REGULATION OF INITIATION OF DNA SYNTHESIS IN RELATION TO MITOSIS IN CULTURED HEPATOMA CELLS

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ABSTRACT

The commitment of Reuber H 35 hepatoma cells to DNA synthesis was studied by exposing cells which were synchronized by serum depletion, to serum. It is shown that the period of commitment is 6-8 hr, independent of the length of the G_1 phase. It can even occur during the G_2 period of the previous cell cycle.

INTRODUCTION

Various events that occur in the cell cycle are regulated relative to each other. In cell cycle studies two events have been studied most intensively: mitosis and initiation of DNA synthesis. The biochemical basis of the M to S transition is still unknown. In recent years the existence of several G_1 ts mutants that complement each other have indicated the necessity of several gene products (Ashihara et al., 1978; Levine, 1978; Pringle, 1978). Moreover, in a number of cell types with a serum-restriction point, the rate-limiting step, after which the cell is ordinarily committed to S, can be located (Todaro et al., 1965; Yoshikura and Hirokawa, 1968; Temin,1971; Pardee, 1974; Lindgren et al., 1975; Brooks, 1976). The cell may employ several different mechanisms so as to order one event in the cell cycle relative to another (Mitchison, 1972; Hartwell et al., 1974; Levine, 1978). Nevertheless the models for the M to S transition propose that the mitotic event is part of the chronological order in the events leading to S so that the serum control point is somewhere in between.
This paper demonstrates that while the serum commitment event occurs at a constant time before S, under certain conditions of growth, this commitment can be located before M. Apparently the G_1 phase is determined by the combination of two parallel pathways, one for the mitotic event and one for initiation of DNA synthesis.

MATERIALS AND METHODS

Reuber H 35 hepatoma cells were subcultured in Eagle's basal medium enriched 4-fold with vitamins and amino acids. Fetal calf serum and new born calf serum were added to a final concentration of 5 % and 10 % respectively. Growth occurred at 37°C in an atmosphere of 5 % CO_2 and 95 % air. In case of transfer to serum-free medium cells were washed previously with Hanks Balanced salt solution. Percentage of cells synthesizing DNA was determined after incubation of cultures with 1 µCi ^{3}H-thymidine (specific radioactivity 3.75 Ci/mole) per ml of medium for 30 min and autoradiography. The capacity of cells to enter the phase of DNA synthesis was determined by continuously labeling with 1 µCi ^{3}H-thymidine (1 Ci/mole specific radio-

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activity) per ml of medium and sampling at variable times. After autoradiography cells were stained with Giemsa.
All tissue culture components were purchased from Gibco Grand Island Biological Co. (Grand Island, N.Y.).

RESULTS

Characterization of two cell cycles of cells synchronized by serum deprivation. Cultures incubated with serum-free medium for 72 hr and subsequently exposed to serum start DNA synthesis after a considerable delay. The first cells are seen in S phase at 18 hr, fifty percent of the cells have reached S at 23 hr and nearly all the cells have reached S only after 28 hr (fig. 1). The data on the continuous labeling and on pulse labeling with ^{3}H-thymidine show that fifty percent of the cells have already passed S phase at 30 hr. The first cells enter mitosis at 28 hr. Curves representing the entry of cells in S, in G_2 or in mitosis, can be compared after correction for the increase in cell number. The corrected curves representing the cumulative entrance of cells into G_2 and in mitosis are shown in figure 2. According to these curves G_2 phase has a length of 3–4 hr. The variability of G_2 phase duration is relatively small. The curve representing cumulative entrance of cells into the second cycle was

