[4] Gresser, I., these PROCEEDINGS, **47**, 1817 (1961).
[5] Henle, G., and W. Henle, *J. Bacteriol.*, **89**, 252 (1965).
[6] Henle, W., personal communication.
[7] Stewart, S. E., and M. L. Irwin, *Cancer Res.*, **20**, 766 (1960).
[8] Dalldorf, G., *J. Am. Med. Assoc.*, **181**, 1026 (1962).
[9] Schmaman, A., B. Gampel, and C. H. Luntz, *S. African Med. J.*, **39**, 741 (1965).
[10] Cockshott, W. P., in *Symposium on Lymphoreticular Tumours in Africa*, ed. F. C. Roulet (Basel: Karger, 1964).
[11] Dalldorf, G., C. A. Linsell, F. E. Barnhart, and R. Martyn, *Perspectives Biol. Med.*, **7**, 435 (1964).
[12] Gluckman, J., *S. African Cancer Bull.*, **7**, 7 (1963).

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*CELL GROWTH AND THE INITIATION OF TRANSFORMATION BY SV40**

BY GEORGE J. TODARO AND HOWARD GREEN

DEPARTMENT OF PATHOLOGY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Communicated by Boris Ephrussi, December 8, 1965

Although the sequence of events involved in the multiplication of viruses in mammalian cells has been extensively studied, the mechanism by which certain of these viruses are able to disrupt cellular growth control is not at all understood. The two processes appear to be quite independent, since oncogenesis may occur in cells which do not support viral replication. This is true of the interaction between the mouse cell line 3T3 and the oncogenic virus SV40; the virus multiplies very little, if at all, but is capable of transforming a large fraction of the population,[1] destroying cellular sensitivity to contact inhibition.[2] The very high contact sensitivity of the line and the absence of a complicating cytocidal effect make it possible to examine the transforming function of the virus on nongrowing as well as growing cells. The experiments to be described here show that the virus cannot initiate transformation in a strictly nongrowing population. The fixation of the transformed state as a heritable cellular property requires one cell generation after infection, while the expression of the transformed state requires several more generations. Cells which are not permitted to grow through a cell division cycle subsequent to infection are altered neither in their genotype nor in their phenotype.

Materials and Methods.—Fibroblast line 3T3, a spontaneously established cell line of mouse embryo origin,[3] was used between the 105th and 200th cell generation. Cells were infected with SV40 strain 776, grown on Rhesus monkey kidney cultures[4] and kindly supplied by Dr. John Easton (National Institutes of Health). The transformation assay using SV40 and cell line 3T3 has been described in detail.[5, 6] The transformed colonies may be scored, after confluence is attained, against a uniform background of untransformed cells.

Results.—(1) *Cell growth and the expression of the transformed state:* The transformation of 3T3 by SV40 has usually been studied by infecting a population of cells and inoculating them on the following day at high dilution into a number

of Petri dishes. The resulting colonies are scored as transformed or unchanged some 12–14 days later, so that a number of cell generations occurs between the time of infection and the time the colonies are scored. These conditions are essentially the same as those employed in other transformation systems.[7–9]

The ability of the virus to transform may also be examined in nongrowing cultures. 3T3 cells stop growing at a cell density of $5 \times 10^4/cm^2$, and may be kept in a nongrowing state for a prolonged period, arrested in stage G-1 of the growth cycle.[10] In such cells DNA synthesis becomes virtually completely suppressed,[11] and as in the case of human diploid fibroblasts,[12] RNA synthesis is reduced to $^1/_{10}$th the rate found in dividing cultures.[13] Cells in this condition were infected with SV40 and kept in the nongrowing state for a period of 2 weeks. In four separate experiments involving cell numbers and viral multiplicity sufficient, under the usual conditions, to produce a total of more than 10^5 transformations, no transformed colonies emerged. Evidently, the phenotype characteristic of the transformed state cannot be induced by the virus in the nongrowing cell, and the virus itself does not induce the cells to resume division.

In order to determine how many cell divisions are necessary for development of the transformed state, nongrowing 3T3 cultures were infected with a stock of SV40, containing $10^{8.0}$ T.C.I.D. per ml, and transferred at various dilutions, each permitting growth through a known number of cell divisions before saturation density was attained. Table 1 shows that, as expected, trypsinization and transfer without dilution (no growth) resulted in practically no transformants. After from one to three generations a small but increasing fraction of the cells gave rise to transformed colonies. At 1:16 dilution, permitting each cell to undergo approximately four cell generations, nearly the full number of expected transformants occurred. The quality of the colonies scored in these experiments also depended on the number of generations through which the cells had grown before confluence was attained. If only one generation was completed, the transformed colonies were small and not very dense; by four generations the colonies resembled those occurring after ten generations.

