Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 4123–4126, July 1980
Cell Biology

Prevalent deficiency in tumor cells of cycloheximide-induced cycle arrest

(animal cells/tumorigenic transformation/protein synthesis/cell cycle/growth control)

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ABSTRACT **Mammalian cell growth is regulated by a process that is completed at a restriction point in the late G_1 part of the cell cycle. This process is highly sensitive to serum concentration and to moderate inhibition of protein synthesis by cycloheximide (CHM) or other agents. We have proposed that a cell must accumulate a labile protein in a critical amount before events related to its DNA synthesis can start. The accumulation of this protein requires conditions suitable for growth, including sufficient amounts of serum-derived factors. An important criterion for attributing a major role to such a regulatory mechanism is that cells whose growth control is modified—e.g., by mutation—should be defective in this process. Cells of this kind are produced by tumorigenic transformation. We show here that mouse 3T3 cells, human fibroblasts, and Chinese hamster CHEF/18 cells have stringent G_1 growth control by CHM. In contrast, tumorigenic lines obtained from these cells by transformation with various agents (DNA tumor virus, RNA tumor virus, chemical carcinogens) or spontaneously all showed relaxed growth control under the influence of CHM. In these lines, growth control was relaxed to different degrees; some lines were held in G_1 by a combination of low serum concentration and CHM, but others were not. Serum concentration showed a synergistic effect with CHM. Low serum concentrations did not limit growth only by affecting the rate of protein synthesis. The labile-protein mechanism is likely to be basic to growth control by serum factors. Transformed tumorigenic cells in general may have relaxed this mechanism.**

Normal cells of higher animals are endowed with control mechanisms that keep their proliferation in balance with the entire organism. Tumor cells are defective in control of their growth (1). Normal cells in tissue culture are arrested mainly in G_1 when grown to high density, when grown in media containing inadequate supplies of serum factors or nutrients, when grown in the presence of some drugs (2, 3), or when put into suspension (4). The cells go into a quiescent state (G_0) unless they can carry out metabolic events somehow dependent on the above external factors (2). Alternatively, if they accomplish these events, they proceed to grow and carry out DNA synthesis and later processes leading to cell division. The "metabolic place" in the cell cycle at which cells have completed their regulatory events has been named the "restriction point" (5).

It was reported recently (6) that concentrations of cycloheximide (CHM) that inhibit protein synthesis in 3T3 mouse cells by up to 70% specifically delay transit through G_1, and in particular through the part of the cycle prior to the restriction point. These results can be explained by proposing that cells must synthesize an adequate amount of labile protein that has a half-life of a few hours in order to proceed and make DNA. Synthesis of this protein was proposed to be the event affected by external growth regulating conditions.

As an independent test of this hypothesis, we have examined the effects of CHM on abilities of variously transformed tumorigenic cells to transit G_1. These cells are relaxed in growth control and, in general, their growth is not as readily arrested by the high density (2), low serum concentration (7), or drugs (3, 8, 9) as are normal cells. If the CHM-sensitive event is basic to normal growth control it should be relaxed in the transformed cells. Furthermore, the stringency of control should depend on conditions that affect growth, such as serum supply. We present evidence, from several lines from mouse, hamster, and human, that cells made tumorigenic by various agents are all relaxed with regard to the CHM inhibition of G_1 transit. These results support the labile-protein hypothesis of growth arrest.

MATERIALS AND METHODS

Cell Culture. BALB/c 3T3 mouse fibroblast clone A31-CL7 (A31) (10), 3T6 cells (11), benzo[*a*]pyrene-transformed A31 cells, BP-3T3-CL7-5 (BP-A31) (12), simian virus 40-transformed A31 cells, SV-A31-CL29 (13), Moloney sarcoma virus-transformed A31 cells, M-A31-CL 71 (M-A31) (14), and two human cell lines, normal FS-2 fibroblasts and tumor epithelial HS0578T (15) were obtained from stocks in this laboratory. The human tumor cell line LNSV40 (16) was from A. Neil Howell (Sidney Farber Cancer Institute). Chinese hamster embryo fibroblasts CHEF/18, CHEF/16 (17), and also the chemically transformed T30-4 fibroblasts (18) were obtained from Ruth Sager (Sidney Farber Cancer Institute).

