CAUSAL RELATIONS AMONG CELL CYCLE PROCESSES IN *TETRAHYMENA PYRIFORMIS*

An Analysis Employing Temperature-Sensitive Mutants

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ABSTRACT

Utilization of temperature-sensitive mutants of *Tetrahymena pyriformis* affected in cell division or developmental pathway selection has permitted elucidation of causal dependencies interrelating micronuclear and macronuclear replication and division, oral development, and cytokinesis. In those mutants in which cell division is specifically blocked at restrictive temperatures, micronuclear division proceeds with somewhat accelerated periodicity but maintains normal coupling to predivision oral development. Macronuclear division is almost totally suppressed in an early acting mutant (mo1[a]) that prevents formation of the fission zone, and is variably affected in other mutants (such as mo3) that allow the fission zone to form but arrest constriction. However, macronuclear DNA synthesis can proceed for about four cycles in the nondividing mutant cells. A second class of mutants (psm) undergoes a switch of developmental pathway such that cells fail to enter division but instead repeatedly carry out an unusual type of oral replacement while growing in nutrient medium at the restrictive temperature. Under these circumstances no nuclei divide, yet macronuclear DNA accumulation continues. These results suggest that (*a*) macronuclear division is stringently affected by restriction of cell division, (*b*) micronuclear division and replication can continue in cells that are undergoing the type of oral development that is characteristic of division cycles, and (*c*) macronuclear DNA synthesis can continue in growing cells regardless of their developmental status. The observed relationships among events are consistent with the further suggestion that the cell cycle in this organism may consist of separate clusters of events, with a varying degree of coupling among clusters. A minimal model of the *Tetrahymena* cell cycle that takes these phenomena into account is suggested.

One way to uncover causal relations between successive or simultaneous cellular developmental processes is to interfere selectively with one process and then observe the effects on the others. Microsurgical dissection provides a paradigm for this experimental logic: for example, Tartar (61) was able to demonstrate conclusively that the oral development of a ciliate is not necessary for the cell division that follows it, by simply cutting out the developing oral apparatus of *Stentor* and then observing that fission proceeds nonetheless. However, where cells are small and not readily operable, and/or the processes under study are pervasive physiological

events, other methods must be resorted to. One such method is to use physiological agents such as sublethal temperatures or chemical inhibitors (e.g., reference 69). Although these do affect cellular development differentially, their effects are broad ranging and sometimes difficult to interpret.

This study is devoted to the application of a third method, genetic dissection through the use of temperature-sensitive mutations. Most such mutations in bacteriophage (15) and probably also in eukaryotic cells (58) represent amino acid substitutions in particular proteins brought about by missense base substitutions in DNA. In this way, their effects are highly specific. However, if the affected gene product and its function are unknown, then one cannot be certain of the specificity of the mutant at the level of gross cellular processes, as pleiotropic effects are often hard to rule out. This limitation can be overcome at least partly by setting up a screening system to select mutations affected in particular cellular processes. This strategy has been successfully employed in budding yeast to dissect causal relations in the cell cycle (33). We apply here essentially the same strategy to the ciliated protozoan *Tetrahymena pyriformis*. Mutants that were blocked in cell division were selected and genetically analyzed (27); other mutants were obtained that brought about a switch in developmental pathways. The first set of mutants was found to permit a substantial number of cell-cycle processes to continue despite the block in cell division, while the second was more restrictive. Information was obtained on the course of nuclear and cortical events in mutant cells shifted to nonpermissive temperature. Such information can be combined with the extensive body of pertinent existing data derived from use of other methods to provide a reasonably detailed picture of the causal interrelations of the grosser aspects of the cell cycle. The experimental outcome shows that some very simple models of the cell cycle are inapplicable to ciliates, and makes possible the construction of a minimal plausible model of the interrelation of cell cycle events in *Tetrahymena*.

MATERIALS AND METHODS

Strains

Inbred strains of *T. pyriformis*, syngen 1, were used in this study. Strains B and D, both in the 18th generation of inbreeding, were received from Dr. David L. Nanney. The mo1, mo3, mo6, and psm mutants were obtained by selection and inbreeding after nitrosoguanidine mutagenesis, as described in detail elsewhere (27). The "psm-like" line is a nitrosoguanidine-induced strain B variant, originally designated 10-1-42, selected by Dr. Peter Bruns who kindly donated it to us.

Media and Cultivation

Long-term stock cultures were maintained at 18°–20°C in 1% proteose peptone, initially without and later with 0.1% yeast extract, in culture tubes with transfer every 2nd wk. Stocks undergoing current experimentation were maintained in slanted tube cultures, transferred daily or every other day in a 1% proteose peptone-0.1% yeast extract medium. Experiments were conducted in a 2% proteose peptone-0.5% yeast extract medium (PPY). A few early experiments were carried out in a tryptone-glucose-vitamins-salts (TGVS) medium (22).

Experimental Protocols

500-ml Fernbach flasks (Jena Glaswerk) containing 150 ml of TGVS or PPY medium were inoculated from a 1-day old tube culture and then incubated at 23°C or 28°C without shaking or aeration for 16–18 h (five to six cell generations). Experimental flask cultures were then transferred to a water bath set at 40° ± 0.2°C, still without shaking or aeration. Cell density ranged from 700 to 30,000 cells per milliliter at the time of the temperature shift. With few exceptions, each experiment involved two cultures of wild-type cells and two of mutant cells, with one of each pair remaining at 23°C or 28°C and the other shifted to the 40°C bath. The actual temperature inside the flask was probably somewhat below 40°C (in one experiment it was measured at 39.6°C). Samples were taken from both control and experimental cultures at the time of the temperature shift of the experimental cultures and at intervals thereafter. These were prepared for cell counting, silver impregnation, and Feulgen staining. Samples were prepared for counting as described earlier (22) and counted in a model A Coulter Counter (Coulter Electronics, Hialeah, Fla.).

Cytological and Cytophotometric Procedures

Feulgen staining and fluorescence cytophotometry were performed by the methods of Bohm and Sprenger (2) as described by Doerder and DeBault (12). Hydrolysis in 1 N HCl at 40°C was performed for 25 min in experiments involving mo1^a, and for 20 min in experiments involving psm. All other general procedures for handling and measurement of slides were performed as in Doerder and DeBault (12).

Measurements of DNA content of macronuclei of mo1^a cells cultured at 40°C posed a special problem

because the macronucleus was generally over- and underlain by several micronuclei. What could be measured, therefore, was the DNA content of the macronucleus *plus* that of the superimposed micronuclei. To obtain an estimate of DNA content of the macronucleus alone, cells were chosen for measurement in which some of the micronuclei were *not* superimposed on the macronucleus (this condition was fulfilled by most cells). The DNA content of two or three such free-lying micronuclei was measured, the cytoplasmic background value (measured in the same cell) subtracted, and the corrected values were averaged. The macronucleus plus superimposed micronuclei were then measured, the number of superimposed micronuclei counted, and this number was multiplied by the corrected estimate of DNA content per micronucleus derived from the free-lying micronuclei of the same cell. This "micronuclear correction" was then subtracted from the measured value of macronucleus plus superimposed micronuclei to give an estimate of the DNA content of the macronucleus alone.

In experiments designed for simple enumeration of nuclei, a conventional Feulgen procedure was used, with 6-min hydrolysis in 1 N HCl at 60°C and counterstaining with 0.1% fast green in 95% ethyl alcohol. Numbers of micronuclei and macronuclei were counted in 200 cells in each sample prepared in this manner.

Silver impregnation was performed by the directions of Frankel and Heckmann (26) with slight modifications that will be reported in detail elsewhere.[1] Tallies of stages of cortical development were accomplished by bright field microscopy, with 200 cells tallied per sample. In some silver-impregnated series all nuclei are clearly visible, and these were used for a combined enumeration of nuclei and assessment of cortical development. This was performed by phase-contrast microscopy, with 100 cells analyzed from each sample.

