THE ROLE OF THE MICRONUCLEUS IN STOMATOGENSES IN SEXUAL REPRODUCTION OF PARAMECIUM TETRAURELIA: LASER MICROBEAM IRRADIATION OF THE MICRONUCLEUS

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SUMMARY
Fifteen amicronucleate cell lines and 22 cell lines with defective micronuclei were obtained following selective laser microbeam irradiation of the micronucleus. The amicronucleate cell lines showed reduced growth rate and formed abnormal oral apparatuses in asexual reproduction, and failed to produce any oral apparatus in autogamy, in agreement with previous observations on amicronucleate cells obtained by micropipetting. The 22 cell lines with defective micronucleus exhibited various abnormalities of the oral apparatus newly formed during autogamy. These abnormalities included the arrest of membranelle assembly, reduction in the length of the buccal cavity and oral membranelles, disruption of the organization of the membranelles, quadrulation of the dorsal peniculus, and failure of addition of membranellar basal body rows. Hence the micronucleus plays multiple roles in sexual stomatogenesis, deciding early steps of oral membranelle assembly and affecting their subsequent patterning. Our results agree with the notion that the micronucleus acts during a critical period between the second meiotic division and up to the formation of the zygotic nucleus to control the early stage of oral membranelle assembly. Laser microbeam irradiation might have created recessive mutations and/or chromosomal aberrations, which were expressed during this critical period with the formation of abnormal postmeiotic nuclei.

INTRODUCTION
Ciliated protozoa are characterized by nuclear dimorphism: a cell possesses one or more micronuclei traditionally thought to play a germinal role only, and a micronucleus that dictates somatic functions. However, in many ciliates, the loss of the micronucleus leads to reduced vigor in vegetative propagation (notably in *Paramecium*) or non-viability (some *Tetrahymena* and *Euplotes* species) (reviewed by Ng, 1986). It is now evident that an intimate relationship exists between the micronucleus and an important somatic process – the development of the oral apparatus (stomatogenesis). The situation in *P. tetraurelia* has been revealed in some detail. The production of a new oral apparatus normally occurs during every binary fission and sexual reproduction (for stomatogenic events, see Ng & Newman, 1984a). However, vegetative cells could not produce a normal oral apparatus during a 'depression period' several fissions after the removal of both micronuclei by

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micropipetting (Ng & Mikami, 1981). Furthermore, when amicronucleates underwent sexual reproduction, a complete failure of oral membranelle assembly resulted. As the old oral apparatus is resorbed during the sexual cycle, these cells were left without an oral apparatus and eventually died (Ng & Mikami, 1981; Ng & Newman, 1984b). These findings demonstrate that the micronucleus plays an important but replaceable role in asexual stomatogenesis, whereas it is indispensable for sexual stomatogenesis. A study of the timing of the micronuclear and stomatogenic events during the sexual process of conjugation has strongly implicated micronuclear activities up to the zygotic nucleus stage affecting stomatogenesis (Ng & Newman, 1984a).

Studies on amicronucleate cells of other *Paramecium* species and a few other ciliates (*Oxytricha fallax, Stylonychia mytilus*) have also provided corroborative support for a stomatogenic role for the micronucleus. The loss of the micronucleus in these ciliates was also found to be associated with production of an abnormal oral apparatus and/or reduced ability to form food vacuoles during both vegetative propagation (*P. caudatum*, Fujishima & Watanabe, 1981; Fokin, 1983a, 1985; *P. bursaria*, Fokin, 1983b) and sexual reproduction (*P. caudatum*, Ossipov & Tavrovskaya, 1969; Ossipov & Skoblo, 1973; Mikami, 1979; *P. multimicronucleatum, P. putrinum* and *O. fallax*, Diller, 1965; *S. mytilus*, Shi et al. 1983).

Although the studies of amicronucleate cells have established a definite stomatogenic function for the micronucleus in sexual stomatogenesis, the full extent of its participation in sexual stomatogenesis remains to be determined, since removal of the micronucleus arrests the process at the very first step of membranelle assembly. The present study aims to determine additional roles for the micronucleus in sexual stomatogenesis, by revealing oral defects resulting from damage to the micronucleus by laser microbeam irradiation.

