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Factors Affecting the Frequency of Transformation of Rat Embryo Cells by Simian Virus 40

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The transformation of primary rat cells into established cell lines by simian virus 40 has been monitored using the different restrictive assays of colony formation in sparse culture, dense colony or focus formation on a confluent cell sheet, and colony formation in semisolid medium. Primary embryonic rat cell cultures are considerably less susceptible to infection and subsequent transformation than the established mouse 3T3 cell line or later *in vitro* passages of rat cells. These embryonic cells show a stage-specific susceptibility to transformation but not to infection with a maximum susceptibility achieved at the 15th to 16th days of gestation. All transformed cell lines derived by SV40 infection of primary rat cells express viral T antigen as detected by immunofluoresence, though they differ greatly in their plating efficiency in semisolid medium containing methylcellulose. Only the assay of colony formation in semisolid medium selects directly for transformants which plate well in that medium while all assays appear to select for cell lines containing viral T antigen.

INTRODUCTION

The transformation of mammalian fibroblasts by simian virus 40 involves the interaction of one or perhaps two viral gene products with unknown cellular components (Benjamin, 1970; Osborn and Weber, 1975; Martin and Chou, 1975; Tegtmeyer, 1975; Brugge and Butel, 1975; Robb, 1973; Crawford *et al.*, 1978). This interaction results in quantitative differences in cellular karyotype (Moorhead and Saksela, 1963; Lehman, 1974), lifetime *in vitro*, enzyme synthesis (Ossowski *et al.*, 1973), and growth rate under conditions restrictive to the growth of normal cells (Holley and Kiernan, 1968; Smith *et al.*, 1971; Todaro *et al.*, 1964; Macpherson and Montagnier, 1964; Stoker, 1968). The end point of this process of transformation is considered to be the production of a tumor in a susceptible animal.

It is now apparent that the various biological assays of *in vitro* transformation monitor different physiological changes within a cell (Oey *et al.*, 1974; Pollack *et al.*, 1974). Furthermore, in the case of SV40-transformed mouse 3T3 cells these transformed properties can be dissociated one from another in various cell lines (Vogel and Pollack, 1973; Vogel *et al.*, 1973; Risser and Pollack, 1974; Pollack *et al.*, 1974). The established, heteroploid nature of 3T3 cells make such observations somewhat suspect and thus it is desirable to investigate the types of transformed cells obtained from more normal cell systems. In this paper we confirm the earlier observation that SV40 induces distinct patterns of transformed behavior on infection of primary rat cells and further explore some factors which influence their generation.

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MATERIALS AND METHODS

Medium. Fetal bovine serum (Reheiss-Rehatuin or GIBCO) was used in the concentrations stated. Medium was Dulbecco's modified Eagle's medium (DME-GIBCO-H21) and unless otherwise specified, contained 100 units penicillin/ml and 100 μg streptomycin/ml. Phosphate-buffered saline (PBS) lacked Ca^{2+}, Mg^{2+}.

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Virus. Two triply plaque-purified samples of simian virus 40 were used in all experiments reported here. The virus was propagated and titered on BSC-1 cells as described elsewhere (Risser and Pollack, 1974). The two samples titered 1.5×10^9 and 1.7×10^9 PFU/ml.

Preparation of primary embryonic cell cultures. Timed pregnant rats of the Fisher inbred strain (CDF albino from Charles River Supply Co., Boston) were sacrificed by rapid CO_2 suffocation and embryos surgically removed. After removal of the cranium, embryos were rinsed and minced into small bits of tissues. In some experiments the viscera were also removed from the embryos before mincing. Tissue was trypsinized for 10 min at 37° in PBS containing 0.25% trypsin (GIBCO). The supernatant was decanted and discarded, and additional trypsinization was carried out on the remaining clumps of tissue for 20–30 min at 37° (50 ml of trypsin solution/ml of minced tissue). The final cell suspension was decanted and trypsin neutralized by the additional of fetal bovine serum to 10% volume. Cells were pelleted by centrifugation at 200 *g* for 10 min and rinsed in PBS. Cells were suspended in 90% DME, 10% fetal bovine serum containing 100 units/ml penicillin, 100 μg/ml streptomycin. Cells were plated at an initial density of 5×10^6 cells/90-mm plate. The next day medium was removed, plates were vigorously rinsed twice with 10 ml of PBS, and fresh medium was added. Approximately $2-5 \times 10^5$ cells remained after this treatment, 90–95% of which showed a characteristic fibroblastic morphology, while the remainder resembled epithelial cells.

