Non-specific elongation of cell cycle phases by cycloheximide in rat 3Y1 cells, and specific reduction of G_1 phase elongation by simian virus 40 large T antigen

ATSUYUKI OKUDA and GENKI KIMURA

Department of Virology, Medical Institute of Bioregulation, Kyushu University 69, Fukuoka 812, Japan

Summary

Partial inhibition of protein synthesis by cycloheximide caused prolongation of G_1, S and G_2 phases in rat 3Y1 fibroblasts. In cells expressing simian virus 40 (SV40) large T antigen, by infection with SV40 in the previous generation, the prolongation of G_1 phase in the presence of cycloheximide was suppressed. However, the prolongation of S and G_2 phases in the presence of cycloheximide was not suppressed in cells expressing large T antigen, by infection with SV40 in the current generation. Similarly, when density-arrested cells (cells in G_0 phase) were infected with SV40 (either wild-type strain or a mutant deleted in the unique coding region for small t antigen) and reseeded sparsely in the presence of cycloheximide, the cycloheximide-induced delay of entry into S phase was suppressed. In this case, the reduction in [^{35}S]methionine incorporation, that in protein accumulation and that in cell volume increase, were not surmounted by SV40 infection. In T-antigen-negative cells, all the regions in G_1 phase seemed to be sensitive to cycloheximide, i.e. they suffered elongation. These results suggest that, in comparison with cells that enter S phase by the action of growth factors, cells expressing large T antigen can enter S phase more efficiently through a quite different process.

Key words: cell cycle, protein synthesis inhibition, SV40 large T antigen, growth control.

Introduction

A number of transformed cell lines and cancer cells are impaired in the normal regulatory mechanism of cell proliferation, especially the process required for initiation of the S phase of the cell cycle (for a review, see Yanishevsky & Stein, 1981). Maintenance of the transformed state in cultured rodent cells transformed with simian virus 40 (SV40) involves the function(s) of large T antigen (for reviews, see Topp *et al.* 1980; Martin, 1981; Graessmann *et al.* 1982). Large T antigen expressed in cells can overcome the inhibition of the cells from entering S phase under suboptimal culture conditions, such as deficiency in serum growth factors (Smith *et al.* 1971; Scher *et al.* 1978; Stiles *et al.* 1979; Okuda *et al.* 1984; Okuda & Kimura, 1986), extreme cell crowding (Okuda *et al.* 1984), presence in culture medium of sodium butyrate (Kawasaki *et al.* 1981; Mitsudomi & Kimura, 1985*a*), and restriction temperatures for temperature-sensitive cell lines (Floros *et al.* 1981; Ohno & Kimura, 1984; Mitsudomi & Kimura, 1985*b*).

Partial inhibition of protein synthesis by cycloheximide results in the inhibition of G_1-phase progression (Kim *et al.* 1968; Schneiderman *et al.* 1971; Highfield & Dewey, 1972; Brooks, 1977; Rønning *et al.* 1981; Campisi *et al.* 1982). Cycloheximide also inhibits the progression of S and G_2 phases (Okuda & Kimura, 1988). In this study, we report that in SV40-infected rat 3Y1 fibroblasts large T antigen overcomes selectively the inhibition of G_1 phase progression in the presence of cycloheximide, and that the effect is not accompanied by an enhancement of general protein synthesis and an increase in cell mass.

Materials and methods

Cell culture

A clonal isolate (clone 1-6) of rat 3Y1-B diploid fibroblasts (Kimura *et al.* 1975) (referred to as 3Y1) was used. The regular culture medium was Dulbecco's modified Eagle's medium (DEM) supplemented with 10% foetal calf serum. Density-arrested, resting cultures were prepared by seeding 1×10^5 cells in 5·2 cm plastic dishes with 5 ml of culture medium, followed by incubation for 5 days. All cultures were incubated at 37 °C in a humidified atmosphere of 10% CO_2/90% air.

Virus infection

Wild-type (wt) SV40, strain SV68C (Todaro & Takemoto,

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1969) and a viable deletion mutant of SV40, *dl*-884 (Shenk *et al.* 1976), kindly provided by P. Berg, which lacks the unique coding region for the small t antigen (Crawford *et al.* 1978), were used. Unless otherwise specified, wt SV40 was used. The density-arrested 3Y1 cells ($1{\cdot}2\times10^6/5{\cdot}2$ cm dish) were inoculated with 1 ml of undiluted virus stock suspension (wt: $1{\cdot}7\times10^9$ p.f.u. ml^{-1}; *dl*-884: $1{\cdot}6\times10^9$ p.f.u. ml^{-1}) prepared as described (Okuda *et al.* 1984), and incubated at 37°C for 2 h for virus adsorption.

Arrest at early S phase

The density-arrested cells uninfected or infected with wt SV40 were dispersed with trypsin–EDTA, and $1{\cdot}4\times10^6$ cells were seeded in 8·6 cm dishes with 10 ml of the regular medium containing $2{\cdot}5\ \mu g\ ml^{-1}$ aphidicolin (Wako Pure Chemical Industries, Osaka, Japan) (or 2×10^5 cells in 3·3 cm dishes with 2 ml of the medium for flow cytometry or 7×10^5 cells in 26 cm^2 culture flasks with 5 ml of the medium for the analysis of G_2 progression) and then incubated for 20 h.

Mitotic cells

Aphidicolin was removed from the cultures arrested at early S phase, and the cells were incubated for 6 h to permit progression through S and G_2 phases. Mitotic cells were detached by mild pipetting and collected by centrifugation.

