

AMOUNT OF DNA PRODUCED DURING EXTRA S PHASES IN *TETRAHYMENA*

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The significance of DNA replication for cell growth and cell multiplication is documented by an increasing number of publications (6). It has been shown that a certain amount of DNA is a necessary prerequisite for nuclear or cellular division to occur (5, 10, 11). The link between one replication round and one cell division, however, can be broken in several ways. In ciliates, S phases may occur without preceding cell division in response to a steady loss of DNA during cell divisions (5) or if cell division is inhibited, e.g., by inhibitors (4) or by repetitive heat shocks (7, 12). DNA synthesis during heat shock treatment has been analyzed by Jeffrey et al. (8, 9). They found that when a six shock treatment was applied, two replication periods occurred. Although these experiments give some information about the initiation of DNA replication, the conditions for progress and termination remain largely unknown. The present study tests whether additional replication rounds induced under experimental conditions result in duplication

of the amount of DNA. For this purpose, DNA synthesis was investigated in *Tetrahymena* treated with heat shocks which were started at known stages of the cell cycle. The experiments to be described were designed to determine by cytophotometry and autoradiography the amount of DNA per macronucleus during heat treatment. It will be shown that DNA synthesis, once it has been started in a given cell, leads to a doubling of the amount of DNA and that the cells in which additional S phases occur are those with the lowest DNA content.

MATERIALS AND METHODS

Tetrahymena pyriformis strain GL (amiconucleate) was grown axenically in test tubes 3 cm in diameter in a 2% proteose peptone medium containing 1 g/liter Liver Fraction L (Wilson Laboratories, Chicago, Ill.) and the following salts (milligrams/liter): MgSO_4 (2,000), $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (500), MnCl_2 (10), ZnCl_2 (1), CaCl_2 (1,000), CuCl_2 (100), FeCl_3 (25). The cultures were maintained in exponential growth at 28–29°C.

Samples of cells in the same known cycle stage were obtained by selecting cells with a constriction pipette and transferring them with approximately 5 μ l of medium to glass capillaries. Samples of 20 synchronous dividers can easily be obtained within 2 min. The capillaries were closed by melting the tips and were kept in a water bath.

The standard heat shock treatment consisted of six 30-min heat shocks separated by 30 min at culture temperature. For rhythmically changing the temperature from 28.5 to 34.5°C, a programming unit which allows every desired schedule was used (B. Braun Apparatebau, Melsungen, Germany). The higher and the lower temperatures were reached in less than 5 min.

For determination of synthesizing times within individual cells and for estimation of synthesizing activity, cells were labeled with [6-³H]thymidine (The Radiochemical Centre, Amersham, England: sp act 27 Ci/mmol). The cells were incubated in depression slides (error by dilution of the isotope less than 5%), the isotope concentration ranging from 5 to 10 μ Ci/ml medium.

At the end of the labeling period, cells were dried onto a gelatin-coated slide, fixed with alcohol acetic acid, washed for 5 min with 5% TCA at 5°C, and washed free of acid by three changes of 70% alcohol. Autoradiography was carried out with Kodak NTB 3 emulsion (Eastman Kodak Co., Rochester, N.Y.). After drying, the slides were stored for exposure at 5°C. The slides were developed for 2 min with Kodak D 11 developer, fixed, stained with toluidine blue, and mounted.

The amount of DNA per macronucleus was determined by scanning microspectrophotometry at 450 nm after Feulgen staining. Hydrolysis was carried out with 1 N HCl for 15 min at 55°C. For cytophotometry, a Zeiss Scanning Microscope Photometer SMP 05 was used. The diameter of the diaphragm was 0.5 μ m. The total extinction was calculated by an accumulation unit (Zeiss).