Infection and transformation assays. When primary or secondary cultures reached a density of $1-2 \times 10^6$ cells/90-mm plate, they were rinsed once with PBS and infected with 0.4 ml of virus ($6-6.8 \times 10^8$ PFU or mock-infected with 0.4 ml of DME for 2–3 hr at 37°. Virus was then removed and medium replaced.

The following day cells were trypsinized and counted in a Coulter counter. One aliquot was used in a density transformation assay in which cells were seeded at 10^5, 10^4, and 10^3 cells/60-mm plate in medium containing 10% fetal bovine serum, 90% DME or 1% fetal bovine serum, 99% DME, in triplicate. In these assays, plates were fed twice weekly and stained at the end of 2 weeks as described (Risser and Pollack, 1974). From this assay the plating efficiency (colonies per cells seeded) and density transformation or focus formation (dense colonies per cells seeded) were determined. In determining the plating efficiency all visible colonies were scored and in determining the focus formation all large dense colonies were scored ($\geq$2 mm). Determination of focus formation was always done by comparison to the mock-infected plate seeded at the same density. Since primary embryonic cells frequently grew to densities of $1-2 \times 10^5$ cells/cm^2 in medium containing 10% fetal bovine serum and showed small areas of piled up cells, only large dense colonies clearly not present on mock-infected plates were scored as virus-transformed foci.

A second aliquot of trypsinized cells was used to determine the transformation frequency in semisolid medium. Cells were suspended at 2.5×10^5 cells/ml in medium containing 2.5% fetal bovine serum. Then 9 vol of medium containing 10% fetal bovine serum, 90% DME, 1.2% methylcellulose were added. Cells were dispersed by gentle pipetting with a 10-ml wide-bore pipet and seeded onto a solid agar-coated dish containing 3 ml of 0.9% agar (Difco-Bacto) in the appropriate medium. Assays were done in quadruplicate or sextuplicate, and cultures were fed once weekly with medium containing methylcellulose. At the end of 3 weeks the number of colonies $\geq$0.2 mm was scored using a dissecting microscope with a stero-light source (Model 281-Sage Instruments).

A third aliquot of infected cells ($2-5 \times 10^5$) was seeded onto 12-mm cleaned glass coverslips in 35-mm dishes. The following day cells were fixed in PBS containing 3.5% formaldehyde and stained for viral T antigen by indirect immunofluorescence as described (Risser and Pollack, 1974). Fixation in formaldehyde prior to staining preserves cellular structure to a greater extent and does not effect the detection of viral T antigen (Osborn and Weber, 1975).

TABLE 1

TRANSFORMATION OF RAT EMBRYO FIBROBLASTS AND 3T3 CELLS BY SV40

Cell	Virus multiplicity (PFU/cell)	T antigen (% positive)	Focal colonies	Density transformation (% of total cells)	Total colonies	EOP	Colonies in semisolid medium	Anchorage transformation (% of total cells)
3T3	0	≤0.1	0, 0, $0/10^5$ [a]	≤0.001	37, 39, $35/10^2$	37	ND[b]	ND
	470	75.5	30, 34, $35/10^3$	3.3	15, 17, $17/10^2$	16	ND	ND
REF 15-day	0	≤0.1	0, 0, $0/10^4$	≤0.01	10, 15, $17/10^3$	1.4	0, 0, 0, 0, $0/10^5$	0.000
	590	8.5	4, 5, $5/10^4$	0.05	40, $32/10^3$	3.6	2, 1, 2, $2/10^5$	0.002

[a] Colonies on each of three dishes seeded at 10^5 cells/dish.
[b] Not determined.

