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Cell Biology

Reversible growth arrest in simian virus 40-transformed human fibroblasts

(homocysteine/methionine/cell cycle)

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ABSTRACT **A reversible growth arrest of simian virus 40-transformed human fibroblasts has been produced by replacement of methionine in the growth medium by its immediate metabolic precursor, homocysteine. Although these arrested cells exhibit a greatly reduced cloning efficiency when plated in methionine-supplemented medium, they resume rapid proliferation without a lag when subconfluent cells are refed with methionine-supplemented medium. This growth arrest is accompanied by a reduction in the percentage of mitotic cells in the cell population. Furthermore, data obtained using fluorescence-activated cell sorting techniques indicate that the cells are arrested in the S and G_2 phases of the cell cycle. This is in contrast to a G_1-phase accumulation of cells, which occurs only in methionine-supplemented medium at very high densities and which is similar to the G_1 block seen in cultures of normal fibroblasts at high density. The apparent relationship between specific events in the DNA-synthetic and premitotic phase of the cell cycle and methionine dependence in these transformed cultures is discussed.**

A number of transformed and malignant cells are unable to proliferate when methionine is replaced by its immediate precursor, homocysteine, in the culture medium. The cell types include murine leukemia (1); the Walker 256 rat breast carcinoma (2); rat hepatoma, melanoma, and mammary tumor (3); human lung adenocarcinoma and human acute lymphoblastic leukemia (4); simian virus 40 (SV40)-transformed baby hamster kidney (5); and SV40-transformed human fibroblasts (6–8). These cells are termed Met^-. Normal cells, on the other hand, grow well when methionine is replaced by homocysteine (9). These cells are termed Met^+.

Rare Met^+ revertants of SV40-transformed human fibroblasts have been selected in medium containing homocysteine in place of methionine. These cells concomitantly have reverted toward normal for other properties associated with transformation, indicating a relationship of the Met^- phenotype and oncogenic transformation (8).

In vivo and *in vitro* studies of methionine biosynthesis do not distinguish Met^+ normal and revertant cells on the one hand and fully transformed Met^- cells on the other (6, 7, 10). This has led us to postulate that Met^- cells are defective in utilization of homocysteine or biosynthesized methionine. To elucidate the biochemical basis of the Met^- phenotype, it is important to know the effects of incubation in homocysteine-containing medium on the traversal of Met^- cells through the cell cycle. We have found that Met^- cells reversibly arrest in homocysteine-containing medium and eventually accumulate in the late phases of the cell cycle; by contrast, as they slow their growth due to high density in methionine-containing medium, they accumulate mostly in G_1-phase. These findings are also of interest with regard to the mechanism of growth arrest in oncogenically transformed cells.

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MATERIALS AND METHODS

Cell Lines and Culture Conditions. P1, P2, and P5 are Met^- subclones of the human SV40-transformed cell lines W18VA2 and SV80, respectively (8). Medium used for these experiments was Eagle's minimal essential medium (lacking methionine and choline chloride) with nonessential amino acids (GIBCO) supplemented with 13.9 mM glucose, 1 mM sodium pyruvate, 0.25 mM ferric nitrate, 0.1 mM folic acid, 1.5 mM hydroxycobalamin, and 10% dialyzed fetal bovine serum (Irvine Scientific, Santa Ana, CA). The serum was dialyzed against a 12-fold excess volume of 0.9% NaCl for 4 hr with three changes. The medium was supplemented with either 100 μM L-methionine or 200 μM DL-homocysteine thiolactone.

Cells were grown in Corning roller bottles (490 cm^2) in either methionine- or homocysteine-containing medium. After the indicated period of incubation, cells were rinsed twice with phosphate-buffered saline and were detached from the plastic with trypsin at room temperature. Cells were then treated with DNase and soybean trypsin inhibitor and were fixed in formaldehyde as described (11). The cells were fixed for at least 24 hr and hydrolyzed in 4 M HCl for varying periods of time. It was found that varying the hydrolysis time from 20 to 80 min did not make a significant difference in the final results, and 20 min at room temperature was chosen as our standard procedure. Immediately following hydrolysis, the cells were stained with acriflavine for 5 min and were washed with acid alcohol as described (11). The cells then were resuspended in water and analyzed at a wavelength of 488 nm with a fluorescence-activated cell sorter built according to the design of the Los Alamos laboratory (11). The data were analyzed by a computer program designed as described (12).