Cells were routinely grown at 37°C in a water-saturated 10% CO_2/90% air atmosphere in Dulbecco's modification of Eagle's medium (Flow, McLean, VA) supplemented with 10% calf serum (Flow), glutamine (4 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). Stocks of CHEF/18, CHEF/16, and T30-4 derived lines were maintained in the α modification of Eagle's medium (Flow) supplemented as above except that calf serum was replaced by fetal calf serum (Flow). Each stock was determined to be free of mycoplasma contamination, on the basis of the ratio of [^{3}H]uridine and [^{3}H]uracil incorporations (19).

Cytofluorometry. Cells grown in duplicate 60-mm culture dishes were trypsinized and suspended in 4 ml of medium; 0.5 ml was removed into a vial containing 10 ml of Hanks' balanced salt solution with 0.5% formaldehyde for a cell count (Coulter Counter), and the remaining 3.5 ml was processed for flow microfluorometry in a Biophysics Systems Cytofluorograf (model 4800A) as described (20).

Materials. CHM was obtained from Sigma, and [^{3}H]leucine was from New England Nuclear. Powdered media were from GIBCO.

RESULTS

Comparison of CHM Effect on 3T3 and on Its Transformed Derivative Lines. The cells were cultured for several days in media containing various concentrations of CHM. Growth was exponential up to 0.1 μg of CHM per ml for all of

Abbreviation: CHM, cycloheximide.
* Address for reprint requests.

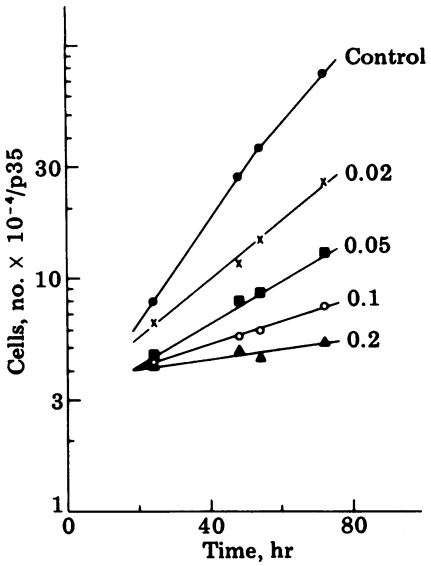

FIG. 1. Growth curves of 3T6 cells with different concentrations (μg/ml) of CHM. Generation time (hr): ●, 14; x, 24; ■, 30; ○, 62; ▲, >120.

the lines and was slower as the CHM concentration increased. Data for 3T3 cells have been presented (6); similar results for 3T6 cells are given in Fig. 1 as typical. Duration of the entire cycle was increased by CHM for all lines (Fig. 2). The distributions of cells in the cycle were determined cytofluorometrically, after growth for 1 or 2 days in the presence of CHM at 0.05 μg/ml and 10% serum. As reported (6) and shown in Fig. 3, 3T3 cells changed their distribution from the exponential pattern to an increase in the fraction of cells in G_1. The behavior of the other four lines was different. All remained distributed through the cycle. Thus, CHM at 0.05 μg/ml was much less able to restrict transit of these transformed cells through the G_1 part of the cycle.