Cloning procedures. Single isolated colonies growing in liquid culture were cloned using steel cloning cylinders. Colonies growing in methylcellulose suspension were drawn into a micropipet and seeded onto 35-mm dishes. To encourage attachment to the petri dish, a sterile coverslip was placed over the colony.

RESULTS

Different Assays for Transformation of Primary Cells.

When a culture of rat embryonic cells from 15-day old embryos was infected with SV40 at a multiplicity of 590 PFU/cell, the data presented in Table 1 were obtained. Results from a transformation assay carried out on 3T3 cells under similar conditions is included for comparison. 3T3 cells are considerably more susceptible to both acute infection as monitored by T-antigen production at 2 days and subsequent transformation as monitored by focus formation than are primary rat embryo cells.

In the assay of SV40 transformation carried out on primary rat cells in liquid culture containing 10% serum, mock-infected plates which received 10^4 cells/plate showed several small areas of greater than monolayer density. These might be interpreted as foci, however, further study showed they are clearly not the same as virus-induced foci. When 7 such dense areas were cloned from mock-infected plates and subcultured at 10^3 cells/dish for recloning, all lacked viral T antigen and all failed to grow into established cell lines (Table 2). In contrast when 10 large dense colonies were cloned from plates receiving infected cells, all clones gave rise to cell lines containing the viral T antigen which were capable of continuous growth *in vitro*. We have used the ability of a culture to be cloned in sparse culture and grow continuously *in vitro* for 3 months as a working definition of cellular establishment, since these properties are necessary if the cells are to be studied *in vitro*. The distinction between mock-infected and virus-infected plates was improved by maintaining the cultures in 1% serum, however, the frequency of virus-induced foci was reduced approximately two- to threefold under these conditions.

TABLE 2

CORRELATION OF T-ANTIGEN PRODUCTION WITH CELLULAR ESTABLISHMENT[a]

Origin of clones	Fraction of clones established into cell lines
From SV40-infected REF	
T-antigen positive	36/36
T-antigen negative	0/12
From mock-infected REF	
T-antigen positive	—
T-antigen negative	0/17[b]

[a] Cellular establishment is defined as continuous division on sparse subculture (50 cells/cm^2) for a period of at least 3 months. Twelve of the established clones have been passaged *in vitro* for over a year with no indication of lowered growth rate.

[b] All 17 mock-infected clones had ceased division by 3 weeks *in vitro*. An additional three mock-infected clones were continuously dividing after 4 weeks *in vitro* at which time the experiment was terminated.

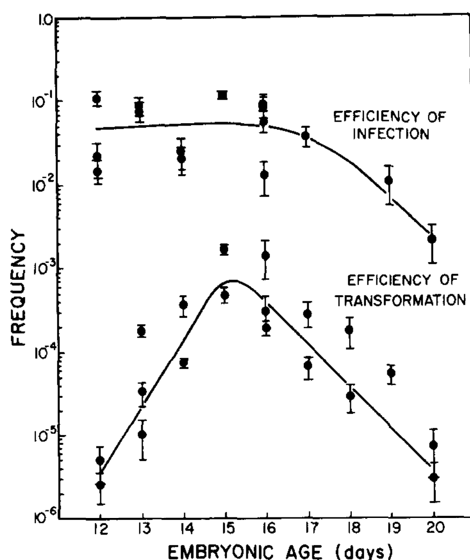

FIG. 1. The effect of embryonic age on transformation induced by SV40. The upper curve is the frequency of T antigen-containing cells 2 days postinfection. The lower curve is the frequency of dense focal colonies formed after 2 weeks of culture in 10% fetal calf serum.

It is also apparent from inspection of Table 1 that plates receiving 10^3 mock-infected embryonic cells showed a relatively high plating efficiency (1–3%). Such colonies are usually of monolayer density on both virus-infected and mock-infected plates. The relatively high plating efficiency of mock-infected secondary rat embryonic cells is not a reproducible phenomenon and we have frequently observed stimulation of colony formation following SV40 infection (Risser *et al.*, 1974). Though particular attention has been given to the degree of trypsinization in preparing primary cells, the serum concentration of the transformation assay (1, 3.5, or 10%), and the initial seeding density of primary cultures, it has not been possible to reproducibly demonstrate an enhanced plating efficiency directly, following SV40 infection of embryonic rat cells.

