The Periodic Loss of Metabolically Unstable DNA from Newly Replicated DNA of Mouse L Cells

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ABSTRACT

The fate of ^{3}H-thymidine incorporated into newly synthesized DNA of mouse L cells was analyzed. Under conditions in which DNA synthesis proceeded continuously, the incorporated radioactivity was periodically lost and regained during a 135-min chase as judged by either labeling index, acid-insoluble counts per cell, or mean grain count. When DNA synthesis was inhibited by hydroxyurea, the periodic changes of the incorporated radioactivity were not observed. The periodical changes were regarded as the result of an actual metabolic changes in ^{3}H-labeled DNA of the viable cells and the loss of radioactivity seems to require active continued DNA synthesis.

Key words: ^{3}H-thymidine incorporation; unstable DNA; hydroxyurea

INTRODUCTION

The technique of DNA labeling with ^{3}H-thymidine has been used for cell kinetic studies. In such experiments, once the incorporated radioactivity is considered to be kept still as long as the cells are alive. Although Zajicek and Gross[1] reported that the release of degraded DNA from cells under radiation damage began 60 min after the start of labeling, other investigators could not confirmed the phenomena[2-6].

On the other hand, Hershey and Taylor[7] reported that approximately 50% of the incorporated radioactivity was released to the acid-soluble fraction during a 15-min chase after pulse-labeling the nuclei of partially synchronized CHO cells in vitro. The release of the radioactivity was observed only when DNA synthesis was maintained.

The present investigation was undertaken to verify the result of Hershey and Taylor. Using a different mammalian cell line growing in

a more natural environmental condition, kinetics of ^{3}H-thymidine incorporation and its fate were studied.

MATERIALS AND METHODS

Cells: L5 cell line, a derivative of mouse L cells (B929-L2J)[8,9] was used. The cell line was donated by Dr. Tsuboi (Department of Physiology and Pathology, National Institute of Radiological Sciences, Chiba, Japan) and has been propagated in our laboratory since 1977 by subculturing at 5-day intervals.

Culture: The culture medium is Ham's F12[10] (Nissui Seiyaku Co., Tokyo) supplemented with 10% calf serum and 60 mg/l of Cefamedin (Fujisawa Pharmaceutical Co., Ltd., Osaka). The culture was screened from time to time for mycoplasm contamination[11]. Three days prior to the experimental use exponentially growing cells in stock culture were harvested by trypsinization using 5 ml of Puck's saline A containing 0.02% EDTA and 0.04% trypsin. Trypsin activity was blocked by the addition of an aliquot of medium containing 10% calf serum. After measurement of cell number in a Coulter counter, 2×10^5 cells were transferred to each of 10 Falcon plastic flasks (surface area=25 cm^2). Culture media with three different levels of radioactivity (0.05, 0.1 and 0.5 μCi/ml) were prepared by adding ^{3}H-thymidine (Radiochemical Center, specific activity=2.0 Ci/mmol) to the thymidine-deprived F12 medium. Hydroxyurea was added to the medium to a concentration of 1 mM.

Pulse-chase experiments: Cells labeled for 15 min in a medium with 0.05 μCi/ml of ^{3}H-thymidine were chased in either normal or hydroxyurea-containing (1 mM) medium and harvested for autoradiography after varying periods as described above. The labeled cells were washed 4 times with 5 ml of pre-warmed PBS before chasing. For measurement of radioactivity with liquid scintillation spectrometer, the cells were labeled in the medium containing 0.5 μCi/ml of ^{3}H-thymidine.

Continuous labeling experiments: Cells were grown in a medium containing ^{3}H-thymidine at a concentration of 0.05 μCi/ml for autoradiography or 0.1 μCi/ml for liquid scintillation counting. In another experiment, hydroxyurea was added 30 min after the culture was transferred to ^{3}H-thymidine containing medium. Cells were harvested at varying periods.

Autoradiography: Cells were suspended in 5 ml of 50% acetic acid with a vortex mixer, centrifuged, and fixed in 5 ml of acetic acid-alcohol (1:3) for 1 hr. They were then smeared on slides using Autosmear (Sakura Seiki Co., Ltd., Tokyo), treated with 5% trichloroacetic acid (TCA), washed successively with 70% alcohol and distilled water. These

slides were coated with liquid photographic emulsion (Type ET2F, given generously by Fuji Photo Film Co., Ltd., Tokyo) by dipping method and exposed in a refrigerator at 4°C for 10 days before development.