When a higher concentration of CHM (0.1 μg/ml) was used, a gradation of relaxed control was clearly observed for the various transformed lines. Relaxation was least in 3T6. In the presence of this higher drug concentration, these cells had more cells in G_1 and fewer in S and G_2 relative to the control; this was similar to 3T3 cells grown with 10% serum and only 0.05 μg of CHM per ml. The next was M-A31 which had a larger G_1 population in the presence of CHM but still had quite large S and G_2 populations. Third were BP-A31 and SV-A31 whose distributions did not change in medium containing 10% serum plus CHM. The duration (G_1) of G_1 for the different generation

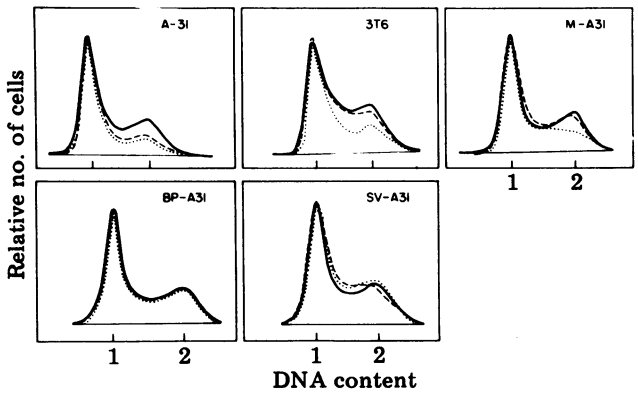

FIG. 3. DNA distribution in 3T6 and A31 and their tumorigenic derivatives, growing with CHM at 0.05 μg/ml (- - -) or 0.1 μg/ml (• • • •) or without CHM (—).

times (T) was calculated from the relative areas under the peaks by using the von Foerster equation which takes into account the age distribution of the exponential population (21). Fig. 4 shows G_1/T for different cell lines as a function of CHM. Those cells that responded most to the drug showed increasing G_1/T values with increasing CHM concentration; BP-A31 and SV-A31 showed the contrary change. The present data reinforce the earlier conclusion (9) that transformation alters growth control to different degrees for various cell lines.

The lines BP-A31 and SV-A31 fall into the most growth-relaxed class, and they are similar in other ways—forming tumors in *nude* mice and colonies in Methocel, and growing to high densities in medium containing 10% serum. BP-A31 cells are more responsive to a low-serum environment, growing to high density and requiring a long time to be arrested in presence of 0.2% calf serum but finally showing growth arrest in G_1 (22). By contrast, SV-A31 cells continue to proliferate in low serum and remain distributed around the cell cycle until they die (9). The difference in regulation of BP-A31 and SV-A31 cells by CHM can be brought out by adding CHM to 2% serum. Under these conditions, BP-A31 cells accumulated in G_1 but the SV-A31 cells did not (Fig. 5). When the SV-A31 cells were subjected to even more stringent conditions of low serum and CHM at 0.1 μg/ml for 48 hr there was an abnormal flow microfluorometry distribution, broadly over the G_1, S, and G_2 fractions. These properties might be due to damage of the cells with irreversible arrest in all parts of the cycle.

The difference between these cells is not because of a decreased overall sensitivity to low serum: the rate of incorporation

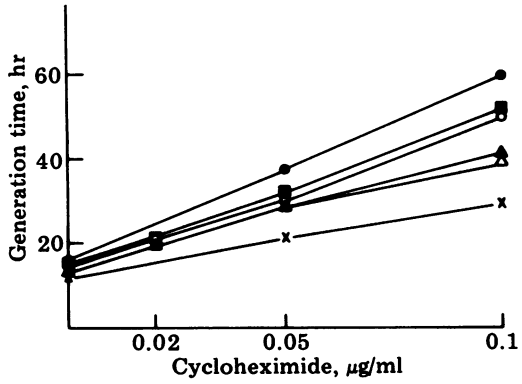

FIG. 2. Generation time as a function of CHM concentration for different cell lines: ●, A-31; ■, SV-A31; ○, 3T6; ▲, BP-A31; △, M-A31; x, CHEF-18.