RESULTS

Cell Cycle of Wild-Type T. pyriformis

The nuclear and cortical events of the cell cycle of *T. pyriformis* have been reviewed elsewhere (6, 28, 44) and will be recapitulated only briefly here. The major cytological events are diagrammatically illustrated in Fig. 1 (top). A new oral primordium develops on the cell surface during the latter half of the cell cycle at a site just posterior to the cell equator. At a late stage of this process an equatorial fission zone develops just anterior to the oral primordium, and the cell then cleaves along this zone. The two nuclei divide at different times. The micronucleus migrates to a peripheral position during a very early stage of oral development and then divides mitotically[2] before the onset of cell division (31, 35; see also Fig. 3). Division of the macronucleus takes place after the completion of micronuclear division and at the same time that the cell itself is dividing; the macronucleus elongates while the fission zone is developing on the surface, and divides during the early part of cytokinesis while maintaining a central position in the cell (35, 65). The S periods of the two types of nuclei also differ, the micronucleus replicating its DNA just after its own division while the macronucleus normally replicates in the middle of the cell cycle (44).

Wild-type *T. pyriformis* also can undergo an alternative mode of oral development known as oral replacement (23, 28). This involves development of an oral primordium just posterior to old oral structures, which are then resorbed and replaced by the newly developed ones. This process is rare in exponentially growing cells, but occurs commonly in stationary phase cells (23, 42) and in cells maintained in non-nutrient medium (23, 50). It also takes place occasionally in cell division mutants that have undergone division arrest at 40°C. It is important to add here that oral replacement primordia can be distinguished unambiguously from oral primordia of the predivision type even in highly distorted cells, because in the oral replacement process a substantial part of the preexisting oral structure (the undulating membrane) disaggregates and contributes to the new oral primordium (23, 42).

Cell Division and Developmental Pathway Mutants

Recessive temperature-sensitive mutations that specifically block cell division are described in detail elsewhere (reference 27).[3] Six relevant loci have been identified, only two of which (mo1 and mo3) will specifically be dealt with in this report. The cortical phenotypes brought about at restrictive temperatures in cells homozygous for the more strongly expressed allele of mo1 (mo1^{a}) and for all alleles of mo3 are schematically illustrated in Fig. 1 (with nuclei omitted for clarity). Cells homozygous for mo1^{a} develop oral primordia at the normal subequatorial position after a shift from 28°C (permissive temperature) to 40°C (restrictive temperature). However, a fission zone does not develop and the cells do not furrow; they instead develop irregular projections in the equa-

[1] E. M. Nelsen, manuscript in preparation.

[2] N. E. Williams, personal communication.

[3] Also J. Frankel, E. M. Nelsen, and L. M. Jenkins, manuscript in preparation.

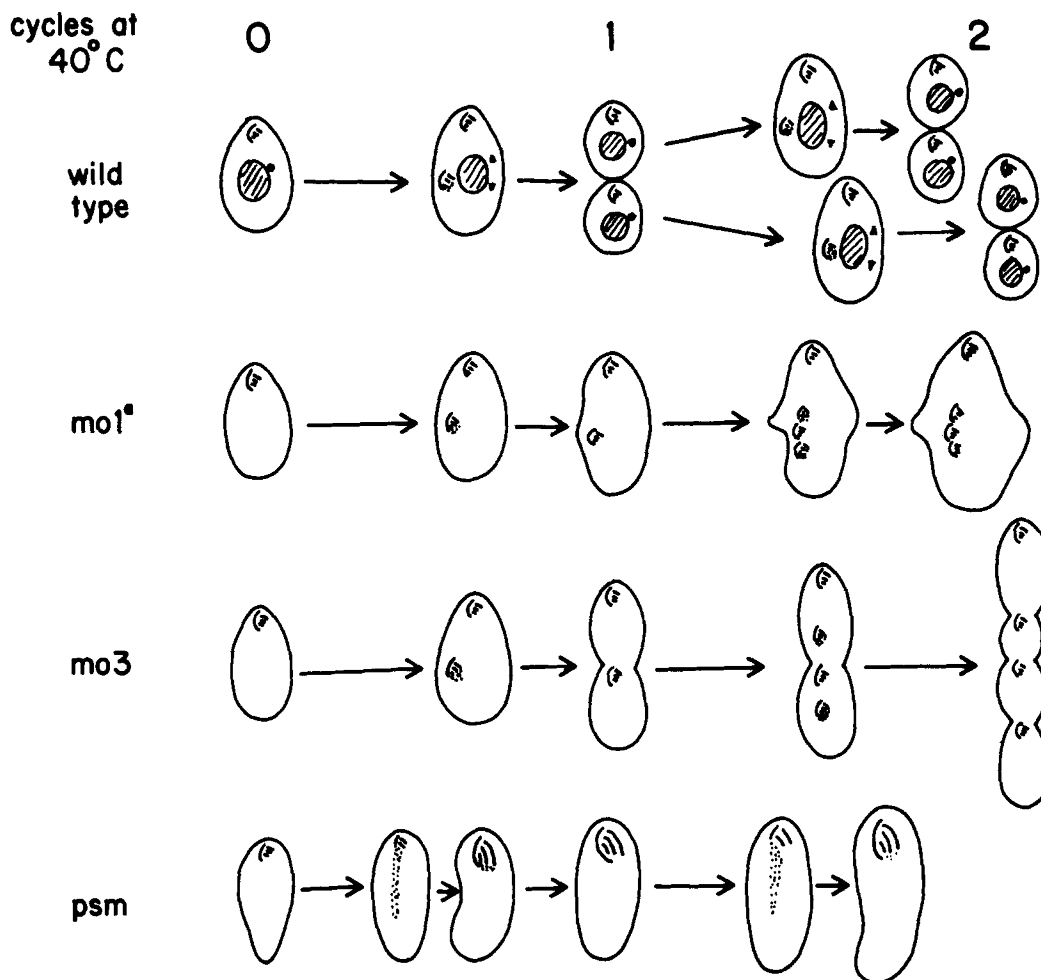


FIGURE 1 Schematic diagrams of the development of wild-type and mutant cells through two cell cycles at 40°C. Cell outlines, oral structures, and developing oral primordia are shown in all four series of diagrams. In addition, the course of division of micronuclei and macronuclei is shown in wild-type cells; nuclei are omitted from diagrams of the mutants. For further explanation, see the text.

torial region in which furrowing would normally have occurred. Despite the prevention of normal cell division, second generation oral primordia are later initiated at sites anterior and/or posterior to the now completed oral primordium of the first arrested cell generation. At the same time, cells become larger and increasingly more irregular in form. The continuation of growth and of periodic cycles of oral development in cells that cannot even begin to divide brings about the eventual formation of large, misshapen monsters.

Cells homozygous for the mo3 mutations (Fig. 1) undergo normal oral development after the temperature shift from 28°C to 40°C, and furthermore are capable of developing a fission zone and of initiating division constriction. However, constriction is arrested and the cells then undergo the elongation that in normal cells takes place after cell separation. Second generation oral primordia then form in each of the two still-connected cell units, and a new attempt at cell division then brings about a chain of four cells. This simple developmental progression is somewhat complicated by a tendency of cell units to pull apart secondarily after several hours of growth, or alternatively for diverse geometric distortions to occur that result in a loss of the simple linear configuration which is shown in Fig. 1. The former process occurs readily in cells homozygous for some of the more weakly expressed mo3 alleles (such as

mo3^a), while the latter complication is prevalent in homozygotes of more highly expressed alleles (such as mo3^b).

An altogether different abnormality is manifested in the "pseudomacrostome" (psm) mutant (Fig. 1, bottom). This recessive nitrosoguanidine-induced mutant, as well as an independent but phenotypically very similar variant ("psm-like"), differs from wild-type cells in cell shape at room temperature, with a more tapered midregion and posterior end. On transfer to 40°C, psm cells switch to the oral replacement mode of development. This switch is unusual in that it takes place in cells that are continuing to feed and are increasing dramatically in cell size. Further, the oral replacement primordia are not of the usual "microstome" type characteristic of *T. pyriformis*, but instead are extremely long and tend to produce unusually large oral apparatuses even in starved cells, reminiscent in some ways of the macrostome oral apparatuses of *T. patula* (57, 62) and *T. vorax* (4, 5); hence the descriptive name "pseudomacrostome." The morphogenetic features of psm will be described in detail elsewhere.

