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# Growth Control of Normal and Transformed Cells

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Both serum factors and protein synthesis are required for normal cell growth. Swiss 3T3 cells require the serum growth factors insulin and EGF (epidermal growth factor) during the initial part of the $G_1$ period, until they pass a restriction point about 2 h before the initiation of DNA synthesis. Concentrations of cycloheximide that inhibit protein synthesis by as much as 70% dramatically lengthen the cell cycle before the restriction point, while the cell cycle after the restriction point remains nearly constant. These results are consistent with a model in which labile proteins are required for transit of cells past the serum-sensitive restriction point. The relation of these findings to the growth control of transformed cells is discussed.

**Key words: 3T3 cells, transformed cells, restriction point, labile proteins, growth factors**

A central question regarding cancer is how normal cells differ from transformed cells. Tumorigenic cells have been reported to differ from nontumorigenic ones in many ways. The differences can be divided into four groups: 1) growth properties in culture (for example, transformed cells require lower serum concentrations for optimal growth, grow to higher density, grow into mutlilayers, do not arrest in a state with a $G_1$ DNA content ($G_0$) at high density or in low serum, and grow without anchorage); 2) metabolic properties (for example, transformed cells produce a protease called the plasminogen activator and have altered surface components and higher transport rates of several nutrients); 3) morphologic changes; and 4) karyotypic changes. Most of these differences have been observed by comparisons of normal and DNA virus-transformed cells, although there have been a few studies with RNA virus-transformed cells, and a very few with chemically transformed cells.

Cell populations in vivo exist in a steady-state condition in which most cells are quiescent and cell replication is balanced by cell death. Two broad models can be considered for the control of normal cell growth and the loss of this control upon transforma-

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tion. The first model proposes that transformed or tumor cells arise in the population as cells that can escape from the quiescent state in some abnormal way. Thus the process of transformation is an initation of growth in an otherwise resting cell. An alternative, equally plausible, model is that cells in the steady-state, resting condition require a starting event as suggested in the first model but the difference is that once in the cycle, the transformed cells are unable to stop growing.

The growth of normal cells is regulated in the $G_1$ phase of the cell cycle. It has been proposed that the regulatory event occurs at a specific "metabolic place" in $G_1$ called the restriction point (R point) [1]. Thus each cell carries out certain early $G_1$ processes and then must undergo a special event at the restriction point in order to carry out further biochemical processes leading to DNA synthesis. If the cell cannot carry out this restriction point event, it passes off into the $G_0$ state. This event can be considered a sort of "switch" at which a choice is made between proliferation and quiscence. A variety of conditions that control growth appear to involve the same restriction event. That is, only one control point exists and is responsive to many growth-restrictive conditions.

Some sort of crucial $G_1$ event was also suggested earlier (see Baserga [2] and Prescott [3]). Temin's experiments [4] showed serum to be dispensable to the growth of chick fibroblasts about halfway through $G_1$; he suggested that all serum-dependent events were accomplished at that time. Hershko et al [5] proposed a pleiotypic control, meaning that when cells are arrested in a variety of ways they always show several characteristic changes, including slower transport of some nutrients, diminished macromolecular synthesis, and enhanced protein breakdown. By analogy with the stringent response of bacteria, Hershko et al proposed that these effects were mediated by a pleiotypic modulator compound of low molecular weight. This compound has yet to be identified. Smith and Martin's concept [6] comes close to the idea of a restriction point; they propose a point (in $G_1$) at which all cells under all conditions become arrested and from which they escape probabilistically, either rapidly or slowly, depending on growth conditions.

Lower organisms also show a cell-cycle specific control event prior to DNA synthesis initiation. Hartwell [7] showed that yeasts are arrested at a specific point, under conditions of poor nutrition, or when they are exposed to mating factor peptide made by the opposite mating type. A temperature-sensitive mutant arrested at this event has been isolated. In Bacilli there is also a critical event at which the decision is made as to whether the bacteria continue vegetative growth or form a spore [8].

