Mechanism of Growth Arrest of Chemically Transformed Cells in Culture

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ABSTRACT

The mechanism of growth arrest was studied in two mouse embryo-derived cell lines and two transformed clones obtained through 3-methylcholanthrene transformation of both of the parent nontransformed lines. The two transformed lines were AKR-MCA derived from the nontransformed AKR-2B cell line and the C3H/MCA-58 derived from the C3H/10T½ cell line. Both transformed clones reach a saturation density approximately 2-fold that of their respective nontransformed parent lines. The chemically transformed cells were found to reach low base-line levels of [3H]thymidine incorporation better if allowed to remain in high serum (10%) than in low serum (0.5%) while the nontransformed cells grew arrested better in low serum. Changing to fresh medium with 10% serum caused the quiescent nontransformed and transformed cells to undergo a wave of DNA synthesis, as determined by [3H]thymidine incorporation, suggesting that the transformed cells, like the nontransformed cells, were growth arrested in the G0/G1 phase of the cell cycle. The time interval between the stimulation to proliferate and the onset of DNA synthesis was considerably shorter in the transformed cells than in the nontransformed cells. The quiescent transformed cells were not stimulated to undergo DNA synthesis by the addition of serum, epidermal growth factor, fibroblast growth factor, or the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate. They were stimulated by the addition of fresh medium containing no serum. The nontransformed cells' growth arrest in the presence of 10% serum were stimulated to undergo a round of DNA synthesis by serum, epidermal growth factor, fibroblast growth factor, 12-O-tetradecanoylphorbol-13-acetate. They were not stimulated by medium without serum. The data indicate that the chemically transformed cells' growth arrest in G1 by a different mechanism than the nontransformed cells. The nontransformed cells' growth arrest due to depletion of essential serum growth factors, whereas the chemically transformed cells appear to growth arrest due to depletion of multiple amino acids and glucose present in the chemically defined medium.

INTRODUCTION

Nontransformed animal cells in culture growth arrest in the G0 phase of the cell cycle when they reach a certain density due to depletion of essential serum growth factors or they grow arrest at subconfluence in serum-deficient medium (17, 20). The quiescent cells can then be stimulated to undergo a round of DNA synthesis by adding serum (26, 29) or purified mitogens including EGF3 (3, 11, 23), FGF (10), or the tumor promoter, TPA (21, 25). DNA virus-transformed cells, on the other hand, do not grow arrest in G0, under usual monolayer culture conditions. They either become arrested throughout the cell cycle or continue proliferating until they die even when placed in serum-deficient medium (18, 19). However, Holley et al. (12) have reported that a chemically transformed line of 3T3 cells does become quiescent when grown in medium with 0.2% serum and that these cells grow arrest in the G1 phase of the cell cycle. O'Brien and Diamond (16) have shown that chemically transformed hamster cells can grow arrest in medium with 10% serum and that the cells can be stimulated to undergo a wave of DNA synthesis beginning 6 hr following stimulation in a manner similar to that of their nontransformed counterparts, suggesting that the transformed cells were arrested in G1.

The present study was carried out in order to confirm that chemically transformed cells, unlike DNA virus-transformed cells, can become quiescent in G0, and to examine the mechanism for growth arrest in the chemically transformed cells. For these studies we have used the nontransformed AKR-2B cell line and a 3-methylcholanthrene-transformed clone (AKR-MCA) derived from the AKR-2B cells (9). In addition, we have examined the C3H/10T½ cell line and a 3-methylcholanthrene-transformed derivative, called C3H/MCA-58 (22). Both chemically transformed cells became quiescent in G0 at a point closer to the onset of DNA synthesis than that for their nontransformed counterparts. The data indicate that the chemically transformed cells' growth arrest due to depletion of multiple amino acids and glucose from the chemically defined medium. In contrast to the nontransformed cells, they are unresponsive to serum, EGF, FGF, and TPA.