**MATERIALS AND METHODS**

**Cells and culture**

*Paramecium tetraurelia* stock 51 was used. Culture and handling of paramecia followed the methods of Sonneborn (1950, 1970). Cells were fed with phosphate-buffered Cerophyl medium (2.5 g l⁻¹, pH 7) inoculated with *Enterobacter aerogenes*, supplemented with 5 mg l⁻¹ stigmasterol. Experiments were performed at 27°C on four post-autogamous clones. Cultures were stored at 15-17°C.

**Generation of unimicronucleate cells**

*P. tetraurelia* possesses two micronuclei. Unimicronucleate cell lines were generated by microsurgical removal of one micronucleus. Details of the micromanipulation method were according to Ng (1981), except that only one needle was used. Unimicronucleates and bimicronucleates showed little difference in stomatogenesis.

**Laser microbeam irradiation of the micronucleus**

The laser irradiation set-up and methods were described by Ng (1980). Four unimicronucleate cell lines of independent clonal origins generated by microsurgery were used. Cells were kept in logarithmic growth phase, at 27°C, for a day prior to the operation at the clonal age of about 50
fissions. They were stained with Acridine Orange dye (8 or 16 µg ml⁻¹ medium) for 10–45 min before irradiation of the micronucleus for 1–4 s. Longer periods of staining (20–90 min) and irradiation (6–10 s) were done with one clone, because an aged Acridine Orange solution was used. The micronucleus was irradiated only when the beam was not interrupted by the macronucleus or any part of the oral apparatus. The position of the micronucleus was also checked immediately after irradiation to ensure that it had not moved away from the predetermined path of the laser beam. These precautions minimized damage to other cellular organelles.

Sampling after irradiation

After irradiation, each cell was expanded into a cell line in depression culture. The presence and morphology of the micronucleus were assessed 2 days (6–7 fissions) after the operation by staining a sample. Surviving cell lines were maintained by serial transfer of about a dozen cells to fresh medium before reaching the stationary phase of growth. The remaining cells were allowed to starve and go into autogamy. Samples of post-autogamous cells were fixed for cytological examination before they underwent division. Sampling was repeated at different clonal ages to ascertain the consistency of expression in each cell line. To ascertain whether the new macronuclear anlagen, formed in autogamy, assumed full somatic functions, a number of cells were isolated from the post-autogamous cultures into fresh medium and the number of survivors was scored after 3 days.

Cytology

Post-autogamous cells were fixed for silver impregnation (Chatton & Lwoff, 1936; Corliss, 1953), which revealed oral structures, micronuclei and macronuclei, and food vacuoles. In favourable preparations, macronuclear anlagen could also be seen. The nuclear configurations indicated by silver impregnation were confirmed by parallel preparations by the temporary acetoorcein method (Beale & Jurand, 1966), without prior fixation with osmium tetroxide, or by indigo carmine staining (Delameter, 1948; Sonneborn, 1950; Butzel, 1953). The micronucleus of vegetative cells was also assessed using these nuclear staining methods.

RESULTS

A total of 55 cells was irradiated and 45 survived the operation. Among the surviving cell lines, three classes were identified according to their micronuclear states.

