Bull. Yamaguchi Med. Sch., Vol. 28, Nos. 1-2, 1981

Change of Thymidine Uptake Rate during Passage through the S Phase: A Study with Retrograde Synchrony Method

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(Received February 10, 1981)

Abstract. Tritiated-thymidine uptake rate at various points within the S phase was measured in exponentially growing hamster kidney carcinoma cells in vitro (HKC 400) by a new retrograde synchrony method. Although the method is analogous to the labeled mitoses technique, it utilizes the data of radioactivity per labeled mitosis obtained by cytophotometric measurement of silver grains in autoradiogram. The uptake rate showed biphasic pattern: the first peak which was the highest was in the mid-S phase and the second was just before the end of S. There was a marked depression in the late S phase and it was as low as 5% of the first peak. This marked depression suggested the possibility that the S phase is divided into at least 2 subphases with regards to the utilization of the exogenous thymidine.

Key Words: cell cycle, S phase, thymidine uptake rate, retrograde synchrony, autoradiogram

Introduction

It is well-known that DNA synthesis, an integral part of cell growth and division, is a highly ordered and genetically programmed biochemical process but thymidine uptake rate is by no means uniform through the S phase. Lajtha et al[1]. remarked on the possible change in the rate of DNA replication during the S phase of normal cell cycle. Hamilton[2] supposed, in an attempt to explain sharp depressions in the labeled mitoses curve, that DNA synthesis is transiently interrupted within the S phase. Many other workers[3-6] have reported that thymidine incorporation rate increases towards the mid-S, and after a short interval of depression it rises again in the late S phase. Remington & Klevecz[5], in the survey of literature, suggested that the S phase can be divided into 2 to 5 distinct periods with respect to DNA synthesis rate. However, most of these data were based on the experiments which utilize synchronized cell population. Normal cell-cycle traverse can not be expected when synchrony is induced by drug. Mitotic selection also may cause derangement from balanced growth because normal cell-to-cell interaction is lost. Furthermore, the degree of synchrony will deteriorate during passage

through the G1 phase.

To avoid these difficulties, the present investigation used a method of retrograde (or retroactive) synchrony[7] which observes synchronized cell cohort within an exponentially growing cell population by pulse-labeling the cells with ^{3}H-thymidine and harvesting mitoses after variable intervals. Prerequisite to this method is that ^{3}H-thymidine taken up by the cells totally incorporated into acid-insoluble fraction before entering mitosis, and it was proved that this condition is satisfied the tumor cell population used in this experiment.

Materials and Methods

Cell: A monolayer culture of diethylstilbestrol-induced kidney carcinoma (HKC 400) of Syrian hamster[8] was used, which has been propagated by subculturing at weekly interval using Ham's F12 medium (Nissui Seiyaku Co., Tokyo) supplemented with 10% calf serum and 60 μg/ml of Cephamedin. The inoculation size in each culture flask (TD-40, Ikemoto Rika Kogyo Co., Tokyo) was 4×10^5 cells. Subculture was made by cropping cells using Puck's saline containing 0.02% EDTA and 0.04% trypsin.

Growth curve: Cells were plated to 10 flasks at a density of 4.3×10^5 cells per flask and sampled at 12 hr interval. The cell number was counted in a Coulter counter (Coulter Electronics, Inc., Florida).

Medium for labeling: Thymidine-deprived Ham's F12 (Nissui Seiyaku Co.) was supplemented with variable amounts of ^{3}H-thymidine (specific activity: 2.0 Ci/mmol, Radiochemical Center, Amersham).

Pulse labeling and chase: Using cells subcultured 2 days in advance, 2 series of experiments (Exp. A & B) were performed. In experiment A, cells were labeled in a medium containing 0.1 μCi/ml of ^{3}H-thymidine for 20 min whereas in experiment B the radioactivity and labeling time were 0.4 μCi/ml and 5 min, respectively, in order to make the product equal for both experiments. The cultures were washed 3 times after pulse, chased for variable times without using Colcemid or any other mitosis-blocker and mitoses were collected by shake-off method[9]. The sampling interval in Exp. B was shorter than that in Exp. A as shown in Figs. 5 & 6.

