The Relation between Length of the Cell Cycle Duration and RNA Content in HeLa S3 Cells

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ABSTRACT. The cell cycle of HeLa S3 cells synchronized by hydroxyurea was investigated by flow cytometry. Metachromatic fluorochrome acridine orange was used to stain the DNA and RNA of the cells differentially. Periodic changes in the cellular DNA and RNA contents were observed through five cell cycles. The G₁ and S phases of synchronized HeLa S3 cells that contained large amounts of RNA became shorter than those of cells that contained smaller amounts of RNA.

The DNA and RNA contents of individual cells can be measured simultaneously by flow cytometry (1, 2, 3, 4, 5, 6). In this technique, cells in suspension are stained with the fluorescent, metachromatic dye acridine orange (AO) under conditions in which the dye is intercalated into native DNA and orthochromatically fluoresces green, whereas single-strand RNA metachromatically stains red. Using this technique, we previously reported the relation between the cellular RNA content and the length of phases for V79 Chinese hamster cells synchronized by hydroxyurea (HU) (5). The cycle time of V79 cells in balanced growth was about nine hours.

To clarify the intracellular regulation of the cell cycle to examine whether a relation between cellular RNA content and the cycle exists even in cells with a long cycle. In this study, changes in the mean cellular DNA and RNA contents of two populations of synchronized HeLa S3 cells were measured by flow cytometry for five generations.

HeLa S3 cells were maintained as growing monolayer cultures in Dulbecco-Vogt, modified Eagle's medium containing 15% newborn bovine serum (NBS, Flow Laboratories) in a humidified atmosphere of 10% CO₂ at 37°C. HeLa S3 cells in logarithmic growth were plated in petri dishes (28 cm², A/S Nunc) so that the total cell number could be maintained at approximately 6×10^5 cells per dish for the measurements.

Cells cultured in petri dishes for 30 h were synchronized by double treatment with HU. At time $t=0$ (hour), the cells were washed once with medium containing 0.2mM HU and 15% NBS (HU medium) then they were cultured again in HU medium. At time $t=19$, cells were washed twice with medium without HU (HU-free medium) then were cultured again in HU-free medium. At $t=29$, cells were treated with HU as described above, and at $t=39$, the cells were released from HU.

To obtain cell populations containing relatively small and large amounts of RNA,

Abbreviations used: NBS, newborn bovine serum; HU, hydroxyurea; CV', modified coefficient of variation; BG, balanced growth; AO, acridine orange.

Fig. 2. Changes in F_{530} and $F_{>600}$. Open circles: F_{530} or $F_{>600}$ values for peaks in the frequency distribution histograms. Closed circles: values for subpeaks less than 80% of the height of the main peak. Longitudinal bars: peak width at 80% of the main peak height. Half arrows labeled HU: the length of the third HU treatment; arrow above the middle line refer to the upper panel and those below to the lower one. The abscissa gives the time after the first HU addition and the ordinate F_{530} or $F_{>600}$ values. Roman numerals I to IV and Ia to IIIa correspond to single cell cycles from G_1 to G_2+M . Solid lines were drawn based on the open circles. Broken lines were added for clarity. Dotted lines represent the normal level (averaged values) of $F_{>600}$ in the G_1 phase (lower) and the G_2+M (upper) phase obtained from the decomposed frequency distribution histograms (5).

Fig. 1. Changes in F_{530} (left) and $F_{>600}$ (right) frequency distribution histograms. These histograms are normalized for peak height. Numbers in the histograms indicate the time (hours) after the third HU release. C: the BG cells (control). The origin of the F_{530} histograms was shifted. The origin of the $F_{>600}$ histograms was corrected by considering a nonspecific $F_{>600}$ value (6).

we divided synchronized HeLa S3 cells into two groups. At time $t=53$, when the cells in each petri dish were in G_1 , one group was treated with HU for 9 h and the other for 24 h. At various times after the third HU release, the cells were washed with PBS⁽⁻⁾, then trypsinized and suspended in medium containing 13% fetal bovine serum (Flow Laboratories).

The amounts of cellular DNA and RNA in these cells immediately were measured as described elsewhere (5). HeLa S3 cells in balanced growth (BG cells) that had been seeded in petri dishes at a relatively low density were prepared and were measured every 12 h as a sensitivity check of the whole system of measurement.

Changes in the frequency distribution for the relative intensities of green (F_{530}) and red ($F_{>600}$) fluorescence from AO-stained HeLa S3 cells after the third HU release are shown in Fig. 1. To evaluate the relationship between $F_{>600}$ and the cell cycle parameters T_{G_1} , T_S , and T_{G_2+M} (the durations of the G_1 , S, and G_2+M phases), F values of peaks in the F_{530} and $F_{>600}$ histograms were plotted against the time after the first HU addition (open circles in Fig. 2). The solid lines for F_{530} and $F_{>600}$ in Figure 2 were drawn based on the open circles for F_{530} taking into account the heights of the subpeaks. The solid lines for F_{530} were drawn so that the T_{G_2+M} in every cell cycle was 3.0 h because it was impossible to detect slight differences in T_{G_2+M} from measurements taken every 2 h.