Grain count: Since background level of autoradiography by this method was very low, most cells that were labeled had more than 5 grains, cells with less than 5 grains were neglected. Mean grain count was determined from observation of 100 labeled cells.

Labeling index: More than 1000 cells were observed to determine the labeling index. The nucleus with less than 5 grains was scored unlabeled.

Counting of acid-insoluble materials: Cells cropped by trypsinization and centrifugation were suspended in 5 ml of ice-cold PBS. One ml of the suspension was used to determine the cell number. The cells in 2 ml of the suspension were collected on Whamtan GF/C filter of 4.1 cm in diameter, washed three times with 1 ml of 5% TCA and once with distilled water, dried, and then treated for 2 hr in 1 ml of Soluene-350 solubilizer (Packard). The radioactivity was counted in 10 ml of Aquasol (New England Nuclear) by a liquid scintillation counter (Packard).

RESULTS

Pulse chase experiment: In the growing cell population, the labeling index, the acid-insoluble count per cell and mean grain count showed cyclic changes with a period of about 45 min, running almost parallel with each other, until 135 min after pulse (Figs. 1, 2 & 3). The cycle was conspicuous and each trough height was about one half of the precedent peak.

In non-growing cell culture in which DNA synthesis was arrested, no cyclic change of these measurements was observed although the labeling index and mean grain count gradually decreased over the period of 90 min after pulse (Figs. 1, 2 & 3).

Continuous labeling experiment: The curves of labeling index and mean grain count derived from growing culture were shown in Fig. 4. Cumulative labeling index did not increase appreciably until the end of this experiment at which time it increased slightly. Mean grain count increased almost linearly with time up to 45 min and stayed unchanged thereafter for at least 30 min to form a plateau. After 75 min grain counting was impossible because of heavy labeling. Fig. 5 illustrated the curves of the acid-insoluble count per cell of the DNA synthesizing and non-synthesizing cell population. The pattern of the DNA synthesizing population showed the similar staircase pattern as in mean grain count

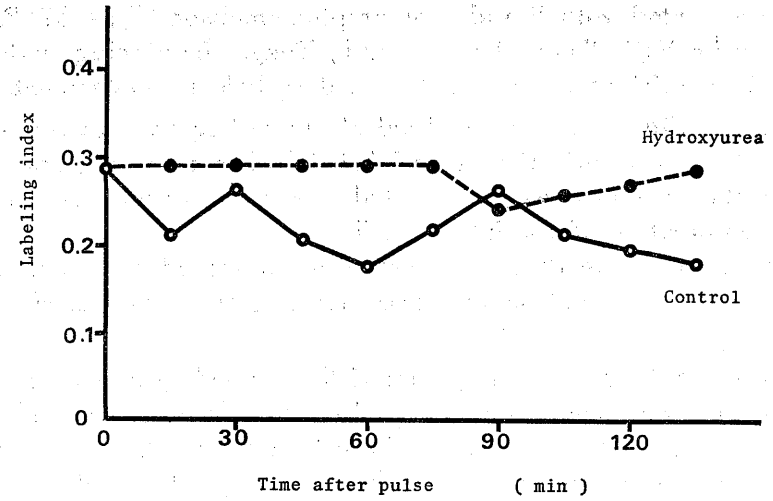

Fig. 1. The fate of ^{3}H-thymidine incorporated into DNA (labeling index): Cells were pulse-labeled for 15 min and chased with control medium —○—, or medium containing 1 mM of hydroxyurea ---●---. Standard deviation of each circle was less than 0.03

