A FEEDBACK CONTROL OF CELL CYCLE PARAMETERS IN *TETRAHYMENA*

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Biochemical studies of growth parameters of the ciliate *Tetrahymena pyriformis* reveal two kinds of variations: (a) variations in the “average cell” according to changes in environmental conditions, and (b) differences between individual cells as shown by statistical distribution of the parameters in question. The studies of our laboratory deal with the latter type of variation. As reported by Cleffmann (1), the DNA content of individual *Tetrahymena* cells varies and is regulated during subsequent cycles. The constant mean generation time of a population is maintained by an oscillatory pattern of successive generation times in individual cell lines (5). These data suggest regulatory mechanisms to be involved in the control of cellular growth parameters.

This communication deals with the questions of whether and how generation time determines growth in proliferating individual cells. Gross amounts of RNA and protein of exdividers were studied cytophotometrically and related to the duration of the preceding cell cycle.

**MATERIALS AND METHODS**

*Tetrahymena pyriformis* HSM was grown axenically in synthetic medium supplemented with 0.04% proteose peptone and 1 mg/liter cholesterol. By daily reincubation, stock cultures were permanently kept in logarithmic growth phase at 29°C. Three days before experiments 0.6 µg/ml of actinomycin D was added. This treatment increases the mean generation time (GT) from 220 to 500 min but substantially extends the amplitude of the oscillatory pattern of subsequent GT’s (4). Young exdividers were isolated by hand from stock cultures (2,000-3,000 cells/ml) and incubated in culture capillaries with 2 µl of fresh medium containing 0.6 µg/ml actinomycin. The time until next division (GT) was recorded, the exdividers were placed on slides and their position registered. Excess medium was removed and equal dryness and flatness of cells achieved by applying a vacuum of 0.2 Torr and a strong desiccant overnight. Individual relative amounts of RNA and protein were measured cytophotometrically. For details of the galloyamine and 1-fluor-2,4-dinitrobenzene (DNFB, Merck) staining and preparation techniques for quantitative RNA and protein assays, refer to references 2, 7, and 9. Time of DNA replication was determined by following the quantitative pattern of [3H]thymidine incorporation into synchronously growing cells.

Pulse-labeled cells (20 µCi/ml, 15 min [3H]thymidine, 21 Ci/mmol, Amersham Buchler) were washed and immediately dried onto cover slides. After TCA treatment (10% at 5°C for 15 min) and ethanol washing, the cells were recounted and dissolved in scintillation vials by adding 0.5 ml Soluene 350 (Packard Instrument Co., Inc., Downers Grove, Ill.). Radioactivity was measured in a 3375 Tricarb (Packard) within 10 ml of Dimilume (Packard). The mixture is free of chemoluminescence, the channel ratio being 0.7. The background was determined by drying five times the experimental volume of 2 µl labeling medium on slides and treating like experiments. This value never exceeded the toluene standard (21 cpm) and was automatically subtracted. Activity was calculated on a per cell basis yielding maxima of 5.0 cpm/cell (n = 44). In fact, this radio assay is based on rates of incorporation rather than on percentage of labeled cells but it essentially produces the same pattern when compared to earlier experiments (4).

**RESULTS AND DISCUSSION**

One out of six experiments on protein content of single cells with known GT’s is shown in Fig. 1. The bimodal distribution of GT’s is due to an oscillation of subsequent individual GT’s (4). The photometric equivalent of the gross protein content per cell appears to be a function of GT (r = 0.53, n = 94; probability of no correlation, less than 0.1%).

Comparison of all experiments reveals the linearity of protein content/GT not to be as good after very long cell cycles. This effect reduces the correlation coefficient of the data presented in Fig. 1 (r = 0.53) and suggests that protein content finally approaches a maximal value.

The photometric equivalent of the RNA content per cell, however, is not affected by the duration of GT but is rather constant at division (Fig. 2). Every column represents 40-50 individuals pooled according to GT’s (270 ± 40; 430 ± 80; 620 ± 95) the standard errors of RNA contents being 8-10%. Cell size is defined by the number of photometric
impulses accumulated by a continuous scan of an individuum at an aperture of 1.25 μm and increases with GT. Size, therefore, is positively correlated with protein content \((r = 0.87, n = 198)\). This seems to be a general aspect of cellular growth in *Tetrahymena* and not to be restricted to the experimental conditions applied in this investigation. In this organism, normally, the protein content per cell is better correlated with size \((r = 0.798, s% = 14.61)\) than is the RNA content \((r = 0.677, s% = 20.98;\) Reuter, personal communication).

Evidently, in short cell cycles a "division-specific" amount of RNA accumulates faster than in longer ones. In *Tetrahymena* the rate of RNA synthesis is discontinuous with a sharp increase related to the event of the macronuclear S phase \((6)\). Therefore, timing of the S phase necessarily would determine the time needed for accumulation of the final amount of RNA.