The standard techniques of enumeration in a Coulter Counter and Giemsa staining were used to quantitate cell growth and cloning efficiency, respectively.

RESULTS

We (5, 8) and others (1) have reported that cultures of various transformed and malignant lines cease growing when methionine is replaced by its immediate precursor, homocysteine. Fig. 1 shows that, for human SV40-transformed lines P2 and SV80 in mass culture, the growth arrest is reversible. Cells were incubated in homocysteine-containing medium for 10 days.

Abbreviation: SV40, simian virus 40.

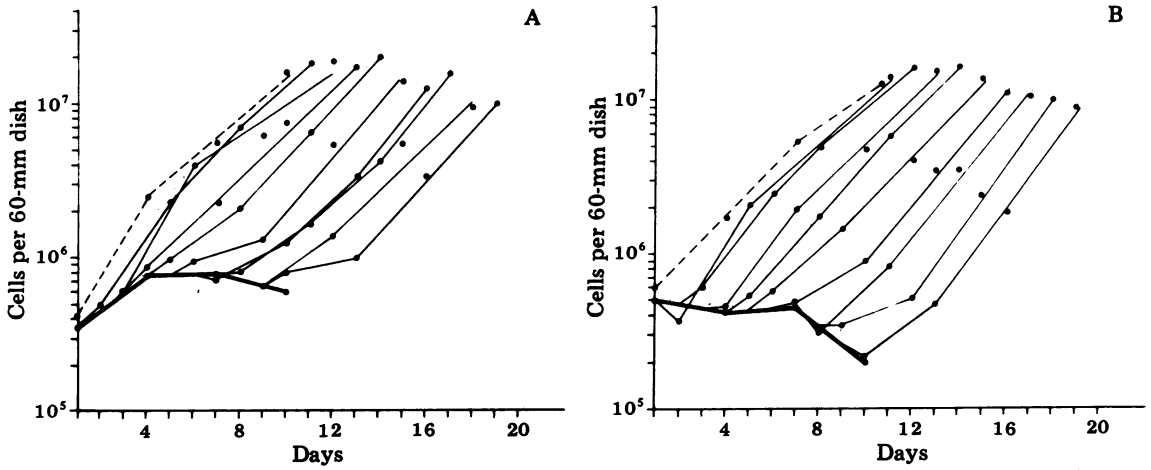

FIG. 1. Ability to grow in mass culture in methionine-containing medium after incubation in homocysteine-containing medium. (*A*) SV40-transformed human line P2 (subclone of W18VA2). (*B*) SV40-transformed human line SV80. —, Cells continuously grown in homocysteine-containing medium; —, cells shifted from homocysteine-containing medium to methionine-containing medium at times shown; ·····, cells continuously grown in methionine-containing medium.

Each day up to day 9 a batch was shifted to methionine-containing medium. At any point through the 9-day incubation period in homocysteine-containing medium, cells could resume rapid growth when shifted to methionine-containing medium.

However, different results were obtained when, after various periods of incubation in homocysteine-containing medium, the cells were trypsinized and plated in methionine-containing medium under cloning conditions at 200 cells per 60-mm dish. The cloning efficiency in methionine-containing medium of P2 decreased rapidly after incubation for 2 days in homocysteine-containing medium (Fig. 2). The cloning efficiency of P5 in methionine-containing medium decreased rapidly after incubation for 4 days in homocysteine-containing medium.