Kinetics of entry into S phase

The mitotic cells (3×10^3) or the density-arrested cells (5×10^3) were reseeded in 1 cm^2 spot areas on the surfaces of plastic dishes with 100 μl of test media containing [^{3}H]thymidine ($1\ \mu Ci\ ml^{-1}$, $20\ Ci\ mmol^{-1}$, Amersham International, UK). When performing parallel experiments with different cell preparations or in the presence of cycloheximide (Sigma, St Louis, MO) with different concentrations, each experimental sample was plated onto the spot areas allocated on dishes in such a way that all the experimental samples shared a common dish. A dish was taken out at 2-h intervals, and cells were then fixed for autoradiography. The time required for 50 % of cells to enter S phase was defined as the time required to enter S phase.

Detection of T-antigen-positive cells

Indirect immunofluorescence for nuclear SV40 T antigen was performed as described (Okuda *et al.* 1984).

Analysis of the progression of S phase

After removal of aphidocolin the cultures arrested at early S phase were transferred to test media, and the distributions of DNA content were determined at intervals as described below. The time required to increase the mean DNA content (mean channel number of the pulse height analyser) to 1·5-fold of the mean DNA content of the density-arrested culture was calculated, and twice the value was defined as the length of S phase.

Flow cytometry

Cellular DNA was stained with propidium iodide ($50\ \mu g\ ml^{-1}$ in 0·1 % sodium citrate), the nuclei were extracted and the DNA content of the individual nuclei was determined in a Cytofluorograph (FC 4800A, Bio/Physics Systems, Inc., New York, NY). The data were processed using a microcomputer-equipped pulse height analyser (MCA/PC98B, Laboratory Equipment Corp., Ichikawa, Japan).

Analysis of the progression of G_2 phase

Cells arrested in early S phase were prepared in 26 cm^2 plastic culture flasks. After removal of aphidicolin the cultures were incubated with the regular medium for 4 h. At this time a large fraction (over 57 %) of cells had a G_2 DNA content. Then the medium was changed to test media (regular medium containing or not containing 0·4 μM-cycloheximide) containing $40\ ng\ ml^{-1}$ of colcemid (Nakarai Chemical, Kyoto, Japan). The caps of the culture flasks were closed tightly and the cultures were incubated in a temperature-controlled box installed on a phase-contrast microscope stage. The cumulative number of round metaphase cells was counted under the phase-contrast microscope for as many as 400 cells over more than five fields. The time required for 50 % of the cells with a G_2 DNA content to enter metaphase was defined as the length of G_2 phase.

[^{35}S]methionine incorporation

The density-arrested cells (7×10^4) uninfected or infected with SV40 were reseeded in glass liquid scintillation-counting vials (diameter: 2·2 cm) with 1 ml of the regular medium and incubated for 11 h (infected cells) or 13 h (uninfected cells). Then, the medium was changed to the test medium (regular medium containing or not containing 0·4 μM-cycloheximide) supplemented with [^{35}S]methionine ($0{\cdot}5\ \mu Ci\ ml^{-1}$, 1100 Ci $mmol^{-1}$, Amersham International, UK), and the cells were incubated. At 2-h intervals acid-soluble materials were extracted with 2 ml of cold 5 % trichloroacetic acid for 30 min and the vials were washed with water followed by addition of liquid scintillator. The radioactivity was determined in a liquid scintillation counter.

Protein-content determination

The density-arrested cells (2×10^5) uninfected or infected with SV40 were reseeded with the regular medium in 3·3 cm dishes, and incubated for 1·5 h for cell anchorage. After medium was changed to various test media (regular medium containing or not containing 0·4 μM-cycloheximide, or lacking serum), the cultures were incubated for another 18·5 h. The cells were dissolved in 0·9 ml of 0·1 M-NaOH and then neutralized with 0·9 ml of 0·1 M-HCl. The protein content was assayed using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) (Bradford, 1976). Bovine plasma gamma globulin was used as a standard.

Measurement of cell volume

Cells were prepared as for protein-content determination. The prepared cells were dispersed with trypsin–EDTA and diluted with DEM containing 1 % serum. The cell volume distribution was obtained from the pulse heights of a cell counter (model ZB, Coulter Electronics Inc., Hialeath, FL) using a sampling strobe generator (NS-457, Northern Scientific Inc., Middleton, WI) and the microcomputer-equipped pulse height analyser.

Results

Inhibition by cycloheximide of entry into S phase after release from density arrest, and its suppression by SV40 large T antigen

As shown in Fig. 1, when density-arrested 3Y1 cells were released from arrest, cycloheximide inhibited entry into S phase in a dose-dependent fashion. The inhibition was partially overcome when cells had been infected with wt SV40. The surmounting effect was observed but to a lesser extent, when cells had been infected with a mutant of SV40 deleted in the unique coding region for small t antigen.

Fig. 2 shows the kinetics of entry into S phase in the presence of cycloheximide after release from density

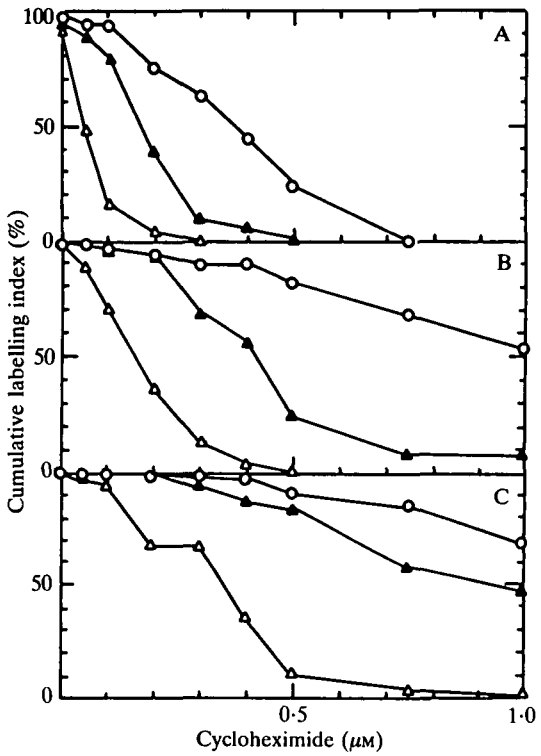

Fig. 1. Dose-dependent inhibition by cycloheximide of entry into S phase after release from density arrest and suppression of the inhibition by SV40 infection. Density-arrested cells were uninfected or infected with SV40, and reseeded sparsely with the regular medium containing the indicated concentrations of cycloheximide and [^{3}H]thymidine. After incubation for the period indicated below, cells were fixed for autoradiography. Incubation time: A, 22 h; B, 30 h; C, 44 h. (△) Uninfected; (○) infected with wild-type SV40; (▲) infected with *dl*-884 (a mutant of SV40 deleted in the unique coding region for small t antigen).