RESULTS

If samples of synchronous cells are treated with six heat shocks, the cells are prevented from division and the average DNA content of the macronucleus doubles within the same time as in untreated cells (Fig. 1). Doubling at the proper time occurs regardless of whether the shock treatment was started during G₁ or during S, provided the first shock does not interfere with the onset of the DNA-synthesizing period. Naturally, one finds a regular doubling of DNA in cells which are submitted to heat shock treatment during G₂, since these cells replicated DNA under normal temperature. After a certain period of time of no change, the average DNA content increases again. The duration of the synthesis-free period varies from 90 min to 3 h. Since in untreated cells the duration of G₂ + M + G₁ is 90 min, this means

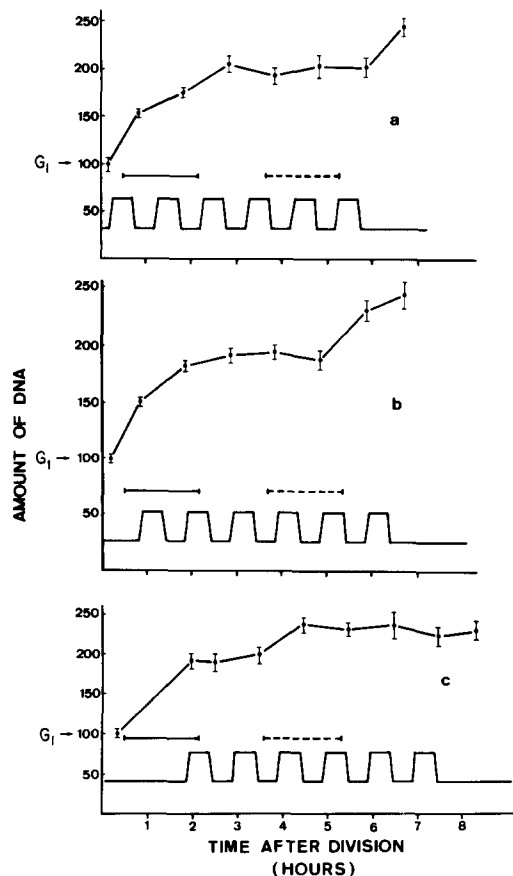


FIGURE 1 Mean DNA content per macronucleus of synchronous *Tetrahymena* cells during treatment with 6 heat shocks (percent increase over G₁ value). At time zero, dividing cells were collected and cultured at the temperature regimen indicated in the graphs. At different times during the treatment, samples were fixed and the amount of Feulgen-stainable material was determined. Each point represents the average of 25–40 cells. The temperature changed from 28.5°C to 34.5°C. Cells did not divide during this treatment. The horizontal lines indicate the periods during which synchronous cultures would have synthesized DNA at normal culture conditions. Vertical bars give standard errors. Heat shock treatment started (a) before the beginning of S phase (macronuclear G₁), (b) during S phase, (c) at the end of S phase (most cells probably in G₂).

that in some cases there is no delay in the onset of the next DNA-synthesizing period (Fig. 1 c) whereas in other cases different degrees of delay occur (Fig. 1 a and b). This delay and a certain reduction in synchrony are due to the variation of timing in individual cells so that some cells may be hit by a heat shock when they are ready to start

their replication period, thereby delaying the initiation of DNA synthesis (9).

The result of this second synthesizing period is a 30–40% increase in the amount of DNA. This degree of increase was found in all cases regardless of the cell cycle stage at which the heat shock treatment started. Extension of the heat shock treatment to 11 heat shocks shows that DNA replication can be initiated a third time with the same characteristics (Fig. 2): at approximately 90 min after the end of the second replication round, the average DNA content increases again at about 30%.

These results clearly demonstrate that DNA synthesis continues in a rhythmical pattern although cell division has been suppressed. Synthesizing periods are separated by synthesis-free intervals. During each additional synthesizing phase, only part of the DNA content of the population is replicated. This is in agreement with the data found by Jeffery et al. with labeling experiments (8). The question whether the result of an additional DNA-synthesizing period is a full duplication in individual cells remains to be answered.