A second method of determining the number of cell generations required for expression of the transformed state consists in infecting an exponentially growing population and at various times thereafter inoculating a known number of infected cells onto confluent monolayers of 3T3, on which only transformed cells will form colonies. The inoculation of cells during the first few days after infection led to the formation of very few colonies, but cells plated six to nine cell generations following infection gave rise to transformed colonies with an efficiency comparable to that of the controls, plated directly on Petri plates. It may be concluded that by both methods of testing the degree of release from contact inhibition of cell division, expression of the transformed phenotype does not become complete until several generations after infection.

(2) *The fixation of the transformed state:* The requirement that cell growth precede the development of the transformed phenotype in 3T3 may be interpreted in two ways: (1) The virus acts on the nongrowing cell to produce an alteration which is genetically transmissible, and only the expression of this alteration requires cell division. (2) The virus produces no genetically transmissible change in the nongrowing cell; cell growth is necessary for this as well as for expression. The

TABLE 1

Dilution	Average number of cell generations required to attain saturation density*	Transformed colonies (% of control)†
None	0	0.2
1:2	1	1.8
1:4	2	10.4
1:8	3	13.1
1:16	4	69.0
1:100	6.8	108

* Assuming uniform distribution and no loss of viability of inoculated cells. Therefore, the numbers of cell generations attained are slightly underestimated.
† Determined by plating at 1:1000 dilution.

following experiments lead to the conclusion that the second of these alternatives is correct.

A number of stationary cultures of 3T3 were infected with SV40. After 3 hr the virus was removed, depleted medium[13] was added, and the cultures were kept in the nongrowing state. At intervals, beginning 1 day later, cultures were trypsinized and plated at several dilutions so that growth could begin, and the transformed colonies were scored 10–14 days later. Curves *1* and *2* of Figure 1 show the relative number of transformants obtained as a function of the time elapsed between the infection and the initiation of cell growth. It is clear that when cells were allowed to resume cell division 3–6 days after infection, most of the transformants expected failed to occur. Ultimately 90 per cent of the transformants were "missed" as a result of the delay in initiation of growth. Other cultures were transferred at 1:2 dilution immediately following infection so as to allow one cell generation to occur. After completing this doubling, the cells remained in the nongrowing state for periods up to 12 days, and were then replated with dilution, the colonies being scored in the usual way (Fig. 1, curve *3*). No diminution in the number of transformants resulted from maintaining the cells in the nongrowing state. It is therefore concluded that one generation is sufficient to fix the transformed state in the infected cell.

When freshly infected cells were prevented from growing through the generation necessary to fix the transformed state, about 10 per cent of the expected number of transformants did occur on replating at high dilution after 10 days in the nongrowing state. It seemed possible that complete viral particles might be retained in the nongrowing cells through this interval, and initiate transformation after growth began. To test this possibility, cell layers were trypsinized immediately after infection and at various times up to 10 days later, the cells were centrifuged at low speed and disrupted by freeze-thawing, and the released cell-associated virus was titered on African green monkey kidney cells. From the time of infection on, virus progressively disappeared from the 3T3 cells, but on the order of 10 per cent was still present in the cell layer 10 days later. Studies by Bourgaux[14] and Fraser and Crawford[15] have similarly shown retention of polyoma virus in infected hamster cells over many days and the transmission of viral particles to the progeny through division. Since the transformation frequency is proportional to infectious virus concentration over the range studied in our experiments,[1] it seems likely that the transformations which occurred when growth was resumed were initiated by viral particles persisting intracellularly, and were really late transformations similar to those which occur in small proportion in growing populations.[16, 1]

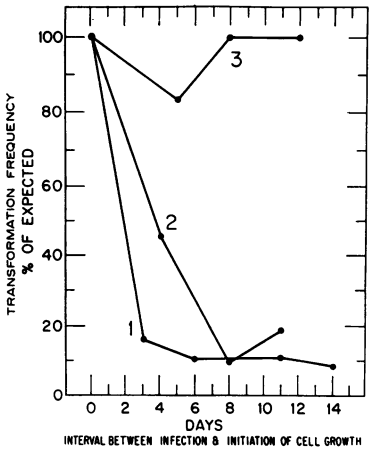

FIG. 1.—Transformation frequency of virus-infected cells after varying intervals in the nongrowing state. Curves *1* and *2*, no cell divisions between infection and arrest of growth. Curve *3*, one cell division between infection and arrest of growth.