MATERIALS AND METHODS

Cell Culture. AKR-2B and AKR-MCA cells are grown in McCoy's Medium 5A supplemented with 10% fetal bovine serum (Reheis Chemical Co., Phoenix, Ariz.) without antibiotics (9). C3H/10T½ and C3H/MCA-58 cells are grown in Eagle's basal medium also supplemented with 10% fetal bovine serum and without antibiotics (8). Experiments were carried out both in 490-sq cm plastic roller bottles (Corning Glass Works, Corning, N. Y.) or Falcon No. 3002 Petri dishes with a 21-sq cm growth surface. All experiments are carried out within 10 passages of the frozen stock from which cells are recovered periodically. These stock cells

1 This investigation was supported by Grant CA 16816 from the National Cancer Institute, Department of Health, Education, and Welfare, and the Mayo Foundation.

2 To whom requests for reprints should be addressed.

Received April 3, 1978; accepted May 30, 1978.

3 The abbreviations used are: EGF, epidermal growth factor; FGF, fibroblast growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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have been shown to be free of *Mycoplasma* contamination by culture methods, by scanning and transmission electron microscopy (9), and by staining with the Hoechst No. 33258 stain (4). Cells are examined after Hoechst No. 33258 staining monthly to ensure that they have remained free of *Mycoplasma*.

The AKR-MCA and C3H/MCA-58 cells are transformed as indicated by growth morphology, growth in soft agar, and tumorigenicity in nude mice (8, 9, 22). The AKR-2B and C3H/10T1/2 cells do not grow in soft agar and do not form tumors in nude mice (8, 9, 22).

**Growth Arrest of Transformed and Nontransformed Cells.** The method for growth arrest of the AKR-2B cells in low serum has been described previously (1, 7). For growth arrest in 10% serum, both transformed and nontransformed cells are subcultured with a dilution of 1:30 for the AKR cells or 1:10 for the C3H cells, medium is changed on Day 4 adding 35 ml for roller bottles and 2 ml for dishes, and [3H]thymidine incorporation is determined as described below on Day 7 after subculture. If the level of [3H]thymidine incorporation is not at base-line level, the cells are incubated for an additional 24 hr and the level of [3H]thymidine incorporation is again determined. In most cases the level of [3H]thymidine incorporation reached base-line levels by 7 days after subculture, and in virtually all cases by 8 days after subculture.

**Stimulation of DNA Synthesis in Quiescent Cells.** Resting cells are stimulated to grow by changing to fresh medium with 10% serum. Since the act of changing medium (i.e., removing and readding the depleted medium) causes some small but significant stimulation of DNA synthesis, experiments concerning the differential effect of serum, purified mitogens, or chemically defined medium on stimulation of DNA synthesis are carried out by adding the factors to the depleted medium. Stock solutions of EGF and FGF (Collaborative Research, Inc., Waltham, Mass.) are either 1 or 10 μg/ml in Hanks' balanced salt solution containing 0.5% bovine serum albumin. TPA (obtained from the IIT Research Institute, Chicago, Ill., through the NCI Carcinogenesis Research Program) is dissolved in acetone at a concentration of 10 μg/ml.

Studies on stimulation of AKR-MCA cells with different components of the chemically defined medium are carried out with the Gibco RPMI-1640 Select-Amine Kit (Grand Island Biological Co., Grand Island, N. Y.). Media are formulated deleting single amino acids, glucose, or vitamins, and 2 ml are added to the 2 ml of depleted medium with serum in Petri dishes. [3H]Thymidine incorporation is determined 18 hr after addition.

**Assay for DNA Synthesis.** Cells are pulsed for 60 min with 1.0 μCi [methyl-3H]thymidine (6.7 μCi/mmol; New England Nuclear, Boston, Mass.) per ml of medium, and the incorporation into acid-precipitable material is determined as previously described (1, 7). DNA is determined by the diphenylamine reaction of Burton (2), and the results are expressed as cpm [3H]TMP incorporated per μg DNA per hr or μg DNA per roller bottle. Light microscope autoradiography is performed as described previously (1).

