic macronucleus which is somatic, being transcriptionally active and responsible for virtually all phenotypes of the cell. During conjugation, the micronucleus becomes transcriptionally active in meiotic prophase I (20). After meiosis, gametic nuclei are formed, and after nuclear exchange and fertilization, the new micro- and macronucleus are formed from products of the fertilized nucleus (15, 19). A complex series of molecular events occurs during the development of the new macronucleus; polyploidization of the micronuclear genome to the 45 C level, amplification of rDNA, DNA sequence rearrangement, and elimination of 10-20% of the micronuclear DNA sequences (for a review see Ref 8). The sequences eliminated from the micronuclear genome are collectively referred to as mic-specific DNA. Genic activity of the micronucleus in vegetative growth phase has not been detected by autoradiography (9) nor by a more sensitive approach using heterokaryons in which the macronucleus and micronucleus have different alleles at specific loci. The phenotype of these heterokaryons in which the macronucleus and micronucleus have different alleles at specific loci. The phenotype of these heterokaryons cells always depends on the genotype of the macronucleus and not of the micronucleus (1, 2, 5). There is a possibility that the inertness of the micronucleus might be due to the difference of DNA content between the micronucleus and the macronucleus. To eliminate the possible gene dosage effect, Mayo and Orias (13) constructed a heterokaryon that has a galactokinase gene (galA+) in the micronucleus and a galAl mutant gene in the macronucleus. They reported that assay for the galactokinase activity of soluble cell extracts prepared from the heterokaryon showed no activity attributable to the micronuclear gene. In vegetative growth, the micronucleus is generally thought to be inert and has no role except its own reproduction. In several species of ciliates, however, a cell line which has no micronucleus (amicronucleate) showed some abnormalities (for a review see Ref 18). In *Paramecium*, Mikami (14) reported that an amicronucleate cell line induced by enucleation had a small oral apparatus (OA), resulting in slow growth. Organization of the OA in amicronucleate cell lines is a major focus of study in several ciliates (for a review see Ref 18). In *T. thermophila*, the vegetative micronucleus is not usually dispensable because an amicronucleate cell line has almost never been established despite repeated attempts. An exception is the mutant amicronucleate cell line BI3840 isolated by Kaney and Speare (10). The BI3840 cell contains some mic-specific DNA sequences in the macronucleus (12). Whether the sequences are responsible for the vegetative micronuclear function is not known.

What is the essential role of the micronucleus for vegetative growth in *T. thermophila*? Very aged clones,
star strains, often have small and genetically defective micronuclei which have abnormal chromosomal constitution. This means that the intact micronucleus is not always necessary. In cell populations of star strains, C* or A*, abnormal shaped cells, so-called crinkled cells spontaneously appear. These cannot give rise to viable clones when isolated. The crinkled cell has been shown to be amicronucleate (16) and lacks the OA (3), suggesting a critical morphogenetic role of the micronucleus.

Since it is rather difficult to determine the presence or absence of the micronucleus of the star strains because of its small size, in the present study we induced amicronucleate cells from young cells with diploid micronuclei. They were easily induced by treatment with an antitubulin drug, nocodazole. In the amicronucleate cell its OA is lost and then even its ciliary rows are disintegrated. Loss of the OA occurred without cell division. These results suggest that a critical vegetative function of the micronucleus is to maintain the cortical structure, especially the OA.

MATERIALS AND METHODS

Strains and growth conditions. The strain B21922 used in this study is derived from the inbred strain B1868 of T. thermophila. Fertility of B21922 is more than 80%. Star strains A*/III, B*/VI and C*/III are very aged clones with defective micronuclei and are genetically sterile. The star strains were kindly provided by P. Bruns (Cornell University). Cells were grown axenically in PYG medium (2% proteose peptone (Difco), 1% yeast extract (Difco), 1% glucose) at 26°C.