Nuclear Division in Cell Division Mutants

Nuclei were enumerated in mo1^a and mo3^b cells shifted from 28°C to 40°C (Table I). There was little increase in cell number in the mutants after the temperature shift (Fig. 2*a*, *c*), indicating total blockage of cell division at 40°C (with some secondary pulling apart in mo3^b between 8 and 12 h). Nonetheless, micronuclei kept on dividing. Further, this division was synchronous, as evidenced by (*a*) the predominance of micronuclear numbers in the progression 2^n (italicized in Table I), and (*b*) synchrony of mitosis in cells with two or more micronuclei, in which exceptions to full synchrony (i.e. all micronuclei within a cell simultaneously in mitosis) were unusual (17 out of 605 cases). Macronuclei, on the other hand, divided rarely in mo1^a cells at 40°C and to a variable degree in mo3^b cells. Further, where divisions did occur they were mostly uncoordinated; in mo3 cells it often happened that after a first successful division one daughter macronucleus later succeeded in dividing a second time while the other did not, which resulted in a substantial class of cells with three macronuclei (Tables I and II). The resulting appearance after long periods at 40°C was of large cells with many micronuclei and typically one or two (mo1^a, Figs. 5 and 6) or two to four (mo3, Fig. 4) macronuclei.[4] Other mutants blocked in cytokinesis (mo1^b, mo6, mo8, mo12; see reference 27 for general characterization) manifested the same basic phenomenon; micronuclear number increased rapidly in all of them, while macronuclear division was variably affected. The degree to which macronuclei could divide appeared to be systematically correlated with the stage of division arrest, with little macronuclear division in a mutant that fails to form a fission zone (mo1^a), more in an allele (mo1^b) that forms a partial fission zone (27), and the most in the mo3 mutants that furrow and form tandem chains.

The simplest explanation of the dramatic increase in number of micronuclei is that this represents a normal continuation of micronuclear cycling despite the absence of consummated cell divisions. This working hypothesis was tested in three different ways. The first was to compare the rate of increase in number of micronuclei at 40°C in wild-type and mutant cells. Wild-type cells underwent rapid cell multiplication at 40°C after a short initial lag, while the mo1^a and mo3^b mutants displayed very little increase in cell number (Fig. 2*a*, *c*). Such differential cell multiplication has to be taken into account before a meaningful comparison of *nuclear* multiplication can be made. This was accomplished by standardizing the data on the basis of number of nuclei per milliliter of culture at 40°C and then expressing these data in terms of increase in nuclear number after the time of the temperature shift. The striking result that is made evident by comparing nuclear multiplication

[4] While micronuclear division always followed the same basic time course, the extent of macronuclear division varied somewhat according to the medium and, in mo3^b, also according to the experiment. In cells of mo1^a, in both B- and D-strain genetic background, fewer than 20% of the macronuclei divided once in PPY medium at 40°C (three experiments), whereas in TGVS medium at 40°C (one experiment) nearly half of the macronuclei divided once, and a few cells attained three macronuclei. In mo3^b, fewer macronuclei were observed in an experiment in PPY medium (Table I) than in another experiment in TGVS (Table II). A second experiment in PPY was not tallied in detail, but cells with three and four macronuclei appeared to be more frequent than in the experiment shown in Table I. The variations in mo3^b are probably due to small differences in the degree and duration in which a tandem orientation is maintained during successive cycles of division-arrest.

TABLE I

Increase in Number of Nuclei in Cytokinesis Mutants Maintained in PPY Medium at 40°C

Mutant designation	Time at 40°C	Relative cell no.*	Number of cells in each micronuclear class																Micronuclear average	Number of cells in each macronuclear class					Macronuclear average
			1	*2*	3	*4*	5	6	7	*8*	9	10–14	15	*16*	17–29	30–31	*32*	33+		1	2	3	4	5+	
	h																								
mo1[a]‡	0	1	*177*	*23*															1.12	195	5				1.03
	3	1.22	*11*	*90*	1	*95*				*3*									2.99	194	6				1.03
	6	–		*1*		*27*		1	4	*148*	2			*17*					8.09	186	14				1.07
	12	1.25		*1*	1	*5*				*28*		2	11	*102*	1	11	*35*	3	18.28	175	25				1.13
mo3[b]‡	0	1	*192*	*8*															1.04	200					1.00
	2	1.00	*129*	*69*		*2*													1.38	179	21				1.11
	4	0.99	*11*	*24*	1	*126*	21	2	2	*8*	3	2							4.04	120	59	19	2		1.52
	6	1.01	*1*	*4*	1	*93*	5	3	2	*75*	3	5	1	*7*					6.26	106	73	19	1	1	1.59
	8	1.09	*1*	*9*		*63*	2	2	1	*94*		3	3	*20*		1	*1*		7.59	117	55	21	2	5	1.65
	12	1.32	*3*	*4*		*32*		4	1	*99*		10	3	*34*	3	1	*5*	1	9.92	98	64	26	9	3	1.78

Italicized numerals represent the class of cells with micronuclear numbers expected from regular and synchronous divisions of micronuclei (2^n). 200 cells were tallied in each sample.

* The cell number at the time of the temperature shift from 28° to 40°C (0 h at 40°C) is arbitrarily set as 1, and the number at subsequent times at 40°C is computed as a decimal fraction of that initial number. The actual initial cell density was 8,155 cells per milliliter in the mo1[a] experiment, 7,810 cells per milliliter in the mo3[b] experiment.

‡ All data on this and the succeeding tables are obtained with mutants in a B strain genetic background, or in wild-type B strain cells.

