HEAT SYNCHRONIZATION OF CELL DIVISION IN TETRAHYMENA THERMOPHILA AND A MUTANT, NP1, WITH A TEMPERATURE SENSITIVE DEFECT FOR ORAL DEVELOPMENT

by

PETER B. SUHR-JESSEN

Biological Institute of the Carlsberg Foundation,
16 Tagensvej, DK-2200 Copenhagen N

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Optimal conditions for induction of synchronous cell division in *Tetrahymena thermophila* have been investigated. Wild type cells and a mutant, NP1, with temperature sensitive oral development have been used. NP1 forms no food vacuoles after long exposures to 37°C or higher temperatures.

According to one temperature regime both clones were grown at 30°C and exposed to five 30 min heat shocks at 42.3°C spaced 30 min apart. This treatment resulted in 80% and in 50% increase in cell number over 15 min intervals placed at the time of the first and the second synchronized cell division, respectively. NP1 formed food vacuoles at the same rate as wild type cells.

According to a second temperature regime the cells were grown at 30°C and heat shocks at 41.8°C were spaced one cell generation apart. This treatment resulted in division of about 95% of the cells within a one hour period between the sixth and seventh shock.

Heat shocks were also applied to cells grown at 37°C. Five shocks at 43.1°C spaced 30 min apart synchronized cell divisions of wild type cells but not of mutant cells.

1. INTRODUCTION

The first induction of synchronous division in mass cultures of animal cells was performed by SCHERBAUM and ZEUTHEN in the early fifties (25). They employed the free-living ciliate *Tetrahymena pyriformis* and about 85% of the cell population underwent at least two syn-

chronous divisions. Such cultures were obtained after a series of exposures to elevated temperature spaced 30 min apart («multi-shock program»), and they allow standard chemical and physiological techniques to be used in studies of the vegetative cell cycle (25). As the effects of the heat shocks became better un-

derstood ZEUTHEN improved the method by spacing the shocks one cell generation apart ("one heat shock per cell cycle", (33)).

In contrast to *T. pyriformis* which is amiconucleate, *T. thermophila* has a macro- and a micronucleus, and it can be induced to exhibit sexual cell cycles; during the latter meiosis and cross fertilization takes place (11, 15, 23). This allows mutant isolation and genetic dissection of the life cycle of the cell. Recent advances in *Tetrahymena* genetics (2, 19, 20, 26) have generated many new mutants some of which are defective in cell division (5, 9), oral development (21, 27, 28) and phagocytosis (28). Previously, cell division in *T. thermophila* has been synchronized by use of the »multi heat shock program« (10, 13) and in this laboratory KIRSTEN HAMBURGER obtained promising preliminary results with »one heat shock per cell generation« (personal communication).

It was the purpose of the present study to optimize the conditions for the induction of synchronous cell divisions in wild type *T. thermophila* by use of heat shocks and to investigate if a mutant defective in oral morphogenesis (NP1) can be synchronized. This was of interest because oral morphogenesis may play a role in allowing cell divisions to be synchronized (6, 10, 17, 30, 35).

This work shows that whereas wild type and the mutant NP1 can both be synchronized with respect to cell division under conditions which permit oral development, the mutant can not be synchronized when grown at a temperature which is restrictive for oral development. It is also demonstrated that synchronized mutant cells form food vacuoles at the same rate as wild type cells, and that food vacuole formation ceases during cell division (4, 12, 14).

2. MATERIALS AND METHODS

Tetrahymena thermophila, (earlier *T. pyriformis*, syngen 1) inbred strain D, mating type III (DIII) (16), originally obtained from D. L. NANNEY, and a nitrosoguanidine-induced mutant NP1 (wt/wt (vac⁻, mating type III)) were employed. NP1 has a macronuclear mutation for a heat sensitive development of the oral apparatus (OA) which allows the

development of a functional OA at 30°C but not at 37°C (21, 27). Stocks of both clones were grown axenically at 30°C and 37°C with daily dilutions to about 400 cells per ml. The stocks at each temperature were tested daily for food vacuole formation using India ink, and were used only if the NP1 stock at 37°C contained less than 1% vacuole-forming cells, while the three other cultures all contained more than 90% vacuole forming cells.