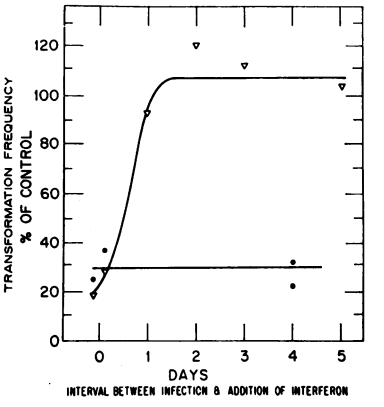

FIG. 2.—Relation between transformation frequency and time of addition of interferon to infected cells. Nongrowing cells, ●; exponentially growing cells, ▽.

(3) *Inhibition of transformation in the growing and nongrowing cell by interferon:* Previous studies have shown that the antiviral agent interferon is an effective inhibitor of the transformation process if added before or shortly after the viral adsorption period.[17] Experiments were undertaken to determine at what point after infection interferon loses its ability to prevent transformation. Exponentially growing cells (generation time 18–19 hr) and nongrowing cells were infected with virus, and at various times either before or after infection 100 units of mouse interferon (kindly provided by Dr. S. Baron) were added and allowed to adsorb for 3 hr. Cultures treated with normal mouse serum served as controls. The following day the cultures were transferred with dilution and the resulting colonies scored for transformation. The results are shown in Figure 2. Transformation was inhibited in both growing and nongrowing cells when the interferon was added just before or just after the 3-hr viral adsorption period. Twenty-four hours later, when the growing cells should have gone through one cell division, the addition of interferon no longer had any protective effect. The interferon-sensitive step in the transformation of growing cells is therefore completed by this time. On the other hand, in identically treated cultures which remained nongrowing for 4 days after infection, the addition of interferon before plating suppressed the late transformants as efficiently as those which were initiated immediately after infection. It may be concluded that the transformation process in the nongrowing cell is unable to proceed past the interferon-sensitive stage.

(4) *Viral infection and DNA synthesis in the nongrowing cell:* An oncogenic virus which leads to release of cells from growth-controlling influences might reasonably be thought to begin its action by the initiation of cellular DNA synthesis. Such synthesis has been observed in cultures infected with polyoma virus by Dulbecco *et al.*[18] and by Weil *et al.*[19] but did not occur in the experiments of Sheinin and Quinn.[20] In saturation density cultures of 3T3 the suppression of DNA

synthesis by contact inhibition is virtually complete; the system therefore lends itself particularly well to the study of viral-induced DNA synthesis in the infected cell. Nongrowing cultures were infected with a multiplicity of 30, which induces a transformation frequency of 3 per cent after dilution and growth. Following infection, the cultures were exposed to H^3-labeled thymidine for 4-hr intervals over a total of 40 hr, and incorporation of tritium into DNA was measured.[21] No DNA synthesis attributable to viral infection occurred in the nongrowing cell, though the sensitivity of the method would have detected initiation of DNA synthesis in less than 0.01 per cent of the population if it were occurring at the rate characteristic of the *S* period.

It seemed possible, however, that viral induction of DNA synthesis at a rate below this level might occur in nongrowing cells and be essential to the subsequent transformation. This was tested by exposing infected cells to high concentrations (500 μg/ml) of the thymidine analogue, IUDR. This agent inhibits DNA synthesis, both cellular and viral,[22] is lethal to dividing cells, but has no effect on resting cells.[6] Stationary cultures were infected and exposed to 500 μg/ml of IUDR for 3 days. If any DNA synthesis essential to transformation were induced during this time by the viral infection, the transformation frequency obtained after the initiation of growth should have been reduced. The results of various experiments have shown that the analogue had no effect as long as the cells exposed were not growing, indicating that no DNA synthesis essential to transformation occurs until the cells resume growth after replating.