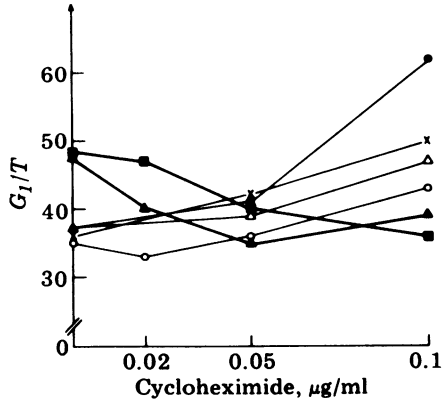

FIG. 4. G_1/T ratios as a function of CHM concentration. G_1 is the average duration of G_1 and T is the generation time. Symbols as in Fig. 2.

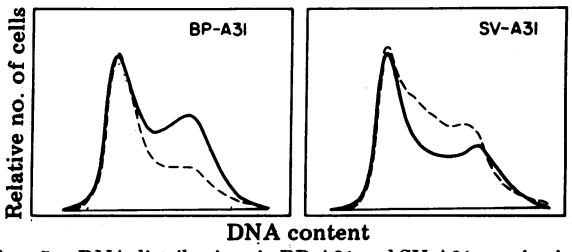

FIG. 5. DNA distributions in BP-A31 and SV-A31 growing in 2% calf serum with (---) and without (—) CHM. Cells were plated at 3×10^3 cells/cm^2 and allowed to grow in 10% calf serum to about 10^4 cells/cm^2. After 24–30 hr the medium was aspirated and fresh medium with or without CHM was added; DNA distributions were measured 24 hr later.

of [^{3}H]leucine by both cell lines during 24 hr in 0.5% serum was similarly decreased by about 20%. However, when these cells were grown for 24 hr with low serum plus 0.05 or 0.1 μg of CHM per ml, there was considerably less [^{3}H]leucine uptake by SV-A31 cells compared to BP-A31 cells (Table 1). This result is the opposite of what would be the case if the arrest, in G_1, of BP-A31 cells relative to SV-A31 cells were due to stronger inhibition by CHM of the BP-A31 cells.

Comparison of CHM Arrest of Other Cell Lines. In order to test if it is possible to extend to other species the conclusion reached with the A-31 line and its tumorigenic derivatives, we studied other cell lines for G_1 growth arrest by CHM. When treated with CHM for only 24 hr, the nontumorigenic diploid Chinese hamster embryo fibroblast line (CHEF/18) showed a flow microfluorometry distribution with approximately the same reduction of S and G_2 cells as shown by 3T3 cells, although more cells were in S (Fig. 6). In 2% fetal calf serum with CHM at 0.05 μg/ml these changes were more evident. The CHEF/18 cells could not be examined after 48 hr in culture in the presence of CHM because they became damaged and showed a broad G_1–S peak. The tumorigenic clonal line CHEF/16, isolated from the same embryo, showed no accumulation in G_1 in 24 hr and only a small accumulation after 48 hr. These cells retain some growth control, as shown by their ability to arrest in G_1 when they are deprived of fibroblast growth factor, epidermal growth factor, and insulin (18). The chemically transformed hamster cell line T30-4 showed no increase of G_1 cells after treatment with CHM (0.1 μg/ml) for 48 hr. Increased S and G_2 peaks were obtained with CHM at 0.2 μg/ml.

Of three human cell lines tested, only the normal fibroblasts FS-2 showed a reduced fraction of cells in S and G_2 with CHM. The tumor cell line HS0578T did not respond at all to the addition of CHM. It recently was reported that these cells have

Table 1. Relative rates of protein synthesis in BP-A31 and SV-A31 growing at different serum and CHM concentrations

Calf serum	CHM, μg/ml	[^{3}H]Leucine incorporation, % of control	
		BP-A31	SV-A31
10%	0	100	100
	0.05	70.0	39.8
	0.01	51.8	35.9
2%	0	100	100
	0.05	73.4	48.1
	0.10	60.4	52.7
0.5%	0	100	100
	0.05	72.3	53.2
	0.10	58.2	46.5

For experimental details see Fig. 5. [^{3}H]Leucine incorporation into trichloroacetic acid-insoluble material was measured over 24 hr, normalized to 10^4 cells, and expressed as percentage of control.

