ANALYSIS OF THE SCHEDULE OF DNA REPLICATION IN HEAT-SYNCHRONIZED TETRAHYMENA

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ABSTRACT

The temporal schedule of DNA replication in heat-synchronized Tetrahymena was studied by autoradiographic and cytofluorometric methods. It was shown that some cells, which were synchronized by selection of individual dividing cells or by temporary thymidine starvation, incorporated [3H]thymidine into macronuclei in a periodic fashion during the heat-shock treatment. It was concluded that supernumerary S periods occurred while cell division was blocked by high temperature. The proportion of cells which initiated supernumerary S periods was found to be dependent on the duration of the heat-shock treatment and on the cell cycle stage when the first heat shock was applied. Cytofluorometric measurements of Feulgen-stained macronuclei during the heat-shock treatment indicated that the DNA complement of these cells was substantially increased and probably duplicated during the course of each S period. Estimates of DNA content also suggested that the rate of DNA synthesis progressively declined during long heat-shock treatments. These results indicate that the mechanism which brings about heat-induced division synchrony is not an interruption of the process of DNA replication. Further experiments were concerned with the regulation of DNA synthesis during the first synchronized division cycle. It was shown that participation in DNA synthesis at this time increased as more cells were able to conclude the terminal S period during the preceding heat-shock treatment. It is suggested that a discrete period of time is necessary after the completion of DNA synthesis before another round of DNA synthesis can be initiated.

INTRODUCTION

Despite a number of recent investigations (38, 39), the temporal schedule of macronuclear DNA replication in Tetrahymena pyriformis synchronized by multiple heat shocks (31) has remained a controversial issue (3). Zeuthen and co-workers (1, 6, 15) have concluded, primarily on the basis of evidence obtained from autoradiographic and density labeling experiments, that DNA synthesis continues asynchronously in the form of periodically occurring S periods while cell division is blocked during the heat-shock treatment. This suggestion explained biochemical DNA measurements (16, 22, 29, 30, 40) which revealed varying degrees of DNA accumulation (1.7- to 4-fold) during the treatment, and microspectrophotometric observations (30) that DNA content rose above the normal
G₂ complement in all cells by the end of the treatment (EST). Nevertheless, Byfield and Lee (3)
have recently proposed that DNA synthesis proceeds only very slowly during the treatment and
that some cells do not complete one S period. Byfield and co-workers (3, 4) have revived the
earlier suggestion of Scherbaum et al. (30) that division synchrony is brought about by the selective
inhibition of the replication of a particular fraction of the DNA which may code for division-related
proteins.

Although there is some disagreement concerning the schedule of DNA synthesis during the multi-
ple heat-shock treatment, it is generally accepted that synchronized division (SD1) eventually re-
sults in the phasing of subsequent DNA synthesis (1, 3, 14, 15, 20). However, a considerable propor-
tion of the cell population does not participate in DNA synthesis at any time between the first two
synchronized divisions (1, 15, 20, 23). The purpose of the present investigation was to determine
the schedule of DNA synthesis during the multiple heat-shock treatment and its relationship to the
occurrence of further replication after synchronized division.

Virtually all previous analyses of DNA replication in heat-synchronized Tetrahymena have em-
ployed mass cultures of cells which were randomly distributed over the normal cell cycle when the
heat-shock treatment was initiated (BST). This approach provided sufficient amounts of material
for biochemical analysis, but since DNA synthesis remained asynchronous the schedule of macronu-
clear S periods could not be determined. Our previous communication (20) described an initial
attempt to employ small groups of synchronous cells to investigate the timing of DNA synthesis. It
was found that some cells (67%) subjected to BST in early G₁ participated in two consecutive S peri-
ods without intervening division, but others (33%) engaged in only one S period during the same in-
terval. The present report continues this investigation by characterizing the schedule of DNA syn-
thesis when the heat-shock treatment is initiated at various times during the cell cycle. We have found