Normal cell lines. Eight cell lines, from three of the four experimental clones, remained normal after the irradiation. In all these cases, the normally vesicular micronuclei were found to be present in >95% of vegetative cells. In addition, >90% of the cells in the post-autogamous population exhibited the normal nuclear constituents of two micronuclei and two macronuclear anlagen. The normal functioning of the new macronuclear anlagen was demonstrated by the high post-autogamous survival rates (5/8, 6/8, 8/8, 8/8, 8/8, 52/56; number of survivors/number of isolates, in 6 cell lines assessed). From these we infer that the micronuclei were normal in the eight cell lines. The formation of the oral apparatus was also normal in the post-autogamous populations (see Fig. 1). Only a few cells showed breaks or misalignment of the basal body rows in the oral membranelles, as is the case with unirradiated cells. Thus stomatogenesis remained normal in cell lines possessing a normal, though irradiated, micronucleus. Grossly abnormal stomatogenesis was found exclusively in cell lines having lost the micronucleus or possessing defective micronuclei, as described below.
Amicronucleate cell lines. Micronuclei were not detected in 15 cell lines (from 2 of the 4 experimental clones). The exact stage at which they disappeared was not established because the nuclear state was not assessed until six to seven fissions after irradiation, but this might well occur before the first fission, according to a previous study by Ng (1980). These amicronucleate cell lines were characterized by reduced fission rate and cell size. Silver-impregnation of vegetative cells in one of these cell lines revealed oral apparatuses of reduced length and with disrupted oral membranelles (as in cell lines DM1 and DM2, see below). After autogamy, cells of this amicronucleate cell line failed to produce any oral membranelle, buccal cavity or food vacuole (see Fig. 15). The other 14 amicronucleate cell lines probably had similar stomatogenic failures, as thin cells arose in vegetative cultures and their post-autogamous cells were mostly thin and non-feeding. The present findings, in agreement with earlier observations on amicronucleate cells generated by laser (Ng, 1980) and by micropipetting (Ng & Mikami, 1981; Ng & Newman, 1984b), showed similar stomatogenic failures following the loss of the micronucleus.

DM (defective micronucleus) cell lines. The main concern of the present investigation is whether the micronucleus participates during autogamy in later
stomatogenic steps beyond the early steps of oral membranelle assembly. Insight into this question is obtained from 22 cell lines (DM1–DM22) derived from all four experimental clones. In each of these DM cell lines, both multiple nuclear and oral defects were found among the post-autogamous populations (see below). Most of these defects were consistently expressed in consecutive autogamies of all DM cell lines, although the frequency of each type of defect in a population might differ. These DM cell lines were classified according to their major oral defects, as described below.

Disruptions to the organization of the oral apparatus

DM1 and DM2 were normal during vegetative propagation, but after autogamy about half of the cells in the population did not possess any micronuclear derivatives (Table 1). The remaining half possessed both micronuclei and macronuclear anlagen, except 10% of cells in DM1, which contained only the macronuclear anlagen. The morphology of these micronuclear derivatives was abnormal in some cases (cf. Figs 2, 3). The functional abnormality of the macronuclear anlagen was also suggested by the low post-autogamous survival rates (Table 1).

The presence of micronuclear derivatives, while indicating that the micronucleus persisted throughout autogamy, nevertheless did not guarantee normal stomatogenesis during this period (for percentages of cells with oral defects, see Table 1). The new oral apparatus formed during autogamy was often reduced in length, for both the buccal cavity and oral membranelles (Figs 4–6). The quadrulus, instead of the usual S shape, became C-shaped due to the loss of the posterior spiral. The organization of the oral membranelles also displayed a range of abnormalities. The longitudinal alignment of basal bodies, especially in the quadrulus, was frequently abnormal so that the rows became irregular (Fig. 4) or even scattered (Fig. 5). It was also not uncommon for the oral membranelles to be broken at one or more sites. The fragments anterior or posterior to the breaks might be shifted laterally relative to each other (Fig. 5). In a few cases, extra rows of basal bodies were found, especially in the quadrulus (Fig. 6). Occasionally, the differentiation of the quadrulus might also be disrupted so that the characteristic spacing pattern of the rows was not established, with the rows remaining tightly packed instead (Fig. 6). Ability to form food vacuoles was retained in 50% (DM1) or 90% (DM2) of cells with these abnormal oral apparatuses. It should be noted that the buccal reduction and similar membranelar abnormalities were also found in vegetative amicronucleate cell lines and the other 20 DM cell lines, together with other more specific oral defects.

In the post-autogamous populations that did not possess micronuclear derivatives an oral apparatus was usually lacking, except in a few cases. This defect will be described in greater length in connection with cell lines DM13–DM22.

