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CHARACTERIZATION OF A CV-1 CELL CYCLE
II. THE ROLE OF CELL-SUBSTRATE ATTACHMENT

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SUMMARY

The availability of synchronous cultures of untransformed CV-1 cells has allowed analysis of the role of cell-substrate attachment in cell cycle progression as well as the ability of serum and SV40 infection to override the requirement for cell-substrate attachment. Attachment to a solid substrate is required for progression through G_1. Prevention of attachment results in cell cycle arrest 30 min after cytokinesis. Serum is required for attachment, but increasing the serum concentration from 2 to 20% does not enhance cell attachment, nor obviate its need for cell cycle progression. SV40 infection does not overcome the requirement of serum for attachment nor the need of attachment for normal cycling.

INTRODUCTION

The ability of mammalian cells to grow without firm attachment to a substrate, e.g. in spinner culture or in soft agar, is usually associated with a phenotypic characteristic of cells transformed by oncogenic viruses and chemical carcinogens, and, while many studies have focused on the acquisition of this capability, fewer have focused upon what effects loss of attachment has on the normal (untransformed) cell. It is generally recognized that normal cells in culture, when seeded at a low density, proliferate at a relatively constant rate, determined by the generation time of the particular cell type. Their rate of growth declines precipitously upon reaching saturation density, after which a majority of cells cycle either irregularly, or not at all. Analysis of these relatively 'quiescent' cells has indicated that their progress has been arrested during the early part of the cell cycle, the G_1 phase. Addition of serum to these cultures usually induces these cells to cycle and initiate DNA synthesis. Almost the same effects are produced when similarly quiescent host cells are infected with simian virus 40 (SV40).

It has also been shown that when untransformed cells lose their attachment to a rigid surface their growth is also arrested (Stoker, O'Neill, Berryman & Waxman, 1968) at a point preceding *S*-phase (Clarke, Stoker, Ludlow & Thornton, 1970; Otsuka & Moskowitz, 1976; Folkman & Moscona, 1978). Using populations of synchronous cells we have investigated the kinetics with which cells attach to a solid substrate in order to learn more about the location of that arrest point and the nature of its regulation.

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Our report is based upon observations of an epithelioid subclone of the CV-1 cell line, of African green monkey kidney origin. This subclone, when used within a limited number of passages, maintains a fairly constant subtetraploid karyotype, and does not appear to have properties generally associated with transformed cells. Experiments were performed in which the substrate attachment of mitotic cells was either prevented for varying periods of time or facilitated by pretreatment of the substrate with collagen. The rate at which cells attached to the substrate and entered *S*-phase under various conditions was measured. The onset of *S*-phase was delayed for an interval of time proportional to the time (less 30 min) that attachment to the substrate was prevented. Conversely, facilitation of attachment proportionally shortened the interval prior to initiation of *S*-phase. Serum concentrations of less than 2% markedly reduced the rate of attachment of cells, while serum concentrations above that required for optimal growth did not enhance attachment. These experiments indicate that, unlike transformed cells, normal cells require serum for attachment and attachment for normal progression through the cell cycle. Moreover, although SV40, like serum, can induce relatively 'quiescent' monolayer cells to cycle, prior infection of these cells with SV40 did not obviate the requirement of serum for attachment, nor did it obviate the requirement of attachment for normal cycle progression.

MATERIALS AND METHODS

Cells and culture techniques

All experiments were performed with a subclone of the CV-1 cell line between passages 12 and 20. Cells were passaged weekly in roller bottles containing Eagle's minimal essential medium supplemented with 10% foetal bovine serum (FBS). Populations of mitotic cells were obtained on consecutive hours by their selective detachment from roller cultures by high-speed rotation. The degree of synchrony was estimated from the time required for over 90% of the cells to enter *S*-phase, as determined by autoradiographic analysis, and undergo mitosis at the end of the first cycle post-mitotic selection, as determined by microscopy. Cells were also labelled for a 24-h period to determine the percentage of cells which were non-cycling. Mitotic cell selections were 98% mitotic. Our procedures for cell growth and synchrony were as previously described (D'Alisa & Gershey, 1979) with the following exceptions:

Determination of serum effect. In order to ascertain the effects of serum on the attachment of selected mitotic cells, roller bottles with a 3-fold greater surface area were used. The initial number of cells seeded, amount of medium used for feeding and the number of mitotic cells collected per hour were also proportionally increased (3-fold). The hourly schedule of high-speed rotation and media change was interrupted and the medium in the roller bottles was removed after 55 min of growth and replaced with an equal volume of serum-free Eagle's medium. After the usual 3-min spin at high speed to selectively remove the mitotic cells, the eluant was divided equally and apportioned into four 75-cm^2 flasks (8×10^5 cells/per flask) (equilibrated with 10% CO_2 in air) which contained different amounts of FBS such that the final concentrations of FBS were 0, 0·5, 1, 2, 5, 7, 10 and 20%. Following removal of the mitotic cells, the roller cultures were again re-fed with Eagle's medium supplemented with 10% FBS.

SV40 infection. In another set of experiments, cells were first infected with SV40 virus and their attachment rates in the presence of various concentrations of serum were scored. Alternatively, SV40-infected cells in the presence of 10% FBS were prevented from attaching for 4 h as described below. The subsequent attachment and incorporation of tritiated thymidine of these infected cells were measured (see below). The production of SV40 has been previously

detailed (Diacumakos & Gershey, 1977). Virus stock had a titre of 10^9 plaque-forming units (PFU)/ml and an equivalent number of T antigen-forming units (TFU). Two of 4 roller bottles, set up according to our protocol for mitotic cell selection, were infected with an input multiplicity of 20 PFU-TFU/cell. Virus was added in a minimal volume of serum-free Eagle's medium and adsorption was allowed to proceed for 2 h at 37 °C. As a control, the other 2 roller bottles received a similar aliquot of serum-free Eagle's medium alone. Following infection, the bottles were washed twice with Eagle's medium supplemented with 10 % FBS. Bottles were returned to an hourly schedule of high-speed rotation and re-feeding for an additional 4–8 h before mitotic cells were collected for the experiment as described in the previous paragraph. As a control for SV40 infection, round 12-mm coverslips were washed in acetic acid, water and absolute ethanol, flamed and inserted sterily into the 75-cm^2 flasks used for the culture of the selected mitotic cells. Forty-eight hours after infection, the coverslips were removed, washed in phosphate-buffered saline (PBS), fixed in ethanol at −70 °C and stained for SV40 T antigen as previously described (D'Alisa & Gershey, 1978).

Promotion of attachment. In order to promote cell attachment, 75-cm^2 culture flasks or microscope slides (precleaned with acetic acid and ethanol and cut in half longitudinally) were coated with collagen. One hundred microlitres of a collagen solution (Ethicon, Batch TD-85) 10 mg/ml, were added to 0·5 % acetic acid (100 ml), which had been filtered through a Millipore filter (0·45 μm) and deaerated to constitute a working solution. Glass slides to be coated were flamed and placed in large, sterile Petri dishes. The diluted, working collagen solution was poured into the Petri dishes and culture flasks to a depth of 4 mm. The liquid was then removed by evaporation in a warm dessicator for 5–6 h.

Prevention of attachment. Following collection of mitotic cells into culture flasks, attachment was prevented by physical means. A shaking apparatus of local design consisted of a rectangular platform, which, under the power of 2 motors with individual speed controllers, maintained both a rotary motion in the plane of its flat surface and a tilting motion about the axis drawn longitudinally through its mid-point. In this way, very gentle but efficient resuspension of the cultures was maintained in the same 37 °C temperature-controlled room that the roller cultures were grown in. Following agitation of cell cultures for 0, 2, 3 or 4 h, cell viability was determined by dye exclusion following a 5-min exposure to erythrocin B. The number of cells taking up the dye (non-viable) was scored with the aid of a haemocytometer. In addition, cultures were labelled for 24 h with [^{3}H]thymidine (55 Ci/mmol, New England Nuclear) at a concentration of 1 μCi/ml in growth medium, following which cells were washed and fixed as described (Gershey, D'Alisa & Zucker, 1979) in preparation for autoradiography.