1 Abbreviations used in this paper are:
BST: beginning of the synchronizing (multiple heat shock) treatment.
EST: end of a standard 6-shock heat treatment.
M + U: methotrexate plus uridine.
TR: thymidine rescue.
SD1, SD2: synchronized division 1, 2, etc.

that supernumerary S periods are periodically initiated subsequent to subjectation to the treatment
in G₁, S, or G₂. Each of these S periods involve a substantial increase and probably a doubling of
the macronuclear DNA complement. Finally, we have established that the occurrence of DNA syn-
thesis between synchronized divisions is dependent on the schedule of supernumerary S periods during
the treatment. These results are discussed in relation to the mechanism of heat-synchronization and
the regulation of DNA synthesis.

MATERIALS AND METHODS

Cells, Growth Conditions, and Synchronization

Axenic stock cultures of Tetrahymena pyriformis (a-
micronucleate strain GL-C) were maintained in slanted test tube cultures at 28°C. The growth me-
dium consisted of a tryptone-dextrin-vitamins-salts mixture (12). These stock cultures were used to inoc-
ulate larger flask cultures which contained 150 ml of growth medium.

Groups of synchronous cells at a particular stage of the cell cycle were obtained by the selection of divid-
ing cells from flask cultures (32) as described previously (20). These cells were subjected to the heat-
shock treatment in capillary micropipettes (25) during early G₁ (5-10 min after the previous cell division),
early S (50-60 min after the previous cell division), and G₂ (120-140 min after the previous cell division).
The timing of cell cycle stages in T. pyriformis GL-C was described previously (20).

Synchronization was also achieved by the temporary thymidine starvation method of Villadsen
and Zeuthen (34). Methotrexate (Lederle Laboratories, Pearl River, N. Y.) and uridine (M + U), at
respectively concentrations of 0.05 mM and 5.0 mM (19, 36), were added to flask cultures of exponentially
growing cells in order to elicit a thymidine deficiency. Addition of thymidine (5.0 mM) 4 h later resulted in
a moderate degree of synchronization. These cultures were subjected to the heat-shock treatment during mid-
S (40 min after thymidine rescue) and early G₂ (100 min after thymidine rescue).

The standard multiple heat-shock treatment (31) was administered by an automatic temperature-regu-
lating water bath (24) and consisted of six 30-min periods of elevated temperature (34°C) separated by
30-min intershock periods at optimal growth temperature (28°C). In some cases the standard heat-
shock treatment was extended beyond six shocks.

Labeling and Autoradiography

DNA was tritium labeled by expelling cells from their micropipettes into depression slides contain-
DNA Synthetic Schedule in Tetrahymena

Cytophotometric Determination of Macronuclear DNA Quantity

Macronuclear DNA content was determined by fluorescence cytophotometry (2). The cells were dried on slides, fixed in 3:1 ethanol-acetic acid for 15 min, rinsed with water, dehydrated through an ethanol series, and stored in absolute ethanol at +4°C until stained. A modification of Elftman's (10) Feulgen procedure was used in which the acid hydrolysis was performed in N HCl at 40°C for 40 min, the cells stained for 2 h in Schiff's reagent, and bleached in three changes of SO2 water for 15 min each. After staining and mounting in Kleermount (Carolina Biological Supply Co., Burlington, N. C.), the relative amount of DNA per cell was measured microfluorometrically. For these measurements the Leitz-MPV-fluorometer (Leitz, Wetzlar, Germany) was used in an optical arrangement that allowed successive and/or simultaneous illumination with transmitted and incident light, and was similar to that described by Ruch (28). The specific optical arrangement in the Leitz-MPV-Fluorometer used for the measurements utilized a high pressure xenon lamp XBO-150W (Osram, Berlin) in combination with an interference heat-protecting filter, a red-absorbing filter 5-mm BG38, and a Hg546 interference filter to produce a near monochromatic light source at 546 nm. This intense green light was reflected and focused onto the specimen by a dichroic mirror (Leitz) TK580 and a PHACO NPL 100/1.30 objective, respectively. The induced red fluorescence was then collected by the objective lens, passed through the dichroic mirror and a K580 barrier filter (both of which transmit red light), the area of the nucleus optically isolated by the measuring diaphragm of the fluorometer; and the image of the nucleus, projected onto the photomultiplier S-20 type 9558AQ with a quartz window. Only a narrow band of the red spectrum was used for the measurement (2) and was selected by a 60-mm interference wedge filter set at 660 nm. Thus, fluorescence intensities at 660 nm were recorded as photomultiplier voltage output and were proportional to the DNA content (2).