Discussion.—The experiments described above have made it possible to distinguish two stages in the transformation process: first, the fixation of the transformed state in the infected cell, and second, its expression as loss of sensitivity to contact inhibition of cell division. Both events require cell growth; one cell generation appears to be sufficient for fixation of the transformed state, while several generations are required for its full expression. These events may therefore be sharply distinguished from another change in the virus-infected cell—T antigen synthesis,[23] which occurs in nongrowing cells[24] and is not prevented by drugs which suppress DNA synthesis.[25, 26] The production of this new antigen may be necessary but is not sufficient to bring about cellular transformation in the absence of subsequent growth.

It may be surmised that what is essential in the requirement of cell growth for fixation of the transformed state is cellular DNA synthesis, since most other macromolecular biosyntheses occur in resting cells (G-1 of the cycle). If this proves to be the case, the possibility would be strong that the viral genome must interact with replicating cellular DNA in order to bring about cellular transformation (see, however, ref. 27). The experiments described here do not give any information concerning the possibility of incorporation of viral DNA into the cellular genome,[28] so that it is not clear whether there is any analogy to bacterial systems, in which the integration of foreign DNA occurs and its relation to cellular DNA synthesis has been extensively studied.[29–31]

In the present experiments the transformed character is defined by the ability of single transformed cells to continue cell division and initiate colonies in the presence of extensive cell-to-cell contact. All transformed 3T3 cells form colonies even when plated on confluent monolayers of the parent-type cell, in this respect

differing from viral transformants of the hamster cell line BHK 21.[32] The number of cell generations required for expression of the transformed state will, however, vary considerably depending on the criterion used to define the transformation. For example, the evolution of neoplastic potential, as measured by ability to produce tumors when injected into animals, is apparently acquired some time after recognizable morphologic transformation and change in growth potential have occurred *in vitro*.[33, 34] It is conceivable that other altered properties, such as the greater resistance of transformants to certain toxic agents,[35] may develop sooner than the virus-induced loss of contact sensitivity.

Since contact inhibition appears to operate through control of the rate of cellular RNA synthesis,[11] the expression of the transformed state requires the destruction of this control. This could be achieved by (1) alteration of the cell surface in such a way as to render cell-cell contact no longer an effective stimulus for the suppression of macromolecular synthesis, (2) destruction of the system in the nucleus that responds to the cell-cell interaction. It seems plausible that at least the first alternative could require a phenotypic lag in order, for example, to dilute during growth normal cell membrane components no longer synthesized in the virus-transformed cell.

The fact that cell growth is necessary for the fixation of the transformed state is consistent with observations *in vivo* that (1) organs containing cells with a high growth rate are usually among those more susceptible to neoplastic change, (2) cells which never divide in adults do not give rise to neoplasms, and (3) young animals are more susceptible than adults to viral oncogenesis. In very young animals the cell divisions necessary for both fixation of the transformed state and for full expression of the transformed character may all be accomplished very quickly. In older animals, where the necessary divisions occur more slowly, the time required for expression of the neoplastic state might be an important factor leading to a prolonged latent period. Finally, the experiments reported here afford a possible basis for the interpretation of the initiator-promotor phenomenon in carcinogenesis.[36]

* Aided by grant CA 06793 and award 1 K6 Ca 1181 (H. G.) from the National Cancer Institute and an institutional grant from the American Cancer Society.

[1] Todaro, G. J., and H. Green, *Virology*, in press.

[2] Todaro, G. J., H. Green, and B. Goldberg, these PROCEEDINGS, **51**, 66 (1964).

[3] Todaro, G. J., and H. Green, *J. Cell Biol.*, **17**, 299 (1963).

[4] Easton, J. M., *J. Immunol.*, **93**, 716 (1964).

[5] Todaro, G. J., and H. Green, *Virology*, **23**, 117 (1964).

[6] *Ibid.*, **24**, 393 (1964).

[7] Macpherson, I., and M. Stoker, *Virology*, **16**, 147 (1962).

[8] Sachs, L., D. Medina, and Y. Berwald, *Virology*, **17**, 491 (1962).

[9] Williams, J. F., and J. E. Till, *Virology*, **24**, 505 (1964).

[10] Nilausen, K., and H. Green, *Exptl. Cell Res.*, **40**, 166 (1965).

[11] Todaro, G. J., G. K. Lazar, and H. Green, *J. Cell. Comp. Physiol.*, **66**, 325 (1965).

[12] Levine, E. M., Y. Becker, C. W. Boone, and H. Eagle, these PROCEEDINGS, **53**, 350 (1965).