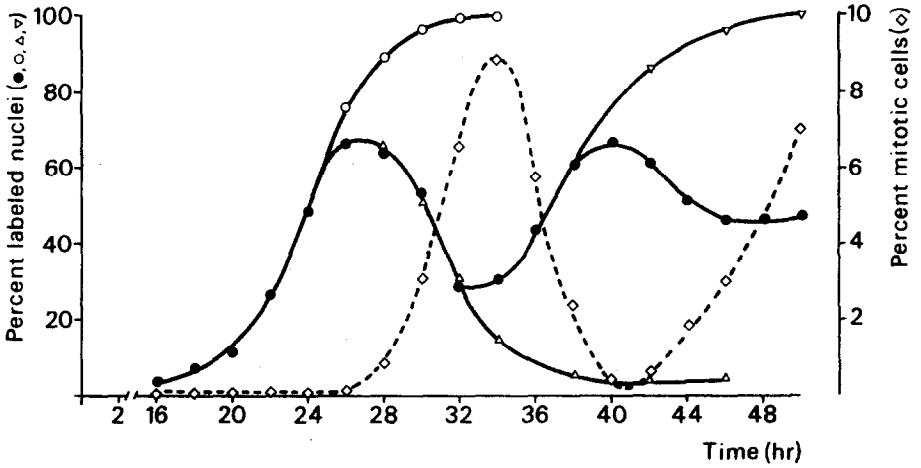

Figure 1. *Initiation of DNA synthesis and mitosis after serum addition.* Reuber H 35 hepatoma cells were synchronized by a 72 hr incubation in serum-free medium. After serum re-addition the percentage of cells in S at various times was determined by ^{3}H-thymidine pulse labeling (-●-). Cumulative entrance of cells in S was determined by continuous labeling with ^{3}H-thymidine during the first round of DNA synthesis (-o-, label added at 22 hr) and during the second round of DNA synthesis (-V-, label added at 36 hr). Effect of removal of serum at 26 hr (-Δ-) was measured by measuring the percentage of labeled nuclei via pulse label with ^{3}H-thymidine incorporation at different times between 26 and 36 hr and continuous label with ^{3}H-thymidine (from 36 to 50 hr).

further used for comparison with the curve representing cumulative entrance of cells into the second round of DNA synthesis. Continuous labelling from 35 hr and onwards show that all cells are capable of performing a second round of DNA synthesis. The serum dependency of this second round of DNA synthesis is most clearly seen by addition of serum-free medium at 26 hr. Based on pulse incorporation of ^{3}H-thymidine no DNA synthesis was observed under these conditions. It is observed that the curves representing the entry of cells into the second cell cycle and entry into the second round of DNA synthesis are nearly parallel, suggesting a relatively low G_1 variability in the second cell cycle.

Commitment period in first and second cell cycle. We considered it to be of interest to study the commitment period in the two consecutive cell cycles which are characterized by large differences in the duration of their G_1 periods.

Determination of commitment to the first round of DNA synthesis is performed at various time points during the G_1 period. Fig. 2 shows that after an exposure to serum for 18 hr only 45 percent of the cells are committed to DNA synthesis as determined by continuous labeling with ^{3}H-thymidine for a period up to 30 hr. Exposure of

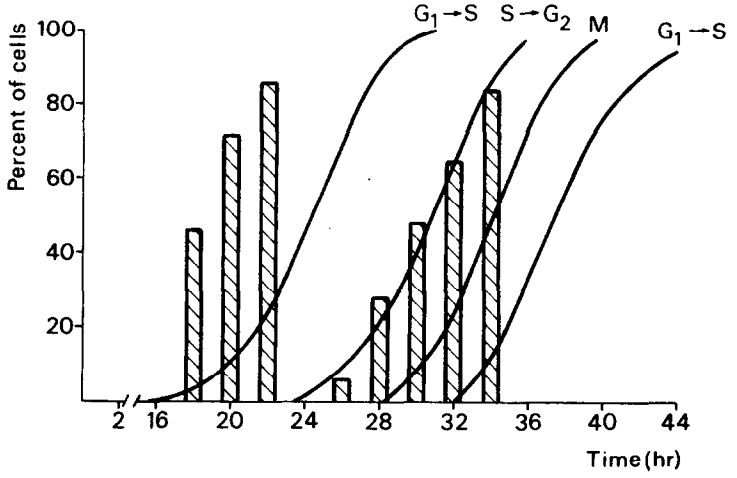

Figure 2. ***Relation between percent of cells committed to DNA synthesis and time of serum removal.*** **Comparison of cumulative entrance into S phase, G_2 phase and mitosis of the first cell cycle, and entrance into S phase of the second cell cycle. Curves were derived from the data presented in Figure 1 and by a correction mentioned in the text. The bars represent the percent of cells committed to DNA synthesis at that particular time. Commitment was determined by addition of serum-free medium and determination of the maximal percent of labeled nuclei using continuously labeling with ^{3}H-thymidine. Maximal values were observed between 30 and 35 hr when serum was removed at 18, 20 and 22 hr (continuous labeling starts at 22 hr). Maximum values were observed between 42 and 50 hr when serum was removed at 26 hr, 28 hr, 30 hr and 34 hr (continuous labeling starts at 36 hr).**

cells to serum for periods of as long as 20 and 22 hr, shows a percentage of cells committed to DNA synthesis of 72 and 86 percent, respectively. Summarizing the data presented in figure 2 we suggest that cells are committed to start DNA synthesis about six hours before the actual start of DNA synthesis.