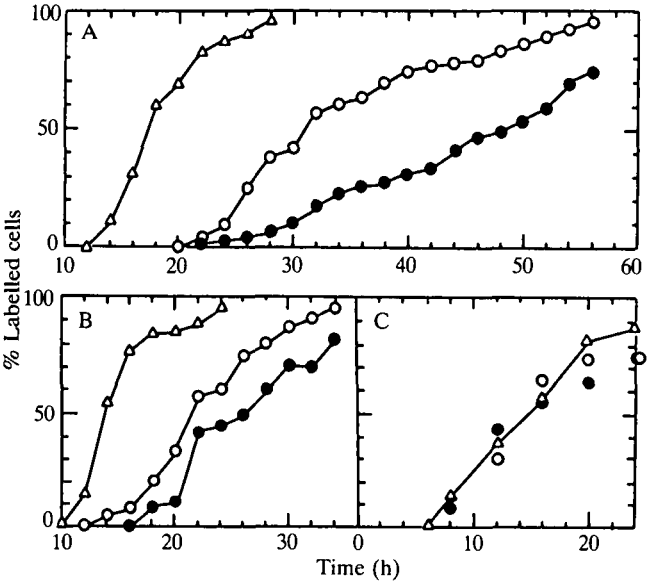

Fig. 2. Delay of entry into S phase in the presence of cycloheximide after release from density arrest. Density-arrested cells were uninfected or infected with SV40 and reseeded sparsely (0 time) with the regular medium containing cycloheximide, at the concentrations indicated below, and [^{3}H]thymidine. After incubation for the indicated periods, cells were fixed for autoradiography or immunofluorescence assay. A. Uninfected, fraction of cells labelled with [^{3}H]thymidine; B, infected, fraction of cells labelled with [^{3}H]thymidine; C, infected, fraction of T-antigen-positive cells. Cycloheximide concentrations: (△) 0 μM; (○) 0·2 μM; (●) 0·4 μM.

arrest. Entry into S phase was delayed in the presence of cycloheximide. The delay was shortened when cells had been infected with SV40. The kinetics of appearance of T-antigen-positive cells were not significantly affected by cycloheximide. From these results, we conclude that, after cells are released from density arrest, cell entry into S phase is delayed in the presence of cycloheximide, and that T antigen reduces the delay.

Prolongation of G_1 phase of proliferating cells by cycloheximide and its reduction in cells expressing large T antigen

As shown in Fig. 3, a delay in entry into S phase was also observed when mitotic cells were exposed to cycloheximide. The delay was shortened when mitotic cells were

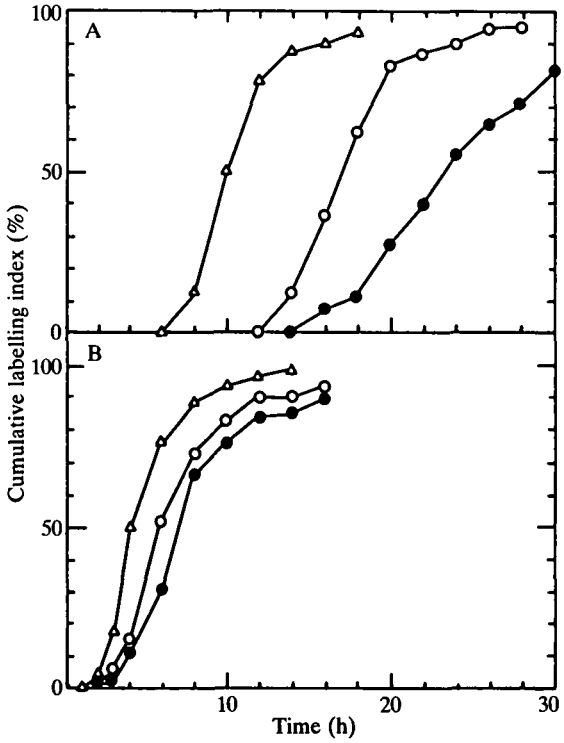

Fig. 3. Reduction in the G_1 phase prolongation in the presence of cycloheximide by infection with SV40. Mitotic cells having been infected or uninfected with SV40 were seeded (0 time) with the regular medium containing cycloheximide with the concentrations indicated below. Cells were continuously labelled with [^{3}H]thymidine for the indicated periods and fixed for autoradiography.
A. Uninfected; B, infected. Cycloheximide concentrations: (△) 0 μM; (○) 0·2 μM; (●) 0·4 μM.