A constant RNA content related to a GT-dependent protein content results in GT-specific RNA/protein ratios at division \((270\ min:0.73;\ 430\ min:0.67;\ 620\ min:0.53)\). Such different ratios are not remarkable per se and do normally occur with different nutritional conditions \((11)\) or different temperatures \((6)\). As the probability of shorter GT's increases with the duration of the previous ones, and vice versa, the same must be true for high and for low ratios. I tried to reduce the ratios experimentally by selective inhibition of RNA synthesis. The low ratio is supposed to mimic the termination of a long cell cycle and to initiate a shorter one by means of a premature stimulation of the S-phase.

In *Tetrahymena*, the highest RNA/protein ratios were observed at 21°C \((6)\). Therefore, and because of the time-lapse effect of low temperature on cycle phases, logarithmically growing cells were incubated at 21°C for 3 days. At time zero, three synchronous subcultures were established by handselecting dividers from stock cultures: one control and two cultures with different times of application of 0.6 μg/ml actinomycin. One time of application was time zero, the other 2 h before zero. The latter was achieved by starting the treatment 2 h before selection of dividers. The corresponding (relative) RNA/protein ratios were measured cytophotometrically by scanning the individual exdividers at 400 and at 600 nm, according to a simultaneous DNFB-galloycyanine staining method developed by Seyfert (University of Giessen, thesis in preparation). Essentially the same results were obtained by calculating the ratios from mean OD readings of separately stained cells. Both methods revealed the 2-h pretreatment to change the normal ratio of 0.612 \((n = 42)\) into 0.588 at division \((n = 40,\ probability\ of\ coincidence\ less\ than\ 0.1\%)\).

The results of pulse labeling these cultures with \[^{3}H\]thymidine are shown in Fig. 3. Evidently, the S phase can be stimulated if the rate of RNA synthesis is reduced during the previous cell cycle. This agrees with the assumption that RNA/protein ratios at division are involved in timing events in the subsequent cycle.

**FIGURE 2** RNA content and cell size after GT's of different duration. One experiment out of six. Abscissa: duration of GT, each column representing 40-50 individuals immediately after division. Ordinate: cytophotometric equivalent of RNA content (left) and cell size (right). The cells were pooled according to GT without overlapping, SE of RNA contents = 8-10%.
FIGURE 3 The effect of a timed reduction of RNA synthesis on the initiation of DNA replication. Abscissa: time of [3H]TdR pulses (15 min). Ordinate: radioactivity incorporated, the maxima of 4.6–5.0 cpm/cell set to 1.0. Synchronous cultures were grown at 21°C and actinomycin D (0.6 μg/ml) was added at time zero (open triangles) and 2 h before zero (filled triangles). Circles: control culture.

Our results are subjected to the general limitations of cytophotometry, especially regarding specificity. But from the selective effect of low doses of actinomycin on rRNA (12) and from very good correlations of our cytophotometric data with quantitative determinations of extracted and purified rRNA it is concluded, that the ratios refer to ribosomal RNA and gross protein. So far, they do not explain the how and why of Tetrahymena replication and division. But compared to yeast where cell cycle control has been attributed to the sequential reading of a developmental program (14), our data suggest that feedback mechanisms as well are involved in timing cell cycle events in Tetrahymena. The individual GT is determined by the biochemical history of the previous cycle. Such control, effectively causing “regression to the mean,” has also been shown to occur in successive generations of bacteria (10).

The final amount of RNA per cell seems to be more precisely regulated than that of protein which, in return, is better correlated with size or GT. The resulting ratios prevent cells from becoming excessively bigger or smaller by permitting compensation for errors within G1 periods of variable duration. In fact, the variation coefficient of protein content (or dry mass) per cell approaches a minimum at the beginning S phase in both Tetrahymena (13) and in mouse fibroblasts (8).

Generation time, however, is not the only variable affecting the RNA/protein ratios as demonstrated by the experimental manipulation of the rate of RNA synthesis. By means of a “gene dosage” effect on the rate of transcription (6), a cell might compensate for its “low” DNA content by premature replication. As shown by Cleffmann (3), there actually is evidence of selective, preferential replication in Tetrahymena cells with DNA contents lower than the mean.

Regulative mechanisms probably become dominant in Tetrahymena because it is a rapidly proliferating organism readily adapting to many environmental conditions. Since the amitosis of its macronucleus does not provide daughter cells with equal amounts of DNA (1), the DNA content per cell is a substantial variable and seems to prevent further generalizations.

SUMMARY

Protein and RNA contents of individual cells were measured cytophotometrically and related to the duration of individual generation times. Constant amounts of RNA per cell at division, and generation time-dependent protein contents, resulted in generation time-specific RNA/protein ratios. Experimental reduction of these ratios by inhibition of RNA synthesis stimulated premature macronuclear S phases.

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