The growth behavior of cells in culture is central to the entire cancer problem, since growth changes characteristic of transformed cells are similar to the altered growth kinetic properties observed with malignant cells in vivo. In this article we describe some of our recent results on the regulation of normal cell growth. We also relate these findings to growth control in transformed cells.

## MATERIALS AND METHODS

### Materials

Epidermal growth factor (EGF) was the generous gift of Dr. Tom Maciag, Collaborative Research, Inc. Radiochemicals were obtained from New England Nuclear. Colcemid was purchased from Gibco. All other chemicals were obtained from Sigma.

### Cell Culture

Swiss 3T3 cells were originally obtained from Dr. Howard Green, Massachusetts Institute of Technology, and Balb/c 3T3 clone A31 were obtained from Dr. Charles D. Sher, Sidney Farber Cancer Institute.

Cells were routinely grown at 37°C in a water-saturated 10% $CO_2$ : 90% air atmosphere in Dulbecco's modified Eagle's medium (DME, Gibco H21, high glucose) supplemented with 10% calf serum (Flow Laboratories), 100 units/ml of penicillin, and 100 μg/ml streptomycin. Both cell lines were determined to be free of mycoplasma by the ratio of $^3$H-uridine to $^3$H-uracil incorporation into RNA [9].

### Cell Counting

Cells were removed from duplicate plates or flasks using trypsin (0.05%)/EDTA (0.5 mM) (in 138 mM NaCl, 2.67 mM KCl, 1.47 mM $KH_2PO_4$, 8.10 mM $Na_2PO_4$) and were resuspended in 20 ml Hanks' balanced salt solution (BSS) containing 0.5% formalin to fix them. Cell numbers were determined with a Coulter Counter model B.

### Cell Synchrony

A modification of the technique of Hamlin and Pardee [10] was used. Cultures in 75-cm$^2$ Falcon flasks were arrested in $G_0$ by exposure to medium containing 0.5% serum for 40 h. These quiescent cells were then stimulated to grow by the addition of medium containing 10% serum, and 8 h later, before cells had begun to synthesize DNA, 0.1 mM hydroxyurea was added for 8 h to block the cells at the $G_1$/S boundary. In order to obtain mitotic populations, the cells were released from the $G_1$/S block by replacing the medium containing hydroxyurea with regular medium. After a further 8 h, cells began to enter mitosis and these cells were shaken off the culture flask every 20 min for 2 h and pooled on ice; $10^4$–$10^5$ mitotic cells were plated into 8-cm$^2$ culture dishes in medium containing 10% serum.

### Cytofluorimetry

Cells were prepared for cytofluorimetry by a modification of the method of Fried et al [11]. Cells were removed from the culture flasks (Falcon, 75 cm$^2$) with trypsin/EDTA and resuspended in DME supplemented with 10% calf serum. After pelleting at 4°C the cells were resuspended in 3 ml of hypotonic staining solution [12] containing 50 μg/ml propidium iodide in 0.1% sodium citrate. The suspension was maintained on ice for at least 15 min prior to cytofluorimetry.

Cytofluorographic analysis was performed with a model 4800 Cytofluorograf equipped with a model 2102 multichannel analyzer with a distribution integration capability (Biophysics Systems, Ortho Instruments).

### Autoradiography

Cells were labeled with 2 μCi/ml methyl $^3$H-thymidine (45 Ci/mmole). The fraction of labeled cells was determined by fixing the cells onto the culture dish with MeOH:HOAc fixative, 2:1. Dried plates were then coated directly with Kodak NTB2 emulsion, exposed for three days, and then developed. A minimum of 400 total cells were counted for each determination.