**RESULTS**

**Saturation Density and Generation Time.** Growth curves of the AKR-2B and AKR-MCA cells showed that the 2 cell lines had virtually identical generation times (Chart 1; Table 1). However, the AKR-MCA cells reached a saturation density that was approximately 2.5-fold greater than that of the AKR-2B cells (Table 1). The same saturation density was achieved in each cell line regardless of whether the initial inoculation was 10⁶ or 5 x 10⁶ cells/dish (Chart 1). The generation times for the 2 C3H cell lines is shown in Table 1. The C3H/10T1/2 cell line had a slightly longer generation time than that of the chemically transformed counterpart; however, the difference was probably not significant. Again the saturation density of the transformed cells was over 2-fold greater than that of the nontransformed cells, and the saturation density of both cell lines was similar to that previously reported for these cells (22).

The saturation density of the AKR-2B cells was slightly greater than that of the transformed C3H/MCA-58 cell line (Table 1). This does not, however, mean that the AKR-2B cells pile up; they form monolayers of closely packed epithelioid-type cells (9).

**Growth Arrest of Chemically Transformed Cells.** The plateau of cell density reached by the nontransformed cells is due to growth arrest of the cells (17, 20). The similar, but higher plateau of cell density achieved by the transformed cells could be due to either growth arrest of the transformed cells or the attainment of a balance between cell replication and cell death. For examination of these possibilities, the level of [3H]thymidine incorporation was determined in the AKR-MCA cells as they reached their saturation density. As shown in Chart 2, the level of [3H]thymidine incorporation

![Chart 1](chart1.png)

**Chart 1.** Growth curves for AKR-2B and AKR-MCA cells. Cells were seeded in 21 sq cm dishes at an initial concentration of 10⁶ or 5 x 10⁶ cells/dish. Cells were harvested by trypsinization and counted in a hemacytometer at the indicated times.

<table>
<thead>
<tr>
<th></th>
<th>Generation time (hr)</th>
<th>Saturation density (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR-2B</td>
<td>16.8 ± 1.6a</td>
<td>1.05 x 10⁶</td>
</tr>
<tr>
<td>AKR-MCA</td>
<td>16.4 ± 0.9a</td>
<td>2.86 x 10⁶</td>
</tr>
<tr>
<td>C3H-10T1/2</td>
<td>30.5b</td>
<td>3.44 x 10⁶</td>
</tr>
<tr>
<td>C3H-MCA-58</td>
<td>27.3b</td>
<td>7.89 x 10⁶</td>
</tr>
</tbody>
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a Mean ± S.D., 3 separate experiments.
b Mean of 2 separate experiments.

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Chart 2. Growth arrest of AKR-MCA cells in 0.5 or 10% serum. Cells were seeded in 490-sq cm plastic roller bottles (1.5 to 2.0 x 10^6 cells/bottle), and the medium was changed on Day 4. The incorporation of [3H]thymidine into DNA following a 1-hr pulse and the DNA content per bottle were determined at 24, 48, and 72 hr after medium change. Virtually identical results were obtained with cells grown in dishes.

All 4 cell lines used in this study generally attained minimal levels of [3H]thymidine incorporation in medium with 10% serum at 7 days following subculture as illustrated at the zero time point in Charts 3 and 4. Incubation for longer times usually gave no further decrease in incorporation. Autoradiographic studies of the 2 AKR cell lines following a 1-hr pulse with [3H]thymidine after 7 days of culture in medium with 10% (3 days following medium change) revealed that 0.8% of the nuclei of the AKR-2B cells and 1.1% of the nuclei of the AKR-MCA cells were labeled. Standard deviations were 0.2 and 0.4% for the AKR-2B and the AKR-MCA cells, respectively, with 6 separate determinations for each cell line.

Stimulation of DNA Synthesis in Growth-arrested Cells.

