CELL CYCLE CONTROLS

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PREFACE

This book reflects the current knowledge of investigators whose chief concern has been to understand cell cycle controls. The material presented stems from studies on many different cell types and cell systems. We have sought to include the most recent studies on the higher eukaryotic cell types as well as many new and exciting insights derived from investigations on lower eukaryotes. We feel that this volume will provide the background for understanding current concepts on cell cycle controls and will stimulate students and researchers to unravel the many exciting questions concerning the cell cycle.

This book should appeal to cellular, molecular, and developmental biologists as well as to many others in the life sciences. It is aimed not just at the specialist in the cell cycle field, but should be of value to all those with a general interest in cellular control mechanisms.

The book is organized into three main sections. Each section begins with a chapter by one of the foremost workers on the cell cycle: Section I includes studies on the regulation and timing of the cell cycle and begins with a chapter by E. Zeuthen of Copenhagen, Denmark; Section II deals with investigations on gene expression in the cell cycle and begins with a chapter by J. M. Mitchison of Edinburgh, Scotland; Section III presents works on the nuclear and chromosomal activity in the cell cycle. It begins with a chapter by D. Mazia of Berkeley, California. Each contributor has thoroughly but concisely reviewed his field and has included new and previously unpublished material to give a critical and up-to-date summary of the subject. Clearly international, the contributors to this book include many of the leading scholars of the cell cycle.

George M. Padilla
Ivan L. Cameron
Arthur M. Zimmerman

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A CELLULAR MODEL FOR REPETITIVE AND FREE-RUNNING SYNCHRONY IN *TETRAHYMENA* AND *SCHIZOSACCHAROMYCES*

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I. Introduction

Some twenty years ago the "induction synchronists" happily brought their results to market. They told the world that synchronous cell divisions could be forced upon populations of cells which thus far had multiplied exponentially with the cells out of phase. This called the "selection synchronists" forth. They selected cells in phase from exponentially multiplying cultures and started synchronous cultures with these. They told us that induction synchrony was no good because the cell cycles studied were distorted, unnatural, not normal. To this no reply was possible. The critics could even be right and in relation to some synchronization work they undoubtedly were.

The heart of the matter is that those who started induction synchrony did this on hope and faith. The hope was that it would be possible to find means to force cells into phase, a situation which should be useful for further work. It would be visibly reflected in periodic displays of synchronized cell divisions. The faith was that normal cell cycle controls and regulations could later be shown to be involved.

So who regulates and sets time in the cell cycle, the cell or the experimenter? The induction synchronist was never quite sure with his cell material, but I believe that on my own behalf, and by this time, I can compromise and say: both in cooperation, and this view shall pervade the following pages.

I want to use this opportunity to report and discuss new results from Copenhagen with a repetitively synchronized *Tetrahymena* system, itself the logical product of careful studies of positive sides, and of weaknesses (58,60). in the earlier multishock system (44). Developments are accounted for in a series of reviews (42,43,52,55,60,62). The fission yeast *Schizosaccharomyces pombe* is now included, and we have started related work on monolayer and suspension cultures of mammalian cells.

II. Tetrahymena

For years our laboratory has worked with an amicronucleate strain of the hymenostome ciliate protozoan *Tetrahymena pyriformis*. The strain used (GL) is Lwoff's original (28): in the United States it has acquired one more capital

letter, C - for Copenhagen. In other laboratories a number of different strains - amicronucleate and micronucleate—, and in our own laboratory a different species, (*T. vorax*, strain V_2S) has been synchronized with versions of the multishock procedure. More recent work on the two species shall be surveyed in separate sections of this chapter.

A. Tetrahymena pyriformis:

1. The system

First I shall present a rather new *Tetrahymena* system, which we find quite promising by itself and as a model for adventures with other cells (59,60). Fig. 1 shows how repetitive synchrony builds up in exponentially multiplying cells when heat shocks are spaced a full cycle apart. The upper tracing shows the temperature regimen found to be optimal: intervals at 34°C for periods of 30 minutes alternated with intervals at the optimum temperature (28°C) for periods of 150 or (as in Fig. 1) 160 minutes. It is likely to be significant that the time interval between shocks equals the doubling time (158 ± 3 minutes) for cells grown exponentially in the rich medium (2% proteose-peptone, 0.1% liver extract) used here. The next curves shows how the fraction of cells in division and the fraction of cells engaged in DNA-replication fluctuated with time. Each symbol represents a separate experiment arranged around a standard convenient population density. Cell densities increased from 2.5×10^3 to 4.8×10^5 cells per ml. As reported before (58) the average synchronous division step adds 85% to the population count.