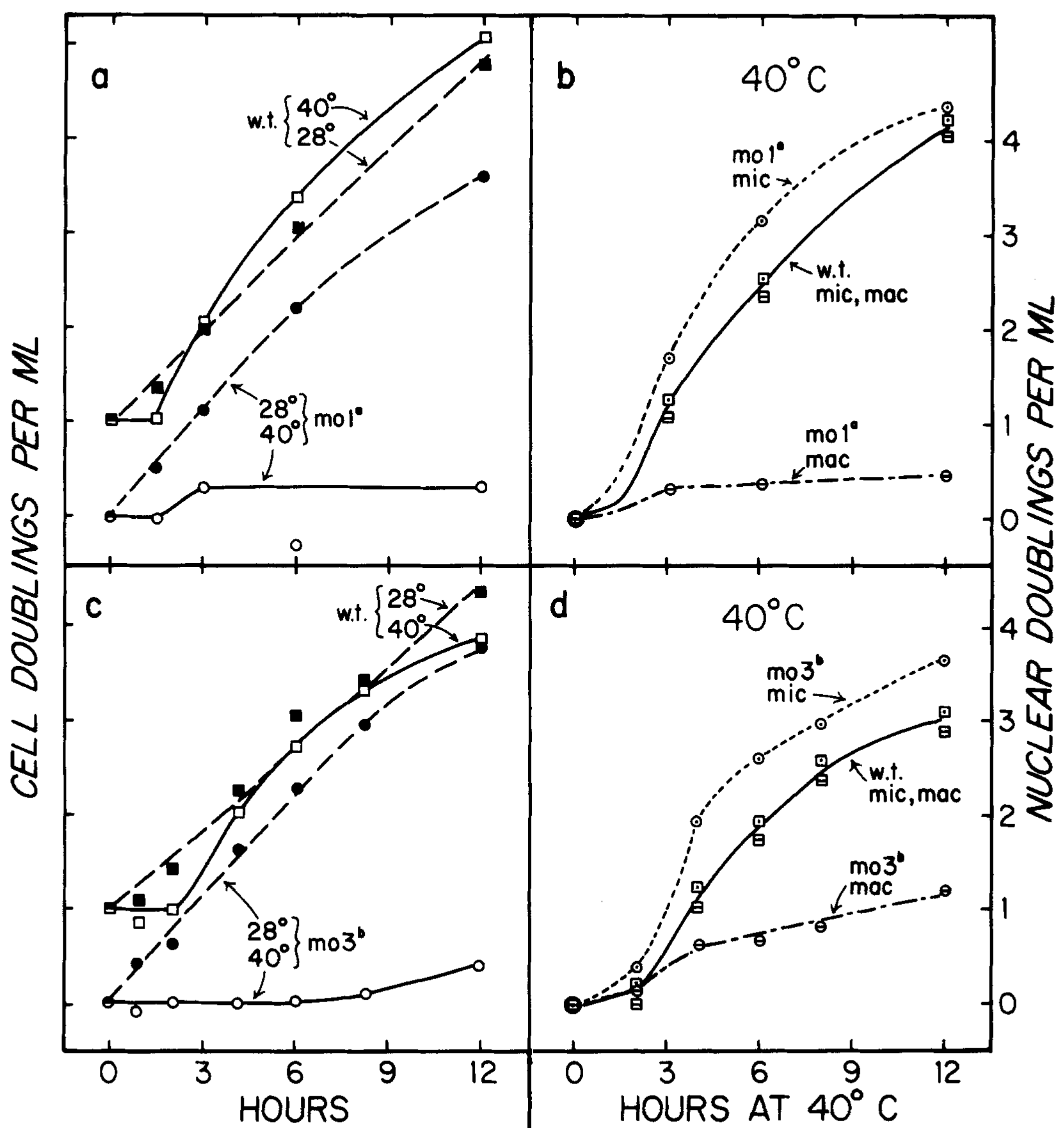
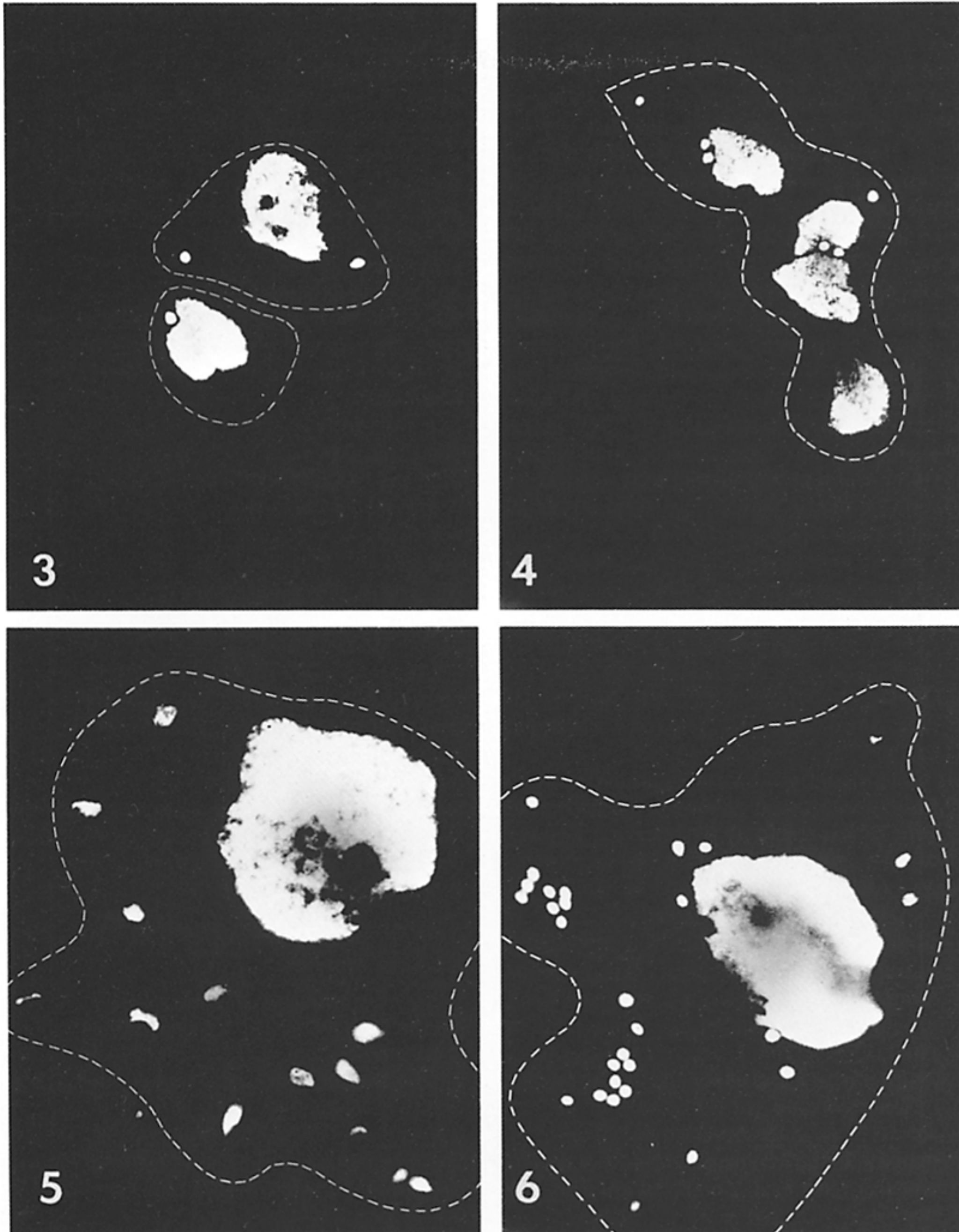


FIGURE 2 Increase in number of cells and of nuclei in wild-type (B strain) cells and in mo1ᵃ and mo3ᵇ mutant cells maintained in PPY medium. Frames *a* and *c* show increase in cell number at 28° and 40°C, frames *b* and *d* increase in number of nuclei at 40°C only. The abscissae represent number of hours of maintenance at 40°C after a shift from 28° to 40°C (all frames), or of growth of control cultures at 28°C (frames *a* and *c*). The ordinates indicate doublings of number of cells (frames *a* and *c*) or nuclei (frames *b* and *d*) per milliliter of culture after the temperature shift. Number of nuclei was calculated by multiplying the number of cells per milliliter by the average number of nuclei per cell. Each interval on the ordinate represents a doubling. The left ordinates are unlabeled because growth curves for wild-type and mutant cultures are displayed with a vertical offset of one doubling in order to increase clarity. Frames *a* and *b* represent an experiment with mo1ᵃ, frames *c* and *d* an experiment with mo3ᵇ. Explanations of symbols: for frames *a* and *c*—■, wild-type cells at 28°C; □, wild-type cells at 40°C; ●, mutant cells at 28°C; ○, mutant cells at 40°C; for frames *b* and *d*—⊡, wild-type micronuclei at 40°C, ⊟, wild-type macronuclei at 40°C; ⊙, mutant micronuclei at 40°C; ⊖, mutant macronuclei at 40°C. The actual initial (0 h) cell numbers in these experiments were: for frames *a* and *b*—wild-type 28°C, 6,925 cells per milliliter; wild-type 40°C, 7,595 cells per milliliter; mo1ᵃ 28°C, 9,945 cells per milliliter; mo1ᵃ 40°C, 8,155 cells per milliliter; for frames *c* and *d*—wild-type 28°C, 11,910 cells per milliliter; wild-type 40°C, 11,360 cells per milliliter; mo3ᵇ 28°C, 8,045 cells per milliliter; mo3ᵇ 40°C, 7,810 cells per milliliter. For further explanation, see the text.



FIGURES 3–6 Wild-type and cell division mutants of *Tetrahymena pyriformis*, syngen 1. Photomicrographs of cells stained with fluorescent Feulgen's (see Materials and Methods for details) before and after transfer from 28°C to 40°C. All figures are printed at the same magnification (× 540).

FIGURE 3 Two mo3^{b} cells grown at the permissive temperature, 28°C. The orientation of the nuclei in the lower cell is characteristic of interphase, while the configuration in the upper cell is characteristic of cells just before the onset of cell division, at which time the micronucleus has divided but the macronucleus has not.

FIGURE 4 An mo3^{b} cell after 4 h of exposure to 40°C. Four macronuclei and six micronuclei are visible on this photograph, with two additional micronuclei most probably superimposed on the macronuclei. This cell has thus gone through two macronuclear divisions and three micronuclear divisions.

FIGURES 5 and 6 Two mo1^{a} cells after 8 h of exposure to 40°C. Each of these cells has one very large undivided macronucleus. The micronuclei have divided many times; in the cell shown in Fig. 5 there are (probably) 16 micronuclei dividing synchronously, whereas in the cell shown in Fig. 6 there are (probably) 32 micronuclei not dividing.

(Fig. 2*b, d*) is that whereas macronuclei multiplied far less in the mutants at 40°C than in wild-type cells, micronuclei multiplied with greater frequency in the mutants during the first 4–6 h at 40°C. This unexpected result was obtained consistently in other experiments as well. Later, the curves for wild-type and mutant micronuclei became roughly parallel. The nonlinearity of both curves probably was primarily due to the slight synchronization of cell cycles that was a nonspecific effect of the temperature shift, as can readily be detected by comparing the cell population growth curves of wild-type cultures shifted from 28° to 40°C with those of cultures maintained continuously at 28°C (Fig. 2*a, c*). The general parallelism of the micronuclear multiplication curves in wild-type and mutant cells suggests that micronuclei in the mutants are cycling normally but with some initial acceleration.