Quadrulation of the dorsal peniculus

DM3, -4, -5 and -6 resembled very closely DM1 and DM2 in their micronuclear and stomatogenic aspects (Table 1). In addition, these four cell lines displayed a unique stomatogenic feature in the ‘quadrulation’ of the dorsal peniculus. Instead of
**Table 1. Summary of the characteristics of cell lines DM1–DM6**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Vegetative micronucleus</th>
<th>Total no. of cells studied</th>
<th>MD absent*</th>
<th>MD present</th>
<th>Proportion of surviving clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM1</td>
<td>+</td>
<td>177*</td>
<td>97(55)</td>
<td>20(11)</td>
<td>4/8</td>
</tr>
<tr>
<td>DM2</td>
<td>+</td>
<td>191*</td>
<td>113(60)</td>
<td>39(20)</td>
<td>1/8</td>
</tr>
<tr>
<td>DM3</td>
<td>+</td>
<td>215*</td>
<td>97(45)</td>
<td>105(49)</td>
<td>3/8</td>
</tr>
<tr>
<td>DM4</td>
<td>+</td>
<td>39</td>
<td>0(0)</td>
<td>27(70)</td>
<td>61/104*</td>
</tr>
<tr>
<td>DM5</td>
<td>+</td>
<td>59</td>
<td>34(58)</td>
<td>9(15)</td>
<td>3/8</td>
</tr>
<tr>
<td>DM6</td>
<td>Minimic.</td>
<td>10*</td>
<td>—</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

*Abbreviations: MD, micronuclear derivatives (new micronuclei and macronuclear anlagen); OA, oral apparatus; Dp quad., dorsal peniculus quadrulating; +, detection of a normal micronucleus in 95% of vegetative cells; minimic., minimicronucleus.

*Total being the sum of columns 4, 5, 6 in the Table.

*Defective oral apparatuses are those with disruption to the regular pattern of the membranelles and reduction in buccal length. See the text and Figs 4–6.

*Proportion of cells exhibiting quadrulation of the dorsal peniculus (see Figs 7, 8), out of the total number of cells in which the dorsal peniculus could be clearly observed.

*These values are pooled from consecutive post-autogamous samples. The number and percentage of cells without micronuclear derivatives, with micronuclear derivatives and normal oral apparatus, with micronuclear derivatives but defective oral apparatus for individual sample in each cell line, showed some variations: DM1: 31(48%), 2(3%); 30(48%); 38(63%), 9(15%), 14(23%); 28(53%), 9(17%), 16(30%) (3 samples). DM2: 34(57%), 6(10%), 20(33%); 45(60%), 26(35%), 4(5%); 34(61%), 7(13%), 15(26%) (3 samples). DM3: 19(17%), 87(78%), 6(5%); 78(76%), 18(17%), 7(7%) (2 samples).

*All survivors showed slow growth rate and eventually died.

*Feeders (the minority) in depression cultures were selected, based on cell size, for fixation for cytological examination; hence the number of cells without micronuclear derivatives, which were often non-feeders, and the percentage of feeding cells could not be ascertained from the stained preparations.

*Not assessed.
the usual compact pattern (Fig. 1A), two to three dorsal penicular basal body rows spread out at their anterior half and extended towards the quadrulus (Figs 7, 8; Table 1).

DM6 possessed micronuclei of reduced sizes (minimicronuclei). In some cells the loss of chromatin material seemed to be complete, leaving only an empty micronuclear envelope or 'ghost' micronucleus. In others, no micronucleus could be detected. Whether the micronucleus was too small to be visualized or was actually absent in the latter cases could not be confirmed. After autogamy, micronuclei (Fig. 9) and macronuclear anlagen remained small.

**Failure in addition of membranellar basal body rows**

DM7 and DM8 also possessed minimicronuclei, similar to DM6, but no 'ghost' micronucleus was detected. DM9 possessed a normal micronucleus, which was faithfully propagated in binary fission. After autogamy, cells of these three cell lines might be left without micronuclear derivatives, or in DM7 and DM8 some contained one to six minimicronuclei, with or without macronuclear anlagen, and in DM9 some possessed only one to four micronuclei, without macronuclear anlagen.