It is possible, however, to demonstrate that many of the flat colonies arising on plates which received 10^3 infected cells have been established into cell lines by the virus. Thus when 20 morphologically flat colonies were picked from plates receiving infected cells and subcultured, 8 contained the viral T antigen in >95% of the cells of the clones and grew into established cell lines. Twelve colonies which showed <1% T-antigen-positive cells after subculture did not continue to grow *in vitro*. An additional 13 clones randomly picked from plates containing isolated colonies (Risser *et al.*, 1974; Pollack *et al.*, 1974) also continued to divide *in vitro* and were uniformly T-antigen positive. In contrast, 10 single flat colonies picked from mock-infected plates and subcultured did not continue to divide *in vitro*.

The frequency of transformation of primary rat cells by SV40 as measured by the anchorage assay, i.e., colony formation in semisolid medium containing methylcellulose, was reduced approximately 20-fold from that detected by focus formation (Table 1). When five such colonies were picked and subcultured, they also gave rise to established cell lines which were uniformly T-antigen positive. From these data (Table 2) it is apparent that, when primary or secondary cells are established into cell lines capable of unlimited growth *in vitro* by SV40, the continued production of SV40 T antigen is a common component of this process. One can select such cell lines in at least three ways, i.e., as isolated colonies on sparse plates, as foci which have overgrown a layer of cells, or as colonies in semisolid medium.

Embryonic Age Affects the Transformation Efficiency.

During the course of these experiments it became apparent that considerable variation in transformation efficiency was observed between cells from 12- and 16-day embryos as measured by focus formation. When this parameter of stage of embryogenesis was systematically investigated, the results presented in Fig. 1 were obtained. Acute infection over the 14-day period, as measured by T-antigen-positive cells 2 days postinfection, varied from about 1 to 10% of the total cells on the plate with no apparent bias to any day. The frequency of dense colonies induced by the virus varied from ≤1 in 3 × 10^6 to 1 in 10^3 of the total cells plated. Hence, the difference in the

TABLE 3

TRANSFORMATION AND INFECTIOUS VIRUS PRESENT IN CELL CULTURES FROM RAT EMBRYOS OF DIFFERENT AGES

Embryonic age	Cells/plate	Infectious virus/culture (PFU)[a]			Transformation (% of total cells)		
		Input	Day 1	Day 9	% T antigen	Density	Anchorage
12	0.6×10^6	6×10^8	5.7×10^4	1.5×10^4	10.7	≤0.0003	≤0.0003
16	1.6×10^6	6×10^8	7.7×10^4	1.0×10^4	8.7	0.0400	0.0020
20	1.8×10^6	6×10^8	2.7×10^4	0.8×10^4	0.2 ± 0.1	0.0010	≤0.0003

[a] Cells were infected as under Materials and Methods. The following day cells were trypsinized and resuspended in 2% fetal calf serum, 98% DME, antibiotics, which is the medium used for virus propagation. An aliquot of suspended cells was frozen immediately and the remainder replated at a density of 10^5 cells/60-mm dish. Eight days later cells and medium were harvested and frozen, and infectious virus was titered on BSC cells. Plaque counts were multiplied by the total dilution factor so that virus titers are directly comparable to virus input.

transformation frequency of 12- and 16-day cells was not due solely to differences in the degree to which cells were infected by the virus. Similar results were obtained when the assay of colony formation in methylcellulose was used, though data here are less extensive (Table 3). It is probable that the resistance of later stages, e.g., 20 days, is due to events prior to T-antigen induction since the ratio of T-antigen-positive cells to transformants is roughly the same at 16 as at 20 days (Fig. 1).