We next asked whether or not cells in homocysteine-supplemented medium were arrested at a specific point(s) in the cell cycle. The cells were grown in roller bottles in either homocysteine- or methionine-containing medium and were analyzed for DNA content and, thereby, cell-cycle position by a fluorescence-activated cell sorter. When homocysteine replaced methionine in the culture medium, P5 cells started accumulating in the late phases of the cell cycle (Fig. 3). By day 7 the

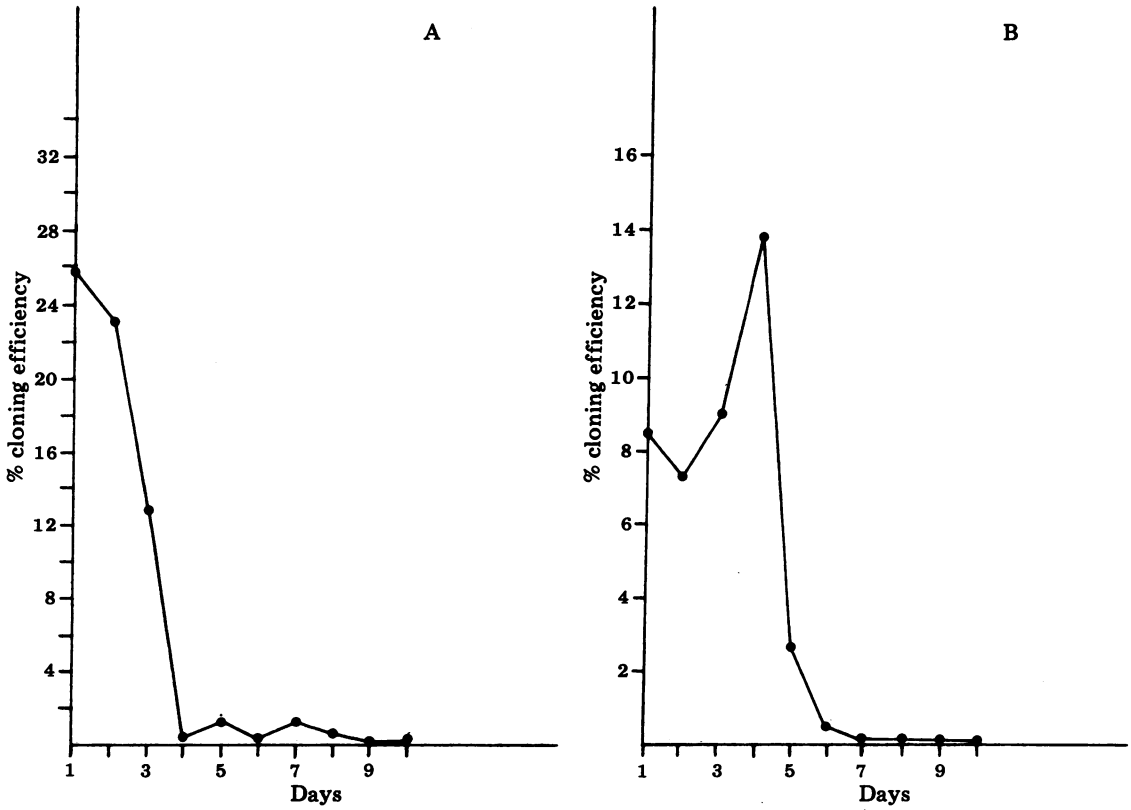

FIG. 2. Cloning efficiency in methionine-containing medium after indicated days of incubation in homocysteine-containing medium. (*A*) SV40-transformed human line P2 (subclone of W18VA2). (*B*) SV40-transformed human line P5 (subclone of SV80).