Autoradiography: Collected mitoses were centrifuged, fixed in ethanol-acetic acid (3:1) for 30 min and transferred to 50% acetic acid to effect thorough spreading of chromosomes. They were then centrifuged onto slide glass at 2000 rpm for 20 min using Autosmear (Sakura Seiki Co., Tokyo). The smears were rinsed in distilled water to remove fixative and coated with photographic emulsion Type ET-2F (generously given by Fuji Photo Film Co., Tokyo) by dipping method at 40°. They were then stored with dessicant in refrigerator at 4° for 7 days before development. A special care was taken to develop all slides under the same condition as to the time and temperature. They were stained very lightly with Mayer's hematoxylin to identify mitoses.

Measurement of grain density: In order to estimate radioactivity, density of silver grains was measured in arbitorary unit (AU) on 50 to 100 mitoses per sample using M85 scanning microdensitometer (Nikon-Vickers Instruments, England) under high-power view (×400). A wavelength of 760 nm was chosen for maximum absorbance with the silver grains and minimum effect from hematoxylin. The flying spot was set at 1 μm in diameter. Absorbance was measured at 50×50 spots per microscopic field and integrated automatically. The background level was practically zero but readings below 1.0 AU were neglected to avoid error from instrumental noise.

Calibration: To examine a linear relationship between grain density in autoradiogram and radioactivity in cell, the following experiment was performed. Culture media with 5 different levels of radioactivity (0.06, 0.1, 0.3, 0.7 and 1.0 μCi/ml) were prepared by supplementing variable amount of ^{3}H-thymidine (specific activity: 2.0 Ci/mmol). Final concentration of total thymidine was adjusted to 0.5 nmol/ml by the addition of non-labeled thymidine (Kohjin Co., Tokyo) (Table 1). An exponentially growing cell population was labeled for 20 min in these media. Mean grain density per labeled cell (mitotics and intermitotics) measured as described above was plotted against radioactivity of the medium (Fig. 4).

Radioactivity per labeled mitosis (RLM): Mean radioactivity of labeled mitoses (as expressed in mean grain density) in each sample was plotted against time after pulse-labeling (Figs. 5 & 6).

Table I. Specific activity and final concentration of culture media for calibration.

specific activity (Ci/mmol)	final concentration (μCi/ml)
0.12	0.06
0.2	0.1
0.6	0.3
1.4	0.7
2.0	1.0

Table II. Cycle time (hr) analysed with FLM method.

TC	TG1	TS	TG2	TM
18.5	7.4	6.0	4.9	0.2

Mitoses with grain density below 1.0 AU were excluded in the calculation of the mean grain density.

Fraction of labeled mitoses (FLM): The ratio of labeled to total mitoses was calculated in each sample using the same criterion as in RLM to differentiate labeled and unlabeled mitoses and plotted against time after pulse. The fraction of labeled mitoses curve constructed in this way was analyzed by computer[10] to extract cell cycle parameters.

Kinetic study of thymidine incorporation: In order to know whether or not thymidine is incorporated completely and instantaneously into acid-insoluble fraction, ramdom-growing cell population was labeled with ^{3}H-thymidine (1.0 μCi/ml) for 10 min. Then cells were harvested at various intervals and washed 3 times with 0.3 N perchloric acid (PCA) at 4° for 10 min. The cell pellet was dissolved in 2 N NaOH at 120° for 1 hr and radioactivities in PCA-insoluble fractions were measured by liquid scintillation spectrometer M 3330 (Packard Instrument, Inc., Illinois). The radioactivity in PCA-soluble fraction obtained immediately after labeling was also measured.

