Accumulation of S Phase Populations of FM3A Cells Growing in the Mouse due to Administration of 5-Fluoro-2'-Deoxyuridine

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ABSTRACT. We have established procedures for the accumulation of an S phase population of FM3A cells grown in the mouse. Mice were kept for 5 days after intraperitoneal inoculation with $1 \times 10^6$ FM3A cells, then they were treated with 100 $\mu$g of 5-fluoro-2'-deoxyuridine for 16 h. When the FM3A cells were taken from the mice and cultured in vitro, more than 70% of the cells synthesized DNA, and the cell number increased 1.8-fold after 14 h. DEAE-cellulose column chromatography of the nuclear extract fraction showed that the synchronized cells contained about three times the activity for DNA polymerase $\alpha$ than that of random cells. Activity in the cytoplasmic fraction remained at the same level.

During our study of the function of mammalian DNA polymerases, we found that HeLa cells contain at least two forms of DNA polymerase $\alpha$; P-1 and P-2 (11). These differ in their degree of affinity for DNA. The variation in the activity of P-1, which has the higher binding affinity for DNA, was correlated with the rate of DNA synthesis in vivo (12). To study this heterogeneity of polymerase $\alpha$ in detail, we used mouse FM3A cells. The cell line had been established both by suspension culture and in the mouse (9). In the mouse, FM3A cells grow to a high concentration in the abdominal cavity, and this provides the researcher with large amounts of rapidly growing cells without having to use expensive equipment. We had already isolated several temperature-sensitive and drug-resistant mutants from FM3A cells (7, 8, 10, 14), and now are isolating more temperature-sensitive mutants. Therefore, it will soon be possible to combine a biochemical study with genetic analysis.

We here describe the procedures and results of the administration of 5-fluoro-2'-deoxyuridine (FUdR) to mouse FM3A cells growing in the mouse. To determine events in the S phase, such as the behavior of the replication enzymes, we have to accumulate as many S populations as possible. This study has provided us with the basis for research on the mechanism of DNA synthesis in mouse FM3A cells.

Abbreviations used: FUdR, 5-fluoro-2'-deoxyuridine; MEM, minimum essential medium; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; CMF-PBS, Ca$^{2+}$, Mg$^{2+}$-free phosphate buffered saline.
MATERIALS AND METHODS

Cells and growth conditions. FM3A cells (clone 28) were obtained from the Cancer Institute (Toshima-ku, Tokyo 170, Japan). These cells can be grown both in mice and in suspension culture, and can be transplanted from one form of culture to the other without difficulty. Transplantation from the suspension culture to the mouse was by an intraperitoneal injection of 0.1 ml of the cell suspension (1 × 10^7 cells/ml) to 7-week old female C3H/He mice. These C3H/He mice were obtained from the Funabashi Farm (Chiba 273, Japan). Cells for explantation to in vitro cultures were obtained after repeated washings of the peritoneal cavities of 3 randomly selected animals with CMF-PBS. Cells were sedimented by centrifugation (5 min, 500 × g), then washed successively with CMF-PBS and the growth medium (Eagle's MEM [Nissui Pharm.] supplemented with 10% calf serum [GIBCO] and Eagle's non-essential amino acids), after which they were resuspended in the growth medium (1 × 10^7 cells/ml). Viability of the cells was checked by the dye exclusion method with 0.06% erythrocin solution. A 0.2 ml portion of the cell suspension was inoculated into 30 mm Petri dishes, and the cells were incubated with 1.8 ml of the growth medium at 37°C.

Radioactive DNA labelling and autoradiography. Samples were incubated for 60 min at 37°C with 1 μCi/ml [3H]thymidine (6413, 5 Ci/mmol: Amersham). After being washed with ice-cold CMF-PBS, 2 portions of 2/5 of the cells (8 × 10^5 cells) were sedimented, then lysed with 0.1% SDS, and acidified with 10% TCA. Acid-insoluble radioactivity was measured as described previously (11).

For autoradiography, 1/5 of the labelled cells (4 × 10^5 cells) were suspended in 0.15 ml CMF-PBS, then 0.15 ml methanol/acetic acid (3 : 1) was added in drops. The cells then were sedimented and suspended in 0.15 ml of this fixative, after which they were allowed to stand at room temperature for 10 min before final transfer to glass slides. Two slides were prepared from each sample. After washing and fixation, the slides were coated with Sakura NR-M2 emulsion and exposed in an air-tight container with a desiccant at 4°C for 10–20 days, then they were developed in Sakura Konidol developer and counterstained with Giemsa. At least 400 cells on each slide were evaluated for the labelling index.