We concluded that (within the experimental error) the solid lines represent the true progression of the HeLa S3 cell population throughout the cell cycle. Roman numerals I, II, III, IV, Ia, IIa, and IIIa in Figure 2 correspond to cell cycles that proceed from G_1 to G_2+M . The duration of each phase in these cell cycles, the $F_{>600}$ values in the early G_1 phase (the smallest $F_{>600}$ value in a cell cycle), the rate of change in F_{600} ($\Delta F_{>600}/1$ hour) and the modified CV (coefficients of variation) values (CV') are summarized in Table 1.

As shown in this table, the cycle time, T_C , was related to $F_{>600}$; the larger the $F_{>600}$ value the shorter the cycle time. Because the T_C and T_S values for cell cycle III (Table 1) agreed with those obtained by the 3H -TdR continuous labeling index method (unpublished data), we concluded that the values for T_{G_1} , T_S , and T_{G_2+M} in cell cycle III were the same as those for BG cells. Since the $F_{>600}$ value in the early G_1 phase for cell cycle III is 1.35 times of that for BG cells, the RNA threshold

TABLE 1. VALUES RELATED TO THE CELL CYCLE OF SYNCHRONIZED HeLa S3 CELLS*

Cycle	Duration (hour)				$F_{>600}$ (early G_1)	$\Delta F_{>600}$ 1 hour	CV'(early G_1)	
	G_1	S	G_2+M	T_C			F_{530}	$F_{>600}$
I	—	(7)	3	—	—	0.11	—	—
II	7	8	3	18	1.40	0.08	0.27	0.45
III	8	10	3	21	1.35	0.07	0.11	0.50
IV	—	—	—	—	1.20	—	0.11	0.53
Ia	—	(5)	3	—	—	0.12	—	—
IIa	6	7	3	16	1.80	0.09	0.15	0.23
IIIa	—	—	—	—	1.67	—	0.15	0.35
					1.00 (BG cells)	0.05 (+HU)	0.16 (mean) 0.06 (S. D.)	0.41 (mean) 0.12 (S. D.)

* The values listed were calculated from Figure 2. The $F_{>600}$ values for BG cells are averaged $F_{>600}$ value obtained from decomposed $F_{>600}$ frequency distribution histograms for the G_1 phase (5, 6). +HU: the presence of HU.

for HeLa S3 cells is within 1.35 times the average RNA content of BG cells in the G_1 phase (4); within the threshold, T_c is constant.

It is clear from Figure 2 that the $F_{>600}$ values of cell populations induced by HU treatment to have relatively large amounts of RNA decrease in subsequent generations. Within the experimental error, $F_{>600}$ increases linearly in the cell cycle, from G_1 to G_2+M , irrespective of the phase. Therefore, the $F_{>600}$ values in the early G_1 phase can be used to examine the relationship between the relative rate of RNA synthesis (the rate of change in $F_{>600}$) and the RNA content ($F_{>600}$).

Although our data consisted of only three points, the rate of change in $F_{>600}$ ($\Delta F_{>600}/1$ hour) and the $F_{>600}$ values of the early G_1 phases were correlated with the correlation coefficient $\gamma=0.91$ and with the regression line, $\Delta F_{>600}/1$ hour = $0.033 + 0.028 \times F_{>600}$. In contrast, the rate in HU-treated cells was constant irrespective of the $F_{>600}$ value and was smaller than that in the absence of HU: It was approximately equal to the value obtained by dividing the cellular RNA content produced during one cycle of these cells in balanced growth by the T_c value.

The extent of variation in the early G_1 peaks in the F_{530} and $F_{>600}$ frequency distribution histograms (a quick guide for the distribution of cellular DNA and RNA in the cell cycle) was calculated by dividing the width of the 80% peak height by the F value of the peak ($CV' = 0.89CV$, for a peak approximated by a normal distribution). The values for CV' indicate that the degree of variation for the RNA content is larger than that for DNA.

Because the properties of a synchronized cell population can be regarded as those of its constituent cells, the data obtained in this study could be summarized as follows: The T_c of individual HeLa S3 cells with an RNA content above a particular threshold (about 1.35 times that of BG cells) in the early G_1 phase is shorter than that of BG cells. HeLa S3 cells induced by HU treatment to have a relatively large amount of RNA have reduced RNA contents in subsequent generations. The rate of RNA synthesis in HeLa S3 cells is correlated with the RNA content in the initial G_1 phase. And, the degree of variation in the cellular RNA content of HeLa S3 cells in the G_1 phase of the cell cycle is larger than that for DNA. These properties of HeLa S3 cells were essentially the same as previously described for V79 cells (5).

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