Determination of newly formed DNA in individual cells is difficult for the following reasons: (a) DNA content per macronucleus varies considerably, and therefore, the average DNA content in a population of cells cannot be taken as representative for individual cells, (b) it is impossible to measure the DNA content in single cells before and after DNA synthesis. Therefore, the following labeling experiments were performed. Samples of 35 cells were treated by six heat shocks beginning during G₁. The first sample was exposed to [³H]thymidine from 0 to 150 min after division, which includes the first S phase, and then fixed. Another sample was labeled from 280

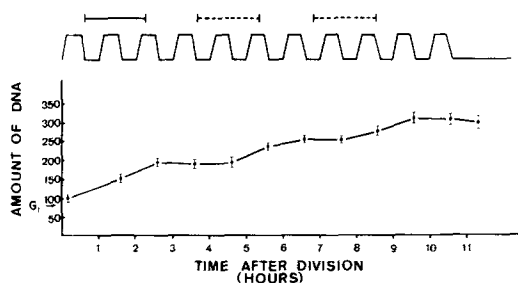


FIGURE 2 Mean DNA content per macronucleus of *Tetrahymena* cells during prolonged treatment with heat shocks (11 shocks). Experimental conditions are the same as described in Fig. 1.

to 380 min after division, which includes the second S phase. Table I shows the results of two experiments. It can be seen that during the first S phase all cells are labeled, whereas between 30% and 40% of the cells participate in the second replication round. There is no significant difference in the amount of label incorporated. The fact that the grain number in the second group tends to be lower is explained by the higher degree of variation in the second replication round. Therefore, the labeling period may not cover the complete synthesizing period in all cells.

In another experiment, cells were incubated with [³H]thymidine during the whole length of the heat shock treatment. After autoradiography, silver grains were counted per macronucleus. The frequency distribution of grain numbers shown in Fig. 3 is not compatible with a normal distribution but rather shows a bimodal pattern.¹ If the whole population is divided into two groups consisting of cells with less and those with more than 64 silver grains, one finds 63 cells with an average of 42.3 silver grains and 21 cells with an average of 86 silver grains. That means that in a population that has increased its DNA content during the experiment by 130–140%, one finds that approximately 30% of the cells have incorporated twice as much isotope as the rest. Similar results have been obtained in three more experiments of this kind. Cells with a higher radioactivity have duplicated their DNA during the second S phase, giving rise to the increase in the whole population.

Analysis of the frequency distribution of DNA contents during the course of heat shock treatment gives further evidence that cells, once they have initiated DNA synthesis, will complete doubling of their DNA content. Fig. 4 shows the frequency distribution of cells before the first, between the first and the second, and after the second synthesizing period. During the first synthesizing period the average DNA content exactly doubles, and the variation coefficient remains constant, confirming earlier results (5). During the additional S phase, the average rises by 30%. Variation strongly increases due to the fact that, after the second S phase, cells between 90 and 120 extinction units (which are present before this replication round) are not to be found. Instead, cells with DNA

¹ Statistical tests performed by Prof. R. König (Fachbereich Psychologie, Universität Giessen) show that the probability for normal distribution is less than 0.5%.

TABLE I
Number of Replicating Cells and Amount of [³H]Thymidine Incorporated during First and Second S Phase

Exp. no.	0-150 min			180-280 min		
	<i>n</i>	Number of cells labeled	Average number of silver grains over macronucl. ± SE	<i>n</i>	Number of cells labeled	Average number of silver grains over macronucl. ± SE
1	35	32 (92%)	17.7 ± 1.3	36	12 (33%)	15.4 ± 1.0
2	66	54 (97%)	30.1 ± 2.8	65	27 (41%)	27.6 ± 2.1

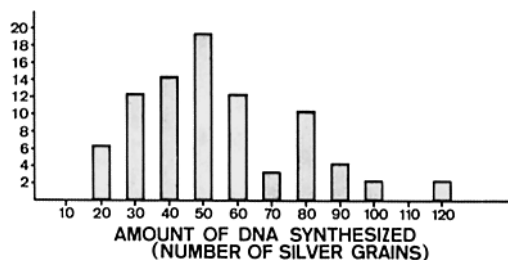


FIGURE 3 Amount of [³H]thymidine incorporated per macronucleus during entire heat shock treatment. 84 synchronous cells were subjected to six shocks beginning during G₁ and simultaneously incubated with 0.2 μCi [6-³H]thymidine/ml of medium. 30 min after the end of treatment, the cells were fixed, prepared for autoradiography, and silver grains of the macronucleus were counted. Abscissa gives the classes for number of silver grains. Ordinate gives the number of cells in each class.

contents between 200 and 250 extinction units, which is about twice as much, are present. This means that the cells with the lowest DNA values doubled their DNA content during the second replication round.