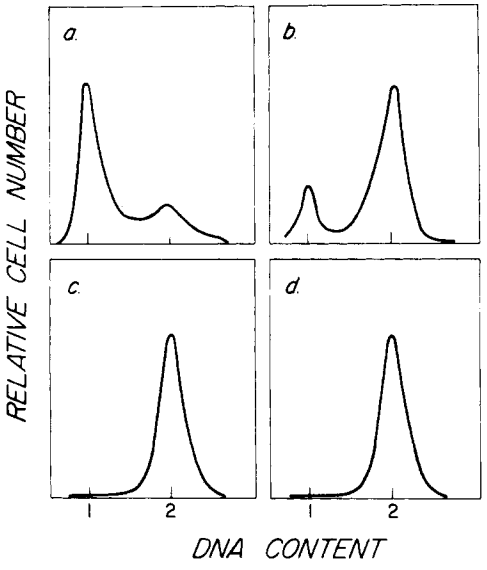


Fig. 1. Effect of cell density on the arrest of Swiss 3T3 cells in $G_1$. DNA histograms of (a) an exponential cell population at a density of $2.4 \times 10^4$ cells/cm$^2$; (b) a cell population, initially at $2.4 \times 10^4$ cells/cm$^2$, 24 h after shiftdown into medium containing 0.5% serum and 0.05 μg/ml colcemid; (c) a cell population, initially at $2.2 \times 10^3$ cells/cm$^2$, 24 h after shiftdown into medium containing 0.5% serum and 0.05 μg/ml colcemid; (d) a cell population, initially at $2.5 \times 10^4$ cells/cm$^2$, 24 h after addition of 0.05 μg/ml colcemid directly to culture medium.

## RESULTS

### Time and Growth Factor Requirements of the R Point

Recently Yen and Pardee [13] have described a technique to differentiate between cells that are located either before or after the serum-sensitive R point in $G_1$. In this technique, exponentially growing cells are placed in medium containing 0.5% serum and 0.05 μg/ml colcemid. Cells that have already passed the serum-requiring point at the time of shiftdown go on to mitosis, where they all stop owing to the presence of colcemid (see Fig. 1a,b). After 20–24 h of this treatment the initial exponential population has separated into two fractions that are easily resolvable by cytofluorography; one has a $G_1$ DNA content representing those cells initially in $G_1$ and prior to the serum-sensitive step, and the other has a $G_2$ DNA content representing cells initially beyond the serum-sensitive step.

Yen and Pardee showed that the R point is about 2 h before S phase in Swiss 3T3 cells. It is important to note, however, that the method requires cells to experience serum starvation immediately following shiftdown. Figure 1b, c shows the results of taking exponentially growing cultures of 3T3 cells at two densities differing nearly tenfold and shifting down as described above. At the higher density, which was in the range used by Yen and Pardee, a comparable arrest was obtained. Thus the small quantities of serum factors provided by the low serum were rapidly utilized by the cells and a large, definite fraction of cells were arrested in $G_1$. Cells at this high density were not already arrested in $G_1$ at the time of shiftdown, since addition of colcemid alone with no serum reduction resulted in the accumulation of all cells in the $G_2$ peak (see Fig. 1d). At tenfold lower

density, all of the cells that had been in $G_1$ phase at the time of shiftdown were able to pass around the cell cycle and were arrested at the colcemid block. In this case, residual serum factors must have permitted those cells prior to the restriction point at shiftdown to traverse the cell cycle. The cell density dependence of growth arrest illustrated by these results suggests that specific serum factors are required for R point transit.

Insulin and epidermal growth factor (EGF) appear to be able to replace the serum-derived growth factors that become limiting when cell growth is arrested. Almost complete relief from the restrictive condition of 0.5% serum was observed after Swiss 3T3 cells were subjected to the above procedure in the presence of insulin (10 μg/ml) and EGF (10 ng/ml) [14]. This suggests that these factors, or factors closely related to them structurally, are required for the transit of $G_1$ phase.