Induction of the amicronucleate cell. To induce amicronucleate cells, the antitubulin drug nocodazole (methyl 5-2-thionylcarbonyl, 1-benzimidazole-2-yl carbamate; Janssen), was added to a dividing cell population (J. Gaertig, pers. comm.). Nocodazole was stored as a 2 mg/ml stock solution in dimethylsulfoxide at 4°C. To obtain a semi-synchronously dividing cell population, late-log phase cells were pelleted by hand centrifugation. An equal volume of fresh PYG medium was added to the cell pellet. This cell suspension was made as shallow as possible and kept overnight at 15°C in a plastic petri dish, and then diluted to about 1/100 by fresh PYG medium containing nocodazole (5 μg/ml) and kept at 26°C. Dividing cells began to appear about 150 minutes after the dilution. The division index at peak was 10–20%. Around 200 minutes from beginning of the treatment, the cells were washed with NKC solution (0.2% NaCl, 0.008% KCl, 0.012% CaCl₂) to remove the drug and were resuspended in NKC solution, or isolated into glucose-rich medium (0.25% proteose peptone, 0.25% yeast extract and 5% glucose).

Staining of cortical structure and nucleus. Cells were fixed with 3% formalin for 30 minutes and transferred to methanol acetic acid fixative (3 : 1). The cells were spread on a glass slide, air-dried, treated with 5 N HCl for 30 minutes and stained with 3% Giemsa (Merk) in 0.02 M Na₂HPO₄. This simple alkaline Giemsa method stains both the cortical structure and the nucleus. To determine the presence or absence of the micronucleus, 2% orcein in 45% acetic acid was used, as this staining showed the micronucleus, even when it overlapped the macronucleus.

RESULTS

Three types of abnormal cells in star strains. We observed the cortical structure of B*/VI, which has not been studied so far, during log phase. Percentages of morphologically abnormal cells varied greatly from culture to culture (0.1–20%). Although the crinkled cells, having disordered ciliary rows and no OA, were observed in them (see Fig. 2F), we found two other types of abnormal cells. Cells of both types were normal in shape but lacked OAs. One type had normal ciliary rows (see Fig. 2D) but the other had disordered ciliary rows (see Fig. 2E). Similar results were obtained on observation of A*/III and C*/III (data not shown). The morphologically abnormal cells seemed to be amicronucleate. But it was difficult to determine unambiguously whether all of these abnormal cells lacked the micronucleus, because of the very small size of the micronucleus of the star strains.

Amicronucleate cells obtained by nocodazole treatment of a young strain. To eliminate error in judging amicronucleates, we induced amicronucleates in the young strain B21922 which has a clearly visible diploid...
micronucleus. For the induction of amicronucleates, nocodazole was used. Nocodazole inhibits nuclear division at a concentration in which cytokinesis is not blocked (J. Gaertig, pers. commun.). Two hundred minutes after treatment with nocodazole, B21922 cells were suspended in NKC solution. Samples were obtained at 2 hour intervals until 20 hours after the end of the nocodazole treatment. With regard to the nuclei, four cell types were observed: 1) amicronucleate cells, 2) cells having a micronucleus but lacking a macronucleus (amacronucleate cells), 3) cells having no nuclei, called empty, and 4) normal cells. Immediately after transfer to NKC solution, at time 0, almost all cells were of normal shape, having apparent ciliary rows as well as OA. Percentages