Results

Growth curve : Culture entered exponential phase of growth from 30 hr after subculturing and it lasted for at least 60 hr that follows (Fig. 1), so that following experiments were started at 48th hr.

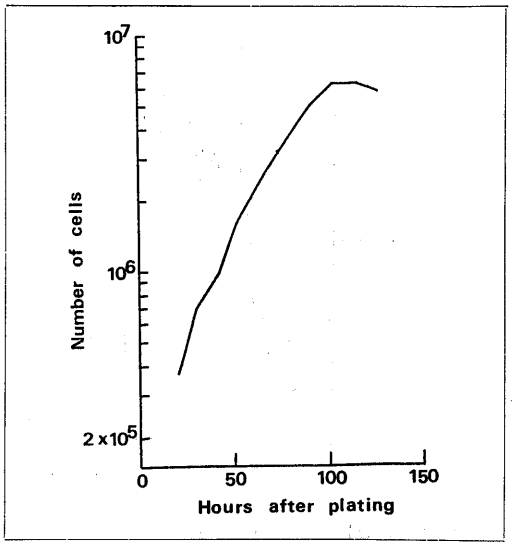

Fig. 1 Growth curve of HKC 400 cells
The cell number in each flask was counted at various intervals. The initial cell number was 4.3×10^5 per flask.

Cell cycle : The FLM curve and the cell cycle parameters in experiment A were shown in Fig. 2. and Table II. The data for experiment B (not shown here) were nearly equal to those for A.

Pool of ^{3}H-thymidine : Radioactivity of cells increased very rapidly to reach 49 cpm/10^3 cell level within 10 min of pulse-labeling. Most of it was in the acid-insoluble fraction and only 1.4% was in acid-extractable fraction. During chase in isotope-free medium, radioactivity in acid-insoluble fraction did no longer increase appreciably (Fig. 3) and it excluded the possibility for free ^{3}H-thymidine being pooled to be incorporated continuously into DNA before entering mitosis.

Calibration of autoradiographic grain density : Mean grain density measured in arbitrary unit was in linear relatioship with the radioactivity of the medium used for labeling (Fig. 4).

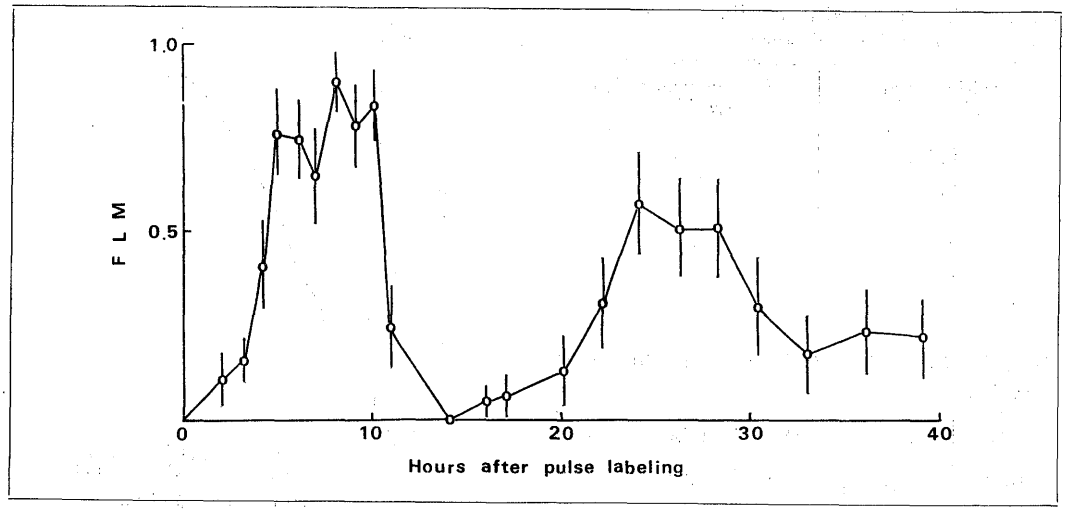

Fig. 2 FLM curve in Exp. A

After pulse labeling with ^{3}H-thymidine (0.1μCi/ml, 20 min), the fraction of labeled mitoses was measured at various times. Confidence intervals (95%) are shown by vertical line. In Exp. B, the curve was almost the same to that of Exp. A.