RESULTS

The Schedule of DNA Synthesis

Synchronization by Cell Selection

The first series of experiments was designed to study the timing of DNA synthesis when groups of synchronous cells were subjected to the multiple heat-shock treatment at different times during the cell cycle. The studies reported in the first three sections are short pulse-fix experiments in which cells were incubated with [3H]thymidine for 12 min and then immediately fixed for autoradiography.

EARLY GI CELLS: As shown in Fig. 1, cells which were in early GI at BST participated in an S period (S1) during the first part of the heat-shock treatment. Another S period (S2), which involved about 50% of the cells, was initiated during the latter part of the treatment. These results supplement those which were obtained previously (20) and have now been confirmed and extended to

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2 The terms S1, S2, and S3 are operationally defined in the following manner: (a) S1 is the normal period of DNA replication which follows the last cell division before BST. During S1, the amount of macronuclear DNA rises from typical GI to G2 values. S1 may occur wholly during, partly during, or wholly before the heat-shock treatment, depending on whether the treatment is initiated in GI, S, or G2. (b) S2 refers to the period(s) of DNA replication which occurs after the completion of S1, but before the first synchronized division. During S2 the macronuclear DNA content rises above the usual G2 values. (c) S3
include the timing of DNA synthesis between SD1 and SD2 (Fig. 1). A third S period (S3) was initiated concomitant with macronuclear division and the beginning of cleavage furrow formation at about 70 min after EST. As shown previously (15, 20), S3 was initiated fairly synchronously and in the absence of a detectable G1 period.

**EARLY S CELLS:** Cells subjected to BST in early S continued DNA synthesis until the second intershock period and subsequently initiated S2 (Fig. 2). Participation in S2 was observed to occur in a bimodal fashion with peaks of macronuclear labeling occurring at the conclusion of the fourth heat shock and the middle of the fifth intershock period.

**G2 CELLS:** As shown in Fig. 3, very few cells which were subjected to BST at 120–140 min after the previous division became labeled during the first heat shock and the following intershock period. The majority of these cells had completed DNA replication before BST and were in G2 at the beginning of the initial heat shock. The proportion of labeled macronuclei (S4) began to gradually increase at the beginning of the second heat shock and reached a maximum (60%) during the third heat shock. Participation in S4 was again bimodal, since a second maximum of labeled macronuclei occurred at the conclusion of the fifth heat shock.

Since cell division is known to occur in some very late G2 cells during the first part of the heat-shock treatment (33), further experiments were conducted in order to determine whether S2 may actually have been preceded by a cellular or macronuclear division. A total of 151 cells, each in an individual capillary micropipette, were subjected to BST during G2 (120–140 min after the previous division). Only 13 (9%) were able to complete cytokinesis during the first 240 min of the heat-shock treatment. In contrast, all control cells treated in a similar fashion, but continuously in-

This pattern may be explained by variation in the frequency of initiation as well as decay in original synchrony. A bimodal second S period was observed by other investigators when *Tetrahymena* cells were treated with low concentrations of actinomycin during G1 and failed to divide (8, 17).