Fig. 2. The process of the change of cortical structure in the amicronucleate cell induced in young cells. Cortical structures and nuclei were stained with Giemsa. A. Normal cell with micronucleus (arrow). B-F. Nocodazole treated cells. B. Dividing cell after nocodazole treatment. The anterior part of the dividing cell has a micronucleus (arrow), while the posterior cell has no micronucleus. In this stage, the OA of the amicronucleate cell has cilia (triangle). C. An amicronucleate cell. Cilia cannot be observed on the OA. D. An amicronucleate cell lacking its OA. E. An amicronucleate cell lacking both its OA and ciliary rows. F. Crinkled cells with bigger macronucleus. Bar = 10 μm.
Fig. 3. Various disintegrated OA in the amicronucleate cells. The disintegration of OA is shown from B to G. A. OA in a normal cell (cell has a micronucleus and a macronucleus). B–G. amicronucleate cells. Three membranelle (M1, M2 and M3) and one undulating membrane (UM) are observed. B. No cilia on the OA are observed. The arrow shows a bend in UM. C. Two interruptions in UM (arrows). D. Disintegration of membranelles of the whole area of the OA. E. OA with indistinct structure. F. OA is vestigial. G. Almost the entire structure of the OA is absent. Bar=10 μm.
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of the amicronucleates, amacronucleates, empty and normal cells were 4\%, 2\%, 1\% and 93\%, respectively. These percentages did not change very much throughout the 20 hour period following the drug treatment, and only the case of the amicronucleate cells is shown in Fig. 1. While only 2\% of amicronucleate cells lacked OAs at the beginning, the percentage of the cells lacking OAs among them gradually increased with time. Twenty hours later most of amicronucleate cells had no OA. Amacronucleates and empty cells were slow to lose their OAs, and only 20\% of them had lost their OAs at 20 hours.

In the amicronucleate cell population, not only were their OAs lost but also abnormalities in cortical structure, like those in star strains were also observed. These were cells without OA but with normal cortical ciliary rows (Fig. 2D), cells without OA and with disordered ciliary rows (Fig. 2E), and crinkled cells which have bigger macronuclei (Fig. 2F). Moreover, cells with defective OAs to various degrees were observed. Deficiency of OA seemed to begin with the loss of cilia on the OA (see Fig. 3B-G). The cells having disordered ciliary rows were never observed before loss of OA. At 20 hours, about 80\% amicronucleate cells also had disordered ciliary rows while all amacronucleates and empty cells had normal ciliary rows.

Amicronucleate cells cannot maintain OAs. When a cell divides, the OA of the posterior part of the cell is newly formed. In the nocodazole treatment, division arrest or unequal distribution of the divided nuclei was observed in about 90\% of dividing cells, and these were not one-sided between the posterior and the anterior. Both parts of the dividing cell had OA, and one of the daughter cells became amicronucleate (see Fig. 2B). Since a cell can divide a few times in the nutrient free solution, NKC solution, two alternative explanations may be possible for the development of the amicronucleate without an OA; 1) when an amicronucleate cell induced by the nocodazole treatment divides, one or both daughter amicronucleate cells are unable to form new OA during subsequent cell divisions, 2) the amicronucleate cells are unable to maintain the OA and eventually lose it. To determine which is correct, dividing cells in nocodazole-containing PYG medium were isolated in a drop of glucose-rich medium without nocodazole to delay the next cell division. The dividing cells soon separate but the next cell division is delayed (> 24 hours) in this medium. Twenty-four hours after transfer to the glucose-rich medium, separated cells in the drop were stained with Giemsa. Ninety percent of the amicronucleate cells lost the OA without cell division but almost all cells with the micronucleus had a normal OA and only 1 of 70 had lost it (Table I). We thus concluded that the second interpretation is correct, i.e., that amicronucleate cells are unable to maintain OAs.

Defective OA in amicronucleate cells. What is the process of the disappearance of the OA in nocodazole-induced amicronucleate cells? The OA consists of three membranelles (M1, M2 and M3) and one undulating membrane (UM). Figure 3 shows the OA of a normal cell (Fig. 3A) and of various different amicronucleate cells (Fig. 3B-G). In amicronucleate cells, various degrees of abnormality of the OA were observed. There were no cilia on the OAs of amicronucleate cells (Fig. 3B-F). Before the membranelles were defective, the UM was bent or interrupted at some points (Fig. 3B and C). When the membranelles were defective, the disintegration of the UM was more extensive than the extent of damage to the membranelles (Fig. 3B-F). Among the three membranelles, M3 was the first to be defective (Fig. 3E and F). Some cells had only vestigial OAs (Fig. 3F and G).