After autogamy, a significant proportion of these three cell lines showed a buccal cavity and oral membranelles, irrespective of the presence or absence of micronuclear derivatives (Table 2). Most striking of all, 50–100% of these in the three cell lines possessed oral membranelles made up of a total of nine rows of basal bodies only, i.e. three instead of the usual four rows in each of the three oral membranelles: the quadrulus, the dorsal and the ventral peniculi (Figs 10, 11). Hence it was a 3×3 pattern rather than the typical 3×4 configuration. In addition, disruption of the regular pattern of the oral membranelles, resembling those found in DM1 and DM2 was also observed, irrespective of the number of rows they contained. Furthermore, in DM7 the membranelles were often broken up into fragments that lay more or less horizontal to the long axis of the buccal cavity (Fig. 11). The ability to form food vacuoles was also lost in many cases, especially in DM7 (Table 2).

**Failure of stomatogenesis even when micronuclear derivatives were formed in autogamy**

DM10–DM12 were distinct from other DM cell lines in that 5–27% of the cells in the post-autogamous population completely failed to form the new oral apparatus, even though the micronucleus in these persisted throughout autogamy, giving rise to micronuclear derivatives (in most cases, new micronuclei only) at the end of autogamy (Table 3). The oral structure left was just a few basal bodies in a shallow vestibular depression (see Fig. 15), identical to the post-autogamous state of amicronucleate cells. Vegetative propagation of the micronucleus and the cells were apparently normal in these three cell lines. Micronuclear aberrations were evident only during sexual reproduction, in which the development of the micronucleus was aborted, or the formation of the new micronuclei or macronuclear anlagen was impaired (Table 3). Furthermore, micronuclei newly formed in autogamy were often morphologically abnormal (Figs 12, 13) and variable in number (up to 4).
In addition to the abnormalities summarized in Table 3, some post-autogamous cells failed to divide normally, with the anterior and posterior daughter cells remaining attached to and bent over each other to form V-chains. Many of these V-
Table 2. Summary of the characteristics of cell lines DM7–DM9

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Vegetative micronucleus</th>
<th>Total no. of cells (1) + (2) + (3)</th>
<th>(1) OA absent&lt;sup&gt;a&lt;/sup&gt;,b</th>
<th>(2) 3x3 row OA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(3) 3x4 row OA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percentage of feeding cells in (2) + (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM7</td>
<td>Minimic.</td>
<td>72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0(0)</td>
<td>15(21)</td>
<td>16(22)</td>
<td>41(57)</td>
</tr>
<tr>
<td>DM8</td>
<td>Minimic.</td>
<td>32</td>
<td>10(32)</td>
<td>2(6)</td>
<td>5(28)</td>
<td>4(12)</td>
</tr>
<tr>
<td>DM9</td>
<td>Normal</td>
<td>92</td>
<td>0(0)</td>
<td>73(80)</td>
<td>4(4)</td>
<td>6(7)</td>
</tr>
</tbody>
</table>

<sup>a</sup>OA, oral apparatus; 3x3 row OA, oral apparatus consisting of three membranelles each made up of three rows of basal bodies, instead of normally four rows (3x4 row OA); minimic., minimicronucleus; +, presence of micronuclear derivatives (new micronuclei and macronuclear anlagen); -, absence of micronuclear derivatives.

<sup>b</sup>Values denote number (percentage of the total number) of cells.

<sup>c</sup>Data for cell line DM7 are pooled from two consecutive post-autogamous samples that are not significantly different from each other.

Table 3. Oral structures of the post-autogamous populations in cell lines DM10–DM12

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total no. of cells</th>
<th>OA&lt;sup&gt;a&lt;/sup&gt; absent&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Defective OA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Normal OA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-, -</td>
<td>+, -</td>
<td>+, +</td>
</tr>
<tr>
<td>DM10</td>
<td>81</td>
<td>50(63)</td>
<td>9(11)</td>
<td>1(1)</td>
</tr>
<tr>
<td>DM11</td>
<td>98</td>
<td>64(65)</td>
<td>5(5)</td>
<td>0(0)</td>
</tr>
<tr>
<td>DM12</td>
<td>164</td>
<td>116(71)</td>
<td>45(27)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>OA, oral apparatus; -, -, no micronuclear derivatives; +, -, possessed either micronuclei (more frequent) or macronuclear anlagen; +, +, both micronuclei and macronuclear anlagen were present.

<sup>b</sup>Values denote number (percentage of the total number) of cells.
chains possessed only one oral apparatus, which was caught on the fission zone (Fig. 14).