DISCUSSION

Tetrahymena cells treated with repetitive sublethal heat shocks are prevented from division but continue to accumulate DNA (1, 3, 7, 12). The present study, in which synchronous cells are subjected to heat shock treatment, shows that they do so in a stepwise manner. Limited synthesizing periods are separated by periods with no increase in DNA amount per cell. Jeffery et al. found in their labeling experiments the same characteristics of synthesizing activity (8, 9). In accordance with Jeffery's 100% labeling index in the first S phase, we find a doubling in amount of DNA per cell. Different from their finding of 65% and higher labeling indices during the second synthesizing period,

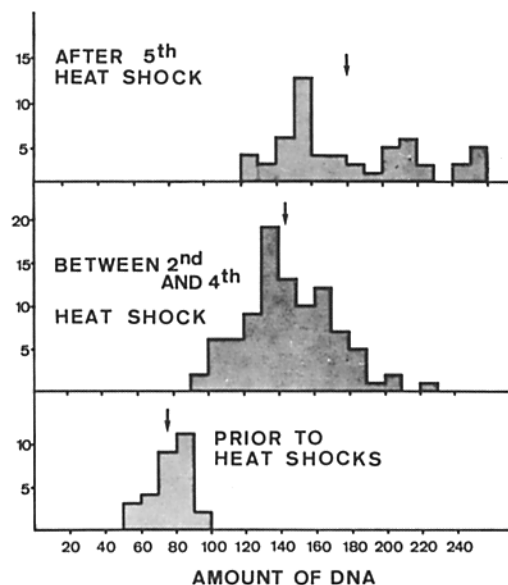


FIGURE 4 Frequency distribution of Feulgen-stainable DNA amount in synchronous *Tetrahymena* cells subjected to heat shocks. Cells were fixed at different phases of the treatment. Treatment started during S (according to the schedule indicated in Fig. 1 b). After Feulgen staining and cytophotometry, the DNA contents of individual macronuclei were grouped in classes as given in the abscissa. Number of individuals in each class is indicated on the ordinate.

we find invariably an increase of 30-40% in the mean DNA amount. Our experiments further show that 30-40% of the cells are labeled in the second S phase. Differences in culture conditions and/or differences in the properties of the strains (both strains originated from the same culture but were maintained at different places for many years) may be the reason for quantitatively different results.

In every case, however, the additional synthesiz-

ing phase is characterized by the fact that only part of the population is involved. Under given conditions, this part is fairly constant. These cells complete the additional S phase by replicating the full complement of DNA just once. Since earlier findings of additional S phases in untreated cells also demonstrated an all-or-none replication of DNA (5), partial replication of the genetic material appears not to occur. Once the cell's mechanism of initiation induces a replication round, it will not come to an end without replicating all parts of DNA. Up to now, no physiological conditions can be found that interrupt a replication process.

The results described also contribute to the problem of initiation of DNA synthesis. The data presented in Fig. 4 suggest that the cells with the smallest DNA content undergo an additional S phase. This confirms our earlier findings concerning additional phases in untreated cells. But, in addition, our present findings show that this threshold of DNA content characteristic for initiation of a new S phase is not absolute, because prolonged treatment results in further S phases, thus increasing the DNA content. Rather, the DNA content in relation to the growing cell mass is critical for initiation. The cell must have the ability to perceive the DNA content and compare it with some other cell constituent. Several lines of evidence suggest that the amount of DNA is determined by the rate of transcriptional activity (which may be proportional to the amount of DNA) or by the amount of transcription products (2, 4, 5; Jauker, personal communication).