Attachment

Attachment of cells following collection of the selected mitotic cells in culture flasks was monitored at 37 °C with phase-contrast optics at magnifications between 100 and 160. A minimum of 10 fields from different areas of the flask were observed and approximately 100–300 cells per field were scored with the aid of a reticle inserted into the eyepiece which superimposed a grid of 400 squares upon the field. In experiments where uncoated and collagen-coated glass slides had been inserted into culture flasks, attachment to both the coated and uncoated glass surfaces could be compared within the same flask. Cells which appeared rounded also occupied space just above the plane of the relatively flat monolayer and in a logarithmically growing asynchronous culture constituted 5 % of the population (as would theoretically be predicted). Attachment was operationally defined as the time required for cells, having reached the 2-cell (or doublet) stage upon completion of telophase, to reassume a morphology typical of the flattened G_1 cell.

Autoradiography

Half microscope slides were flamed and inserted into 75-cm^2 culture flasks prior to seeding of these flasks with mitotic cells at hourly intervals. The flasks also contained tritiated thymidine (50 Ci/mmol, New England Nuclear) in growth medium at a final concentration of 1 μCi/ml. At hourly intervals following cell attachment, slides were sterily withdrawn and processed for autoradiography as described previously (D'Alisa & Gershey, 1979). Fluorographic exposure

of NTB[3] emulsion (Kodak) permitted us to evaluate the results of an experiment within 1 day. In this way, time course experiments could be performed under constant labelling conditions on a single population of synchronous cells with a minimum of handling, and different conditions for attachment could be compared. All experiments were performed in triplicate.

Scanning electron microscopy

Round coverslips (12 mm diameter) (Corning) were coated with poly-L-lysine (Miles Labs.) as described (Sanders, Alexander & Braylan, 1975). Coverslips which had been cleaned with ethanol and then air dried, were coated with 50 μl of the poly-L-lysine solution (1 mg/ml in PBS) and allowed to stand at room temperature for 1 h following which they were washed in distilled water and stored in a moist chamber. Mitotic cells and cells which had been prevented from attaching for 0·25, 0·5, 1, 2, 3, and 4 h as described above were then centrifuged at 4 °C at 500 *g* for 3 min and the pellets were fixed at 4 °C in 1 % glutaraldehyde (EM Lab.) in PBS overnight. Following fixation, 30 μl of a suspension (5×10^5 cells/ml) was pipetted onto each coverslip from which excess water had been removed with filter paper. These coverslips were then stored in a moist chamber (Petri dish) at 4 °C overnight. The coverslips were subsequently dehydrated in a series of graded ethanols and then in acetone. They were then transferred to a holder containing acetone and critical point dried with CO_2 (Sorval, Dupont). The coverslips were shadowed with gold-palladium in a high-vacuum evaporator on a rotating stage (Edwards). An ETEC Autoscan scanning electron microscope (ETEC Corp.) was subsequently used to examine the samples at 20 kV and 45° tilt.

RESULTS

Using populations of mitotic cells (98% mitotic), under conditions where their growth and proliferation proceed synchronously, we have studied the attachment of SV40-infected and uninfected cells to a solid substrate in the presence of various concentrations of foetal bovine serum. In addition we have studied the ability of these cells to traverse the G_1 phase and initiate DNA synthesis.

As previously described, the degree of our synchrony was generally high. Over 95% of the selected cells were labelled within a 24-h labelling period with tritiated thymidine and over 90% of the population had incorporated the label within 3 h of the time the first cells entered *S*-phase. Scoring of the second wave of mitosis, 19–22 h following the establishment of synchronous cultures, by phase microscopy, likewise revealed that cells were dispersed throughout a 3-h period.