Changing to fresh medium with 10% serum resulted in stimulation of a wave of increased thymidine incorporation in all 4 cell lines (Charts 3 and 4). The prereplication interval between stimulation and the onset of DNA synthesis was 12 hr for the AKR-2B cells (Chart 3) as previously reported (1, 7). However, the prereplication interval for the AKR-MCA cells was shortened considerably to approximately 8 hr (Chart 3). A similar shortening of the prereplicative interval for the chemically transformed C3H cells (4 hr) relative to the nontransformed C3H/10T^1/2 cells (10 hr) was also observed (Chart 4). Autoradiography following a 1-hr pulse with [3H]thymidine at 20 hr following stimulation in the AKR-2B cells revealed that 61% of the nuclei were labeled with a standard deviation of 13% for 3 separate determinations. In

the AKR-MCA cells labeled for 1 hr, at 18 hr following stimulation 58 ± 3% of the cell nuclei were labeled.

Effect of Medium, Serum, Growth Factors, and TPA on Stimulation of DNA Synthesis in Growth-arrested Nontransformed and Chemically Transformed Cells. The data presented in Charts 3 and 4 indicate that the chemically transformed cells are growth arrested at a point closer to the onset of DNA synthesis than that for the corresponding nontransformed cells. In order to examine the possible mechanism of growth arrest in the transformed and nontransformed cells, we added various factors to quiescent cells, and evaluated their effect on DNA synthesis at 18 or 20 hr following addition by determining [3H]thymidine incorporation. As shown in Chart 5, the AKR-2B cells were stimulated by fetal bovine serum alone; the growth factors, EGF and FGF; and the tumor promoter, TPA. The AKR-2B cells were not stimulated by the chemically defined medium without serum. In contrast, the chemically transformed

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AKR-MCA cells were not stimulated by serum, EGF, FGF, or TPA (Chart 5). These cells were stimulated very effectively by the chemically defined medium without serum (Chart 5).

In order to determine whether the presence of serum was necessary for stimulation of DNA synthesis in the AKR-MCA cells, the experiment shown in Chart 6 was performed. These data show that, if the depleted medium containing 10% serum is removed from the cells by rinsing with medium without serum at room temperature, DNA synthesis is not stimulated by the medium. If medium containing 10% serum is added after rinsing, the cells are stimulated (Chart 6). However, the addition of EGF instead of serum to the medium does not cause stimulation. These data indicate that some factor present in serum is necessary for stimulation of DNA synthesis in the AKR-MCA cells but that this factor is not depleted by the cells as they become growth arrested.

Chart 7 shows that the C3H/10T½ cells behave essentially the same as do the AKR-2B cells being responsive to serum, EGF, and TPA. Similar to the AKR-MCA cells, the chemically transformed C3H/MCA-58 cells are stimulated only by medium without serum. The C3H/10T½ cells are also stimulated by FGF (data not shown); the effect of FGF on C3H/MCA-58 cells was not determined.

**Determination of Factors Depleted in Medium by AKR-MCA Cells.** The observation that growth-arrested AKR-MCA and C3H/MCA-58 cells are stimulated by addition of chemically defined medium and not serum indicate that growth arrest in these cells results from depletion of some essential factors from the medium. In order to determine those components responsible for growth arrest, we determined the degree of stimulation of quiescent AKR-MCA cells obtained by adding medium deficient in specific single amino acids or other components. The addition of media...
deficient in cystine, glutamine, isoleucine, leucine, lysine, methionine, tryptophan, or valine to growth-arrested AKR-MCA cells cause virtually no stimulation of the cells, while media deficient in the other amino acids stimulate to a level 43 to 100% of that obtained with a complete medium (Table 2). The degree of stimulation of AKR-MCA cells with media deficient in glucose, phosphate, or the vitamin mixture was also determined (Table 2). The deletion of glucose resulted in no stimulation of DNA synthesis in the AKR-MCA cells while the deletion of vitamins or phosphate resulted in stimulation almost equal to that obtained with complete medium. The data indicate that at least 8 amino acids and glucose are depleted by the AKR-MCA cells to the point that their replacement is essential for stimulation of growth. The addition of these 8 amino acids (cystine, glutamine, isoleucine, leucine, lysine, methionine, tryptophan, and valine) in balanced salt solution with glucose did stimulate the cells to a level 34% of that obtained with complete medium.