At the time of macronuclear fission, S4 was initiated, and at about 135 min after EST 60% of the cells were engaged in DNA synthesis (Fig. 2). The duration of S4 was similar to that observed in exponentially growing cells.

**EARLY S CELLS:** Cells subjected to the 6-shock heat-synchronization treatment in early G1. Synchronous groups of cells were obtained by cell selection methods. Each point represents the percentage of cells with labeled macronuclei after a 12-min pulse of [3H]thymidine. This graph represents a total of 14 experiments involving 341 cells all in early G1 at BST (time 0). The heat-shock regimen is indicated above. The vertical arrows represent the range of time in which cell separation occurred during the first synchronized division.

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cubated at 28°C, were observed to divide during the same time interval. In order to determine whether macronuclear division could have occurred in the absence of cytokinesis (11), the cells subjected to the treatment which remained undivided were stained with methyl green. It was found that each had a single macronucleus. These results support the conclusion that the majority of cells which are in $G_2$ at BST initiate an additional S period during the heat-shock treatment without participating in intervening macronuclear or cellular division.

The results presented in Fig. 3 also show that $S_3$ was initiated at the time of macronuclear division and a maximum of 85% of the cell population was engaged in DNA synthesis at 100 min after EST. In summary, the results of the short pulse-fix experiments indicate (a) that heat-shocked cells periodically initiate macronuclear S periods in the absence of cell division, (b) that DNA synthesis ceases immediately before synchronized division, and (c) that more cells engage in $S_2$ and $S_3$ when BST occurs at progressively later times during the cell cycle.

2 Occasionally multinucleate cells were observed in other experiments. Six binucleate cells and one with an apparently fragmented macronucleus were found among 283 cells that had been subjected to the heat treatment in early $G_1$ and then Feulgen stained and prepared for cytophotometry.

### Table I

The Proportion of Cells Subjected to the Heat-Shock Treatment at a Particular Cell Cycle Stage Which Participate in Each Macronuclear S period

| Cell cycle stage at BST | Macronuclear S period | Assay time | Number of experiments | Macronuclear label | Percent macronuclei labeled |
|-------------------------|-----------------------|------------|-----------------------|-------------------|----------------------------|
| Early $G_1$             | $S_1$                 | BST to BST + 150 min | 4 | 73 | 0 | 100 |
|                         | $S_2$                 | BST + 200 min to EST + 50 min | 6 | 61 | 33 | 65 |
|                         | $S_3$                 | EST + 50 min to EST + 150 min | 6 | 28 | 90 | 24 |
| Early $S$               | $S_1$                 | Isolation to BST + 120 min | 2 | 61 | 0 | 100 |
|                         | $S_2$                 | BST + 120 min to EST + 50 min | 5 | 177 | 25 | 88 |
|                         | $S_3$                 | EST + 50 min to EST + 150 min | 6 | 148 | 49 | 75 |
| $G_2$                   | $S_1$                 | Isolation to BST | 2 | 47 | 0 | 100 |
|                         | $S_2$                 | BST to EST + 50 min | 2 | 47 | 2 | 96 |
|                         | $S_3$                 | EST + 50 min to EST + 150 min | 2 | 45 | 7 | 86 |

BST = beginning of heat shock treatment; EST = end of standard 6-shock treatment.

### Proportion of Cells Involved in Each S Period

Additional experiments, involving longer labeling periods (long pulse-fix experiments), were conducted in order to accurately determine the proportion of cells which participated in each of the $S$ periods. The results indicated that all cells participated in $S_1$ (Table I). This was found to be true whether $S_1$ occurred completely before BST ($G_2$ cells), was initiated before BST and completed during the treatment (early $S$ cells), or took place entirely within the treatment (early $G_1$ cells). It was also confirmed that the proportion of cells which participated in $S_2$ and $S_3$ gradually increased as BST occurred at later times during the cell cycle and the interdivision time was correspondingly extended.