**Failure of stomatogenesis related to the loss of the micronucleus at a certain stage of autogamy**

The presence of the micronucleus was demonstrated in >95%, 20% and 40% of vegetative cells in DM13–DM20, DM21 and DM22, respectively. Nevertheless, after autogamy only 0–8% of the population possessed micronuclear derivatives (except DM13 and DM14, which showed a greater variation of 0–25% among different autogamies). The low incidence of micronuclear derivatives in post-autogamous cells indicated that in most of the cells micronuclear development was aborted at some stage of autogamy. The minority, which possessed micronuclear derivatives after autogamy, usually developed oral apparatuses possessing largely abnormal membranelles.

The main interest in these 10 cell lines is in those cells that lacked micronuclear derivatives, because most of these (96–100% in DM14–DM19, DM22; 85% in DM20, DM21) possessed neither oral membranelles nor a buccal cavity (Fig. 15), resembling the complete stomatogenic failure of amicronucleate cells put through autogamy. Although the same defect was also detected in all other DM cell lines, these 10 cell lines were notable for the particularly high expression of this abnormality. These cell lines thus offered an opportunity for determining the stage at which the micronucleus was lost during autogamy. This might provide some information as to the timing of micronuclear activity in controlling the early stage of membranelle assembly.

Four cell lines, DM13–DM16, were used for such investigation. Samples were fixed for staining with basic fuchsin and indigo carmine at half-hourly or hourly intervals from cultures in autogamy. A sample for silver impregnation was also fixed in parallel with the last fuchsin sample. Since the sexual cycle was not synchronized within the population, each nuclear-stained sample contained cells at different stages of autogamy. For a given cell, the morphology of the old macronucleus was used as an approximate indicator of the stage reached in autogamy (according to Ng & Newman, 1984a). Observations were made as to whether the micronuclei were present at a particular stage. The results are summarized in Table 4.

Micronuclear development proceeded up to telophase II stage in most of the cells of all four cell lines (100% in DM13–DM15, 83% in DM16). Beyond this stage, the micronucleus disappeared in the majority, if not all, of the cells. From this it may be deduced that the development of the micronucleus terminated after telophase II of meiosis in most of the cells. Such a termination can be explained by the elimination of all four haploid nuclei, as opposed to the retention of one of these nuclei in normal circumstances. This was evident in many cases as four degenerating pycnotic nuclei were detected in one cell. The absence of oral structures in post-autogamous cells that lacked micronuclear derivatives was confirmed in the silver-impregnated samples. In addition, in favourable basic fuchsin samples in which the presence of oral structures can be ascertained, no sign of elaborate oral development was
observed for those autogamous cells that did not possess micronuclear derivatives. Therefore the absence of oral structures after autogamy was not due to resorption of newly formed oral parts, but was a result of a complete arrest of membranelle assembly.

**DISCUSSION**

The present study shows that stomatogenesis during sexual reproduction became abnormal only when the micronucleus was lost (15 amicronucleate cell lines) or had become defective (22 DM cell lines) subsequent to irradiation of the micronucleus. Furthermore, eight irradiated cell lines possessed normal micronuclei and they developed normal oral apparatuses during sexual reproduction. Evidently, the micronucleus plays an important role in stomatogenesis.

*Timing of micronuclear activity for an early stage of oral membranelle assembly*

When amicronucleates were put through autogamy, stomatogenesis completely failed and astomatous cells were produced. This is in good agreement with the previous findings in amicronucleate *P. tetraurelia* generated by micropipetting (Ng & Mikami, 1981; Ng & Newman, 1984b). Our previous study established that the complete stomatogenic failure of amicronucleates during autogamy stemmed from a developmental arrest at an early stage of oral membranelle assembly, i.e. stomatogenic stage 2 (Ng & Newman, 1984b). This early stage of oral membranelle assembly, marked by the alignment of basal bodies into longitudinal rows in the oral