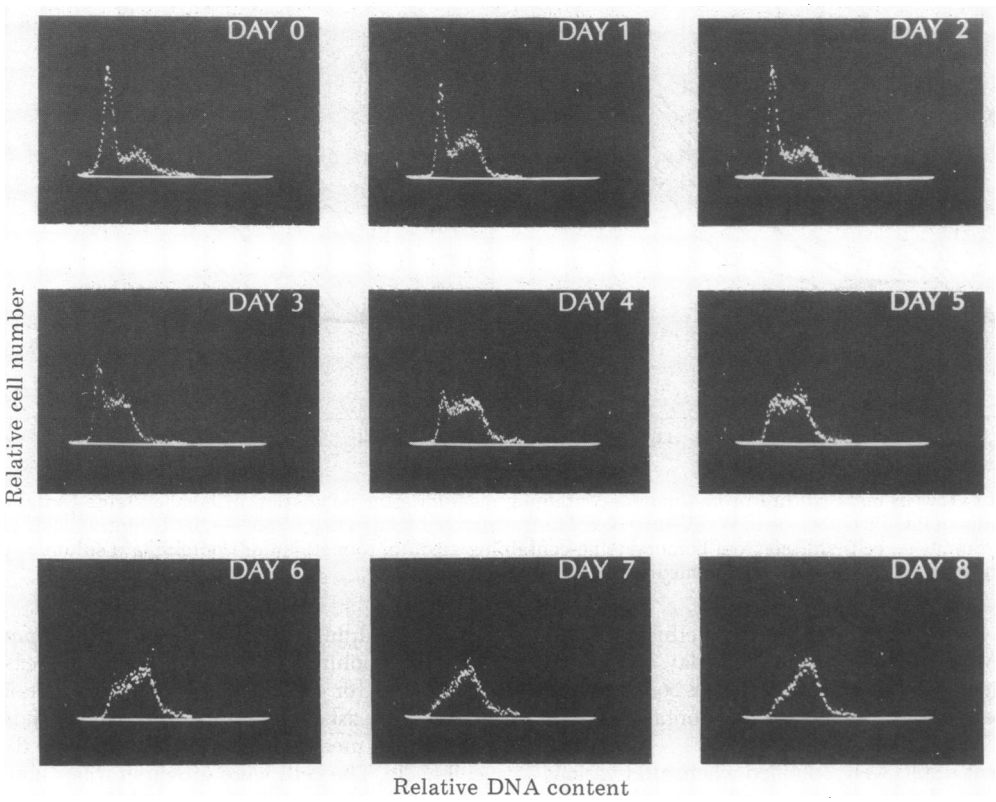

FIG. 3. Fluorescence-activated cell sorting to determine cell-cycle position in SV40-transformed human line P5 (subclone of SV80). Days indicate time of incubation in homocysteine-containing medium.

phase G_1 peak had disappeared, leaving a large asymmetric peak phase at G_2/M. This G_2/M peak probably contains a significant fraction of cells in S phase. The P1 cell line behaved somewhat similarly in homocysteine-containing medium (Fig. 4). However, in the 8-day testing period, the G_1 peak did not completely disappear, but the asymmetric peak at G_2/M grew with time.

In contrast to their behavior in homocysteine-containing medium, most of the Met^- P1 cells accumulated in G_1 phase when cultures were grown to high density in methionine-containing medium. Fig. 5 compares dense P1 cells with confluent normal diploid human cells. Although most of the P1 cells are in G_1 phase, the arresting point(s) seems slightly leaky in comparison to the normal cells.

To distinguish between the G_2 and M phases of the cell cycle, cells incubated in homocysteine- and methionine-supplemented medium for various time periods were fixed and stained to observe metaphase chromosomes. When cells were in the logarithmic phase of growth in methionine-containing medium, 4% of the cells were in M phase, whereas when they were in homocysteine-containing medium, the percentage of the cells that were in M phase was 1.5% or less, indicating that most of the cells in the G_2/M portion of the plots in Figs. 3 and 4 were in G_2 phase (data not shown).

A computer program modeled after Dean and Jett (12) was used to calculate percentages of cells in the various phases of the cell cycle. For P5 in methionine-containing medium or at the beginning of the incubation period in homocysteine-containing medium, about 50% of the cells were in G_1, 30–35% in S, and 10–15% in G_2; by the end of the incubation period in homocysteine-containing medium, about 10–15% of the cells were in G_1, 30–35% in S, and 50% in G_2. For P1 in methionine-containing medium or at the beginning of the incubation period in homocysteine-containing medium, about 50% of the cells were in G_1, 30–35% in S, and 15–20% in G_2; by the end of the incubation period in homocysteine-containing medium, about 20% of the cells were in G_1, 40% in S, and 40% in G_2.