In order to determine whether all cells participated in DNA synthesis during $S_2$ or $S_3$, experiments were conducted in which early $G_1$ cells were continuously incubated with $[^3H]$thymidine from the beginning of the fourth heat shock (first initiation of $S_2$) until 150 min after EST (conclusion of $S_3$). Six experiments of this type were performed and in each the majority of cells had labeled macronuclei. A total of 223 out of 228, or 98%, of the cells had engaged in DNA synthesis. These data establish that each cell participates in either $S_2$ or $S_3$, but do not exclude the possibility that some cells may participate in both $S$ periods. The latter
Figure 4  The timing of macronuclear DNA synthesis in T. pyriformis synchronized by temporary thymidine starvation and afterwards: (A) incubated continuously at 28°C, (B) subjected to the 6-shock heat-synchronization treatment during mid-S (40 min after time 0), (C) subjected to the 6-shock heat-synchronization treatment in early G2 (100 min after time 0). Time 0 represents the time of reversal of starvation by addition of thymidine to the medium. Each filled point (●●●) represents the percentage of macronuclei labeled (from at least 900 cells) during a 12-min pulse of [3H]thymidine. Cell number (○○○) was assayed using a model A Coulter Counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). The heat-shock regimen is indicated in the upper portion of frames B and C.

is obviously true for most cells which were in early S or G2 at BST (Table I).

Synchronization by Thymidine Starvation

Synchronization of DNA synthesis can also be achieved by treatment of mass cultures of exponentially growing cells with M + U, which block the uptake and synthesis of thymidine (36), followed by addition of excess thymidine (34). This method was utilized in an attempt to determine whether the results obtained by the more precise method of cell selection could be confirmed with mass cultures of cells. Cultures of exponentially growing cells were incubated with M + U for 4 h after which thymidine was added to the medium. As shown in Fig. 4 A, cells treated in this manner, and labeled during 12-min exposures to [3H]thymidine, exhibited a moderate degree of synchronization of DNA synthesis. Nearly 80% of the population exhibited labeled macronuclei immediately after thymidine rescue (TR). This proportion could not be increased using [3H]thymidine alone as a rescue agent. DNA synthesis was completed within the next 100 min and cell division occurred within 150–250 min after TR. The temporal pattern of DNA synthesis illustrated in Fig. 4B and C was observed when thymidine-starved cells were subjected to the standard heat-shock treatment after TR. After completing one S period, cultures which consisted of predominantly mid-S and early G2 cells at BST (40 min and 100 min after TR, respectively) participated in further DNA synthesis in a bimodal fashion during the remainder of the heat-shock treatment. Furthermore, cell numbers did not increase during the treatment (Fig. 4), and it was concluded that supernumerary S periods occurred in the absence of intervening cellular di-
vision. These results indicate that the pattern of DNA synthesis which occurred when cells were synchronized by cell selection, and subjected to the heat-shock treatment in capillary micropipettes, is similar to that which was observed for mass cultures synchronized by thymidine starvation. Thus this pattern actually reflects the alterations in the DNA synthetic schedule produced by subjecting exponentially growing cells to a multiple heat-shock treatment.

**Changes in Macronuclear DNA Content**

Changes in macronuclear DNA content during the multiple heat-shock treatment were measured by fluorescence cytophotometry. DNA content was measured after both asynchronous mass cultures and small groups of synchronous G1 cells were subjected to the treatment. The evidence obtained from these studies is compatible with our autoradiographic results.

Measurements obtained from originally asynchronous cells showed a progressive increase in DNA content throughout the treatment (Fig. 5). The mean DNA content doubled shortly after the end of the third heat shock and tripled just before synchronized division (Table II). Application of additional heat-shock cycles resulted in a further increase with fourfold DNA values being reached between the end of the ninth and twelfth shocks (Table II). These results indicate that the process of heat-synchronization does not block the continuation of DNA duplication.