2. Chemistry

a. *DNA, RNA, Protein, Enzymes.* Rather early results (Fig. 2A) indicated that in this system each division step and each replication phase resulted in close to doublings in the number of cells and in the amount of DNA. Enzyme activities followed so far increased discontinuously and mostly in steps. This is true of deoxycytidinemonophosphate deaminase, of α- and β-glucosidase (22). Acid phosophatase (27), and DNA-polymerase (23) show patterns of specific activities resembling those of the glucosidases. On the other hand, RNA and protein increased smoothly (log linearly) through the matching temperature and cell cycles (59).

In Fig. 3, B are shown experiments in which α-and β-glucosidase activities were followed in a system which ran freely at 28°C after synchrony induction with 4 shocks. Two synchronous division are displayed, and each of them is correlated with steps in α- and β-glucosidase activity (22). Thus a stop in the increase in enzyme activity at the end of an enzyme step is not a direct effect of heat. It is an indirect effect the same way as other synchronized phenomena seen in this system.

The chemical data shown in Fig. 2A are fully reflected at the level of accumulation in acid precipitable macromolecules of ^{14}C-thymidine, ^{14}C-leucine, but not of ^{14}C-uridine. Incorporation of the latter came to a stop at every shock, and there were recovery periods after each shock. This way ^{14}C-uridine accumulation tended to lag behind what was seen in normal cells,

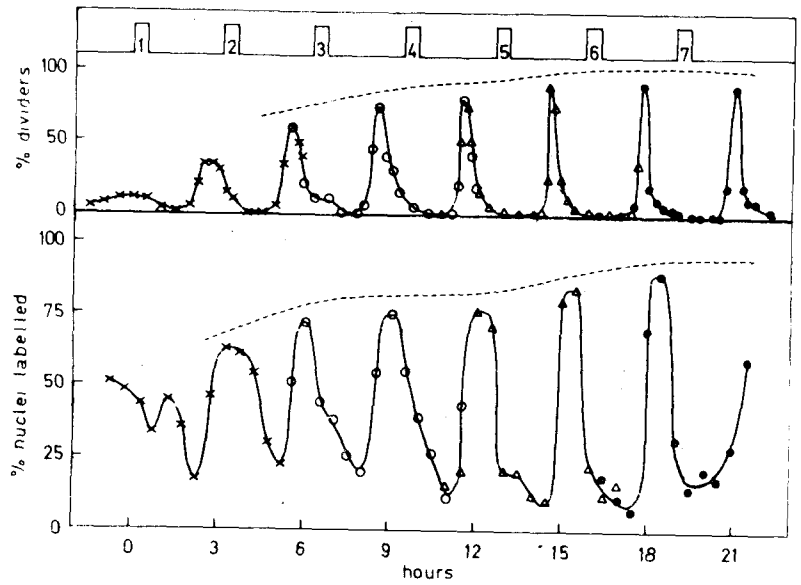

Fig. 1. Temperature induced division synchrony in *Tetrahymena*. The temperature cycle is shown on top and the time is given below. The temperature was changed between 28°C (optimal, held for 160 minutes between shocks) and 34°C (division-blocking, held for 30 minutes in each of the 7 shocks shown). *Upper ordinate:* percentage of cells in division. *Lower ordinate:* percentage of cells in replication. (Indicated as % cells having a labeled macronucleus after a 15 minute pulse with [^{14}C]-thymidine. Points are in the middle of the incorporation intervals). From (59).

and roughly by the duration of the shock, 30 minutes, for every cycle time of 190 minutes.

In terms of label accumulation, repetitively synchronized and free-running synchronized cells were similar in showing log-linear incorporation of ^{14}C-leucine, and stepwise increases in ^{14}C-thymidine incorporation. However, there were signs that synchrony of DNA-synthesis goes wrong after the second free-running division. In free-running cells, C_{14}-uridine accumulation was log linear through two division cycles, indicating that discontinuities in uptake or incorporation in the repetitive system were indeed direct results of the elevated temperature. Effects on RNA re relevant to discussions about chemical mechanisms in the synchrony induction. I refer the reader to papers by Byfield and Scherbaum (11,12), Christenson (14-17), and to the review by Zeuthen and Rasmussen (62).