A second approach to the analysis of cell cycles in division-blocked mutants was to study nuclear and cortical events in the same cells. This was possible in some silver-impregnated preparations in which nuclei were clearly visible (see Materials and Methods). 297 out of 299 cells observed with dividing micronuclei also possessed developing oral primordia; conversely, all cells with *predivision* oral primordia at the stages invariably associated with micronuclear division in normally dividing cells (stages 3 and 4; see reference 28) were undergoing micronuclear division in division-blocked cells. On the other hand, virtually all (28 out of 29) of the relatively few cells that manifested *oral replacement* primordia at appropriate stages were not undergoing micronuclear division. Macronuclei in mo3 cells arrested in cytokinesis underwent partial or complete division at the same time that the cells were attempting to constrict.

A third way to assess quantitatively the relationship of nuclear and cortical events in division-blocked cells is to determine, in each silver-impregnated cell, the number of cycles of oral development that have been completed after the temperature shift and then count the number of micro- and macronuclei in that same cell. Unambiguous assessment of the number of oral developmental cycles is possible for two cycles after the shift to 40°C (after that such assessment becomes more difficult because frequent resorption of oral apparatuses accompanied by extensive distortion of cell contours occurs). The results presented in Table II indicate that micronuclear and oral development cycles remained in register in most cells; by contrast, macronuclear cycles lagged behind oral cycles to varying degrees, with the greatest lag in mo1^a, the least in mo3^a (chains of cellular units tend to be more extended in mo3^a than in mo3^b, with more pulling apart and less tendency to telescope and become distorted, which probably accounts for the more successful macronuclear division in mo3^a).

A precise estimate of the time between the first and second oral-micronuclear cycle at 40°C was made for mo1^a in an experiment in which sampling was very frequent: this estimate was between 90 and 95 min. This extremely short cycle contrasts with a generation time of roughly 120 min, estimated for wild-type cells in the same medium (PPY) during the corresponding period (2–4 h after the 28°–40°C temperature shift).

Several lines of evidence thus indicate that when cell division is mutationally blocked, cell cycles consisting of a periodic and integrated execution of micronuclear division and oral morphogenesis continue at an initially somewhat accelerated pace. Macronuclear division, by contrast, is impeded virtually from the start in division-blocked cells, and eventually ceases as cells become larger and more distorted; even in tandem mo3 chains it is rare to see more than four macronuclei in cells that commonly have 8 or 16 micronuclei (e.g., Fig. 4). Macronuclear division thus need not be closely integrated with the cycling of oral development and micronuclear division.

Nuclear DNA Accumulation in Cell Division Mutants

The mo1^a mutant was subjected to a microfluorimetric analysis of changes in nuclear DNA content after a temperature shift from 28° to 40°C. The salient results are presented in Table III. The wild type was analyzed only at 0 and 8 h after the temperature shift, during which interval the average macronuclear DNA content per cell increased nearly twofold, while the micronuclear DNA content increased by about 50%. In the mo1^a homozygote, cell division was totally arrested after the shift to 40°C, and, as described earlier, the number of micronuclei increased rapidly while most cells continued to possess only a single macronucleus. The DNA content per micronucleus increased gradually and by 12 h attained an average value about twice that at 28°C. The DNA content of the single undivided macronucleus increased, on the average, 18-fold during 12 h at 40°C.

TABLE II

Association between Completion of Cortical Developmental Cycles and Divisions of Micronuclei and Macronuclei in Cytokinesis Mutants at 40°C

	Number of cortical cycles completed at 40°C								
	0			1			2		
No. of nuclei	mo1^a	mo3^b	mo3^a	mo1^a	mo3^b	mo3^a	mo1^a	mo3^b	mo3^a
Micronuclei									
1	*346*	*121*	*139*	28	2	3	2		
2	20	30*	12	*168*	*63*	*93*	13		
3				4	3	3	8	4	3
4				20	11	7	*64*	*49*	*31*
5					1	1	5	4	1
6								4	1
7									1
8					1		1	6	4
12								1	
Macronuclei									
1	*366*	*151*	*151*	210	27	7	84	3	
2				*10*	*53*	*100*	9	20	4
3					1			39	13
4								*6*	*24*

Data from silver-impregnated slides. The mo1^a mutant was grown in PPY medium and sampled at 0.5-h intervals from 0.5–4 h after the shift from 28°C to 40°C; the mo3^b and mo3^a mutants were grown in TGVS medium and sampled at 1.5, 3, and 6 h after the shift from 28°C to 40°C. The number of cortical cycles completed was gauged according to the number of completed oral apparatuses plus clearly delineated resorption sites (cf. Fig. 1). During the interval of investigation oral replacement was negligible, so that virtually all oral apparatuses developed at 40°C were of the type that, in the absence of the cytokinesis block, would have been accompanied by cell division.
Italicized numerals indicate cases of precise correspondence of number of nuclear and cortical cycles. The proportion of such cases is underestimated for micronuclei because they divide before the completion of oral development.
* About half of these represent bimicronucleate cells, i.e. cells that had two micronuclei while in the nondeveloping state before the temperature shift.

Virtually all cells showed this dramatic increase in macronuclear DNA content. Although the absolute spread in DNA values became enormous after long periods at 40°C, the *relative* variation showed rather little systematic increase, especially if five cells with exceptionally low macronuclear DNA contents are excluded from the analysis of the 8-, 12-, and 24-h samples (Table III). Hence, despite the failure of macronuclear division in mo1^a cells placed at a nonpermissive temperature, extensive macronuclear DNA replication continued in the great majority of the cells.

A quantitative comparison of micronuclear division with macronuclear DNA synthesis at 40°C within the same wild-type and mo1^a cells is presented in Fig. 7. All data were reduced, as in Fig. 2, to a value per milliliter of culture standarized relative to that observed at the time of the shift from 28° to 40°C. A comparison of mo1^a to wild-type cells was made only at the 8-h point, at which time the mutant and wild-tvpe were rather similar, suggesting that the disturbance in macronuclear DNA synthesis as well as in micronuclear division resulting from blockage of cell division is slight.[5] The time course of increase of macronuclear DNA in mo1^a paralleled that of micronuclear accumulation, with one difference in detail: micronuclear

[5] Figs. 7 and 8 underestimate the *total* increase in macronuclear DNA content in dividing wild-type cells, as the DNA that enters the macronuclear extrusion bodies formed during macronuclear division is not represented. However, extrusion bodies make up a small portion of the total macronuclear DNA in exponentially dividing amicronucleate tetrahymenas (8, 66), and observations by Doerder and DeBault, and by Nanney (see reference 12, p. 486), indicate that DNA extrusion occurs infrequently during macronuclear division in exponential phase syngen 1 cells.

TABLE III

Nuclear DNA Measurements in Wild-Type (wt) and mol[a] Mutant Cells Maintained in PPY Medium at 40°C

Strain	Time at 40°C	Relative cell no.*	No. of cells analyzed	Macronuclear DNA content: $\bar{X} \pm 95\%$ CI	Macronuclear DNA content: s/$\bar{X}$	Micronuclear values: Mean no. per cell	Micronuclear values: DNA content $\bar{X} \pm 95\%$ CI	(n)
	h							
wt	0	1	30	433 ± 63	0.39	1.17	19.3 ± 2.1	(35)
	8	9.90	50	845 ± 84	0.35	1.08	30.5 ± 2.6	(54)
mol[a]	0	1	40	637 ± 66	0.31	1.10	27.7 ± 3.1	(45)
	2	0.97	20	1,171 ± 184	0.34	1.30	39.1 ± 6.5	(22)
	4	0.88	20	2,787 ± 632	0.49	3.45	37.6 ± 2.1	(35)
	6	0.94	30	7,696 ± 1,140	0.40	9.20	48.8 ± 3.7	(61)
	8	1.00	26	7,609 ± 1,263	0.41	16.34	45.2 ± 6.5	(52)
			(24)	(8,081 ± 1,159)	(0.34)‡	(17.50)	[41.6 ± 4.2	(50)]§
	12	1.55	31	11,289 ± 1,593	0.39	24.55	55.3 ± 3.9	(62)
			(30)	(11,649 ± 1,462)	(0.34)‡	(25.33)		
	24	–	20	15,744 ± 3,810	0.52	28.20	61.5 ± 12.1	(47)
			(19)	(17,319 ± 3,337)	(0.40)‡	(29.63)		

$\bar{X}$ = mean DNA content (arbitrary values); 95% CI = 95% confidence interval of the mean, i.e. $\pm t_{0.05}\, s/\sqrt{n}$ (reference 55, p. 61); s/$\bar{X}$ = standard deviation divided by the mean. The number of macronuclei measured is equal to the number of cells analyzed, with each cell possessing a single macronucleus (with one exception, a mol[a] 4-h cell with two macronuclei, both measured, and the sum of the two used in the computations). Micronuclei were measured in each of the cells: all visible micronuclei were measured in the wt samples and in the mol[a] 0-h sample, whereas one to three (generally two) randomly chosen free-lying micronuclei were measured within cells in later mol[a] samples; *n* gives the total number of micronuclei measured, and the averages give the mean DNA content per micronucleus.