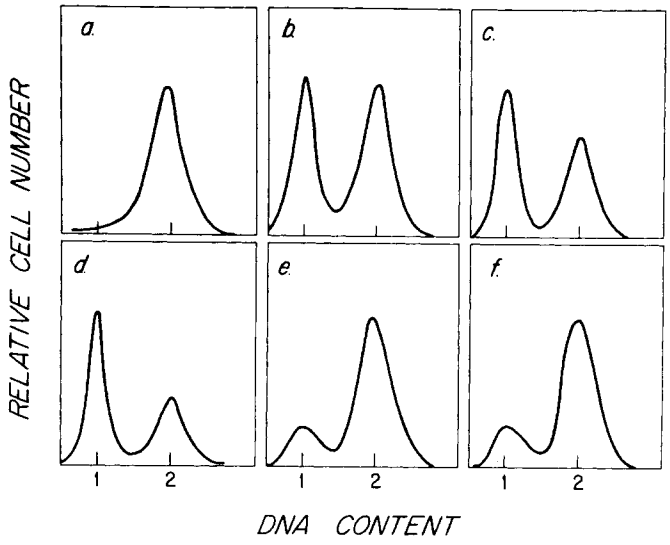


Fig. 2. Effect of serum and purified growth factors on the $G_1$ transit of synchronized Swiss 3T3 cells. DNA histograms of $G_2$ cells ($1.2 \times 10^4$ cells/cm$^2$) placed in medium containing (a) 10% serum; (b) 0.5% serum; (c) 0.2% serum; (d) 0% serum; (e) 0.5% serum, 10μg/ml insulin, and 10 ng/ml EGF; (f) 0.2% serum, 10 μg/ml insulin, and 10 ng/ml EGF. In all cases 0.05 μg/ml colcemid was added 4 h after the $G_2$ cells were shifted into the media indicated above and cells were prepared for cytofluorography 20 h after addition of colcemid.

**TABLE I. Effect of Serum and Purified Growth Factors on $G_1$ Transit of Synchronized Swiss 3T3 Cells**

| | Fraction of cells with $G_1$ DNA content |
|---|---|
| 0% serum | 0.58 |
| 0.2% serum | 0.50 |
| 0.5% serum | 0.43 |
| 0.2% serum + insulin + EGF | 0.23 |
| 0.5% serum + insulin + EGF | 0.23 |

These values were obtained by integrating the peaks in the DNA histograms shown in Figure 2.

The insulin and EGF requirement for Swiss 3T3 cells to pass the R point can be demonstrated with a larger fraction of the population using synchronized cultures. Cells were synchronized by slight modification of the hydroxyurea (HU) technique developed for CHO cells [10]. At 6 h after release from HU, when the cells were in $G_2$, they were shifted into serum-deficient medium; and 4 h later, when most of the cells had completed division and were in early $G_1$, 0.05 μg/ml colcemid was added to arrest at mitosis all cells able to escape the serum deprivation. When $G_2$ cells were shifted into medium completely lacking serum factors, nearly 60% of the cells were arrested in the $G_1$ phase after 20 h (Fig. 2 and Table I). However, as little as 0.2% or 0.5% serum in the shiftdown medium provided sufficient amounts of the limiting growth factors to permit a further 8% or 15% of the cells, respectively, to escape arrest and synthesize DNA. Addition of insulin (10 μg/ml) plus EGF (10 ng/ml) to medium containing either 0.2% or 0.5% serum allowed approximately 75% of cells to escape $G_1$ arrest. It is not known why 25% of the cells were arrested in $G_1$ even in the presence of insulin and EGF. Possible explanations – for instance, that concentrations of insulin and EGF were not optimal for R point transit of $G_2$ cells – will require further study.

The observation that both 0.5% and 0.2% serum were equally effective when supplemented with insulin and EGF is of special interest. This implies that only these two factors and no others became limiting when the serum concentration was reduced to 0.2%. If other factors had become limiting, then the fraction of cells able to escape from the 0.2% restriction would have been smaller than the fraction able to escape 0.5%, upon the addition of insulin and EGF. From this result it follows that other essential growth factors in serum besides insulin and EGF do not become limiting until their concentration is at least two times lower than in 0.5% serum

### Effect of Protein Synthesis Inhibition

Transit throughout the $G_1$ phase depends not only on serum factors but also on the synthesis of proteins. Inhibitors of protein synthesis added at high concentration block entry into S phase [2, 3]. Low doses of streptovitacin A specifically arrest the transit from $G_0$ to S of baby hamster kidney (BHK) cells [15], whereas low concentrations of the related compound cycloheximide slow the transit of Swiss 3T3 cells though the same process [16]. Schneiderman et al [17] presented evidence, from the effects of time exposures to high concentrations of cycloheximide, that progress of CHO cells through $G_1$ depends on the synthesis of labile protein(s) (half-life 2 h) that must be made in sufficient amount before the cells are able to proceed to make DNA.