b. *Nucleosidetriphosphates.* Nexø (34,35) has followed amounts and concentrations of the normal ribo- and deoxyribonucleoside-triphosphates (r- and dNTP) in *Tetrahymena* cells under normal growth and under repetitive and free running synchrony. The average synchronized cell was slightly larger (59)

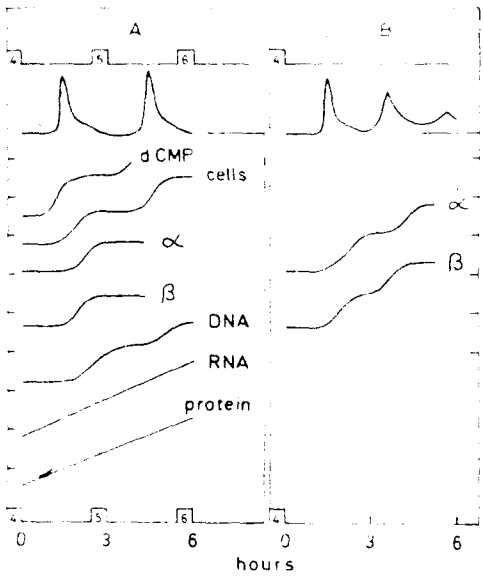

Fig. 2. A. Parameters of repetitively synchronized *Tetrahymena* cells from the 4th to the 6th heat shock. Enzyme activities of deoxycytidinemonophosphate (dCMP); α-glucosidase (α) and β-glucosidase (β) are given in activity/ml of culture. Changes in cell number, DNA, RNA, and protein content are also shown (see text). B. The activities of α- and β-glucosidase in free running system in which synchrony was established with 4 heat shocks. At the top and bottom of both figures are shown the temperature regimens. Just below the top line the changes in the percentage of cells showing division figures are also given (cf. Fig. 1). Enzyme data are from (22), other data from (59).

than the normal cell. This tended to be reflected in the amounts of NTPs per cell. Concentrations hardly differed in normal and synchronized cells. Midway between two divisions 10^6 repetitively synchronized cells (total volume 24 µl) contained 24 nmole ATP. Thus the concentration of ATP was 1 mM. GTP, UTP and CTP range around 200 µM. Concentrations of dTTP, dATP, dCTP and GTP were much lower, around 2 - 8 µM. These values are approximate. There were variations between experiments and through the cell cycle.

There was a fairly smooth accumulation of the four ribonucleotides through the repetitive (from an hour before shock 6 through shock 7) and the free running cycle (through two divisions and 2 S-periods). Concentrations tended to become lower for one half hour after a heat shock. This phenomenon was not fully eliminated if a shock was not applied (free run). Thus, there were faint and probably significant changes in concentration of all four nucleotides through cycles of division and replication at constant temperature. The faint

drop in ATP, GTP, UTP and CTP concentrations in free-running cells correlated with the passage of the cells from S into G2. It is relevant here to mention that Hamburger (20) recently found a transitory respiratory decrease mid-way between divisions in normal single cells taken from the exponential population growth phase. The decrease was seen around the time when the cell would be expected to move from S into G2. It would seem that levels of rNTP are controlled by respiration rates, themselves independently controlled, and not by levels of rNDP in the cell as a whole. Cellular compartments might be considered, but this is not the place.

The dNTP data are perhaps more interesting. There was some DNA synthesis between the S-periods (mostly due to imperfections in the synchrony), but synthesis was 10 - 20 x faster during the nuclear S-periods. This situation was reflected on the amounts per cell and on the concentrations of the dNTPs, though not much. They all persisted through the cycle. Fig. 3 shows the *amounts* in cells in a standard volume of culture. The fully drawn curves are for cells which received shocks 6 and 7 and divided (D) and replicated (S) two times. The dotted curves are for cells allowed to run freely after shock 6. First let us disregard the discontinuities in the curves and observe that the overall growth with respect to dNTP paralleled the cell growth. The amounts doubled with each generation. The next observation is that amounts of dTTP, dATP and dCTP, increased during, and are minimal just after both shocks. However, as with rNTPs, these were not clean effects of the temperature increases. Even prior to the time when free-running cells could have "expected" shock 7 - the shock they do not receive - they had passed a point in the S-period when amounts of dNTPs leveled off; in the case of dATP this course lead to a dip in the amounts per cell which was coincident with the beginning of the next S-period, and which repeated what was seen at the initiation of preceding S-periods in the repetitively synchronized system. Thus dATP builds up in amounts during S which stay constant or drop between S-periods. Common for all dNTPs, the amounts and concentrations were maximal somewhere in S.