* Computed as in Table I. The actual cell density at the time of the 28°–40°C temperature shift (0 h at 40°C) was 1,720 cells per milliliter in the wild-type culture and 2,255 cells per milliliter in the main mol[a] culture. The 12-h value in the latter is probably spurious owing to the very low culture volume remaining at that time. The 24-hour mol[a] sample was derived from a separate culture flask that received a low initial inoculum; cell density was 760 cells per milliliter at the time that the 24-h sample was taken.

‡ Results obtained when a few exceptional cells with extremely low macronuclear DNA values are excluded from the analysis.

§ Results obtained when two micronuclei (in a single cell) with exceptionally high DNA values are excluded from the analysis.

number increased very little during the first 2 h after the temperature shift, while macronuclear DNA content increased rapidly from the start. The early lag in micronuclear division was part of the general transient arrest of cellular development that is a nonspecific response to the temperature shift, observed even in wild-type cells (seen clearly in Fig. 2*a* and *c*). The machinery of macronuclear DNA synthesis was, however, unaffected by the cellular conditions that brought about this arrest.

Limits of Nuclear Division and DNA Synthesis in Nondividing Cells

The 24-h time points in the experiment shown in Table III and Fig. 7 represent an attempt to ascertain the intrinsic cellular limit of micronuclear number and macronuclear DNA content. Before describing the result, a crucial distinction must be made. As cells blocked in division continue to grow, they may reach a limit imposed by culture conditions and enter stationary phase at a level of cell number that is far lower than that in ordinary stationary phase cultures. Hence, if one wishes to ascertain an intrinsic cellular limit of growth, one must initially arrest cell division at an extremely low cell density, to make it possible for cells to reach their intrinsic cellular limits before running out of the external perquisites of culture growth. For this reason, the 24-h sample in the experiment shown in Table III was taken from a separate flask that was shifted from 28° to 40°C at

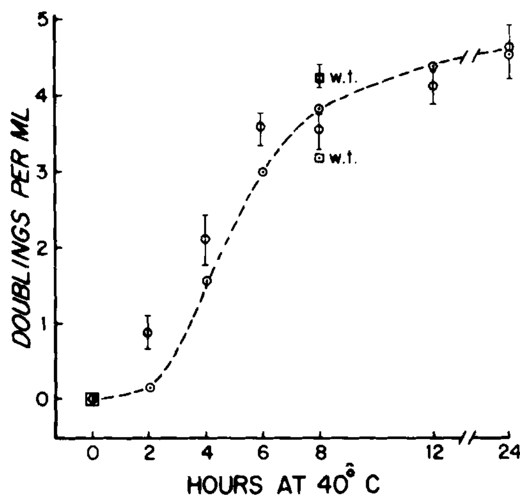


FIGURE 7 Increase in number of micronuclei and of macronuclear DNA content in wild-type and mol[a] mutant cells maintained at 40°C in PPY medium. The abscissa gives the number of hours of maintenance at 40°C after a shift of exponentially growing cells from 28° to 40°C, while the ordinate represents the number of doublings per milliliter of micronuclear number (⊡, ⊙) and of macronuclear DNA content (◫, ⦶). Explanation of symbols: ⊡, number of wild-type micronuclei; ◫, wild-type macronuclear DNA content; ⊙, number of mol[a] micronuclei; ⦶, mol[a] macronuclear DNA content. Vertical bars represent 95% confidence intervals. The initial (0 h) cell numbers in these experiments are given in the footnote of Table III. In computing the nuclear parameters for mol[a], it was assumed that cell density remained at the initial value (cf. Table I). As each measured mol[a] cell had only one macronucleus, the plotted values for mol[a] macronuclear DNA doublings are the same whether expressed on a per milliliter, per cell, or per macronucleus basis. For further explanation, see text.

a very low cell density and was not opened until the 24-h sample was taken (hence this sample is not strictly comparable to all earlier samples which were taken from another flask shifted to 40°C at a higher density). The final cell density of 760 cells per milliliter is probably only slightly if at all higher than the initial density at the time of temperature shift, as mol[a] cultures on the average show only a slight increase in cell number at 40°C (cf. Fig. 2*a*). The cells thus could have undergone about eight doublings of cell mass at 40°C before reaching a level of total protoplasmic mass equivalent to early stationary phase (about 200,000 normal cells per milliliter). However, both micronuclear number and macronuclear DNA content underwent an average of somewhat less than five doublings, at most six in any individual cell. Further, a large proportion of cells in the 24-h sample had irregular numbers of micronuclei, differing from those expected on the basis of division of all micronuclei, and many had two obvious size classes of micronuclei, which rarely occurred in earlier samples. Hence there appears to be an intrinsic cellular limit of about five nuclear cycles that can be carried out normally within an individual *Tetrahymena* cell.

Micronuclear Division and Macronuclear DNA Synthesis in the psm Mutant

The two independent "pseudomacrostome" (psm) isolates increase dramatically in cell size when shifted to nonpermissive temperature, yet proceed through repeated oral replacement cycles instead of dividing (see above). They therefore offer a unique opportunity to assess nuclear behavior in developmentally active cells that are growing but are not going through division cycles. Both psm isolates, however, manifest properties that somewhat hinder analysis: the sterile psm-like variant is normal at 28°C but shows slow and incomplete expression of the developmental pathway switch at 40°C; the psm mutant, on the other hand, is rapidly and completely penetrant at 40°C, but is also variably expressed at 28°C. Nonetheless, studies of number and DNA content of nuclei after a shift to high temperature were conducted with both psm-like and psm. In the experiment with psm, the "permissive" temperature was 23°C instead of 28°C, as the morphogenetic abnormality was expressed in only about 10% of the cells during exponential growth at 23°C.

In both psm-like and psm, the number of nuclei did not increase after cells were switched from the cell division to the oral replacement pathway, and the oral replacement process was not accompanied by any nuclear division. Yet in both mutants there was a clear-cut increase in average DNA content of macronuclei. The results with psm will be presented in detail below; those with psm-like were similar for macronuclei (the micronuclei of psm-like cells have too little DNA for reliable measurement).

In the wild-type controls, all parameters measured remained approximately constant on a per cell basis at 40°C, with an increase of only about 20% in mean micronuclear DNA content and no increase in average macronuclear DNA content

(Table IV). This result is discrepant with that in the earlier experiment (Table III) in which average micronuclear DNA content increased by 50% and macronuclear DNA content doubled over an 8-h time span at 40°C. This discrepancy is difficult to evaluate because of differences in cells (a new inbred sexual generation of the B strain) and in experimental conditions (a more radical temperature shift and considerably higher cell densities in the second experiment than in the first).