[13] Fresh serum contains a substance which reduces the effectiveness of contact inhibition in 3T3 cells. Therefore it is necessary, in order to keep cell division completely suppressed in saturation density cultures, that any medium added to cells be depleted of this substance by previous exposure to 3T3 cells. Such medium is referred to as depleted medium (see ref. 11).

[14] Bourgaux, P., *Virology*, **23**, 46 (1964).

[15] Fraser, K. B., and E. M. Crawford, *Exptl. Mol. Pathol.*, **4**, 51 (1965).

[16] Stoker, M., *Virology*, **20**, 366 (1963).
[17] Todaro, G. J., and S. Baron, these PROCEEDINGS, **54**, 752 (1965).
[18] Dulbecco, R., L. H. Hartwell, and M. Vogt, these PROCEEDINGS, **53**, 403 (1965).
[19] Weil, R., M. R. Michel, and G. K. Ruschmann, these PROCEEDINGS, **53**, 1468 (1965).
[20] Sheinin, R., and P. Quinn, *Virology*, **26**, 73 (1965).
[21] Schmidt, G., and S. J. Thannhauser, *J. Biol. Chem.*, **161**, 83 (1945).
[22] Brockman, R. W., and E. P. Anderson, in *Metabolic Inhibitors* (New York: Academic Press, Inc., 1963), vol. 1, p. 261.
[23] Black, P. H., W. P. Rowe, H. C. Turner, and R. J. Huebner, these PROCEEDINGS, **50**, 1148 (1963).
[24] Black, P. H., *Virology*, in press.
[25] Rapp, F., J. S. Butel, L. A. Feldman, I. Kitahara, and J. L. Melnick, *J. Exptl. Med.*, **121**, 935 (1965).
[26] Gilden, R. V., R. I. Carp, F. Taguchi, and V. Defendi, these PROCEEDINGS, **53**, 684 (1965).
[27] Basilico, C., and G. Marin, *Atti Assoc. Genet. Ital. Pavia*, **10**, 69 (1965).
[28] Dulbecco, R., *Science*, **142**, 932 (1963).
[29] Ephrussi-Taylor, H., in *Deoxyribonucleic Acid*, Proceedings of the 11th Reunion of the Société de Chimie Physique (New York: Pergamon Press, 1961), p. 212.
[30] Fox, M. S., *Nature*, **187**, 1004 (1960).
[31] Voll, M. J., and S. H. Goodgall, these PROCEEDINGS, **47**, 505 (1961).
[32] Stoker, M., *Virology*, **24**, 165 (1964).
[33] Vogt, M., and R. Dulbecco, these PROCEEDINGS, **49**, 171 (1963).
[34] Todaro, G. J., K. Nilausen, and H. Green, *Cancer Res.*, **23**, 825 (1963).
[35] Diamond, L., *J. Cell. Comp. Physiol.*, **66**, 183 (1965).
[36] Berenblum, I., in *Viruses, Nucleic Acids and Cancer* (Baltimore: Williams & Wilkins, 1963), p. 529.

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*AMYLOID, III. A PROTEIN RELATED TO THE SUBUNIT STRUCTURE OF HUMAN AMYLOID FIBRILS**

BY EARL P. BENDITT† AND NILS ERIKSEN

DEPARTMENT OF PATHOLOGY, UNIVERSITY OF WASHINGTON

Communicated by Shields Warren and read before the Academy, October 12, 1965‡

Amyloid is the name applied to deposits of material in the intercellular spaces of many organs and tissues. Most frequently seen in liver, spleen, and kidney, it may occur in any organ. Three main varieties are recognized: (1) primary, (2) secondary, and (3) associated with multiple myeloma. The secondary variety is the most common and is found in association with chronic infections or inflammations such as osteomyelitis, tuberculosis, and rheumatoid arthritis. The primary occurs unaccompanied by other evident disease. Many primary cases have a familial and presumably genetic relationship.[1]

In tissue sections amyloid is recognized by its location and by its several staining reactions. Characteristic is the metachromatic reaction with crystal violet and similar dyes.[2] With Congo red it gives a pink color and exhibits a characteristic green dichroic birefringence.[1] Amyloid deposits stain fairly intensely with reagents for indole, indicating the presence of significant amounts of tryptophan.[2, 3]

In 1959, it was reported that amyloid had a characteristic fibrillar component when examined in the electron microscope.[4, 5] These fibrils differ in appearance