Determination of the commitment to the second round of DNA synthesis was performed by changing the medium for a serum-free medium in series of cultures at 26 hr and at regular (2 hr interval) times thereafter. From each series of cultures cells are pulse labeled with ^{3}H-thymidine at different times in order to establish the time course of leaving the first S phase. Addition of ^{3}H-thymidine at 35 hr and later times only labels cells which are in the second round of DNA synthesis. Therefore, continuous labeling with ^{3}H-thymidine from 35 h - 50 hr leads to a constant value between 45 and 50 hr which is considered to be the percentage of cells committed at the time of serum deprivation. Figure 2 shows the relation between the percent of cells committed to DNA synthesis and the time of serum removal. Commitment occurs 6-7 hr before the cells enter the S phase. Interestingly, at time points 26 and 28 hr, the percentages of commitment to the second round of DNA synthesis are 8 and 35 percent, respectively, although hardly any cell had started the second cell cycle.

Commitment period in logarithmically growing cell cultures. Commitment of cells in logarithmically growing cultures is determined from the rate at which pre-S phase cells are capable of entering S after removal of serum. Continuous labeling with ^{3}H-thymidine shows that during a 7-8 hr period the number of cells arriving in S is similar for cultures growing in serum containing and serum-free medium (fig. 3). We found no further increase in the percentage of labeled cells after that time in serum-free medium, even not after a period of 24 hr.

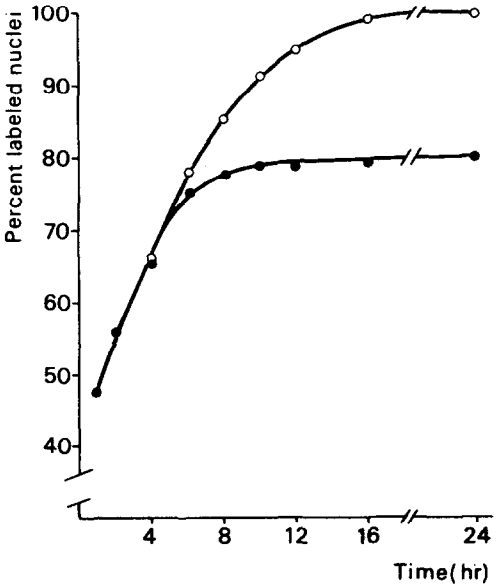

Figure 3. *Effect of serum depletion on entry of logarithmically growing Reuber H 35 cells in S phase.* Cells were grown for 3 days. Then, at the zero time of the figure, the medium was changed and cultures were continuously labeled with ^{3}H-thymidine in the presence of fresh serum containing medium (-o-) or serum-free medium (-•-). At regular times three dishes of each series were fixed and the percentage of labeled nuclei was determined.

DISCUSSION

Two cell cycle-specific temperature sensitive mutants of BHK cells, which are known to arrest in G_1 when shifted to the non-permissive temperature, have been used for determination of the average shift up time from the average time of entry into S. This shift up time was remarkably constant, regardless of the growth conditions (Ashihara et al., 1978). Similarly, the time that removal of serum affects cells with a serum-restriction point in entering the S phase, represents a G_1 event whose occurrence takes place at a specific time before S. Using stationary cell cultures of chicken, rat and mouse fibroblasts, such a serum dependent G_1 event has been observed by Todaro et al. (1965), Yoshikura and Hirokawa (1968), Temin (1971), Pardee (1974) and Brooks (1976). In human glia and glioma cells such a G_1 event was demonstrated by Lindgren et al. (1975). In rat hepatoma cells we showed that the serum dependent event occurred 6-7 hr before S either in logarithmically growing cells with an average G_1 duration of 9 hr, or in a synchronized culture during the first cell cycle with an average G_1 duration of 23 hr, or in a synchronized culture during the second cell cycle in which the average G_1 duration of 3-4 hr. So far the data favour a certain chronological order in the events leading to DNA synthesis, i.e. at least some events are sequential and interdependent of each other. Moreover, our data lead to the conclusion that commitment can occur during the previous cell cycle. A similar conclusion can be drawn from data obtained with BHK cells by Bürk (1970). This means that the events leading to mitosis are independent and sometimes partially parallel to the events leading to S.

As pointed by Mitchison (1972), Hartwell et al. (1974) and Levine (1978) the cell may employ several different mechanisms to order one event in the cell cycle relative to another: (a) a pathway in series in which events are dependent upon each other, (b) pathways in parallel, so that events are dependent upon some sort of a clock (for instance cell mass), (c) a combination such as two parallel pathways each of which contains events in series. Apparently the regulation of the G_1 phase depends on two independent, sometimes parallel, pathways, at least one of them containing events in series. Such a model can explain a number of earlier observations. Thus, the possibility that the regulatory signal for entering S in serum dependent cells can be localized before M agrees with the observation that there are cells with no detectable G_1 (Robbins and Sharff, 1967). It can also explain that the variability in duration of the G_1 phase of sister cells can be less than the high variability in G_1 times of unrelated cells (Van Wijk et al., 1977). Finally, it might explain that under certain conditions cell polyploidy can occur (Brodsky and Uryvaeva, 1977).

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