<table>
<thead>
<tr>
<th>Macronuclear cycle</th>
<th>Micronuclear cycle</th>
<th>No. (%) of cells with micronuclear derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DM13</td>
</tr>
<tr>
<td>Intact</td>
<td>Vesicular</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Prophase</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Cresent</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Triangular</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Metaphase I</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Metaphase II</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Early skein</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Telophase II*</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Mid skein</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Mitosis</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Synkaryon</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Late skein</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Post-zygotic I, II</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Mi, Ma differentiation(^b)</td>
<td>8</td>
</tr>
</tbody>
</table>

*Table 4. Presence of micronuclear derivatives at different stages of autogamy in cell lines DM13–DM16*

* A few cells with telophase II haploid nuclei possessed mid-to-late skein macronuclei, in contrast to the normal situation that these haploid nuclei are restricted to the early macronuclear skein stage.

\(^b\) Mi, micronucleus; Ma, macronuclear anlagen.

\(^c\) Values in square brackets denote sample sizes.
anlage field, is normally first seen with the exchange of gametic nuclei and formation of the zygotic nucleus (see Fig. 16) (Ng & Newman, 1984a). These observations suggest that the activities of the micronucleus up to the zygotic nucleus stage must be decisive in the early stage of oral membranelle assembly.

In the present study, when cells possessing defective micronuclei were put through autogamy, astomatous post-autogamous cells arose, and these did not possess any new micronuclei or macronuclear anlagen derived from the micronucleus. Thus these cells must have lost the micronucleus at some stage of autogamy. This shows, consistent with the previous findings, that the micronucleus **must be present during sexual reproduction** to execute its stomatogenic function. In this connection, the observations on cell lines DM13–DM16 are particularly revealing. In these four cell lines, the micronucleus completed meiosis, but oral membranelle assembly was arrested. Micronuclear development was terminated only after the second meiotic division, as all the haploid nuclei disappeared. We postulate that the
disappearance of all haploid nuclei is the immediate cause for the arrest of oral membranelle assembly. This means that micronuclear activities up to the second meiotic division stage are insufficient for the first steps of membranelle assembly, but that those occurring after the second meiotic division and possibly up to the formation of the zygotic nucleus must be decisive. On the other hand, we cannot at present rule out the possibility that stomatogenic signals for the early stage of membranelle assembly are made available before the completion of meiosis, and that these might be impaired as a result of irradiation, resulting in stomatogenic failure. If so, this would make the disappearance of all post-meiotic haploid nuclei not causative, but merely coincidental. However, the good correlation between stomatogenic failure and termination of the micronuclear cycle after the second meiotic division in all four cell lines makes our first postulate particularly attractive. More cell lines of this nature would have to be analysed to see if this correlation holds. We would argue further (see below) that even though stomatogenic signals might be accumulating in the micronucleus during meiosis, they would not be executing their function until the post-meiotic stages.

Our postulation that the post-meiotic micronuclear derivatives are important in the first steps of oral membranelle assembly is of interest in another consideration. In the first place, as all four haploid nuclei became pycnotic and disappeared after the second meiotic division, it is very likely that none of them has resided in the paroral cone area, which normally protects one haploid nucleus from destruction (Sonnborn, 1954; Yanagi & Hiwatashi, 1985). There is evidence that factors vested within the micronucleus are important in such spatial interaction between the haploid nucleus and the paroral cone area (Ng, 1981); in the four cell lines (DM13–DM16) irradiation of the micronucleus could have damaged these factors. More importantly, there is a close spatial relationship between the oral anlage field and the post-meiotic micronuclear derivatives. The oral anlage field is situated to the immediate left of the paroral cone area where the haploid nucleus and its mitotic products, the gametic nuclei, reside; exchange of gametic nuclei between the mates takes place here and the zygotic nucleus also stays in the vicinity for a while before moving elsewhere. Temporally, these close spatial interactions immediately precede oral membranelle assembly in the anlage field (see Fig. 16). It has been suggested that the close proximity between the oral anlage field and the post-meiotic micronuclear derivatives might be important for the deliverance of the micronuclear stomatogenic signals, even though these might have started to accumulate in the micronucleus during meiosis (Ng & Newman, 1984a). In this light, the failure of the haploid nuclei in cell lines DM13–DM16 to reside in the paroral cone area not only resulted in their elimination, but also upset their spatial interaction with the oral anlage field, leading to the arrest of oral membranelle assembly.