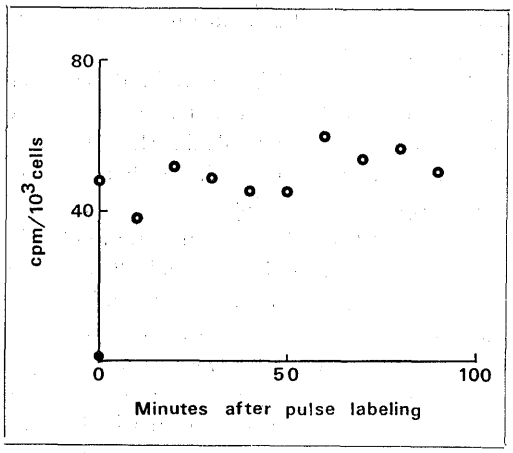

Fig. 3 ^{3}H-thymidine pool effects

Cultures were labeled for 10 min and chased in non-radioactive medium for various intervals. The data points showed the radioactivity of acid-insoluble fraction (○) and soluble fraction (●).

Grain density over the mitoses : Density of silver grains on the individual mitosis was greatly variable even in the same sample. However, the mean for each sample, when plotted against time after pulse, exhibited a rather regular pattern of damped oscillation (Fig. 5 & 6). The first and the second waves were approximately 19 hr apart as in the corresponding FLM curves but the second wave was smaller than in the FLM curve because radioactivity was halved as the consequence of cell division. The waves were not rectangular as would be expected when constant thymidine uptake rate throughout the S phase is assumed. In each experiment, there was a marked depression of the curve at 6 hr or 5.5 hr after pulse, the time period that corresponds to the late S phase.

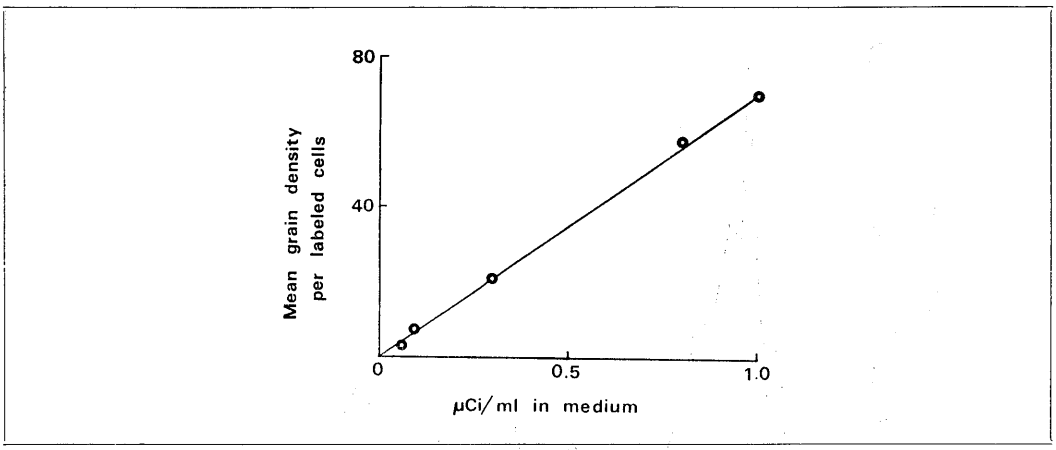

Fig. 4 Calibration line

Five cultures were labeled with ^{3}H-thymidine in various concentrations for 20 min. Harvested cells were smeared, autoradiographied and measured a mean grain density over cells.