When populations of mitotic cells were prevented from attaching to a solid substrate by gentle but constant shaking, the synchronous populations entered *S*-phase after a delay almost equivalent to the interval during which they were not permitted to attach. Curves *C*, *D*, and *E* in Fig. 1 represent synchronous populations of cells whose attachment to the substrate was prevented for 2, 3 and 4 h, respectively after mitotic selection. When cultures were shaken for 4 h, cells began to attach in spite of the shear force that was generated. When the curves in Fig. 1 are compared at a point when 50% of the cells have attached, it can be noted that the entry into *S*-phase of the population shaken for 2 h is delayed by 1·55 h, that of the population shaken for 3 h by 2·5 h and that of the population shaken for 4 h by 3·35 h. The delay in *S*-phase entry is, in all cases, 30 ± 5 min less than the length of time during which attachment was prevented.

Gentle agitation of the cultures does not seem to have an adverse effect on the cells.

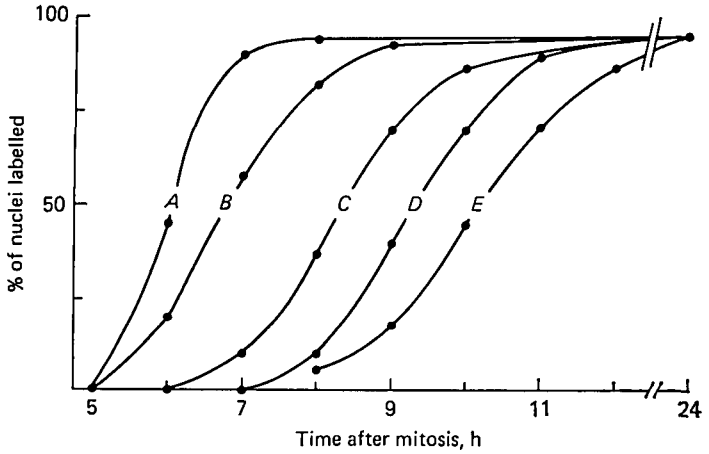

Fig. 1. Kinetics of *S*-phase entry. The increase in the percentage of cells incorporating tritiated thymidine following their selective detachment in mitosis was measured when the cells were allowed to attach to a collagen-coated surface (*A*) or allowed to attach to uncoated surfaces after attachment was prevented for 0 h (*B*), 2 h (*C*), 3 h (*D*), or 4 h (*E*). Note that collagen coating of the surface allowed cells to attach to the substrate and to enter *S*-phase at a faster rate than control cultures. When cell attachment was prevented, the delay in *S*-phase entry was proportional to the length of time during which attachment was prevented minus 30 min.

Over 95% of the cells were recovered, found viable (as judged by erythrocin B dye exclusion) and incorporated tritiated thymidine with kinetics similar to the unshaken cultures. Moreover, as is shown in Fig. 2, when cells were allowed to attach following 2 h of constant resuspension (curve B), they attached more rapidly (30 min faster on the average) than did cells in unshaken cultures (curve *C*).

Scanning electron microscopy revealed that cells which had been shaken for 2 h were rounded like the newly selected mitotic cells following division, but appeared to have fewer microvilli on their surfaces. They did, however, have pseudopodal structures characteristic of G_1 cells (to be described in detail in a subsequent publication).

Pretreatment of the solid substrate, both glass and plastic, with collagen resulted in enhancement in the rate of cell attachment as is illustrated in Fig. 2. Curve *C* represents the rate of attachment to uncoated substrate following selective detachment of mitotic cells from the roller bottles. Curve *A* represents the rate of attachment of the same population of cells to collagen-coated substrate. In both cases, many cells appeared as doublets and sedimented to the substrate within 20 min. However, by 30 min, only 16% of the cells attached to the uncoated substrate, whereas 40% had attached to the collagen-coated substrate. Moreover, 90% of the cells had attached to the collagen-coated substrate within 60 min, whereas almost 2 h were required for the same number of cells to attach to the uncoated surface.