Therefore, the following conclusions concerning regulation of S phases are drawn: Two different mechanisms are involved in the regulation of DNA replication. The initiation of replication, on the one hand, is dependent on the balance of several cell constituents, as for example the amount of DNA (or transcriptional activity) in relation to cell size. For this reason, conditions may be found by which replication is initiated untimely or is omitted during certain cell cycles, or supernumerary replications may occur. These events result in restoration of proper ratios of cell constituents. On the other hand, the continuation, intensity, and termination of DNA replication appear to be rather independent of overall parameters of the cell. Intensity and termination are regulated by the synthetic machinery directly involved in DNA synthesis such as precursors, enzyme activity, and the state of templates.

SUMMARY

Increase in the macronuclear DNA content in *Tetrahymena* subjected to heat shock treatment has been followed by cytophotometry and autoradiography. Heat shocks have been applied to samples of cells of known cell cycle age starting at different points in the cycle. The treatment prevented cells from dividing. DNA content increased rhythmically. The first synthesizing period occurred at the same time as in untreated cells and resulted in a doubling of the DNA content. After a period with no increase, a second rise in DNA content by an average of 30–40% occurred. This is due to synthesis in 30–40% of the cells each of which doubled its DNA content. Since additional replication is initiated in cells with the lowest DNA content, it is concluded that initiation of DNA replication depends on the ratio of DNA amount to cell size, whereas progress and termination of replication are rather independent of the situation in the cell and proceed until every part of the DNA has been replicated.

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REFERENCES

1. ANDERSON, H. A., C. F. BRUNK, and E. ZEUTHEN. 1970. Studies on the DNA replication in heat synchronized *Tetrahymena pyriformis*. *C. R. Trav. Lab. Carlsberg*. **38**:123–131.
2. BERNSTEIN, E., and E. ZEUTHEN. 1966. The relationship of RNA synthesis to temperature as an inducer of synchronous division. *C. R. Trav. Lab. Carlsberg*. **35**:501–517.
3. CERRONI, R. E., and E. ZEUTHEN. 1962. Asynchrony of nuclear incorporation of tritiated thymidine into *Tetrahymena* cells synchronized for division. *C. R. Trav. Lab. Carlsberg*. **32**:499–511.
4. CLEFFMANN, G. 1966. Bildung zusätzlicher DNS nach Blockierung der Zellteilung von *Tetrahymena* durch Actinomycin. *Z. Zellforsch. Mikrosk. Anat.* **70**:290–297.
5. CLEFFMANN, G. 1968. Regulierung der DNS-Menge im Makronucleus von *Tetrahymena*. *Exp. Cell Res.* **50**:193–207.
6. GOEDEKE, R., and L. RENSING. 1974. Regulation des Zellzyklus. *Naturwiss. Rundsch.* **27**:4–16.
7. HJELM, K. K., and E. ZEUTHEN. 1967. Synchronous

- DNA synthesis induced by synchronous cell division in *Tetrahymena*. *C. R. Trav. Lab. Carlsberg*. **36**:127-160.
8. JEFFERY, W. R., J. FRANKEL, L. E. DE BAULT, and L. M. JENKINS. 1973. Analysis of the schedule of DNA replication in heat synchronized *Tetrahymena*. *J. Cell Biol.* **59**:1-11.
9. JEFFERY, W. R., K. D. STUART, and J. FRANKEL. 1970. The relationship between deoxyribonucleic acid replication and cell division in heat-synchronized *Tetrahymena*. *J. Cell Biol.* **46**:533-543.
10. JONES, N. C., and W. D. DONACHIE. 1973. Chromosome replication, transcription and control of cell division in *Escherichia coli*. *Nat. New Biol.* **243**:100-103.
11. SACHSENMAIER, W., and H. P. RUSCH. 1964. The effect of 5-fluoro-2'-deoxyuridine on synchronous mitosis in *Physarum polycephalum*. *Exp. Cell Res.* **36**:124-133.
12. SCHERBAUM, O. H., A. L. LOUDERBACK, and T. L. JAHN. 1959. DNA synthesis, phosphate content and growth in mass and volume in synchronously dividing cells. *Exp. Cell Res.* **18**:150-166.