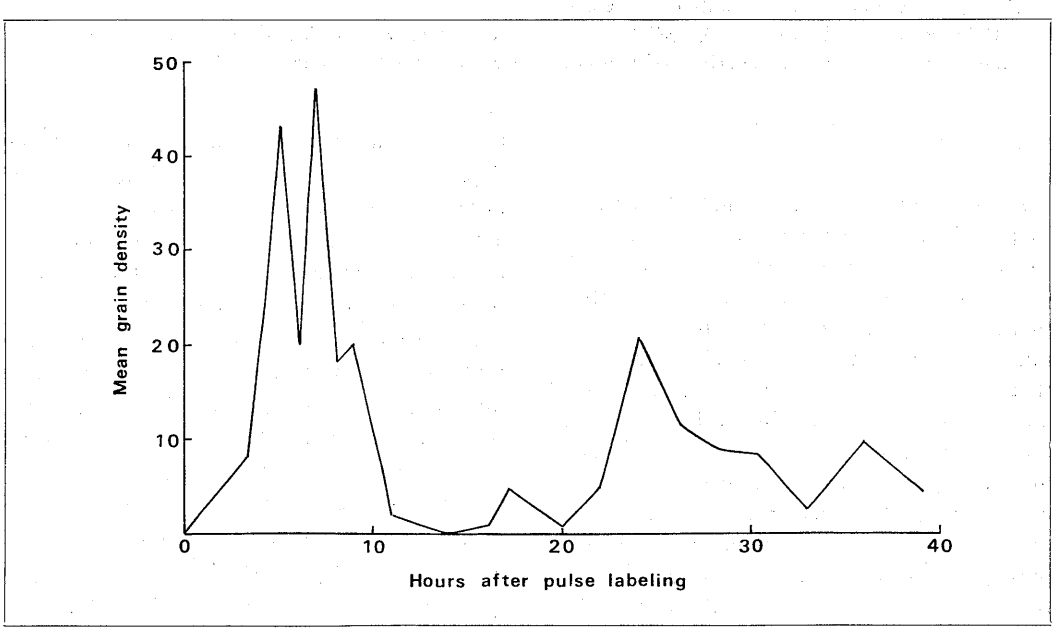

Fig. 5 ^{3}H-thymidine incorporation in Exp. A

Asynchronous cultures were pulse-labeled (0.1 μCi/ml, 20 min) and chased up to the time at mitotic selection. Collected mitoses were autoradiographied and cytophotometried to measure a mean grain density