If transit through $G_1$ depends on the synthesis of labile proteins, we might expect this part of the cell cycle to be preferentially inhibited by low doses of cycloheximide. We therefore examined the effect of low doses of cycloheximide on Swiss 3T3 cell cycle transit. Growth remained exponential, but there was a progressive increase in the cell doubling time as the concentration of cycloheximide increased (Fig. 3). This effect was observed with cycloheximide concentrations that inhibited protein synthesis by up to 70%. The diminution of the growth rate was as might be expected from the inhibition of protein synthesis. Surprisingly, however, the elongation was not uniform around the cell cycle.

The elongation of the cell cycle occurred mainly in $G_1$. When cytofluorographic DNA histograms were obtained from Swiss 3T3 cells growing exponentially in the presence of various concentrations of cycloheximide, the proportion of cells with a $G_1$ DNA content increased as the cycloheximide concentration increased (Fig. 4). The aver-

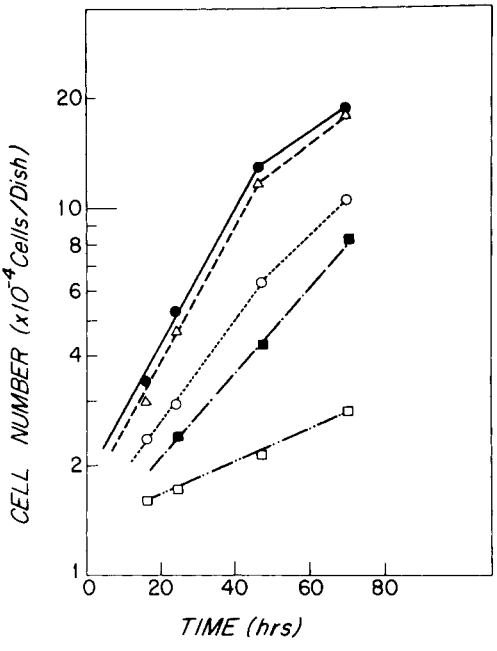


Fig. 3. Effect of cycloheximide on cell doubling time. Swiss 3T3 cells were plated in 8-cm$^2$ culture dishes in the presence of cycloheximide and the doubling time was obtained from the increase in cell number, determined using a Coulter Counter, over time. •, no cycloheximide; △, 0.01 μg/ml cycloheximide; ○, 0.03 μg/ml cycloheximide; ■, 0.05 μg/ml cycloheximide; □, 0.1 μg/ml cycloheximide.