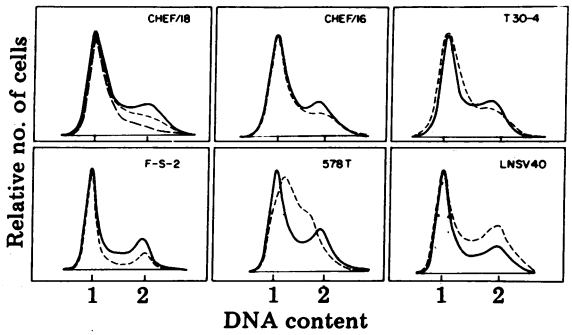

FIG. 6. DNA distributions of hamster and human cell lines with and without CHM at 0.1 μg/ml. —, In 10% calf serum without CHM; ---, in 10% calf serum with CHM; – –, in 2% calf serum with CHM.

a highly relaxed control mechanism because quiescent cultures responded as well to serum-free Dulbecco's modified Eagle's medium as to medium containing serum (23). In the same conditions, the other tumor line (LNSV-40) showed more cells in S and G_2 than did controls at all concentrations of CHM tested. This pattern was enhanced in low serum and CHM.

DISCUSSION

Results of recent studies using low concentrations of CHM as a probe suggest that control of growth at the restriction point in G_1 depends on the ability of cells to accumulate a labile initiator protein (6). We have tried to determine whether the relaxed growth regulation observed in various tumorigenic cells is due to a relaxation of this CHM-sensitive growth control. We have examined nontumorigenic mouse, Chinese hamster, and human cells. These three lines accumulated cells in G_1 when exposed to CHM at 0.05 μg/ml in medium containing 10% serum. In contrast, all the tumorigenic lines examined—four mouse lines obtained with four different agents of transformation, two hamster lines, and two human lines—did not accumulate cells to the same extent in G_1 under the same conditions.

The stringency of G_1 arrest fell into three classes. Normal cells increased the fraction of time spent in G_1 as the concentration of CHM was increased, 3T6 and M-A31 cells maintained about the same fraction in G_1, and BP-A31 and SV-A31 cells had a decreased fraction in G_1 as the CHM concentration was increased. The last two lines can be distinguished by growing them with low serum, particularly when CHM is also present, BP-A31 cells then accumulated in G_1, but SV-A31 cells did not. We conclude that the restriction point control that depends on CHM is relaxed to different degrees in variously transformed cells. Furthermore, the data provide independent evidence of a mutational nature for this hypothesized major growth control mechanism for mammalian cells.

There is a parallel between these results and ones obtained earlier (9, 24) in which cell lines were compared with regard to their G_1 arrest in media containing low serum concentrations. The similiarity is not surprising because serum concentration and CHM affect the same process (6). However, serum concentration has a more specific effect than reducing the general rate of protein synthesis because the decrease in [^{3}H]leucine incorporation at low serum concentration was quite small under conditions that resulted in accumulated (normal) cells in G_1. We surmise that the growth factors provided by serum specifically affect the synthesis or degradation of the putative labile restriction protein.

Many reports have established that protein synthesis is required during G_1 phase by most transformed and normal cells,

except for simian virus 40-transformed lines (25–29). In an interesting example (30) of phenotypic reversions of a transformed line toward more normal states, human osteosarcoma cells transformed by Kirsten murine sarcoma virus of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine achieve flat shape and low saturation density in the presence of CHM. The results in general suggest that the G_1 regulatory mechanism of most cells involves their ability to make proteins. In contrast, simian virus 40-transformed cells appear to have bypassed this protein requirement and, presumably, the entire mechanism.

We thank Gail Morreo for providing excellent technical assistance and Eileen Fingerman for preparing the manuscript for publication. This work was supported by U.S. Public Health Service Grant GM 24571 (to A.B.P.) and U.S. Public Health Service International Research Fellowship TW02800 (to E.E.M.).

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