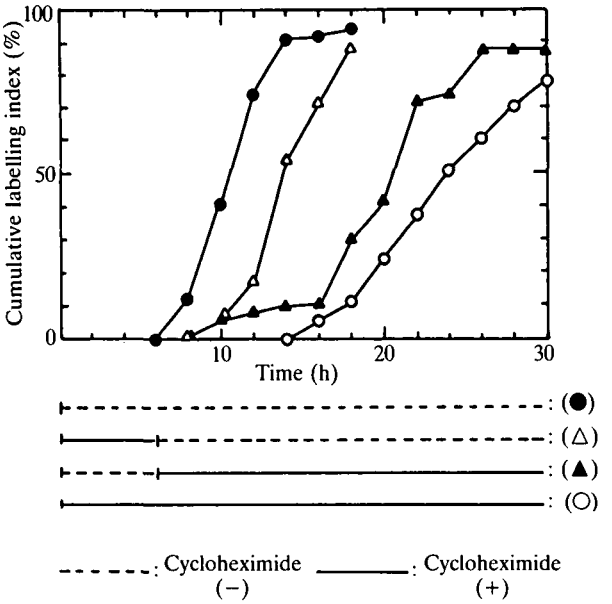

Fig. 4. Effect of exposure to cycloheximide during various intervals after mitosis on the kinetics of entry into S phase. Mitotic cells were reseeded (0 time) sparsely with the regular medium and 0·4 μM-cycloheximide was added during the intervals indicated below the graph with symbols used. Cells were continuously labelled with [^{3}H]thymidine for the indicated periods, and fixed for autoradiography.

prepared from resting cells infected with SV40. As many as 90 % of the infected cells were positive for T antigen 2 h after mitosis, as determined by immunofluorescence. Since the cells not expressing T antigen (uninfected cells) had a longer G_1 length (post-mitosis time by which 50 % of cells had entered S phase: 10 h) than the cells expressing T antigen (4 h), the possibility remains that the difference in the cycloheximide-induced delay time between infected and uninfected cells simply reflects the difference in the G_1 length. To exclude this possibility, the time between the start of cycloheximide administration and the time by which 50 % of cells would enter S phase in the absence of cycloheximide was equalized between infected and uninfected cells. For this purpose, exposure of uninfected cells to cycloheximide was started at 6 h (time equivalent to the difference in the G_1 length between infected and uninfected cells) after mitotic selection. As shown in Fig. 4, more marked delay in entry into S phase was also observed in this case, compared with the case when infected cells were exposed to cycloheximide at mitosis (see Fig. 3B). Therefore, the longer delay induced by cycloheximide in the T-antigen-negative (uninfected) cells does not simply reflect a longer G_1 length.

No effect of large T antigen on the cycloheximide-induced delay of the progression of S and G_2 phases

As shown in Fig. 5, after cells were released from arrest at early S phase, the DNA content distribution shifted towards the G_2 peak and then returned to the G_1 peak. The shift was markedly delayed in the presence of cycloheximide. Similar delay was seen in SV40-infected cells, of which 85 % were T-antigen-positive at the time of the release from arrest at early S phase.

This and additional data on the progression of S phase is presented more quantitatively in Fig. 6. It is also obvious that the expression of T antigen did not affect the retardation of the progression of S phase in the presence of cycloheximide.

The progression of G_2 phase is presented in Fig. 7. The populations with 57 % (uninfected cells) and 64 % (SV40-infected cells) of cells with a G_2 DNA content were incubated in the presence or absence of cycloheximide, and cumulative mitotic cells were counted. It is clear that the G_2 progression was delayed in the presence

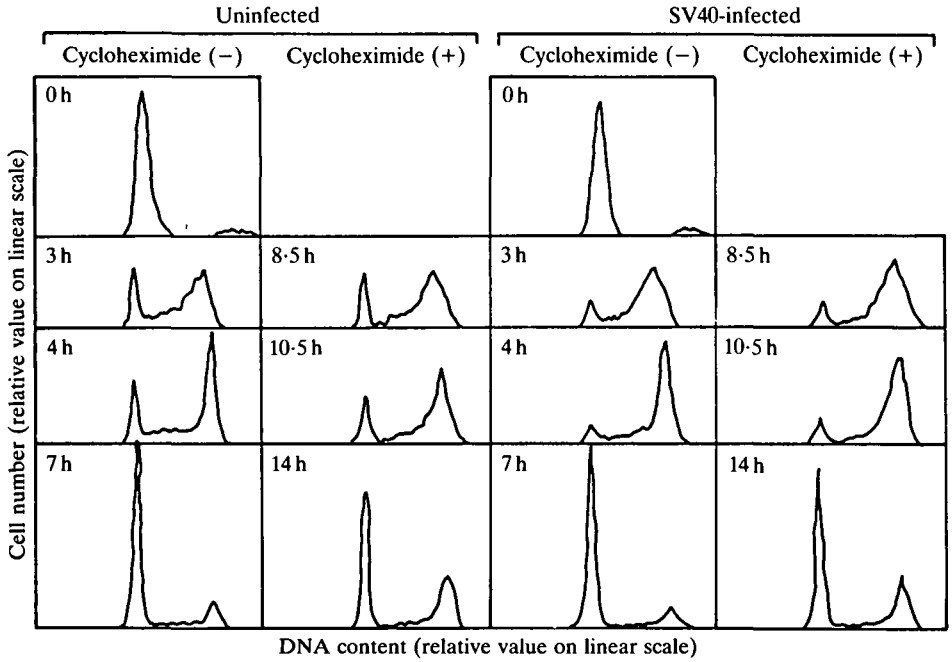

Fig. 5. Prolongation of S–G_2 progression by cycloheximide in uninfected and SV40-infected cells. Cells uninfected or infected with SV40 were arrested at early S phase with aphidicolin. After removal of aphidicolin, cells were incubated for the indicated periods in the presence or absence of 0·4 μM-cycloheximide. Then, nuclei were stained and extracted for determination of the distribution of DNA content by flow cytometry. All cytograms are normalized so that the total cell number is the same.