The hypothesis that micronuclear activities subsequent to the second meiotic division and up to the zygotic nucleus are of stomatogenic importance is consistent with some observations in *P. caudatum*. In conjugation between amicronucleates and micronucleates, stomatogenesis was reported to have concluded normally in the amicronucleate mate if the male gametic nucleus from the micronucleate mate
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migrated successfully to the former (Ossipov & Skoblo, 1973). On the other hand, if no migratory nucleus reached the amicronucleate partner, the latter reportedly failed to produce any vestibular and buccal cavities and perished without division, even if normal pairing occurred (Ossipov & Tavrovskaya, 1969; Ossipov & Skoblo, 1973). These observations suggest that the activities of the gametic and/or zygotic nuclei are of stomatogenic importance, though the stomatogenic details are not available in these reports.

It is perhaps not coincidental that in several hypotrichs the main events of the first round of reorganization of the oral membranelles (adoral zone membranelles) during conjugation take place after the formation of the zygotic nucleus: *Euplotes* (Diller, 1966); *Stylonychia mytilus* (Shi, 1976); *Pseudourostyla cristata* (Zhang et al. 1985) and possibly *Paraurostyla weissei* (Jerka-Dziadosz & Janus, 1975). However, treatment of conjugation of *Stylonychia* with hydroxyurea, which blocks gametic nuclei formation, reportedly allowed the first round of oral reorganization to proceed (Sapra & Ammermann, 1974).

The micronucleus participates in the patterning of the oral apparatus

In addition to the role in the early stage of oral membranelle assembly in sexual stomatogenesis, the present findings show that the micronucleus also takes part in subsequent stomatogenic events. In the 22 DM cell lines, many post-autogamous cells exhibited disruption of the regular pattern of the oral membranelles: namely, breaks and displacement of the membranellar basal body rows, misalignment of basal bodies along these rows, loss of the posterior spiral of the membranelles and the corresponding posterior part of the buccal cavity. Interestingly, some specific stages of stomatogenesis were affected. Normally, during stages 5 and 6 the three pro-membranelles develop the mature pattern: the quadrular basal body rows become spread out at the anterior whereas the two peniculi remain closely packed (see Ng & Newman, 1984a). Nevertheless, in several laser-irradiated cell lines (DM3–DM6) some of the dorsal penicular basal body rows became separated from each other and transformed into a quadrulus-like pattern. Finally, in stage 4, the six-row oral primordium normally develops, by addition of basal body rows, into three pro-membranelles each comprising four basal body rows (see Ng & Newman, 1984a). However, the process of addition of basal body rows must have been interrupted in three cell lines (DM7–DM9), so that in these each membranelle came to possess only three rows.

These abnormal oral apparatuses produced during autogamy, apart from the three-row membranelles of DM7–DM9, are largely similar to those seen in vegetative amicronucleates shortly after their generation, either by laser microbeam irradiation in the present study, or by micropipetting (Ng & Mikami, 1981). Thus in both sexual and asexual reproduction the micronucleus is actively determining the final pattern of the oral apparatus. If the responsible micronuclear factors reside in the DNA sequences it should be possible to isolate micronucleus-acting stomatogenic mutations that disrupt the process at various points. An attempt in this direction has been made (Tam & Ng, unpublished data).
The nature of micronuclear defects as a result of irradiation

The present results also permit some speculation on the nature of the micronuclear stomatogenic signal. The 22 DM cell lines produced apparently normal oral apparatuses during asexual reproduction before the first autogamy since irradiation. This is suggested by their near-standard growth rate and the normal outline of their oral apparatuses revealed by basic fuchsin staining and silver-impregnation of two cell lines (DM7, DM8) at about 40 fissions after irradiation. This is in marked contrast to the normal stomatogenesis they exhibited during autogamy. The formal explanation we offer is genetic. If the stomatogenic factors are borne on micronuclear chromosomes, they may be expressed in the irradiated micronuclei during binary fission. But during sexual reproduction, meiosis may generate chromosomal aberrations and genomic imbalance, resulting in impaired stomatogenic functions. This problem is being pursued and will be discussed further in a future communication.

References


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