Autoclaved 2% proteose peptone broth (Difco laboratories) was supplemented with sodium citrate (2mM), FeCl₃·6H₂O (1mM), CuSO₄·5H₂O (30 μM) and folic acid (1.7 μM). This medium (EPP) allows NP1 to grow without forming food vacuoles (22) with a generation time which is only about 20 min longer than that for wild type cells (unpublished).

The equipment and methods have been previously described by ZEUTHEN and co-workers (34). Cells were grown axenically in 100 ml Erlenmeyer flasks with screw caps containing 10 to 30 ml nutrient medium. The flasks were submerged in a water bath and constantly shaken at a moderate speed; under these conditions oxygen was not a growth limiting factor. The temperature in the water bath was controlled to 0.1°C. A 1,000 Watt heater and a cooling loop were coupled to the temperature controller. Temperature changes from 30°C to 44°C and from 44°C to 30°C took place in less than 5 minutes. Inoculum size was usually between 400 and 1,000 cells per ml and resulted in 10-50,000 cells per ml at the end of the heat shock treatment.

Population growth was followed by withdrawal of duplicate samples of known volumes at 10 or 15 min intervals. One of these was fixed immediately with formaldehyde (final conc., 2% v/v) and counted later by use of an electronic cell counter. The other was immediately studied microscopically and the division index of the live culture was estimated (32). These two methods agreed well in all cases.

Food vacuole formation was followed by the use of ink tests (21). Samples were incubated for 10 min at 30°C with an equal volume of a prewarmed 0.4% ink solution (India ink in EPP) and then fixed with formaldehyde (final conc.,

2% v/v). From each sample, 200 cells were examined by use of a Reichert compound light microscope (12.5×40) and scored as dividers or non-dividers; the number of vacuoles within each cell was also determined.

3. RESULTS

Two heat shock techniques for the induction of synchronous cell divisions in *T. thermophila* have been used (25, 33). Both involve five parameters: the shock- and intershock temperatures, the duration of the shock and intershock periods, and the number of shocks.

NP1 is characterized by its ability to form a functional oral apparatus (OA) at 30°C but not at 37°C, while wild type cells form functional OAs at both temperatures; therefore these were intershock temperatures used with both clones. Throughout most of the study the durations of the shock and intershock periods were 30 min.

Figure 1 show results from experiments in which NP1 was grown at 30°C and treated with five 30 min heat shocks spaced 30 min apart. The shock temperature was varied, and it is

Figure 1. Effects of different shock temperatures on NP1 cells grown at 30°C and treated with five 30 min heat shocks spaced 30 min apart. Time zero is the end of the fifth heat shock.

Figure 2. Effects of different numbers of shock temperatures. NP1 cells were grown at 30°C and treated with 30 min heat shocks at 42.3°C spaced 30 min apart. Time zero is the end of shock treatment.

judged that 42.3°C induces the best division synchrony. Increase or decrease of the shock temperature by as little as 0.1°C influences the division synchrony negatively (Fig. 1, and unpublished).

In another series of experiments the number of heat shocks was varied while the shock temperature was kept at 42.3°C. Figure 2 suggests that five or six heat shocks result in the best division synchrony of NP1.

ZEUTHEN and collaborators showed that the "multi heat shock program", when applied to *T. pyriformis*, resulted in cellular amounts of DNA and protein which were higher than those found in logarithmically growing cells (1, 24, 25). Such effects were minimized when the cells were synchronized with the "one heat shock per cell cycle" procedure (33). Figure 3A, B and C show data from experiments in which NP1 was grown at 30°C and treated with heat shocks spaced one cell generation apart. The increases in population densities at the time of

Figure 3. The best synchronizations of cell divisions obtained with NP1 cells grown at 30°C using "one heat shock per cell cycle" (panels A, B and C) compared to the synchronization regularly obtained using the "multi shock procedure" (Panel D). The numbers in the temperature profile indicate number of heat shocks.

the synchronous divisions are 95%, but cell divisions occur over a long period (more than one hour). Figure 3D shows the results obtained when NP1 cells are treated with 5 heat shocks at 42.3°C spaced 30 min apart. About 80% of the cells divide within 15 min at the time of the first division maximum and about 50% within 15 min at the time of the second. The optimal shock temperature in the "one heat shock per cell cycle" regime is 41.8°C while it is 42.3°C for the "multi shock" program. The reason for this difference is unknown.