In the psm mutant, the number of micronuclei remained approximately constant on both a per cell (Table IV) and a per milliliter of culture (Fig. 8) basis, while mean DNA content per micronucleus increased by about 30%. Virtually all cells retained single macronuclei (only 1% or 2% of any sample had two macronuclei), and the mean macronuclear DNA content increased 2.3-fold during the 12-h experimental period (Table IV). This value is probably an underestimate due to some expression of the abnormal condition even at 23°C; the increase becomes 2.5-fold if compared to the 23°C psm averages from which two cells with abnormally high macronuclear DNA contents were subtracted (Table IV, in parentheses), and 3.7-fold if compared to the macronuclear DNA values of the parallel sample from the wild-type culture. In whatever way these data are regarded, they do establish that a substantial increase in macronuclear DNA content does take place during oral replacement sequences in psm cells maintained in nutrient medium. This increase, however, is considerably less than had been observed in mol[a] cells at 40°C (compare Tables III and IV), and does not keep pace with the increase in macronuclear DNA content of the parallel controls if expressed on a per milliliter of culture rather than a per cell basis (Fig. 8). No comparisons have yet been made with other macromolecular parameters to assess the meaning of this difference in extent of excess DNA accumulation.

DISCUSSION

Cell-Cycle Events in Mutants with Altered Cell Division and Developmental Pathways

Investigation of aspects of the cell cycle of *T. pyriformis* in temperature-sensitive mutants

TABLE IV

Nuclear DNA Measurements in Wild-Type (wt) and psm Mutant Cells Maintained in PPY Medium at 40°C

Strain	Time at 40°C	Relative cell no.*	Macronuclear DNA content: $\bar{X} \pm$ 95% CI	Macronuclear DNA content: s/$\bar{X}$	Micronuclear values: Mean no. per cell	Micronuclear values: $\bar{X} \pm$ 95% CI
	h					(*n*)
wt	0	1	362 ± 28	0.27	1.13	10.3 ± 1.1 (28)
	2	1.15	454 ± 42	0.33	1.12	12.3 ± 1.6 (18)
	4	2.52	364 ± 36	0.34	1.17	11.7 ± 1.6 (17)
	6	4.38	356 ± 32	0.31	1.12	11.7 ± 0.7 (27)
	8	6.66	399 ± 36	0.32	1.11	13.6 ± 1.5 (21)
	12	11.14	327 ± 36	0.39	1.10	12.6 ± 1.1 (33)
psm	0	1	533 ± 88	0.58	1.12	13.7 ± 1.7 (15)
			(486 ± 56)	(0.41)‡		
	2	0.96	687 ± 74	0.38	1.17	14.2 ± 3.9 (6)
	4	0.97	761 ± 124	0.58	1.14	15.4 ± 2.6 (10)
	6	1.12	1055 ± 140	0.47	1.08	16.1 ± 1.7 (19)
	8	1.10	1019 ± 126	0.44	1.09	17.0 ± 1.5 (21)
	12	1.09	1222 ± 154	0.45	1.06	17.8 ± 3.2 (5)

All symbols have the same meaning as in Table III. Macronuclear values are based on measurements within 50 cells, each with a single macronucleus, in each sample. Micronuclei were measured in only some of these cells, and also in some additional cells not included in the sample of macronuclear measurements; one, or rarely two micronuclei were measured per cell; *n* gives the total number of micronuclei measured.

* Computed as in Table I. The actual cell density at the time of 23°–40°C temperature shift (0 h at 40°C) was 8,270 cells per ml in the wild-type culture and 7,163 cells per milliliter in the psm culture.

‡ Results obtained when two cells with exceptionally high macronuclear DNA content were excluded from analysis.

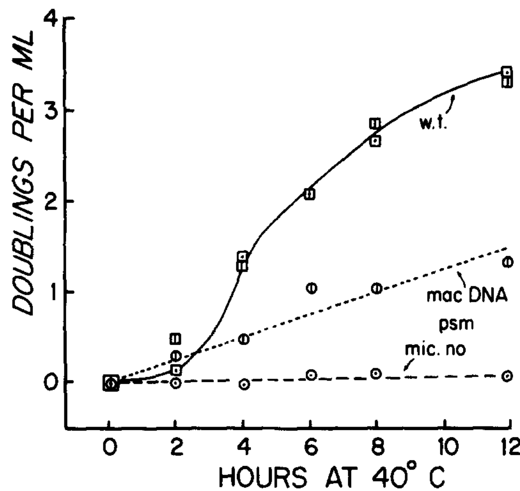


FIGURE 8 Increase in number of micronuclei and of macronuclear DNA content in wild-type and psm mutant cells maintained at 40°C in PPY medium. Ordinates and abscissae as in Fig. 7. Explanation of symbols: ⊡, number of wild-type micronuclei; ⊞, wild-type macronuclear DNA content; ⊙, number of psm micronuclei; ⦶, psm macronuclear DNA content. The initial (0 h) cell numbers in these experiments are given in the footnote of Table IV. Each measured cell had only one macronucleus.

blocked in cell division, and in a mutant that brings about a developmental pathway switch, revealed certain clear-cut dissociations and other patterns of a striking association of events. The major findings may be summarized in the form of six conclusions: (*a*) the micronucleus continues to divide periodically and synchronously in mutants in which cell division is blocked at the nonpermissive temperature (40°C); (*b*) the temporal coordination of micronuclear division with predivision oral development is retained in an apparently unaltered form in these nondividing cells; (*c*) cycles of coordinated micronuclear division and oral development are accelerated in mutants relative to wild-type controls, at least during the first two cycles after onset of cell division blockage at the restrictive temperature; (*d*) division of the macronucleus is inhibited to a varying extent in cell division mutants at 40°C. This inhibition is more severe the earlier the stage in the cell division process at which the mutationally imposed blockage occurs; (*e*) when cells go through development of the oral replacement type, neither the micronucleus nor the macronucleus divides; (*f*) cells growing in nutrient medium accumulate macronuclear DNA, regardless of whether they are in the cell division or oral replacement pathway.

The above conclusions may be divided into those of a positive (*a, b, c, f*) and others of a negative (*d, e*) type. The positive demonstrations are unambiguous, while the negative ones are somewhat more problematical, as they may reveal either intrinsic causal connections between different events or, alternatively, may just reflect pleiotropic effects that uniquely characterize particular mutants. The pleiotropic hypothesis is insufficient to explain the absence of micronuclear division during oral replacement, because failure of micronuclear division is observed not only in psm cells that are capable only of undergoing an unusual form of oral replacement, but also in the cell division mutants at the sporadic occasions when they go through oral replacement instead of the more usual predivision oral development. The occurrence of micronuclear division is thus almost certainly a function of the type of development rather than of the nature of the mutation. An analogous but somewhat weaker argument can be made for the relation of the division of the macronucleus to cytokinesis, as not only was there a clear association between the failure of macronuclear division and the stage of onset of fission arrest in the cell division mutants, but some evidence is also available of a possible similar effect in nonmutant amicronucleate tetrahymenas in which division blockage was imposed by a sublethal high temperature shock (21); in the earlier study the number of macronuclei was shown to be correlated with success of the cell division that followed the temperature shock (see Table II in reference 21).

The temporal coordination of micronuclear division and oral development cycles within cells blocked in cell division is not surprising in view of the many prior observations of nuclear synchrony in natural and artificially created multinucleate systems (41) and of nuclear-cortical coordination revealed by microsurgical manipulation of larger ciliates (11, 40, 53, 60). What is somewhat more surprising is the acceleration of these coordinated cycles in mutant cells blocked in cell division compared to identically treated nonmutant cells. This observation is, however, not without precedent. Fournier and Pardee (16) have recently reported acceleration of cell cycles in Syrian hamster fibroblasts rendered binucleate by cytochalasin-induced division blockage. Our parallel observation made on a very different cell type placed under comparable circumstances suggests that the phenomenon may be general.

There is a gradual increase in micronuclear

DNA content in both mol[a] and psm cells at 40°C. The micronuclear increase is very much less pronounced than the macronuclear accumulation in both mutant lines, but is nonetheless surprising in view of the diploid nature of micronuclei. Such systematic variation of measured micronuclear DNA content has been observed before: Schwartz and Meister (54) noted a variation over a fourfold range in the mean micronuclear DNA content, measured by Feulgen microspectrophotometry, during the entire clonal life cycle of *Paramecium bursaria*. The limitations of the measurement techniques do not allow us to distinguish whether the increase is due to total or partial replications of micronuclear genomes, or some of each. Another possibility that cannot be excluded is that cells may change in some characteristic that affects the uptake of the Feulgen dye, and that the differences in measured micronuclear DNA content may be artifactual. If we were to assume that the average micronuclear DNA content remained the same at all times, then the micronuclear measurements could be used as an internal standard to assess the extent of increase in macronuclear DNA content. Computed in this way, the increase would be 8.9-fold over a 12-h period at 40°C in mol[a], and 1.8-fold over the same period in psm – lesser increases than shown in Tables III and IV, but still substantial.