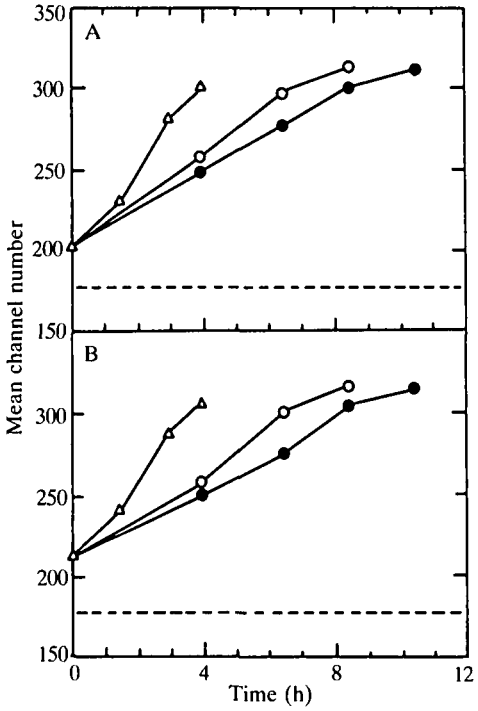

Fig. 6. Kinetics of the progression of S phase in the presence of cycloheximide in uninfected and SV40-infected cells. The mean channel number of the flow cytogram, shown in Fig. 5, was calculated and plotted against the time after release from aphidicolin arrest at early S phase. A. Cells not infected; B, cells infected with SV40. Broken lines indicate the mean channel of the density-arrested culture. Cycloheximide concentration: (△) 0 μM; (○) 0·2 μM; (●) 0·4 μM.

of cycloheximide, and that T antigen did not cause shortening of the delay.

Capability of SV40-infected cells to enter S phase without increase in protein content and cell volume

Density-arrested cells were uninfected or infected with SV40, reseeded sparsely and incubated for 13 h or 11 h, respectively. After similar treatments, S phase cells began to appear at the respective times (see Fig. 2A,B). A considerable fraction of infected cells expressed T antigen by 11 h (see Fig. 2C). These cells were exposed to cycloheximide, and incorporation of [^{35}S]methionine into the acid-insoluble fraction was measured. As shown in Fig. 8, there was no difference in the inhibition of

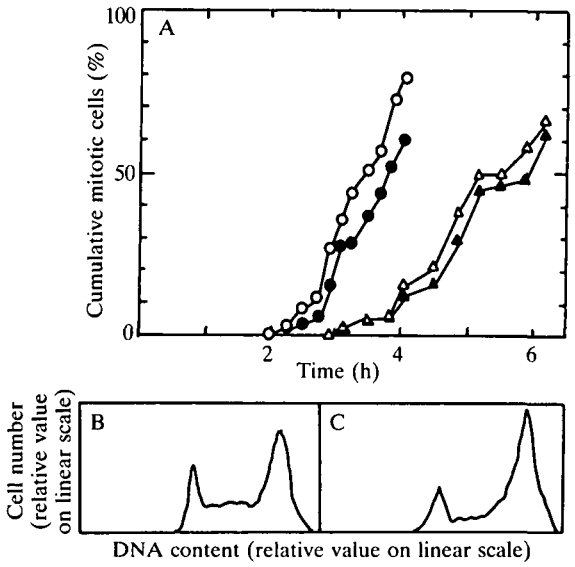

Fig. 7. Prolongation of G_2 progression by cycloheximide in uninfected and SV40-infected cells. Cells uninfected or infected with SV40 were arrested at early S phase by aphidicolin. A cell population mainly consisting of cells with a G_2 DNA content was obtained by incubating for 4 h after removal of aphidicolin. At this point, the flow cytograms of the DNA content distribution are presented in B and C for uninfected and infected cultures, respectively. The fractions of cells with a G_2 DNA content were 57 % (B) and 64 % (C). Then, the cultures were supplemented with colcemid and the incubation was started in the presence or absence of 0·4 μM-cycloheximide (0 time). The fraction of accumulating mitotic cells was determined under a phase-contrast microscope at the times indicated in A. (○) SV40-infected, cycloheximide (−); (△) SV40-infected, cycloheximide (+); (●) uninfected, cycloheximide (−); (▲) uninfected, cycloheximide (+).

incorporation between uninfected and infected cells. In the control experiment without exposure to cycloheximide, SV40-infected cells incorporated more [^{35}S]methionine than uninfected cells, probably reflecting the more rapid entry of infected cells into S phase (see Fig. 2A,B).

Density-arrested cells were uninfected or infected with SV40, and incubated at low cell density with cycloheximide or in the absence of serum for 18·5 h, by which time more than 85 % of cells had entered S phase under

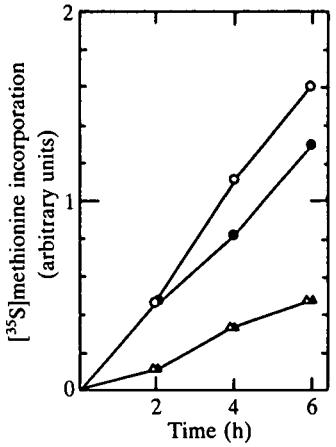

Fig. 8. Inhibition of [^{35}S]methionine incorporation by cycloheximide in SV40-infected and uninfected cells. Density-arrested cells were uninfected or infected with SV40, and reseeded sparsely with the regular medium. After 11 h (infected cells) or 13 h (uninfected cells) of incubation, [^{35}S]methionine was added to the cultures (0 time), and they were incubated in the presence or absence of 0·4 μM-cycloheximide for the indicated periods. Then, the radioactivities in acid-insoluble materials were determined. Each point shows the mean value for three samples. The standard errors was less than 5 % of the mean values. (●) Uninfected, cycloheximide (−); (▲) uninfected, cycloheximide (+); (○) SV40-infected, cycloheximide (−); (△) SV40-infected, cycloheximide (+).