Figure 4 shows time between the end of heat shock 5 (EH_5) and the appearance of the first newly divided cell (ID), as a function of the shock temperature in the "multi heat shock program". It is seen that shock temperatures between 42.0°C and 42.6°C give rise to a 60 min period with no cell divisions. The time between

Figure 4. Time between EH_5 and observation of the first dividing cell as a function of the shock temperature for cells grown at 30°C.

EH₂ and division of 50% of the cells was 10 to 20 min longer (not shown). Temperatures lower than 41.8°C do not fully block cell divisions while temperatures above 42.8°C block cell divisions for several hours or kill the cells. The results are consistent with those for *T. pyriformis* (29, 33).

The function of the OA as measured by rate of food vacuole formation in wild type (wt) and NP1 cells grown at 30°C is shown in Fig. 5. In logarithmically growing cultures, 10% of the cells (the dividers) form no vacuoles during a 10 min incubation period with India ink while the

Figure 5. Food vacuole formation in cells grown logarithmically at 30°C (panel A) and in cells treated with five 30 min heat shocks at 42.3°C spaced 30 min apart (panels B, C and D). The first synchronous division takes place around 65 min after end of shock treatment (65'EH₅). Closed circles: DIII. Open circles: NP1.

rest form 1 to 11 vacuoles (Fig. 5A). The average rate of food vacuole formation among vacuole formers is 0.61 vac·min⁻¹ (wt) and 0.60 vac·min⁻¹ (NP1). In heat synchronized cultures, vacuole formation is somewhat disturbed. Immediately after shock treatment (0-10 min after EH₅) 20% of the cells are non-vacuole formers and the average rate among vacuole formers is

0.35 vac·min⁻¹ (wt) and 0.30 vac·min⁻¹ (NP1) (Fig. 5B). During the first synchronous division more than 50% of the cells (the dividers) are non-vacuole formers. The rest of the cells have almost regained normal function of the OA and form in average 0.52 vac·min⁻¹ (wt) and 0.45 vac·min⁻¹ (NP1) (Fig. 5C). Between the first and the second synchronous division the fraction of non-vacuole formers decreases to about 15% and the rest of the cells form in average 0.67 vac·min⁻¹ (wt) and 0.59 vac·min⁻¹ (NP1) (Fig. 5D). Oral morphogenesis prior to the first division must be normal in NP1, otherwise we would have found a higher fraction of NP1 cells which did not form food vacuoles than the fraction found in wild type cells (compare panels D and A in Fig. 5).

NP1 was grown at 37°C before, between and after five successive half-hour heat shocks to the temperatures indicated in Fig. 6, which shows cell multiplication after the shocks. By and large, growth is undisturbed by temperatures below and at 43.1°C but is completely

Figure 6. Effects of different shock temperatures on NP1 cells grown at 37°C and treated with five 30 min heat shocks spaced 30 min apart.

Figure 7. Optimal conditions for induction of synchronous cell divisions in cultures grown at 30°C (panel A) and at 37°C (panel B).

blocked by temperature shocks at 43.4°C and 43.5°C. As indicated also in Fig. 7B no synchrony has been induced, which is contrary to what is obtained with the wild type (DIII) after 5 heat shocks (43.1°C, optimal for DIII grown at 37°C). As shown in Fig. 7A, DIII shows somewhat better synchrony when grown at 30°C and shocked at 42.3°C (optimal for this growth temperature). NP1 cells exposed to similar treatment exhibit equally good synchrony. The final comparison is between mutant cells treated as in panels B and A of Fig. 7; this is lack of synchrony versus synchrony and at the same time it is lack of OA formation versus OA formation. Capacity for synchrony induction appears to be tightly coupled to the development of a new OA in each cell generation. It is gratifying to be able to note this because description of oral morphogenesis has served as one of several monitors of cell syn-

chrony induction in much of the earlier work on heat shock induced cell division in *T. pyriformis*.