The enhanced rate of attachment of cells to the collagen-coated substrate was also accompanied by an accelerated entry of these synchronous cells into *S*-phase, as judged by autoradiographic analysis and illustrated in Fig. 1. Curve *B* depicts the

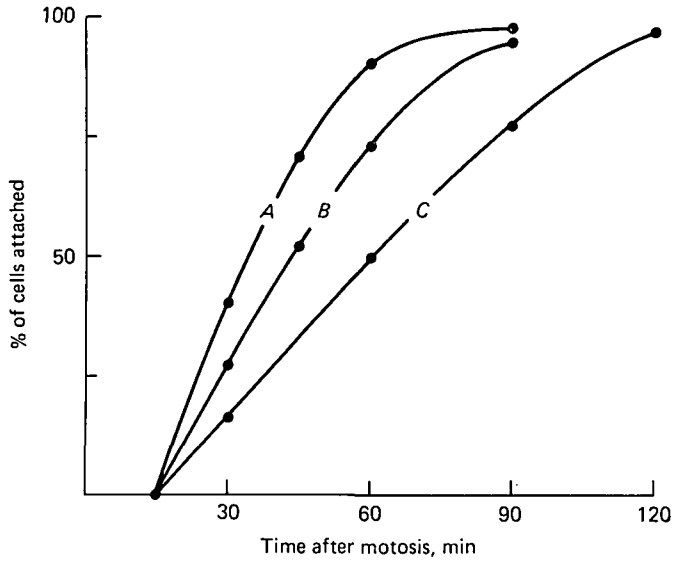

Fig. 2. Rate of cell attachment. The time required for cell-substrate attachment was determined for selectively detached mitotic cells (*A*) when they were allowed to attach to a collagen-coated surface, (*B*) when their attachment was initially prevented for 2 h and then allowed to proceed on an uncoated surface, or (*C*) when allowed to attach to an uncoated surface immediately after selection.

rate at which an increasing number of cells in the population incorporated labelled thymidine when allowed to attach to an uncoated substrate. Incorporation of the radiolabel was first noted 6 h after the initiation of synchronous cultures with mitotic cells, and over 90% of the cells were labelled within the subsequent 3-h period. The addition of collagen to the substrate (curve *A*) resulted in an initial rate of labelling that was 2-fold higher and over 90% of the cells incorporated labelled thymidine within a 2-h period.

Cultures initiated in medium containing less than 1% FBS did not attach to glass slides or the culture vessel, while in the presence of 2–20% FBS at least 95% attached. No difference in the rate of cell attachment was observed with 2–20% FBS. Consequently, 10% FBS (the minimal concentration with which optimal growth of our cells is achieved) was generally employed, except for studies with SV40 as described below.

Forty-eight hours after inoculation with SV40 over 90% of the mitotic cells selectively removed from roller cultures 6–11 h post-infection were infected as measured by immunofluorescent staining for SV40 T antigen. In spite of the presence of the SV40 genome, infected mitotic cells which were incubated in less than 2% FBS also did not attach to a solid substrate. Likewise, in the presence of 2% FBS (or greater) SV40-infected cells attached at the same rate as uninfected cells. When uninfected and SV40-infected cultures, which had been in the presence of low serum for 2 h,were supplemented with 10% FBS, cells began to attach. However, although judged viable by dye exclusion, 30% of both uninfected and infected cells

did not attach in the ensuing 2-h period. SV40-infected cells which were physically prevented from attachment for 4 h in the presence of 10% FBS entered *S*-phase at the same time as their uninfected but 4-h shaken counterparts.