c. *Experimental Control of Thymidine Supply, Effects on Nucleosidetriphosphates.* The complex medium we used is optimal for cell synchronization but it contains purine and pyrimidine compounds, among them thymidine. At one time I wanted to control DNA replication and cell division independently in the same culture (57,58). I decided to attempt this by controlling the supply of thymidine for new DNA and independently playing with the temperature. These cells can methylate a nucleoside monophosphate and get thymidylate, but they can also take thymidine from the medium. The methylation mentioned is catalyzed by tetrahydrofolic acid and can be inhibited with 0.05 mM methotrexate (M). The flow of exogenous thymidine into new DNA can be checked by addition to the medium of uridine (U) in 20 mM concentration. The two compounds combined (M + U) gave good inhibition of the synthesis of DNA (2,47,56). Together they should cut the cells off from new dTTP, and this occurred (35,36).

Fig. 4 shows the *concentrations* of dTTP and dATP. These fit the curves

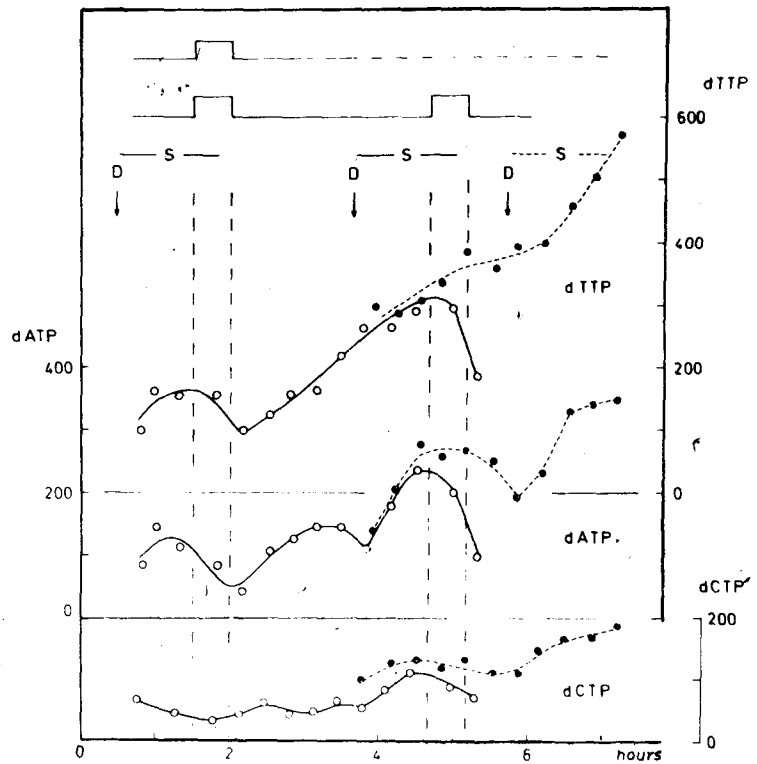

Fig. 3. Changes in deoxynucleosidetriphosphates (dNTP) in *Tetrahymena* cultures with repetitive synchrony around shocks 6 and 7 (open circles), and with synchrony which runs free after 6 shocks (filled circles). Temperature regimens, DNA synthetic periods (S), and times when maximal numbers of cells show division furrows (D) are indicated at the top. Ordinates refer to amounts per unit volume of culture. Calculations were adjusted so that the first sample shows pmole/10^6 initial cells. The abscissa gives the time in hours. From (34,35).

(Fig. 3) for *amounts* per cell nicely. Figure 4 also demonstrates what happened if M + U were added to the system between S-periods (first arrow) and during an S-period (second arrow). When the supply of thymidine was blocked while cells were not in S, a normal increase in dTTP concentration was checked, but there was no decrease. Such a decrease occurred later at the time when an S-period was expected. The S-period came at a normal time and it emptied the cells of available dTTP: thus the cells were stopped early in S, as shown earlier (56). There were also effects on dATP. After a lag, this compound increased to *high levels. Such interdependence between* dTTP and dATP concentrations is

known from other systems (24,25,31,32,41). If the supply of thymidine is blocked during an S-period dTTP siphons out quickly, DNA synthesis stops (2), and dATP goes up. Nexø calculated half-lives for dTTP and dATP to be at around 10 minutes for cells not in S and around 1 minute for cells in S. This is in fair correspondence with the results of Fig. 4, considering that no inhibitor and no synchrony is perfect.