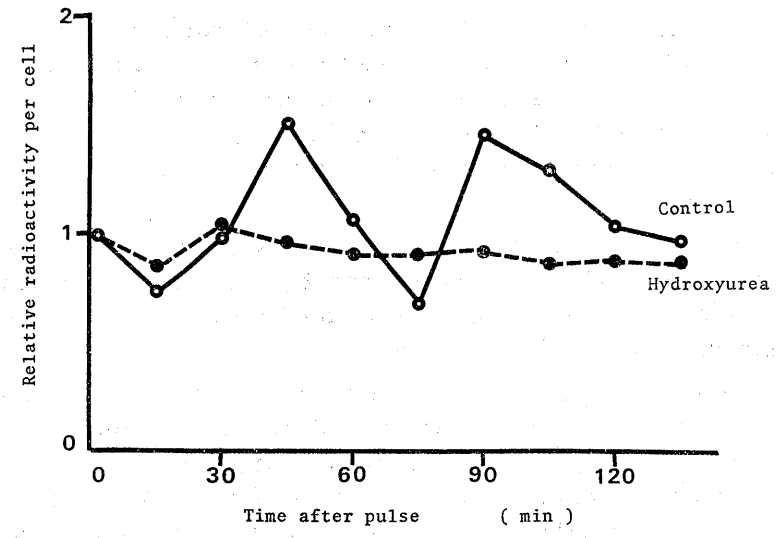

Fig. 2. The fate of ^{3}H-thymidine incorporated into DNA: Cells were pulse-labeled for 15 min and chased with control medium —○—, or medium containing 1 mM of hydroxyurea ---●---. The relative increase in radioactivity per cell is graphed as a function of time. The initial radioactivity was 1238 cpm per 1.8×10^{5} cells.

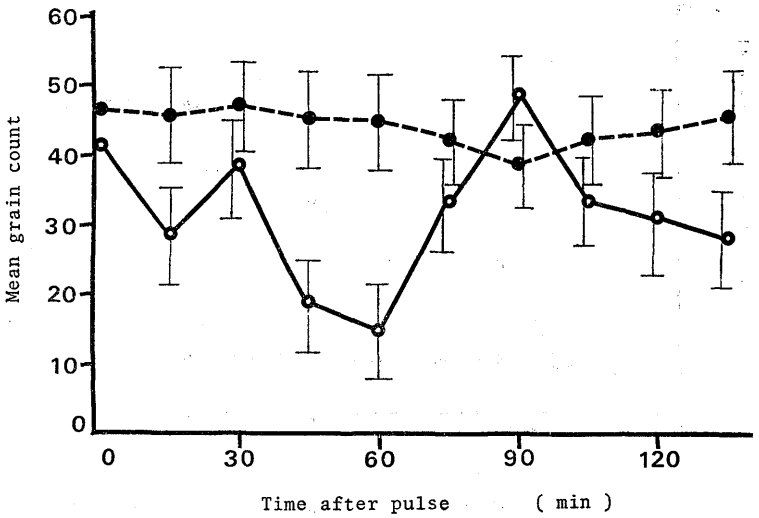

Fig. 3. The fate of ^{3}H-thymidine incorporated into DNA (mean grain count): Cells were pulse-labeled for 15 min and chased with control medium, —○—, or medium containing 1 mM of hydroxyurea---●---.The vertical bar of each circle indicates standard deviation.

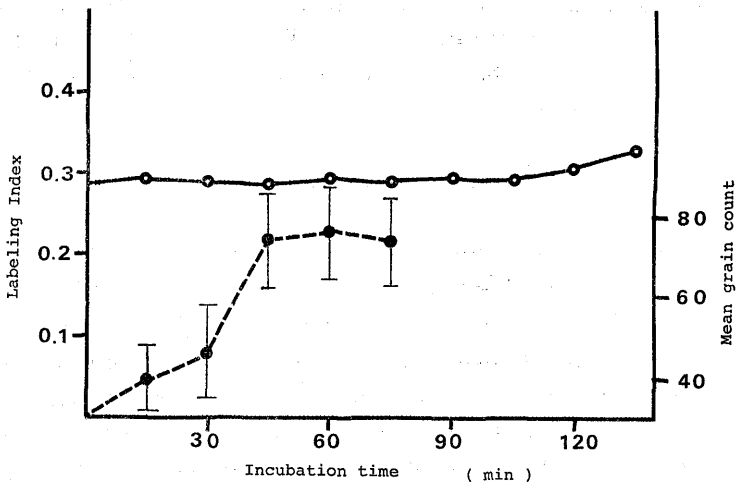

Fig. 4. The kinetics of ^{3}H-thymidine incorporation into DNA (labeling index and mean grain count): Cells were continuously exposed to ^{3}H-thymidine-containing medium. Labeling index and mean grain count were represented by —○— and ---●--- respectively.