Table 1. *Lack of protein accumulation in the presence of cycloheximide or in the absence of serum after release from density arrest*

Conditions	Fold increase* in: Protein content/cell	Cell volume	Labelling index (%)
Uninfected			
Regular medium	1·48 ± 0·16	1·51 ± 0·10	85
Cycloheximide	1·05 ± 0·10	1·10 ± 0·12	2
Serum (−)	0·75 ± 0·10	0·80 ± 0·08	2
SV40-infected			
Regular medium	1·75 ± 0·10	1·56 ± 0·10	95
Cycloheximide	1·28 ± 0·16	1·04 ± 0·06	10
Serum (−)	0·98 ± 0·10	0·89 ± 0·06	40

Density-arrested cells were uninfected or infected with SV40 and reseeded sparsely with the regular medium. On completion of cell anchorage after 1·5 h incubation, the medium was changed to either the regular medium, that supplemented with 0·4 μM-cycloheximide or that lacking serum, and incubated for 18·5 h. To estimate the fraction of cells that had entered S phase during the incubation of 18·5 h, [^{3}H]thymidine was added to a portion of the cultures at the time of medium change, and the cultures were incubated for 18·5 h followed by fixation for autoradiography.

* Ratio compared with the value at the time of anchorage. Each value shows the mean and the standard error for four samples.

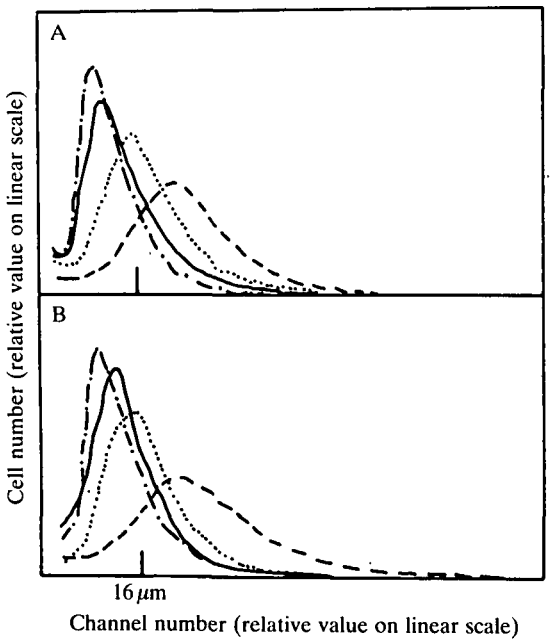

Fig. 9. Inhibition of the increase in cell volume in the presence of cycloheximide or in the absence of serum in uninfected and SV40-infected cells. Density-arrested cells were uninfected or infected with SV40 and reseeded sparsely with the regular medium. On completion of cell anchorage after 1·5 h incubation, the medium was changed to either the regular medium, that containing 0·4 μM-cycloheximide, or that lacking serum, and incubated for another 18·5 h. A. Uninfected; B, SV40-infected. (——) Cells just after anchorage; (– – –) cells incubated in the regular medium; (· · · · ·) cells incubated in the presence of cycloheximide; (— · —) cells incubated in the absence of serum. All histograms are normalized so that the total cell number is the same. Theoretically, the channel number is proportional to the cell volume. The mean channel number for the calibration particles with a mean diameter of 16 μM is indicated.

normal culture conditions, but none of them entered mitosis. In Table 1, the rate of increase in the protein content per cell and that in the mean cell volume during the incubation are presented with the fraction of cells that had entered S phase during the incubation period. The typical cell volume distribution is presented in Fig. 9. In neither uninfected nor infected cells did the cellular protein content and the cell volume significantly increase in the presence of cycloheximide or in the absence of serum (in the absence of serum both were slightly reduced). Nevertheless, infected cells entered S phase in the presence of cycloheximide or in the absence of serum (see also Fig. 2). This was in contrast to cases in which entry into S phase was accompanied by an increase in cell protein content and volume under the normal culture conditions (with serum and without cycloheximide). Thus, the increase in cell mass (cell protein content and volume) is not a prerequisite for cells expressing T antigen to enter S phase. There are a number of similar examples of cells entering S phase without increasing cell mass (for a review, see Baserga, 1984).

Discussion

The inhibition of entry into S phase in the presence of cycloheximide after stimulation of resting cells to proliferate was overcome by infection with wt SV40. The effect was smaller with *dl*-884 than with wt SV40. This indicates that although large T antigen is involved in the surmounting effect, the contribution of small t antigen is not ruled out. Other groups have noted the effects of small t antigen in complementing the action of large T antigen in the maintenance of the transformed state (Frisque *et al.* 1979; Bikel *et al.* 1986, 1987). In any case, it is reasonable to conclude that large T antigen plays a major role in overcoming the inhibition of entry into S phase in the presence of cycloheximide in wt SV40-infected cells.

The prolongation of the length of each cell cycle phase in the presence of cycloheximide is summarized in Table 2. In the absence of cycloheximide, the lengths of cell cycle phases differed from each other, and between uninfected and SV40-infected cells. In order to compare the effects of cycloheximide on the progression of each cell cycle phase between uninfected and SV40-infected cells, the fold increase in the length of each cell cycle phase during administration of cycloheximide was calculated and the values are presented in Table 2 (in parenthesis). Generally, cellular functions are influenced by inhibition of protein synthesis, and our results indicate that the progression of each cell cycle phase is no exception. Large T antigen in SV40-infected cells reduced the cycloheximide-induced elongation of G_1 phase, but did not reduce that of S and G_2 phases. Protein synthesis, cellular protein accumulation and increase in cell size were all inhibited in the presence of

Table 2. *Increase in the length of cell cycle phase in the presence of cycloheximide*

Cell cycle phase	Length of cell cycle phase* (h), and fold increase compared with the length without cycloheximide† at cycloheximide concentrations: 0 μM	0·2 μM	0·4 μM	Corresponding data
Uninfected cells				
G_0–S‡	17	31 (1·8)	48 (2·8)	Fig. 2
G_1	10	17 (1·7)	23·5 (2·4)	Fig. 3
G_1§	10·5	–	24 (2·3)	Fig. 4
Early G_1§·¶	2·5¶	–	6 (2·3)	Fig. 4
Late G_1§·‖	4·5	–	14·5 (3·2)	Fig. 4
S	4	8·5 (2·1)	10·5 (2·6)	Fig. 6
G_2	3	–	5 (1·7)	Fig. 7
SV40-infected cells				
G_0–S‡	13·5	21·5 (1·6)	26 (1·9)	Fig. 2
G_1	4	6 (1·5)	7 (1·8)	Fig. 3
S	4	8·5 (2·1)	10·5 (2·6)	Fig. 6
G_2	3	–	5 (1·7)	Fig. 7

* For the derivation of each length of cell cycle phase, see Materials and methods.