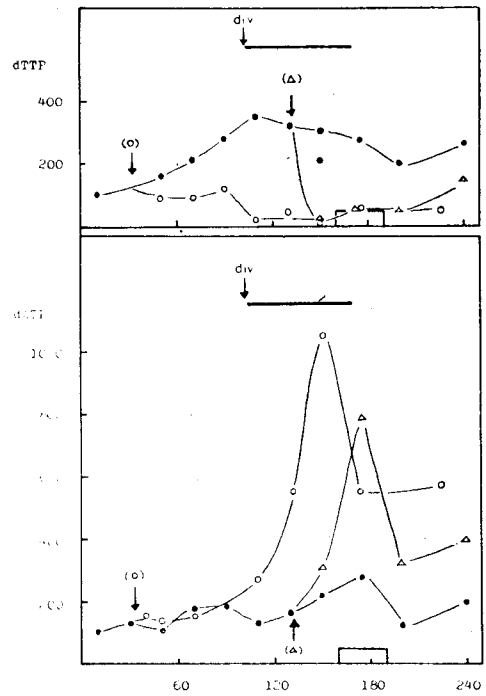

Fig. 4. Amounts (ordinate, filled circles) of dTTP10^6 initial cells and of dATP/10^6 initial cells (cf. Fig. 3), both from the termination of heat shock 6 through shock 7. At arrows, M + U were added to create starvation for thymidine compounds. Open circles show the effects when addition was well ahead of a cell division (div) and the S-period which followed. Open triangles show the stronger immediate effects from addition of M + U when synthesis of DNA synthesis was in progress. The abscissa shows the time in minutes. From (35,36).

d. *Studies on DNA.* Each repetitively synchronized cell division leads to a synchronized S-period exceeding the S-period in normal single cells by 10 - 20 minutes, probably an indication of imperfect synchrony. Studies based on incorporation of BudR into hybrid DNA showed that more than 95% of the

cells' DNA replicates in some order in each S-period. The resolution of the method did not permit us to distinguish between replication units smaller than genomic units of which the *Tetrahymena* macronucleus may have many. The replication order in one generation differs completely from the preceding one, which reminds us of the macronucleus of *Euplotes* (itself a ciliate), not of eucaryotes with diploid sets of chromosomes (5). My collaborator, Dr. Helge Andersen and I view the situation as illustrated in Fig. 5. We suggested that the *Tetrahymena* macronucleus possesses units of replication [perhaps Dr. Hytte Nilsson's haploid segregation units (37)] which replicate partially out of phase. Andersen found that an effective block for replication can be established with M + U, two-thirds or three-fourth into the synchronized S-period, and without consequences for the following cell division (2). However, at the time of this division much DNA is extruded. He placed pulses of ^{3}H-thymidine at various segments of the S-period to be interrupted, and found label extrusion to the cytoplasm only of DNA which was pulse labelled immediately before the supply of dTTP was cut off (1). This extruded DNA replicated in the cytoplasm in the next cell generation (4).

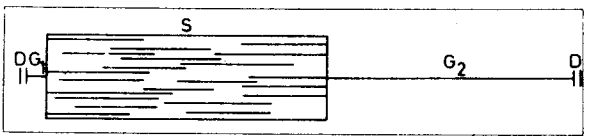

Fig. 5. Time from division, D, to division, D. Between a short G1 and a long G2 the macronucleus replicates its DNA in S. On the basis of the DNA content, the ploidy is 40 - 50. The graph is drawn to suggest that single identical units replicate out of phase. Note that in this illustration G2-functions are initiated before S is yet finished. Drawn in accordance with (5).

We shall see shortly that the point late in S after which DNA replication can be stopped, but not the cell's progression to division, marks a time when structural cytoplasmic changes which normally lead to division are initiated. It thus marks a time when the cell decides that it has enough DNA to go ahead in the cycle. Therefore, the last part of S becomes a period with overlapping S and G2 activities.

Working in our laboratory, Westergaard and Pearlman found that thymine-starvation established with M + U, induced DNA polymerase activity (48) in asynchronous cultures of *Tetrahymena*. Keiding and Andersen (23) now find that this inducibility of DNA polymerase is higher during than between S-periods.

3. Structural Markers of the Cell's Position in G2

The studies of metabolites with fast turnover, which were reported above, gave signs of small enzyme disfunctions, and of their correction, within the