DISCUSSION

We have presented data indicating that the nontransformed and chemically transformed cells growth arrest in the G0 phase of the cell cycle due to different mechanisms. As with other nontransformed cell lines, the AKR-2B and C3H/10T/1/z cell lines growth arrest due to depletion of essential serum growth factors; the quiescent cells are stimulated by the addition of growth factors (EGF or FGF), serum, or the tumor promoter, TPA. The transformed cells (AKR-MCA and C3H/MCA-58) either do not require or do not deplete the serum growth factors and are insensitive to stimulation by these factors when in the quiescent state. The AKR-MCA cells appear to growth arrest due to depletion of multiple amino acids plus glucose from the medium and are stimulated to proliferate by the addition of the chemically defined medium without serum. Some factor present in serum appears to be necessary for stimulation of resting AKR-MCA cells to proliferate; however, this factor is not depleted when transformed cells are growth arrested (see Chart 6).

It is well known that artificial deprivation of low-molecular-weight nutrients in the cell culture medium can cause growth arrest of cells (15, 17, 20). Leucine and methionine deprivation has been reported to cause cessation of growth at random points in the cell cycle (14, 24). On the other hand, phosphate or a combination of histidine and glutamine deficiency leads to growth arrest in G0 (13, 14, 24). The net effect of the multiple amino acid plus glucose deficiency observed in the chemically transformed cells in this study appears to be G0 arrest.

Cells arrested in G0 due to amino acid deficiency are different from cells arrested in G0 due to depletion of serum growth factors. Enger and Tobey (6) have reported that Chinese hamster ovary cells growth arrested by isoleucine deficiency maintain high levels of rRNA and mRNA synthesis as well as protein synthesis in the resting state, whereas the same cells growth arrested due to growth factor depletion show a marked reduction of RNA and protein accumulation. We have shown that the AKR-MCA cells maintain a higher level of polyadenyllic acid-containing polysomal RNA in the resting state than do AKR-2B cells (9). The shorter lag phase between the stimulation to proliferate and the onset of DNA synthesis observed in the chemically transformed cells could possibly result from these cells having to accomplish less macromolecular synthesis than the nontransformed cells in order to replicate their DNA.

It has also been shown that 3T3 cells growth arrested due to amino acid deficiency are not responsive to FGF (14). As reported in this paper, the quiescent chemically transformed cells are not stimulated to proliferate by the addition of growth factors to growth arrested cells. Whether this lack of responsiveness is due to absence or alteration of the EGF receptor is not known at present. Todaro et al. (27, 28) have reported that murine or feline sarcoma virus transformation of mouse cells specifically blocks binding of EGF to cells. However, this effect appears to be specific for sarcoma virus transformation since DNA virus-transformed cells, spontaneously transformed cells, and chemically transformed cells show EGF receptor levels comparable to those of their normal counterparts (5, 27, 28).

The data suggest that the major alteration present in the transformed cells observed in this study is in their response to growth factors. The transformed cells could either be hypersensitive to serum growth factor effect so that much lower concentrations are required for growth stimulation, or the cells could have an altered capability for degradation of the growth factors so that they do not become depleted under normal culture conditions. It can be hypothesized that the growth factor response remains sustained in the transformed cells, causing the cells to continue proliferating beyond the point that nontransformed cells would growth arrest until amino acids are depleted from the medium. This results in growth arrest of the transformed cells at a much higher saturation density than the nontransformed cells.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Wallace Rowe and Dr. Natalie Teich for seed stocks of the AKR-2B cell line, Dr. Charles Heidelberger for seed stocks...
of the C3H/10T1/2 and C3H/MCA-58 cell lines, and Patricia H. Hart for assistance in preparation of the manuscript.

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