Similar results were obtained when early G1 cells were subjected to the treatment (Fig. 6). As would be expected, the macronuclear DNA contents of these cells corresponded to those of the lower half of the distribution in the asynchronous population at BST (compare Figs. 5 A and 6 A).

**Table II**

| Fixation time | Asynchronous cells | Synchronous G1 cells |
|---------------|--------------------|----------------------|
|               | N | X  | SD (±%) | SE | F | N | X  | SD (±%) | SE | F |
| BST           | 50 | 1396 | 343 (24.6) | 48.5 | 1.0 | 41 | 1010 | 201 (±20.0) | 31.4 | 1.0 |
| HS 3 + 20 min | 50 | 2864 | 679 (23.7) | 96.0 | 2.1 | 95 | 1818 | 433 (±24.9) | 46.5 | 1.8 |
| HS 6          | - | - | - | - | - | 88 | 2611 | 990 (±35.6) | 99.1 | 2.6 |
| EST + 45 min  | 50 | 4003 | 903 (22.1) | 127.7 | 2.9 | 26 | 3162 | 779 (±24.6) | 152.8 | 3.1 |
| HS 9          | 50 | 4929 | 1503 (30.5) | 212.6 | 3.5 | 61 | 3654 | 924 (±25.3) | 118.3 | 3.6 |
| HS 12         | 50 | 7021 | 2869 (40.9) | 405.7 | 5.0 | - | - | - | - | - |
| HS 15         | - | - | - | - | - | 13 | 5892 | 1684 (±28.6) | 467.0 | 5.8 |

BST = beginning of heat shock treatment; HS = heat shock; EST = end of standard 6-shock treatment; N = cell number; X = mean DNA content (arbitrary units); SD = standard deviation; SE = standard error; F = DNA content fold difference from mean BST quantity (1.0).
The variation observed in measurements of G1 macronuclei is comparable to that found in T. pyriformis HSM by Cleffmann using scanning microspectrophotometry (9), and probably reflects true variation of DNA content in different macronuclei (9). The mean DNA content of the G1 cells was found to increase 1.8-fold between BST and 20 min after the end of the third heat shock (Table II). This result indicates that at least in most cells the total complement of macronuclear DNA is replicated during the period shown by the autoradiographic experiments (Fig. 1) to encompass S1. By the end of the sixth shock, the mean DNA content was intermediate between two- and fourfold values, but variability was very great (Table II, Fig. 6), with the lower part of the distribution coincident with that observed at the end of S1 (compare distributions B and C in Fig. 6).

This result is consistent with the findings of the autoradiographic studies, in which by the end of the sixth shock a new S period (S2) was completed or underway in a portion of the cell population (Fig. 1). Further increases in DNA content to above the fourfold value were noted when the treatment was extended to include fifteen shocks (Fig. 6 D-E; Table II). These results indicate that DNA replication is also complete during S2, but the extensive time required for doubling also suggests that the rate of replication is markedly decreased.

The Regulation of DNA Synthesis

Although DNA synthesis becomes synchronized at the time of SD1, a large number of cells do not participate (1, 15, 20, 23). Our autoradiographic experiments indicated that the proportion of cells which engaged in S1 could be increased by lengthening the preceding interdivision period (i.e., subjecting cells to BST at later times during the cell cycle). Further experiments were conducted to determine if the number of cells engaged in S2 could be altered by manipulating the length of the heat-shock treatment. Early G1 cells were subjected to treatments consisting of from three to nine shocks. After the conclusion of the terminal shock, the cells were incubated at 28°C, exposed to [3H]thymidine at SD1 (initiation of S2), and fixed for autoradiography at 160 min after EST (end of S2). The results of these experiments are shown in Fig. 7 (Curve 1). It was found that engagement in S2 was indeed related to the length of the treatment. Almost all the cells engaged in S2 when the treatment lasted for only three or four shocks. However, a dramatic decline in participation was observed in treatments consisting of five and six shocks. Treatments consisting of from seven to nine shocks were followed by a gradual increase in engagement in S2. The abrupt decrease in S2 participation after five and six shock treatments is correlated to the timing of initiation of S2 (Fig. 7, Curve 2). It is suggested that the initiation and continuation of a new S period late during a particular heat-shock treatment abolishes the ability to initiate another S period as a response to the synchronized division that occurs shortly thereafter. This would imply that a discrete time period must lapse from the termination of one replication round to the beginning of another.