**DISCUSSION**

Amicronucleate cells of star strains A*, B* and C* appeared to have common characteristics, i.e., they not only lacked OAs but also had some disordered ciliary rows. These characteristics of amicronucleates were not peculiar to aged star strains as they were also observed in nocodazole-induced young amicronucleate cells, suggesting that these characters are caused by the absence of the micronucleus and not by aging of the strains. Misbehavior and division arrest of the micronucleus are also observed in aged strains (16).

When nocodazole was used to induce amicronucleate cells, new OA formations apparently were not inhibited by incubation for about 3.5 hours in the drug and almost all cells possessed OAs immediately after the end of nocodazole treatment (see Fig. 1). This result is different from that of colchicine reported by Nelsen (17) in *Tetrahymena pyriformis* (an amicronucleate species). Colchicine blocks new OA formation resulting in the absence of functional OA only on the posterior daughter cell in spite of no observable effect on the mature OA of the anterior daughter cell (17). Blocks of OA formation are also obtained by using several drugs such as fluorooacetate, azide, p-fluorophenylalanine, puromycin, chlo-

**Table 1. Oral apparatus in four cell types in glucose-rich medium 24 hours after the end of nocodazole treatment.**

<table>
<thead>
<tr>
<th>Cell types(a)</th>
<th>Number of cells observed</th>
<th>Cells with</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>no OA</td>
</tr>
<tr>
<td>Amicronucleate</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td>Amacronucleate</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Empty</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>70</td>
<td>1</td>
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\(a\) See the text for details.
ramphenicol, 2,4-dinitro-phenol, anerobiosis, mercaptoethanol and cycloheximide (6, 7). In the present results, the amicronucleate cell was produced from both the anterior or the posterior part of a cell in a random fashion, and the sister cell with micronucleus did not lose the OA but grew normally when it was transferred to the culture medium (data not shown). This suggests that loss of pre-existing OA and disorder of ciliary rows observed in an amicronucleate do not result from the aftereffects of nocodazole but from the absence of the micronucleus.

Effects of encucleation of a micronucleus by microsurgery have been reported in several ciliates as mentioned in the introduction. In *Paramecium* an amicronucleate cell possesses a small OA but is still able to grow with a longer generation time (14). Our results in *T. thermophila* suggest that one vegetative role of the micronucleus is the maintenance of the cortical structure, especially that of the OA and may be to avoid defects in the formation of new OA, because amicronucleate cells possessed OAs of normal appearance at the beginning but they lost their OAs gradually without cell division. The fact that amicronucleates induced by nocodazole never give rise to clones (J. Gaertig, pers. commun.) may be due to a complete loss of OA.

When the vegetative micronucleus plays a critical role for cell integrity, it may perform the role either through the expression of some not yet detected essential gene(s), or through a function as a kind of developmental center of cytoskeletal structure. The hypothesis that it is the developmental center (11) obviously contradicts the existence of the mutant amicronucleate strain (10). When the number of micronuclei was increased artificially, the cytoskeletal structure was not disturbed (unpublished results). If a mutant of a gene expressed in the vegetative micronucleus is obtained, it would give the solution to this problem. We are now trying to obtain such mutants.

Nocodazole also induced amacronucleates and empty cells in which only a small fraction of cells lacked an OA but no cells had disordered ciliary rows 20 hours after transfer from nocodazole to NKC solution as shown in the RESULTS. Irrespective of the fact that the macronucleus responsible for transcriptional activity was absent, delay in disintegration of OA and ciliary rows was observed. The reason is not clear. As depletion of the cellular ATP pool reduces the rate of microtubule disassembly in cultured mouse fibroblasts cells (4), the evidence observed may suggest that some active mechanisms are involved in disorganization of cortical structure.

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