DISCUSSION

Normal (untransformed) cells from a subclone of the CV-1 cell line, which do not proliferate in agar suspension or in low serum concentrations, do not progress through the cell cycle unimpeded when their attachment to a solid substrate is prevented. Under conditions where a fair comparison can be made, the addition of collagen to the substrate facilitated attachment and resulted in a decrease in the time required for over 90% of the cells to enter *S*-phase. Conversely, delaying the attachment of cells to the substrate resulted in delays in the onset of *S*-phase that were proportional (less 30 min) to the time during which attachment was prevented. The delays in *S*-phase entry were not correlated with any loss of synchrony as judged by the time required for over 90% of the cells to incorporate labelled thymidine at *S*-phase and/or the time required for 90% of the cells to undergo the first mitosis following mitotic selection. It is unlikely that these observed delays were due to handling artifacts since the cells remained viable and incorporated tritiated thymidine with normal kinetics. Moreover, the recovery of viable cells in the shaken cultures was similar to that in the unshaken ones. Furthermore, in attempts to establish CV-1 spinner cultures, we have observed that when cells of similar passage were placed in spinner medium, they remained viable for 10 days at 1×10^5 cells/ml without proliferation, and when these cells were placed in flasks, without medium change, over 80% of the cells attached to the substrate (after a delay of 10–16 h: (unpublished observations)). This result is similar to that reported for 3T3 cells held in suspension for 3 days (Otsuka & Moskowitz, 1976). Lastly, further evidence that our methods for maintaining cells in suspension were not damaging is given by their increased rate of attachment when agitation of the cultures ceased. This observation of a shorter attachment time suggests that mitotic cells, whose attachment is prevented, do continue to cycle for a short period of time following cytokinesis, but are then arrested. This observation is further supported by analysis of these cells by scanning electron microscopy (data not shown). The cells have a morphological appearance that is intermediate between that of mitotic and G_1 cells. In the absence of any reliable G_1 marker, the constancy of time between mitotic selection and *S*-phase, in our system, leads us to consider that unattached cells are probably arrested at a point 30 min after mitosis and prior to the normal initiation of G_1, for which attachment to a solid substrate may be required.

This arrest point may be related to the G_0-like point(s) studied by others (Temin, 1968; Pardee, 1974; Augenlicht & Baserga, 1974). Cells have been noted to reach non-cycling state(s) in response to a variety of conditions such as serum insufficiency (Clarke *et al.* 1970; Temin, 1968; Holley & Kiernan, 1968) or high cell density (Clarke *et al.* 1970; Dulbecco & Stoker, 1970). These conditions may induce changes in cell shape and thereby surface area which in turn may influence the ability of the

cell to grow and proliferate. O'Neill, Riddle & Jorden (1979) have suggested that attachment directly affects the exposed surface area of a cell which is also dependent upon the concentration of serum in the culture medium. Folkman & Moscona (1978) have suggested that density-dependent arrest may result from changes in cell shape induced by a reduction in the number of available cell-substrate contacts.

Cells can be released from G_0-like state(s) by a variety of means. The addition of serum induces cells to cycle and initiate DNA synthesis (Clarke *et al.* 1970). In our system, serum is required for attachment although excess serum (beyond that required for optimal growth) does not appear to enhance cell attachment. Another characteristic of cells arrested by low serum or high cell density is that infection with SV40, without supplements of serum, also serves to induce cells to cycle and initiate DNA synthesis (for general discussion see Weil, Salomon, May & May, 1974). Moreover, cells transformed by SV40 continue to cycle without the usual growth constraints (Dulbecco, 1970; Eagle *et al.* 1970; Paul, Lipton & Klinger, 1971). SV40 infection of CV-cell cultures grown to saturation density, induces levels of DNA synthesis that are 6-fold higher than that of control cultures (unpublished observation). Yet, infected cells, actively producing T antigen (itself an initiator of viral DNA synthesis and promotor of transformation and cellular DNA synthesis), did not overcome the requirement of serum for attachment, nor was the presence of the viral genome able to impart to the cells the ability to progress through their cell cycle in the absence of serum and attachment. These results imply that the mechanism by which SV40 virus and serum act to induce quiescent cells to cycle are clearly different and that infected populations of permissive cells do not exhibit, even transiently, a transformed property which would enable them to grow in low serum and/or suspension.