The continuation of cycles of micronuclear division in temperature-sensitive cell division mutants parallels the continued mitoses observed by Hartwell in cytokinesis mutants of budding yeast. However, the yeast mutants are more limited than *Tetrahymena* in this regard as they go through only two or three mitotic cycles under nonpermissive conditions before terminating both nuclear division and DNA synthesis, often with accompanying lysis (32).

Relationships of Clusters of Cell-Cycle Processes: a Minimal Model of the Cell Cycle in Tetrahymena

Patterns of association of cell cycle events must be interpreted more cautiously than patterns of dissociation, as new mutants may disconnect events hitherto thought to be invariably connected. Nonetheless, the combined mutational, physiological, and microsurgical evidence provides some basis for proposing the existence of three clusters of events in the *Tetrahymena* cell cycle. These three clusters comprise: (*a*) micronuclear division plus the oral development that precedes cell division; (*b*) constriction of the cell and division of the macronucleus; and (*c*) macronuclear DNA replication and some process or event associated with cell growth. The close association of micronuclear division and predivision oral development has been demonstrated positively in this study and negatively in studies on heat-synchronized cells (31, 36) in which both processes are coordinately arrested; a comparably tight association has been demonstrated by microsurgical manipulation of another ciliate species (40). An association between cell constriction and macronuclear division was observed not only in this study but also in an earlier study on physiologically imposed division arrest (21). It should, however, be cautioned that, unlike the probable situation in *Stentor* (10), macronuclear division in *T. pyriformis* is *not* simply achieved by mechanical bisection of the macronucleus by the constricting cell: the division of the macronucleus is normally completed by the middle of cytokinesis, can be differentially affected by colchicine (59, 65), and can occur at a site that does not correspond to that of the cell division furrow (13). Thus, any possible causal link between macronuclear and cell division must be mediated by physiological signals of some kind. Finally, little can be said about the control of macronuclear DNA synthesis except that it is readily dissociable from other cell cycle events (see below) and that it is in some way systematically associated with conditions that promote cell growth. In all conditions thus far investigated in which *T. pyriformis* cells are growing in nutrient medium – regardless of their developmental status – macronuclear DNA accumulation takes place, whereas in all nongrowing situations it does not (e.g., reference 24). This relationship also holds in a dramatic way in polymorphic *Tetrahymena* species (14, 30). The relationship between cell growth and macronuclear DNA synthesis may, however, be highly indirect.

In the remainder of this discussion we will make the assumption that at least the first two of the three clusters postulated above represent reasonably tightly associated sets of events, and consider the relationship *between* clusters. Several such possible relationships are presented, in the form of cell cycle models, in Fig. 9 (derived, with modification, from Fig. 1 of reference 46); the capital letters within the diagrams represent the above-delineated event clusters (C being macronuclear DNA synthesis by itself). We will argue that

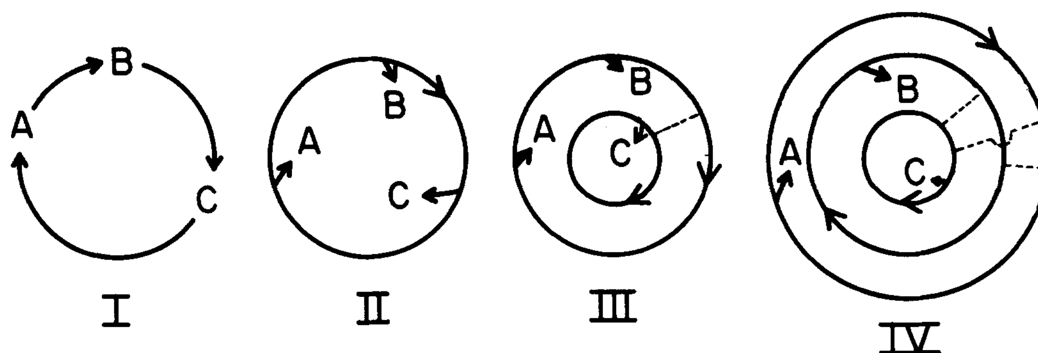


FIGURE 9 Four models for relations of sequential events in the cell cycle of *T. pyriformis*. The capital letters indicate event clusters described in the text: *A*, micronuclear division and oral development; *B*, macronuclear division and cytokinesis; *C*, macronuclear DNA synthesis. Model I, single dependent sequence; model II, independent, single-timer sequence; model III, two parallel independent timer sequences with "check points" (dashed line) affecting phase relations; model IV, three (or multiple) parallel independent timer sequences with check points. Terminology and basic concepts derived from Mitchison (reference 46).

model I is ruled out, model II is unlikely, and model III is the simplest model that is easily reconciled with available data.

We consider first the relationships between clusters A (predivision oral development and micronuclear division) and B (cytokinesis and macronuclear division). Results of this study have shown that A can continue repeatedly while B is blocked, hence A is not sufficient for B. Numerous other observations have shown that normal completion of oral development is not necessary for cytokinesis in *T. pyriformis* (20, 48, 49, 51) or in other ciliates (17, 61)[6]; this strongly suggests that consummation of A is not a prerequisite for B. Hence the direct dependent sequence postulated in model I is without experimental foundation. However, when both sets of processes do occur they follow each other in a relatively close sequence, with only limited temporal slippage thus far observed (63). Further, a wide variety of physiological disturbances affect oral development and division of micronuclei coordinately with macronuclear and cell division (18, 28, 31, 36, 63, 64). Hence, a single timer coordinating event clusters A and B, as shown in models II and III, is suggested, although the available information could still be fit into a concept of separate timers (model IV).

All available evidence strongly suggests that the relationship between macronuclear DNA synthesis ("cluster C") and the other event clusters is exceedingly loose. As shown in this study and earlier in other strains of *T. pyriformis* by Jeffery et al. (38, 39) and Cleffmann (7–9), completion of a round of macronuclear DNA synthesis is not sufficient for cell division; two successive rounds of macronuclear DNA synthesis within one cell division cycle may occur even in undisturbed wild-type cells (8). Conversely, cell division cycles (including oral development) have been observed in the absence of ongoing macronuclear DNA synthesis after heat-shock synchronization in *T. pyriformis* (39, 43) and normally in *T. patula* (30) and *T. paravorax* (14). A temporal setback of cell division brought about by manipulation of temperature affects the events of clusters A and B more severely than macronuclear DNA synthesis, as shown both in this study and in earlier work with the amicronucleate strain GL-C (compare reference 39 to reference 19). The result is that macronuclear DNA replication is brought into new temporal relations to other events in the cell cycle; it is therefore not surprising that two methods of synchronization of strain GL-C (phenoset A; cf. reference 3) by heat shocks both bring about an alteration of the phase relations between macronuclear DNA synthesis and the events of clusters A and B (34, 39, 68, 69). It is these altered phase relations that suggest that DNA synthesis of the macronucleus is timed separately from other events, although physiological mechanisms exist that set certain limitations on the phase relations that are possible (1, 34, 37, 67, 68). Thus it is virtually certain that a separate timer controls mac-

[6] Also T. M. Sonneborn, personal communication.

ronuclear DNA synthesis, and that model II is ruled out. This makes model III the simplest that can reasonably be assumed to apply to the cell cycle in *T. pyriformis*.

Our results and those of others make it obvious that the rigid causal relationships of nuclear DNA synthesis with nuclear and cell division found in other cells (see references 33, 41, and 45) do not apply to ciliate macronuclei. Hence, the relation between the cell cycle and the replication of the nucleus that provides the cell with its major phenotypic support (52, 56) is fundamentally different in ciliates from the relation observed in other cells. This difference is probably related to the fact that the macronucleus is compound, and also to the phenomenon of nuclear dimorphism, which relieves the macronucleus of the burden of competence for meiosis and therefore allows it to assume unconventional genomic organizations. Since the macronucleus is engaged exclusively in current service to the cytoplasm, it is not surprising that its replication should somehow be controlled by a size-related feedback mechanism. There are several types of experimental evidence that such a feedback mechanism does exist in ciliates (29, 47, 66).

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