DISCUSSION

The evidence presented indicates that Tetrahymena cells subjected to a multiple heat-shock treatment participate in supernumerary S periods without intervening cell division. It is also shown that during these S periods the macronuclear DNA complement is substantially increased and probably com-
partly replicated. These findings do not support the proposal that division synchrony is induced by a selective inhibition of a particular DNA fraction (3, 4). A convincing argument against this hypothesis is the observation that cells that are in the G2 period at BST subsequently participate in further DNA synthesis but do not divide. The hypothesis predicts that these cells would divide during the treatment since they had completed a normal DNA duplication before BST (9). It is still possible that the hypothetical DNA fraction could be a G2-replicating DNA species, such as nuclear DNA (7), which would still be partially unreplicated at BST. However, the demonstration that cells are capable of undergoing division if the heat-shock treatment is terminated and further DNA synthesis is blocked with M + U treatment after S1 (19) makes this possibility unlikely. Thus it appears that the original conclusion of Zeuthen (35), that division synchrony is based on the disruption of thermo-labile processes directly related to cytokinesis, rather than to DNA synthesis, remains the most tenable hypothesis.

A modification in both the frequency of initiation of DNA synthesis and the rate of DNA replication in progress during the heat-shock treatment was observed in this study. The consequence of initiation modification was to alter the timing of S2 so that it occurred in a bimodal fashion. McDonald (23) and Prescott (26) first demonstrated the existence of a variation in the individual generation times of *T. pyriformis*, and recent studies by Jauker and Cleffmann (18) have established that this variation may be described by regular oscillations of long and short generation times. It appears that the bimodality of S2 during the treatment may be explained by a retention of this oscillatory pattern, probably in a somewhat exaggerated form. The modification in rate of DNA replication was found to be extensive only late in the heat-shock treatment and resulted in a substantial extension in the duration of later S periods.

One of the most interesting questions related to the timing of DNA synthesis in heat-shocked *Tetrahymena* is why only a part of the population undergoes replication between SD1 and SD2. Hjelm and Zeuthen (15) suggested that engagement in S2 may be related to the time of completion of S1. Subsequent experiments showed that cells involved in DNA synthesis late in the treatment failed to take part in S2 (37). The results of our experiments indicate that cells which engage in S1 have either not participated in S2 or have concluded S2 before the beginning of a critical time late in the treatment. During this time period certain preparations necessary for the initiation of DNA synthesis, such as relevant RNA and protein synthesis (3), could be completed. These events apparently cannot be executed while DNA synthesis is in progress. The above hypothesis emphasizes a fundamental difference which distinguishes *Tetrahymena*, and possibly other eukaryotes, from prokaryotes in which, under conditions of rapid growth and proliferation, additional S periods (replication forks) are initiated before the preceding replication round is completed (13). The only indication of overlapping S periods in a eukaryote is the rare observation of a double set of replication bands in the ciliate *Euplotes eurystomus* (21; J. Ruffolo, personal communication). The rarity of the phenomenon, however, makes thorough investigation and interpretation of this curious exception very difficult.

Since it has been shown that cells which do not participate in S2 between SD1 and SD2 next engage in DNA synthesis between SD2 and SD3 (1), the signal to respond to macronuclear division by initiating DNA synthesis must be short-lived. Cells that do not immediately respond are required to wait one complete cell cycle to eventually initiate replication.

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