Subcellular fractionation. FM3A cells collected from the ascites fluid of mice were washed once with CMF-PBS and twice with buffer 1 (20 mM K-PO₄, pH 7.5, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.25 mM phenylmethansulfonyl fluoride). The resulting pellet (ca. 2.5 × 10^9 cells) was suspended in the same buffer (1.2 × 10^8 cells/ml) and sonicated at 4°C 5 times for 10 sec each with 30 sec intervals in a Branson Sonifier model 185 (25–30 w). The sonicate was centrifuged for 30 min at 15,000 × g, and the supernatant (the cytoplasmic fraction) was separated from the pellet. The pellet then was suspended in 8.3 ml of buffer 1 containing 300 mM KCl, and allowed to stand at 4°C for 60 min, after which it was centrifuged at 15,000 × g for 30 min. The supernatant was designated the nuclear extract.

DEAE-cellulose column chromatography. The cytoplasmic and nuclear extract fractions were dialyzed against buffer 2 (buffer 1 plus 20% ethylene glycol) containing 50 mM KCl, then they were centrifuged at 15,000 × g for 30 min to remove any precipitate formed during dialysis. Their clear supernatant fractions (10 OD₂₈₀; ca. a 5 × 10^8 cell equivalent for the cytoplasmic fraction and ca. a 1 × 10^9 cell equivalent for the nuclear extract fraction) were applied to the DEAE-cellulose (Brown Co.) columns (5 ml bed volume) which had been equilibrated with buffer 2 containing 50 mM KCl. After washing the columns with 2–3 bed volumes of the same buffer, proteins were eluted with a 40 ml-linear gradient of 50 to 400 mM KCl in buffer 2.

DNA polymerase assay. The assay for DNA polymerase activity was carried out under conditions conducive to the detection of DNA polymerase α. The final reaction volume of
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30 µl contained 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 4 mM 2-mercaptoethanol, 200 µg/ml of bovine serum albumin, 500 µg/ml of activated calf thymus DNA (1), 100 µM each of dATP, dCTP, dGTP and 10 µM [³H]dTTP (0.5 Ci/mmol). Incubations were carried out at 37°C for 30 min in plastic immunological microtitration trays. Then the reaction mixture was transferred onto a WhatmanDE81 filter paper disk. This disk was washed by the method of Lindell et al. (6), after which radioactivity was determined with a liquid scintillation spectrometer.

RESULTS

Growth curve of FM3A cells in the mouse. The growth curve of FM3A cells in the mouse is shown in Fig. 1. When 1 × 10⁶ FM3A cells in the logarithmically growing phase were inoculated into the peritoneal cavity, they grew rapidly after a short lag period. The doubling time at the log phase was about 13 h, which is about the same as the doubling time in a suspension culture at 37°C. After reaching 5 × 10⁸ cells per mouse, cell number decreased rapidly. All the mice died of tumors 12 to 13 days after inoculation.

Effect of FUdR on partial synchrony. In preliminary experiments, we tested the effects of hydroxyurea, amethopterin and FUdR on the accumulation of S phase FM3A cells; FUdR was the least toxic agent. FUdR has been reported to synchronize

![Fig. 1. Growth curve of FM3A cells in the mouse. 1 × 10⁶ FM3A cells in the logarithmic growth phase were injected intraperitoneally. Cells were removed daily from 3 randomly selected mice, and cell number was determined. Values are the means of the 3 samples ± S. D.](image-url)
mammalian cells (2), by inhibiting thymidylate synthetase with its phosphorylated form (5). We used this agent on FM3A cells growing in mice. Mice were kept for 5 days after an intraperitoneal inoculation of $1 \times 10^6$ FM3A cells, and then they were treated with various doses of FUdR to the peritoneal cavity for 16 h. The FM3A cells were removed from the mice, and the cell number and $[^3]H$thymidine labelling index were checked. High doses of FUdR decreased the total cell number in a dose-dependent

![Graph](image1)

**Fig. 2.** Effect of various doses of FUdR on the cell number of FM3A cells per mouse. Cells were injected into mice as described in Fig. 1. On the 5th day after injection, various amounts of FUdR were injected into their peritoneal cavities, and the animals were kept for 16 h. Mean cell numbers (± S. D.) are given.

![Graph](image2)

**Fig. 3.** Effects of various doses of FUdR on the labelling index of FM3A cells. Cells were treated with FUdR as described in Fig. 2, then removed from the mice. The removed cells were labelled with $[^3]H$-thymidine in vitro for 60 min, then put through autoradiography as described in MATERIALS AND METHODS.
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manner (Fig. 2). By contrast, FUdR treatment increased the labelling index from 40% to 70% (Fig. 3). The viability of FM3A cells ranged from 85 to 95% for all the doses of FUdR used. The minimum FUdR dose that produced the maximum labelling index was 100 µg per mouse, therefore, we used this as the administration dose.