† Indicated in parenthesis.

‡ The period between release from density arrest and entry into S phase.

§ Results from a set of experiments.

¶ Mitotic cells were incubated for 6 h in the absence or presence of cycloheximide to allow progression of early G_1 phase, followed by incubation in the absence of cycloheximide until entry into S phase. The time t_1 required to complete, in the absence of cycloheximide, the early G_1 process that can be completed in the presence of cycloheximide by 6 h after mitosis was calculated as follows. $t_1 = t_{G_1} - (t^*_{G_1} - 6)$, where t_{G_1} stands for the G_1 length (h) in the absence of cycloheximide, and $t^*_{G_1}$ for the G_1 length (h) when mitotic cells were exposed to cycloheximide for 6 h followed by its deprivation.

‖ Mitotic cells were incubated for 6 h in the absence of cycloheximide, and the time thereafter required to enter S phase in the presence or absence of serum was determined.

cycloheximide, and none of the inhibition was diminished in cells expressing large T antigen. Therefore, the shortening of the cycloheximide-induced elongation of G_1 phase cannot be explained by the reduction in inhibition of general protein synthesis in cells expressing large T antigen.

3Y1 cells expressing large T antigen can enter S phase in the absence of serum growth factors (Okuda *et al.* 1984). The G_1 length is shorter when proliferating cells enter S phase by the action of large T antigen than when they do so by the action of serum growth factors (Okuda & Kimura, 1986). The elongation of G_1 phase by partial inhibition of protein synthesis was reduced in cells expressing large T antigen. These results suggest the two following possibilities for the involvement of large T antigen in the process required for entry into S phase: first, that the process required for entry into S phase is divided into two parts, the process mediated by growth factors and the subsequent process that is triggered by the completion of the earlier process. The later process can be activated by large T antigen without the trigger of the earlier process. The second possibility is that the process mediated by growth factors is quite different from that mediated by large T antigen.

If the first possibility is correct, the later process should be less sensitive to the inhibition of protein synthesis than the earlier process. Otherwise, the fold increase in G_1 length would not have been reduced in cells expressing large T antigen. However, as can be seen in Table 2, when cells not expressing large T antigen entered S phase, the late G_1 period was more sensitive to cycloheximide than the early G_1 period. In addition, the fold increase in the time between cycloheximide administration and entry into S phase was greater when exposure to the drug was started 6 h after mitosis (i.e. the exposure period was late G_1 phase) than at 0 h (i.e. the exposure period was the whole of G_1 phase). This also indicates that late G_1 phase is more sensitive to cycloheximide than the other (early) part of G_1 phase. We, therefore, prefer the second possibility that large T antigen mediates the initiation of S phase through a mechanism quite different from the one that operates when cells are exposed to growth factors. On this basis, the surmounting effect of large T antigen on the inhibition of G_1 progression under various suboptimal culture conditions (Smith *et al.* 1971; Scher *et al.* 1978; Stiles *et al.* 1979; Floros *et al.* 1981; Kawasaki *et al.* 1981; Okuda *et al.* 1984; Ohno & Kimura, 1984; Mitsudomi & Kimura, 1985*a*,*b*; Okuda & Kimura, 1986) can be easily explained.

We can definitely say that the process required for entry into S phase is more efficient and quicker when mediated by large T antigen than when mediated by growth factors. SV40 requires cellular functions for viral DNA replication (for a review, see Acheson, 1980). Cells of most parts of a living organism are not in a proliferating state but in a non-proliferating state. Therefore, SV40 ought to induce cellular DNA synthesis in the non-proliferating cells prior to their own viral DNA replication. The most efficient way for viruses to multiply is by awakening directly the cellular machinery required for the initiation of DNA synthesis. Non-permissive rat 3Y1 cells lack other cellular function(s) required for SV40 multiplication. Therefore, in the abortive transformation of 3Y1 cells with SV40 only the process required for initiation of cellular DNA synthesis becomes efficient.

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References

ACHESON, N. H. (1980). Lytic cycle of SV40 and polyoma virus. In *Molecular Biology of Tumor Viruses*, 2nd edn, part 2: *DNA Tumor*

Viruses (ed. J. Tooze), pp. 125–204. New York: Cold Spring Harbor Laboratory Press.

BASERGA, R. (1984). Growth in size and cell DNA replication. *Expl Cell Res.* **151**, 1–5.

BIKEL, I., MAMON, H., BROWN, E. L., BOLTAX, J., AGHA, M. & LIVINGSTON, D. M. (1986). The t-unique coding domain is important to the transformation maintenance function of the simian virus 40 small t antigen. *Molec. cell. Biol.* **6**, 1172–1178.

BIKEL, I., MONTANO, X., AGHA, M. E., BROWN, M., MCCORMACK, M., BOLTAX, J. & LIVINGSTON, D. M. (1987). SV40 small t antigen enhances the transformation activity of limiting concentrations of SV40 large T antigen. *Cell* **48**, 321–330.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72**, 248–254.

BROOKS, R. F. (1977). Continuous protein synthesis is required to maintain the probability of entry into S phase. *Cell* **12**, 311–317.

CAMPISI, J., MEDRANO, E. E., MORREO, G. & PARDEE, A. B. (1982). Restriction point control of cell growth by a labile protein: Evidence for increased stability in transformed cells. *Proc. natn. Acad. Sci. U.S.A.* **79**, 436–440.