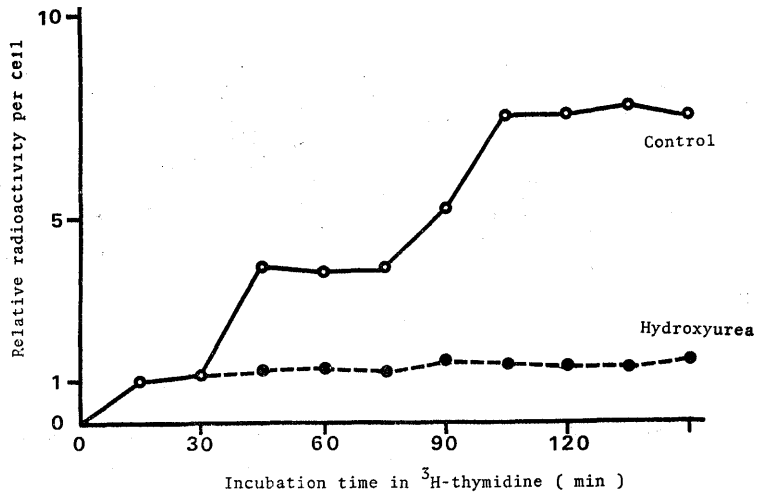

Fig. 5. The kinetics of ^{3}H-thymidine incorporation into DNA: Cells were continuously exposed to ^{3}H-thymidine —○—, or after 30 min label hydroxyurea was added to a final concentration of 1 mM ---●---. The relative increase in radioactivity per cell is graphed as a function of time. The initial radioactivity was 1324 cpm per 2.1×10^{5} cells.

pattern (Fig. 4). The plateau phase of the mean grain count and the acid-insoluble count per cell curves corresponded to the phase when labeling index had dropped after pulse. When 1 mM of hydroxyurea was added to the culture, the cell growth ceased and the acid-insoluble count did not increase at all.

DISCUSSION

Contrary to our expectation, radioactivity per cell ceased increasing 45 min after the start of labeling and the plateau lasts for at least 30 min (Fig. 5). We have repeatedly confirmed this phenomenon.

When Cleaver and Holford[2] and Adams[3] reported a linear increase of radioactivity in a similar experiment, they did not present the data for the period from 30 to 60 min. On the other hand, the data of Kuebbing and Werner[12] (Fig. 1 in reference 12) appears to be a stepwise increase. Several other investigators[13,14,15] also noticed this phenomenon. A question arises that the loss of the radioactivity is due to cell disruption. The possibility seems unlikely because grain count increased during continuous labeling. If it were the case, a preferential loss of heavily labeled cells should occur. Furthermore, a rapid return to the previous level from 50% drop of labeling index (and of radioactivity) during

chasing periods after short pulse can hardly be explained by the disintegration and regrowth of cells. Conversely, the absence of a noticeable depression in the continuous labeling index curve or in the radioactivity-per-cell curve favors another interpretation that some fraction of DNA is released at the same rate as new DNA replication. Temporal coincidence between the plateau of radioactivity-per-cell curve in cumulative labeling and the drop of that observed in pulse chase experiment suggests that radioactivity is released periodically, whereas the DNA synthesis proceeds continuously. It is of interest to note, in this connection, that periodic change of labeling index occurs only when the active DNA synthesis continues. The possibility that it represents some type of repair synthesis after degradation of DNA due to internal radiation is excluded since it has been reported that hydroxyurea did not inhibit repair synthesis[16]. Therefore, a release of DNA moiety from actively growing cell population seems to be an integral part of a normal process of semiconservative DNA replication. Similar phenomenon due to the degradation of metabolic DNA has been reported in non-dividing or highly differentiated cells[17].

Although the mechanism for the periodic loss of ^{3}H-label from newly replicated DNA is not clear, Hershey and Taylor[7] have proposed one possible explanation based on the sedimentation analysis of newly replicated DNA isolated from CHO cell nuclei. They found that ^{3}H-label observed in 4S DNA fragments after pulse label becomes acid-soluble during a chase performed under conditions which permit continued active DNA synthesis. Their interpretation is as follows: The 4S fragment may be a normal fragment in DNA replication and still be metabolically unstable. If one assumes that one strand is replicated more or less continuously in the direction of the fork movement, the other strand would be single-stranded for up to 1000 nucleotides if the only other fragment possible were the gene-sized 10S Okazaki fragment. To reduce the amount of single-stranded DNA which is apt to undergo irreparable damage, DNA synthesis may proceed in the opposite direction from the fork movement in short 100–150 nucleotide pieces. However, these 4S pieces are not ligated into larger fragments, but are eventually replaced by the 10S fragment and degraded. Okazaki et al[18]. reported similar results in prokaryotes and discussed the secondary structure of the replicating region. Huberman and Horwitz[19] suggested the possibility that DNA is synthesized in excess and some undergoes degradation. Our present results seem to support the hypothesis of Hershey and Taylor and, further, suggest that the degradation process of newly replicated DNA occurs periodically.

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