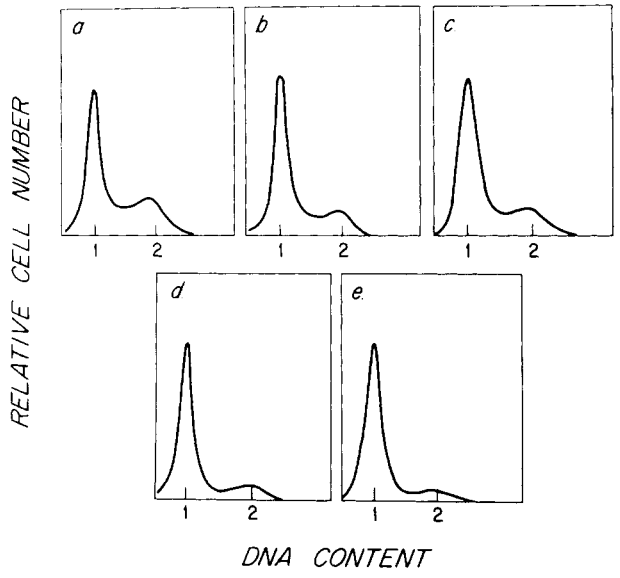


Fig. 4. Effect of cycloheximide on cell-cycle distribution. DNA histograms of exponential populations of Swiss 3T3 cells in (a) no cycloheximide; (b) 0.01 μg/ml cycloheximide; (c) 0.03 μg/ml cycloheximide; (d) 0.05 μg/ml cycloheximide; and (e) 0.1 μg/ml cycloheximide.

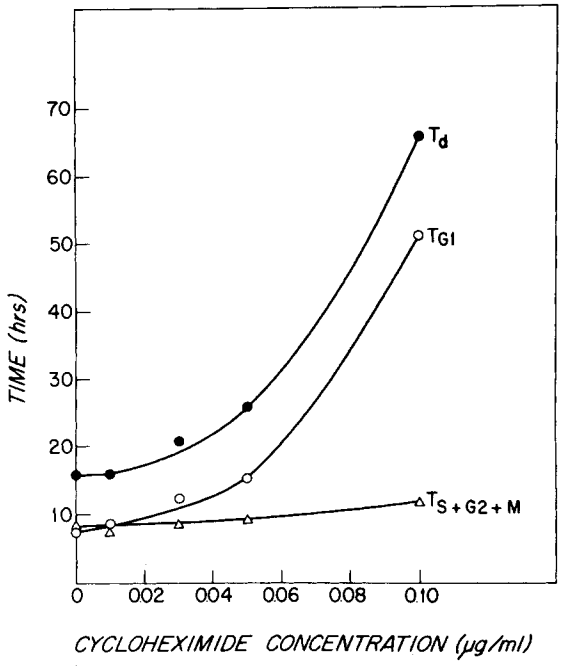


Fig. 5. Effect of cycloheximide on cell-cycle parameters. •, doubling time, $T_d$; ○, duration of $G_1$, $T_{G1}$; △, duration of S + $G_2$ + M, $T_S$ + $G_2$ + M.

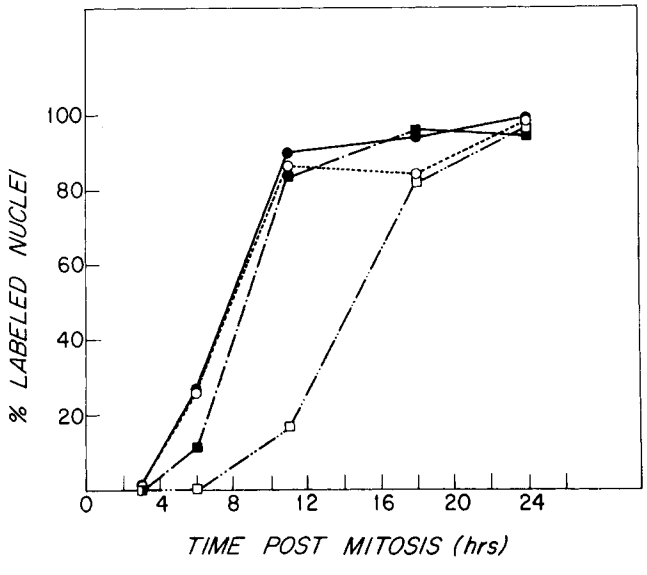


Fig. 6. Effect of cycloheximide on the entry of mitotic Balb/c 3T3 cells into DNA synthesis. Mitotic cells ($10^4$) were plated into 8-$cm^2$ culture dishes in the presence of cycloheximide and 2 μ Ci/ml $^3$H-thymidine. Plates were processed for autoradiography at the indicated times. •, no cycloheximide; ○, 0.03 μg/ml cycloheximide; ■, 0.05 μg/ml cycloheximide; □, 0.1 μg/ml cycloheximide.

age durations of $G_1$ and of the remainder of the cycle were calculated from these histograms using the Von Foerster equation [18], which takes into account the decreasing number of cells with increasing age in the cycle. The values are shown in Figure 5. Almost all of the increase in cycle duration caused by cycloheximide occurs in the $G_1$ period, while the $S + G_2 + M$ duration is only slightly changed. Using the shiftdown technique of Yen and Pardee, we have shown elsewhere that this elongation of the $G_1$ phase occurs primarily before the serum-sensitive R point [19]. These experiments support the idea that labile proteins are required for cell passage past the serum-sensitive R point.