CRAWFORD, L. V., COLE, C. N., SMITH, A. E., PAUCHA, E., TEGTMEYER, P., RUNDELL, K. & BERG, P. (1978). Organization and expression of early genes of simian virus 40. *Proc. natn. Acad. Sci. U.S.A.* **75**, 117–121.

FLOROS, J., JONAK, G., GALANTI, N. & BASERGA, R. (1981). Induction of cell DNA replication in G_1-specific ts mutants by microinjection of SV40 DNA. *Expl Cell Res.* **132**, 215–223.

FRISQUE, R. J., RIFKIN, D. B. & TOPP, W. C. (1979). Requirement for the large T and small t proteins of SV40 in the maintenance of the transformed state. *Cold Spring Harbor Symp. quant. Biol.* **44**, 325–331.

GRAESSMANN, A., GRAESSMANN, M. & MUELLER, C. (1982). Regulation of SV40 gene expression. *Adv. Cancer Res.* **35**, 111–149.

HIGHFIELD, D. P. & DEWEY, W. C. (1972). Inhibition of DNA synthesis in synchronized Chinese hamster cells treated in G_1 or early S phase with cycloheximide or puromycin. *Expl Cell Res.* **75**, 314–320.

KAWASAKI, S., DIAMOND, L. & BASERGA, R. (1981). Induction of cellular deoxyribonucleic acid synthesis in butyrate-treated cells by simian virus 40 deoxyribonucleic acid. *Molec. cell. Biol.* **1**, 1038–1047.

KIM, J. H., GELBARD, A. S. & PEREZ, A. G. (1968). Inhibition of DNA synthesis by actinomycin D and cycloheximide in synchronized HeLa cells. *Expl Cell Res.* **53**, 478–487.

KIMURA, G., ITAGAKI, A. & SUMMERS, J. (1975). Rat cell line 3Y1 and its virogenic polyoma- and SV40-transformed derivatives. *Int. J. Cancer* **15**, 694–706.

MARTIN, R. G. (1981). The transformation of cell growth and transmogrification of DNA synthesis by simian virus 40. *Adv. Cancer Res.* **34**, 1–68.

MITSUDOMI, T. & KIMURA, G. (1985*a*). Effects of sodium *n*-butyrate on entry into S phase in resting rat 3Y1 cells infected with simian virus 40. *J. Virol.* **56**, 951–957.

MITSUDOMI, T. & KIMURA, G. (1985*b*). Abortive transformation of temperature-sensitive mutants of rat 3Y1 cells by simian virus 40: Effect of cellular arrest states on entry into S phase and cellular proliferation. *J. cell. Physiol.* **123**, 353–360.

OHNO, K. & KIMURA, G. (1984). Genetic analysis of control of proliferation in fibroblastic cells in culture. II. Alteration in proliferative and survival phenotypes in a set of temperature-sensitive mutants of rat 3Y1 cells after infection or transformation with simian virus 40. *Somat. Cell molec. Genet.* **10**, 29–36.

OKUDA, A. & KIMURA, G. (1986). Serum-dependent control of entry into S phase of next generation in rat 3Y1 fibroblasts: Effect of simian virus 40 large T antigen. *Expl Cell Res.* **163**, 127–134.

OKUDA, A. & KIMURA, G. (1988). Elongation of G_1 phase by transient exposure of rat 3Y1 fibroblasts to caffeine during the previous as well as present generations. *J. Cell Sci.* **89**, 379–386.

OKUDA, A., SHIMURA, H. & KIMURA, G. (1984). Abortive transformation of rat 3Y1 cells by simian virus 40: Viral function overcoming inhibition of cellular proliferation under various conditions of culture. *Virology* **133**, 35–45.

RØNNING, W., LINDMO, T., PETTERSON, E. O. & SEGLEN, P. O. (1981). Effect of serum step-down on protein metabolism and proliferation kinetics of NHIK 3025 cells. *J. cell. Physiol.* **107**, 47–57.

SCHER, C. D., PLEDGER, W. J., MARTIN, P., ANTONIADES, H. & STILES, C. D. (1978). Transforming viruses directly reduce the cellular growth requirement for a platelet derived growth factor. *J. cell. Physiol.* **97**, 371–380.

SCHNEIDERMAN, M. H., DEWEY, W. C. & HIGHFIELD, D. P. (1971). Inhibition of DNA synthesis in synchronized Chinese hamster cells treated in G_1 with cycloheximide. *Expl Cell Res.* **67**, 147–155.

SHENK, T. E., CARBON, J. & BERG, P. (1976). Construction and analysis of viable deletion mutants of simian virus 40. *J. Virol.* **18**, 664–671.

SMITH, H. S., SCHER, C. D. & TODARO, G. J. (1971). Induction of cell division in medium lacking serum growth factor by SV40. *Virology* **44**, 359–370.

STILES, C. D., CAPONE, G. T., SCHER, C. D., ANTONIADES, H. N., VAN WYK, J. J. & PLEDGER, W. J. (1979). Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1279–1283.

TODARO, G. J. & TAKEMOTO, K. K. (1969). "Rescued" SV40: Increased retransforming efficiency in mouse and human cells. *Proc. natn. Acad. Sci. U.S.A.* **62**, 1031–1037.

TOPP, W. C., LANE, D. & POLLACK, R. (1980). Transformation by SV40 and polyoma virus. In *Molecular Biology of Tumor Viruses*, 2nd edn, part 2: *DNA Tumor Viruses* (ed. J. Tooze), pp. 205–296. New York: Cold Spring Harbor Laboratory Press.

YANISHEVSKY, R. M. & STEIN, G. H. (1981). Regulation of the cell cycle in eukaryotic cells. *Int. Rev. Cytol.* **69**, 223–259.

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