DISCUSSION

It has been known for some time that the human SV40-transformed lines W18VA2 and SV80 and subclones derived from them ceased growing when methionine was replaced by homocysteine in the culture medium (6). We show that under conditions of mass culture this growth arrest is reversible even after as long as 9 days of incubation in homocysteine-containing medium. However, when incubated in medium containing homocysteine in place of methionine, the cells rapidly lost the ability to clone when plated back into methionine-containing medium. Therefore, it seemed that the growth arrest in homocysteine was only partially normal, in that the cells could resume growth in methionine in mass culture but rapidly lost the ability to clone in methionine. This indicated to us that in homocysteine-containing media, the cell cycle was not arrested in the "normal" resting position, G_1/G_0. Instead, analysis with a fluorescence-activated cell sorter shows that the cells in medium containing homocysteine in place of methionine were cycling out of G_1 phase, and growth was eventually arrested mostly in the S and G_2 phases of the cell cycle (Figs. 3 and 4). This type of arrest apparently disrupts cellular metabolism to the extent that the cells lose the ability to resume cycling under the stressful conditions of cloning but not to the extent that they are prevented from resuming growth in mass culture in complete medium. Apparently, cell–cell interactions or conditioning effects under the latter conditions in methionine-containing medium permit the cells to overcome whatever damage had occurred to them in homocysteine-containing medium.

Other experimental conditions have a direct effect on the G_2 phase of various types of cells. CV-1 monkey cells in culture productively infected with SV40 have 90% of their cells blocked in G_2 (13). It has been shown that when cultured rat liver cells

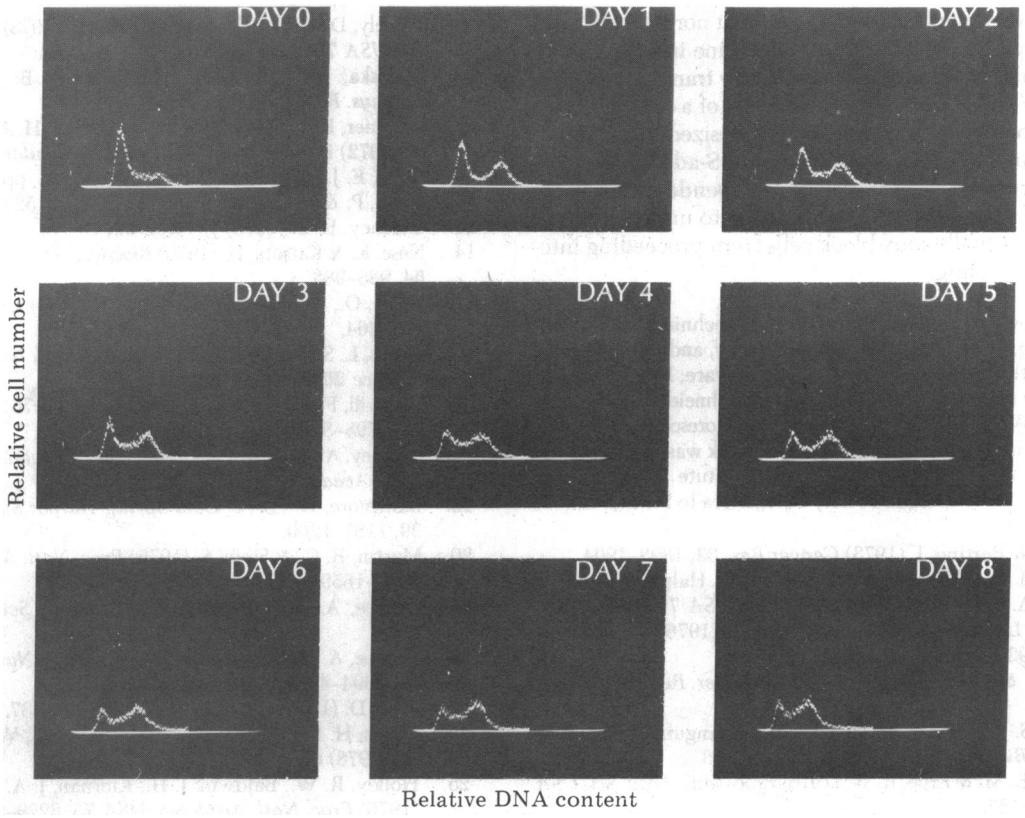

FIG. 4. Fluorescence-activated cell sorting to determine cell-cycle position in SV40-transformed human line P1 (subclone of W18VA2). Days indicate time of incubation in homocysteine-containing medium.