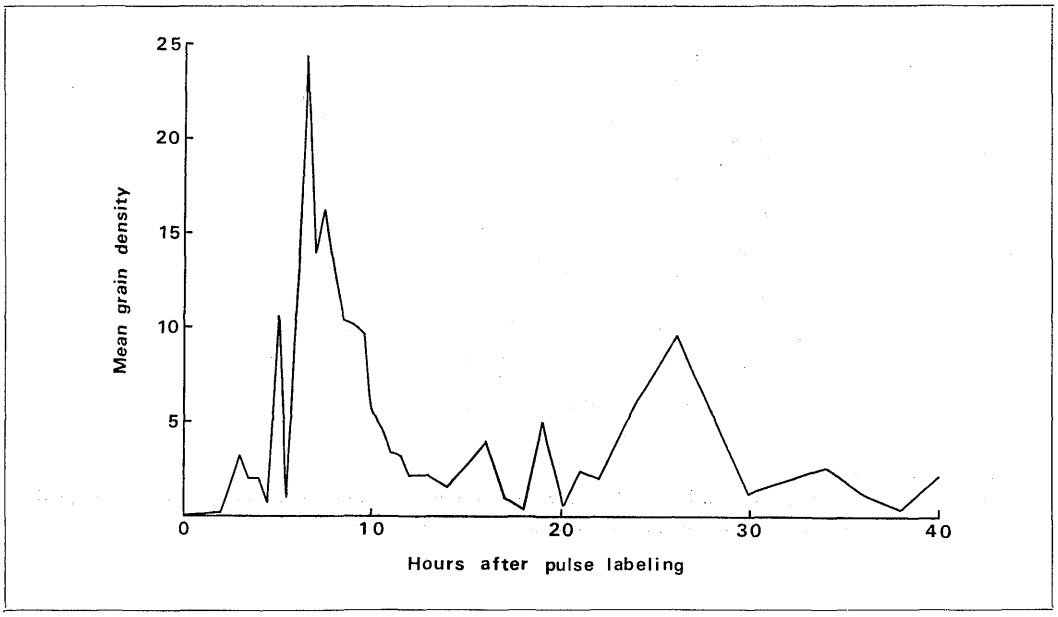

Fig. 6 ^{3}H-thymidine incorporation in Exp. B
Cultures were pulse-labeled (0.4 μCi/ml, 5 min) and mitoses were collected with the more narrow interval than in Exp. A. Experimental procedure was the same to that in Exp. A.

Discussion

A retrograde synchrony method is similar in principle to the procedure of Bostock & Prescott[11] used to characterize DNA molecules synthesized in the early and late S phase. It is also analogous to the labeled mitoses method for cell cycle analysis in that asynchronous culture is pulse-labeled and chased for variable intervals and mitoses are observed. In the present method, mean radioactivity per labeled mitosis was measured and plotted against time after pulse, instead of counting the number of labeled mitoses, to estimate thymidine uptake rate in various portions of the S phase. This method is advantageous over the others which utilize drug-induced synchrony for it does not interfere with normal cell-cycle progression. A block at G1/S boundary is in itself a perturbation. Any measurement made under such circumstances will not exactly reflect normal biochemical sequences in the S phase. The use of shaked-off mitoses also poses problems for normal cell-to-cell interaction is abolished and synchrony will decay rapidly as they pass through G1 phase which is most variable in duration.

Incorporation into DNA of exogenous thymidine proceeds at least in two steps: thymidine enters the cells and is incorporated into macromolecular DNA. Lag time, when exists, complicates the analysis. Cleaver[12] observed gradual increase of grain count in mouse L cells for a few hours after pulse and stated that a part of ^{3}H-thymidine remains free for some time before being incorporated. But, it was not the case with the tumor cell line used in the present experiment. If ^{3}H-thymidine is completely incorporated into acid-insoluble fraction before entering mitosis, the RLM curve should exactly represent thymidine uptake rate. If

some fraction of ^{3}H-thymidine remains free in the late S phase, a true thymidine uptake rate should be even higher than described in Figs. 5 and 6. Pulse-labeled cells enter mitosis but the sequence will be disturbed as they traverse the G2 phase due to variable sojourn time so that actual uptake rate should be more sharply oscillating than would be imagined from raw data. An experimental point with low RLM value may actually indicate the time at which cells stop utilizing exogenous thymidine temporarily.

Thymidine uptake rate does not represent DNA synthesis rate because unknown fraction of thymidine is supplied by de novo synthesis. GC-rich DNA is synthesized early in the S phase and AT-rich DNA in the late S phase[11,13,14]. However, heterogeneity of DNA molecules with respect to nucleoside is not enough to explain the marked difference in the thymidine uptake rate.

Many reports[3-6,9,15-18] have appeared on this subject and despite different methods of analysis employed, most data indicated marked variations of the uptake rate and some[3-6] showed biphasic pattern. The marked depression, even if not the cessation, of thymidine uptake rate should have profound implication in the interpretation of the FLM curve and the present results seem to render support to the view of Hamilton[2].

Acknowledgment

I am grateful to Prof. M. Takahashi for his continuous encouragement, helpful advice and valuable discussion.

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