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REFERENCES

AUGENLICHT, L. H. & BASERGA, R. (1974). Changes in the G_0 state of WI-38 fibroblasts at different times after confluence. *Expl Cell Res.* **89**, 255–262.

CLARKE, G. D., STOKER, M. G. P., LUDLOW, A. & THORNTON, M. (1970). Requirement of serum for DNA synthesis in BHK 21 cells: Effects of density, suspension and virus transformation. *Nature, Lond.* **227**, 798–801.

D'ALISA, R. M. & GERSHEY, E. L. (1978). On the quantitation of SV40 virus by plaque assay and immunoperoxidase technique. *J. Histochem. Cytochem.* **26**, 755–758.

D'ALISA, R. M. & GERSHEY, E. L. (1979). Characterization of a CV-1 cell cycle: I. Definition of a synchrony stem (submitted for publication).

DIACUMAKOS, E. G. & GERSHEY, E. L. (1977). Uncoating and gene expression of simian virus 40 in CV-1 cell nuclei inoculated by microinjection. *J. Virol.* **24**, 903–906.

DULBECCO, R. (1970). Topoinhibition and serum requirement of transformed and untransformed cells. *Nature, Lond.* **227**, 802–806.

DULBECCO, R. & STOKER, M. G. P. (1970). Conditions determining initiation of DNA synthesis in 3T3 cells. *Proc. natn. Acad. Sci. U.S.A.* **66**, 204–210.

EAGLE, H. G., FOLEY, E., KOPROWSKI, H., LAZARUS, H., LEVINE, E. M. & ADAMS, R. A. (1970). Growth characteristics of virus-transformed cells. *J. exp. Med.* **131**, 863–879.

FOLKMAN, J. & MOSCONA, A. (1978). Role of cell shape in growth control. *Nature, Lond.* **273**, 345–349.

GERSHEY, E. L., D'ALISA, R. M. & ZUCKER, R. M. (1979). Characterization of a CV-1 cell cycle: IV. No critical size for S phase entry. *Expl Cell Res.* **122**, 9–14.

HOLLEY, R. W. & KIERNAN, J. A. (1968). Contact inhibition of cell division in 3T3 cells. *Proc. natn. Acad. Sci. U.S.A.* **60**, 300–304.

O'NEILL, C. H., RIDDLE, P. N. & JORDAN, P. W. (1979). The relation between surface area and anchorage dependence of growth in hamster and mouse fibroblasts. *Cell* **16**, 909–918.

OTSUKA, H. & MOSKOWITZ, M. (1976). Arrest of 3T3 cells in G1 phase in suspension culture. *J. cell. Physiol.* **87**, 213–220.

PARDEE, A. B. (1974). A restriction point for control of normal animal cell proliferation. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1286–1290.

PAUL, D., LIPTON, A. & KLINGER, I. (1971). Serum factor requirements of normal and simian virus 40-transformed 3T3 mouse fibroblasts. *Proc. natn. Acad. Sci. U.S.A.* **68**, 645–648.

SANDERS, S. K., ALEXANDER, E. L. & BRAYLAN, R. C. V. (1975). A high-yield technique for preparing cells fixed in suspension for scanning electron microscopy. *J. Cell Biol.* **67**, 476–484.

STOKER, M., O'NEILL, C., BERRYMAN, S. & WAXMAN, V. (1968). Anchorage and growth regulation in normal and virus-transformed cells. *Int. J. Cancer* **3**, 683–693.

TEMIN, H. M. (1968). Carcinogenesis by avian sarcoma viruses. X. The decreased requirement for insulin-replaceable activity in serum for cell multiplication. *Int. J. Cancer* **3**, 771–787.

WEIL, R., SALOMON, C., MAY, C. & MAY, P. (1974). A simplifying concept in tumor virology: Virus-specific pleiotropic effectors. *Cold Spring Harb. Symp. quant. Biol.* **39**, 381–396.

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