are treated with dibutyryl cyclic AMP, the fraction of cells with a G_2 amount of DNA increases from 19% to 40% (14). In Ehrlich ascites tumor cells, polyamine synthesis inhibitors interfere with both S and G_2 progression (15). In the pea plant, *Pisum sativum*, *N*-methylnicotinic acid increases the percentage of cells in G_2 from approximately 20% to 40% or more (16). Interferon seems to have an arresting effect on cultured human and mouse cells in various phases of the cell cycle, including G_2 (17, 18).

Fig. 5 also shows that as the SV40-transformed cells in methionine-containing medium become dense and slow their growth, they accumulate in G_1 phase almost to the extent that human diploid fibroblasts do at confluence. The data suggest that the resting point in G_1 in the transformed cells is slightly leaky.

There have been various reports in the literature concerning the ability of oncogenically transformed cells to arrest growth reversibly. It has been stated that transformation is an abrogation of the resting state (19) or that SV40-transformed hamster cells do not reversibly arrest growth because of nutritional or serum deprivation (20–22). Other studies have indicated that certain transformed or malignant cells can reversibly enter various resting states depending on the conditions of culture (23–26). Our data taken with these reports indicate that most, if not all, transformed and malignant cells have resting states, but the mechanism of growth arrest as well as the means of entry into the resting state may differ in normal and transformed cells.

The biochemical basis of the methionine dependence in SV40-transformed human fibroblasts is not yet clear. *In vivo* and *in vitro* measurements of methionine biosynthesis do not

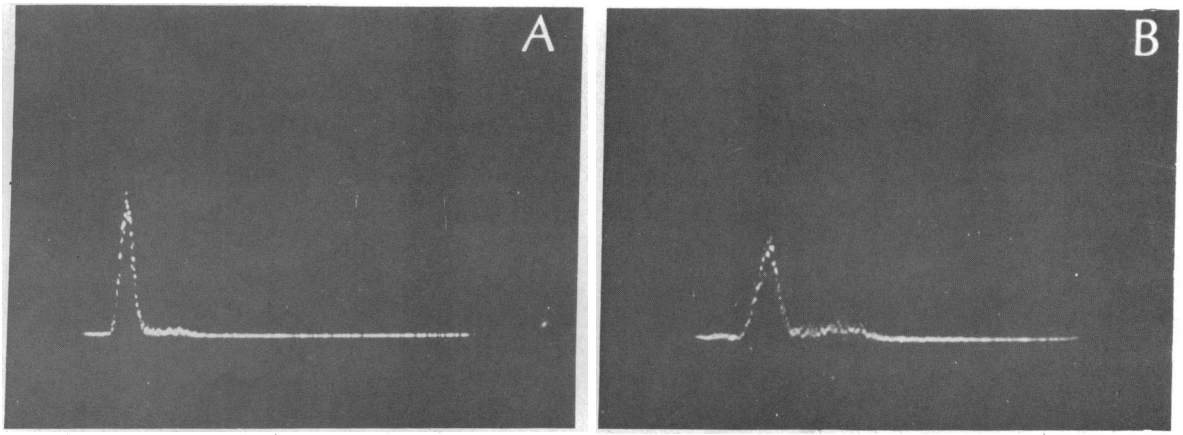

FIG. 5. Fluorescence-activated cell sorting to determine cell cycle position. (*A*) Confluent culture of diploid human embryonic fibroblast line AF2. (*B*) Dense culture of SV40-transformed human line P1 (subclone of W18VA2).

distinguish between methionine-independent normal cells and SV40-transformed cells reverted to methionine independence on one hand and methionine-dependent, fully transformed cells on the other. We have discussed possibilities of a defect in cellular compartmentation such that biosynthesized methionine is not fully available to the cell (7), or that S-adenosylhomocysteine is overproduced in methionine-dependent cells (27). Either of the above mechanisms could lead to under-methylation, which eventually may block cells from proceeding into G_1 phase and recycling.

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