One possible explanation for this stage specificity is an increased sensitivity to viral growth and cell lysis during various stages of embryogenesis, thus reducing the number of potential transformants. If this were the case, one would expect considerably higher titers of virus in the culture fluid or in cell lysates from 12-day infected cells than from 16-day infected cells. As shown in Table 3, this is not the case. SV40 is known to persist in a number of different primary rodent cultures (Black and Rowe, 1963), however, in these embryonic rat cells, no apparent cytopathic effects were detected.

An additional factor investigated was the effect of *in vitro* passage on susceptibility to infection and subsequent transformation. As can be seen from Fig. 2, *in vitro* passage of primary embryonic rat cells increases their susceptibility to both infection and transformation. Concurrent with this increase is a decrease in cellular growth rate and an increased number of chromosomally aberrant mitoses. Such factors may contribute to the increased susceptibility of 3T3 cells to infection and transformation by SV40 (Table 1).

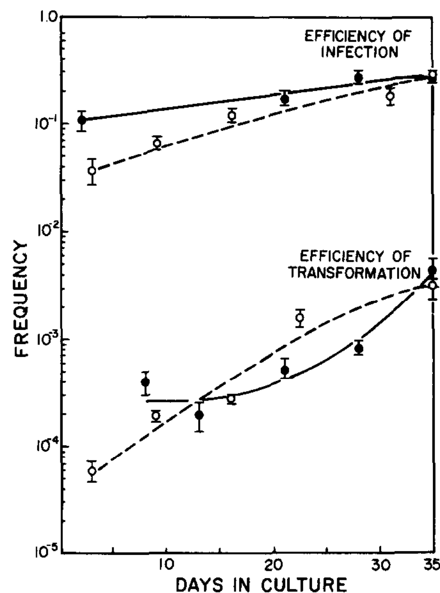

FIG. 2. The effect of *in vitro* passage on transformation efficiency. The solid curves represent a culture of 16-day embryonic cells and dotted curves represent a culture of 17-day embryonic cells. The upper curves are the frequency of T antigen-containing cells 2 days postinfection, and the lower curves are the frequency of dense focal colonies 2 weeks post infection.

Characteristics of SV40-Transformed Rat Clones.

The growth characteristics of both randomly picked and selected SV40 rat lines are presented in Table 4. As can be seen the growth rates and saturation densities of cell lines selected or randomly picked are similar. Most lines grow to relatively high saturation densities in 10% fetal bovine serum. Many lines do not show a well-defined cessation of division after reaching a given density but continue to divide until the entire cell sheet detaches from the petri dish. All lines grow rapidly in 1% serum and to relatively high densities. These results confirm those presented elsewhere (Risser *et al.*, 1974; Pollack *et al.*, 1974) and demonstrate that most primary rat cell lines established by SV40 are insensitive to the growth restrictions of high cell density or low concentrations of serum.

The data in Table 4 also demonstrate that rat cell lines established by SV40 do not necessarily acquire the property of anchorage independence. Thus a spectrum of plating efficiencies in suspension culture containing methylcellulose was observed, ranging from about 0.01% for SVRE clones 12, 15, 17, and 20 to 2–5% for SVRE clone 9 and SVREMc clone 2. These data also demonstrate that if the initial transforma-

TABLE 4

GROWTH PROPERTIES OF REF CLONES ESTABLISHED BY SV40

Cell or clone	Saturation density[a] (10% FCS)	Doubling time in 1% FCS (hr)	Viral T antigen	EOP in methylcellulose (%)
Primary cultures				
REF 12 day[b]	1	NP[c]	–	≤0.001
16 day	23	100	–	≤0.001
20 day	15	134	–	≤0.001
Randomly selected[d]				
SVRE 3	I[e]	33	+	0.03
5	I	27	+	2.3
9	I	27	+	2.5–7.5
12	25	33	+	0.02
Selected by flat morphology				
SVRE 15	36	14	+	0.004
17	15	19	+	0.001
20	19	19	+	0.010
Selected by focus formation				
SVREFo 1	47	22	+	0.001
2	ND	ND	+	0.036
3	I	22	+	0.003
Selected in methylcellulose suspension				
SVREMc 1	34	34	+	0.4
2	I	24	+	5.0
3	ND	ND	+	0.85

[a] Units are cells/cm^2 × 10^4.