### Timing of Cycloheximide-Sensitive Events

We were interested in determining whether synthesis of the labile proteins is accomplished only during the $G_1$ period in which they are utilized or whether some synthesis of the proteins occurs during the preceding cycle. If some of the labile proteins are produced in the preceding cycle, then cells collected at mitosis should be less delayed by low concentrations of cycloheximide present only during the following $G_1$ transit than are cells inhibited throughout the entire cycle. If, however, the synthesis of these proteins does not start until after mitosis, then mitotically collected cells should be just as delayed as cells grown for several generations in cycloheximide. Balb/c 3T3 cells were used for these experiments because Swiss 3T3 cells adhere to culture dishes more tenaciously and thus give extremely low yields of mitotic cells following a shake procedure. Labile protein synthesis similar to that described in Swiss 3T3 cells has also been demonstrated in Balb/c 3T3 cells [19]. Figure 6 shows the entry into S phase as determined autoradiographically for Balb/c 3T3 cells collected by a mitotic shake procedure. The duration of $G_1$ after shifting into low doses of cycloheximide was little changed. At the highest concentration of cycloheximide (that inhibits protein synthesis by 70%), the duration of the first $G_1$ after exposure was almost doubled. This elongation, however, was still not as great as that found in cells grown for several generations with the same concentration of cycloheximide. Thus at least some of the protein synthesis involved in regulation of R point transit may occur in the preceding cycle.

## DISCUSSION

Both serum factors and protein synthesis are required for normal cell growth. Lowering the serum concentration to 0.2% stops the growth of Swiss 3T3 cells mainly by limiting their supply of insulin and EGF. Under these conditions cells are unable to pass a growth restriction point located 2 h before the initiation of DNA synthesis. Experiments using the protein synthesis inhibitor cycloheximide indicate that labile proteins are involved in cell transit past this serum-sensitive restriction point in $G_1$ phase. Indeed, results from experiments with cycloheximide can be used to quantitiatively support a model in which an initiator protein with a half-life of 2.2 h has to be synthesized at a constant rate and built up to a specific level before a cell can pass the restriction point [19].

Transformed cells are generally thought to have a markedly lowered serum requirement. Recent work, however, shows that this is not always so. Dubrow et al [20] found that the ability of Balb/c 3T3 cells and seven transformed derivatives (DNA virus, RNA virus, and chemical) to grow in media containing low serum concentrations was a function of the mode of transformation. DNA virus-transformed lines remained distributed throughout the cell cycle whereas RNA virus-transformed and chemically transformed

cells accumulated in $G_1$. Cherington et al [21] have examined the growth requirements of various hamster cell lines using a chemically defined medium containing the purified growth hormones insulin, EGF, fibroblast growth factor (FGF), and transferrin. They found that a DNA virus-transformed line had lost the requirement for insulin, FGF, and EGF. In contrast, chemically and spontaneously transformed cell lines retained the insulin requirement and had a diminished requirement for EGF. Transformed cells requiring insulin but not EGF were arrested in $G_1$ on withdrawal of insulin.

The mode of transformation appears to determine the amount of growth control exhibited by transformed cell lines. The behavior of a single transformed line cannot be taken to represent the behavior of all transformed lines. In particular, transformation by DNA tumor viruses such as polyoma and SV40 is an extreme sort, with abolished growth control and few growth factor requirements. In contrast, transformation with RNA viruses and chemicals generally has much less drastic consequences for growth regulation.

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