4. DISCUSSION

Cell division in *T. thermophila* grown at 30°C can be synchronized by the use of heat shocks spaced either 30 min or one cell cycle apart; the former method yields the highest degree of synchrony (Fig. 3). The shock temperatures inducing synchronous cell divisions range from 42.0°C to 42.6°C with 42.3°C as the optimum (Fig. 4). This interval is narrow compared to the 3°C interval (32-35°C) found for *T. pyriformis* (29, 33), thus temperature control is critical. Under optimal conditions about 80% of the cells divide during the first synchronous division between 60 and 75 min after EH₁ and about 50% of the cells divide again (second synchronous division) 60 to 75 min later (Fig. 7A). The degree of synchrony is lower than that found for the amiconucleate *T. pyriformis* (25) though as high or higher than that found earlier for *T. thermophila* (10, 13). The reason for this difference between species is unknown.

The rates of food vacuole formation in wild type and NP1 cells are identical at 30°C both when log phase cells and when synchronized cells are compared (Fig. 5). The cells are able to form food vacuoles after the shock treatment with the exception of a 20-30 min period around cell division coincident with the remodeling of the deep fiber (3, 4, 6, 17). This is consistent with similar observations on logarithmically growing wild type *T. thermophila* and on *T. pyriformis* (4, 12, 14, 18, 31, and unpublished observations).

Is development of a functional OA obligatory for heat synchronization of cell division in *Tetrahymena*? Such a connection has been suggested by ZEUTHEN and WILLIAMS (35) and others (3, 6, 7, 10, 17, 30). NP1 does not have a functional OA when grown at 37°C and it keeps multiplying almost completely asynchronously during and following heat treatment (Fig. 7), which agrees with the cited model (35). However, the present results make it difficult to accept the model of ZEUTHEN and WILLIAMS in its primary form. Morphological studies of NP1

Figure 8. A model for regulation of oral development and cell division in *Tetrahymena*.

growing at 37°C suggest that oral development is blocked in stage 2 (21, unpublished results). Thus development of a functional OA can not be a condition for the cell to go from division to division, such as the primary model of ZEUTHEN and WILLIAMS suggests (35). A slight modification of their model which accounts for the additional data presented in this report is shown in Fig. 8. Compounds *a* and *b* (probably polypeptides) form a heat-labile precursor molecule *ab* which polymerizes to a heat-stable protein complex, $(ab)_n$, which leads to the formation of a functional OA. In a parallel pathway polypeptides *c* and *d* are required for cell division (CD). During heat shock treatment of wild type cells *ab* is modified to ab^+ , which has a high affinity for *c* and complex $(ab)^+c$ is formed. Before oral development and cell division can take place compounds *a*, *b* and *c* have to be re-synthesized. At 37°C NP1 makes defective *a* and complex *ab* can not be formed. Thus cell division can proceed even during heat shock treatment. The model predicts that mutants with defects in late oral morphogenesis can be heat synchronized with respect to cell division. This remains to be tested.

The above considerations build on the assumption that the mutation(s) in NP1 reflects only at the level of oral development. This, unfortunately, is not fully correct. At 37°C NP1 has a generation time 10% longer than wild type cells and other yet uncovered defects can not be excluded at this stage.

The heat shock system described will be useful for studies on the timing and regulation of DNA synthesis in macro- and micronuclei (11) and for studies on the timing of the functioning of the temperature sensitive gene product(s) in NP1 and other mutants (9, 29). In addition, it provides a tool for screening for mutants super- or insensitive to heat shocks. Such mutants may provide insight into the control mechanisms for oral development, nuclear division and cell division (8, 9, 17, 28, 30, 35).

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