We next checked the exposure time to FUdR. After the injection of 100 µg of FUdR to mice on the 5th day after the inoculation of FM3A cells to the peritoneal cavity, the mice were killed at 4 h intervals and their ascites fluid taken. The cells were washed and incubated in vitro with [³H]thymidine. Figure 4 shows changes in the radioactivity per 8 x 10⁵ cells and Fig. 5 the labelling index; both indices reached maximums at 12 to 16 h of incubation. Thus, FUdR treatment for 16 h was adopted as the optimum period for the accumulation of S phase cells in the mouse.

Changes in cell number and the rate of DNA synthesis in culture were measured after a 100µg FUdR treatment for 16 h in the mouse (Fig. 6). The rate of DNA synthesis increased gradually, but was followed by a decrease which corresponded to the period of the increase in cell number. Before reaching its lowest level, incorporation of [³H]thymidine again increased. Thus synchronization of the cells was partial, but our results indicate that FM3A cells, treated with FUdR in vivo, continue the normal growth cycle in vitro for at least one generation. In the control experiment in which no FUdR was administered, the incorporation of [³H]thymidine per cell did not change significantly, and the increase in cell number was small (data not shown). This indicates that partial synchrony was caused by the administration of FUdR.
Fig. 5. Effect of exposure time to FUdR on the labelling index of FM3A cells in mouse. Conditions for this experiment were the same as described for Fig. 4, except that the labelling index was measured. Mean percentages of the labelled cells (± S. D.) are given.

Fig. 6. Changes in the rate of DNA synthesis and in the number of FM3A cells in vitro after FUdR treatment of the mouse. Sixteen hours after the administration of 100 µg of FUdR to mice on 5th day after the inoculation of $1 \times 10^6$ FM3A cells to the peritoneal cavity, cells were removed and incubated in vitro. The rate of DNA synthesis and the cell number were measured every 2 h. Values given are means ± S. D.
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not by the simple transfer of cells from mice to a suspension culture. The incorporation of \[^3H\]thymidine per labelled cell was similar for both FUdR-treated and non-treated cells, evidence that the overall rate of DNA synthesis in FUdR-treated cells does not differ from that in non-treated cells.

Effect of FUdR treatment on the pattern of DNA polymerase α. To test the applicability of our procedures to the study of the heterogeneity of DNA polymerase α, we examined the chromatographic pattern of the DNA polymerase α from FM3A cells on a DEAE-cellulose column. The patterns of DNA polymerase α in the cytoplasmic and nuclear extract fractions from both radomly growing and FUdR-treated FM3A cells were compared. As shown in Fig. 7a, c the activity and pattern of DNA polymerase α in the cytoplasmic fraction did not differ much in the random and synchronized cells. By contrast, the DNA polymerase α in the nuclear extract from FUdR-treated cells had about three times as much activity as that from the extract from random cells (Fig. 7b, d).

DISCUSSION

The effectiveness of FUdR on the accumulation of S phase populations of FM3A
cells grown in the mouse has been presented. A single administration of 100 µg of FUdR caused the accumulation of 70–80% of FM3A cells in the mouse at S phase. FUdR is known to locate selectively in tumors and to be incorporated into RNA, but not into DNA (4). Thus the level of FUdR in the peritoneal cavity may have decreased during incubation. Continuous infusion of FUdR as described by Tice et al. (13) would improve synchrony, but the method is not suitable for a large scale experiment such as one using one hundred mice. Our procedure gives a reasonable accumulation of the S population.

We have no direct evidence on the point of accumulation of FM3A cells in the cell cycle. Possibly, FUdR inhibits de novo thymidylate synthesis in FM3A cells, and cells traverse the S phase slowly depending on the limited amount of thymidine in the ascitic fluid. If this is the case, cells should be distributed randomly in the S phase. Judged from the results shown in Fig. 6 (especially the changes in cell number), most cells are at the G1/S boundary because cell number did not increase for 6 h after inoculation, but it subsequently increased 1.8-fold semi-synchronously.

Multiple forms of DNA polymerase α have been found in various sources (3), although the degree of heterogeneity differs somewhat between the species or cellular type. In HeLa cells the cytoplasmic fraction exclusively contains P-2, which has lower an affinity for binding to DNA and is eluted from the DEAE-cellulose column at a higher salt concentration than the other form of polymerase α (P-1) (11). By contrast, the cytoplasmic fraction of mouse FM3A cells contains very high level of P-1 (eluted from DEAE-cellulose column at ca. 200 mM KCl) and low level of P-2 (eluted from DEAE-cellulose column at ca. 250 mM KCl) (Fig. 7). The ratio of P-1 to P-2 in the whole cell also differs; the major DNA polymerase α in HeLa cells is P-2, whereas that in FM3A cells is P-1. We presently are studying the molecular differences in these two forms of DNA polymerase α as well as the physiological significance of their forms.

The procedures described in this report provides a good way to study the biochemical and genetic mechanisms of chromosome replication in the same mammalian cell.

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