[b] Rat embryo fibroblasts (REF) were prepared from embryos of 12, 16, or 20 days of gestation as described under Materials and Methods.

[c] NP, 13-day embryonic cells do not divide when seeded at 2 × 10^3 cells/cm^2 in 1% serum. ND, Not determined.

[d] From Pollack *et al.* (1974)

[e] Indeterminate; cells continue to divide until the cell sheet detaches from the petri dish.

tion assay is carried out in suspension culture containing methylcellulose, the transformants recovered have a relatively high plating efficiency in the medium. Furthermore, such transformants have also acquired the additional transformed properties of growth in low concentrations of serum and growth to high saturation density.

DISCUSSION

The observations presented in this paper demonstrate that *in vitro* transformation even by a simple virus such as SV40 depends on a number of interactions involving viral functions, cellular history *in vivo* and *in vitro*, and the cultural milieu. They further confirm the results obtained in a nonselective analysis of SV40-transformed 3T3 cells (Risser and Pollack, 1974) in that all lines do not have the same collection of *in vitro* growth properties. These results differ considerably from those obtained with 3T3 cells in that no T-antigen-negative or -intermediate lines were obtained.

The process of establishment of a primary cell into a cell line following SV40 infection was invariably associated with the continued production of viral T antigen (Table 2), and thus, it seems likely that the coincidence of SV40 T-antigen production with establishment by the virus is not fortuitous, but reflects viral functions necessary for this phenotype. In addition all rat embryo lines transformed by SV40 showed lowered serum requirements for growth and increased saturation densities. In this regard the use of 3T3 cells to study the dissociation of growth properties in SV40 transformation may well have allowed us to recognize a class of transformant (minimal transformants, Risser and Pollack, 1974) which is obtainable only from established cell lines.

The results in Table 4 clearly demonstrate that anchorage-independent growth is by no means an invariable consequence of SV40 transformation of primary rat embryo cells. Of a total of 16 SV40-transformed rat embryo cell lines that were not selected for growth in methylcellulose [which we have analyzed here and elsewhere (*Risser, et al.*, 1974)] 6 lines show relatively high plating efficiencies in that medium while the other 10 do not. This dissociation of growth properties in transformation may have its molecular basis in the expression of different early region gene products of SV40, for which there is accumulating evidence (Prives *et al.*, 1977; Crawford *et al.*, 1978), differential expression of host enzymes (Ossowski *et al.*, 1973), or a combination of both.

It is clear that several factors influence the frequency with which an SV40-infected cell proceeds to transformation (Figs. 1 and 2). Thus primary embryonic cells of various stages show differential susceptibilities to SV40-induced focus formation and colony formation in methylcellulose suspension, though the infection of these cells as monitored by T-antigen induction is approximately the same. Several explanations for the time-dependent susceptibility of embryonic cells to SV40 transformations *in vitro* can be considered. It may be that 16-day cells are simply more capable of *in vitro* growth and hence more likely to be transformed than are cells from earlier stages; however, plating 12-day embryo cultures in 30% fetal calf serum, where they grow considerably better, does not result in focus formation on infected plates. It could also be that the most common transformations are of a subclass of those cells which attach to the petri dish and that this subclass changes in relative proportion during embryogenesis. Aside from these explanations, one might think that the differentiated state of fibroblastic cells changes during development and only certain stages are particularly susceptible to interactions with viral products which lead to unrestricted growth in liquid or semisolid culture.

Several precedents exist for developmental control of processes related to oncogenesis. The induction of teratocarcinomas by transplantation of primordial germ cells is dependent on the developmental stage of the cells used (Stevens, 1970). The onset of spontaneous leukemia in AKR mice and radiation-induced leukemia in C57BL mice is preceded by characteristic changes of thymus structure (Metcalf, 1966) and of expression of cell surface differentiation antigens (Old

and Stockert, 1977). Indeed recent results on the Abelson murine leukemia virus indicate an integral involvement of this virus with cellular differentiation antigens (Risser, Stockert and Old, 1978). In this regard serologic relationships have been observed between embryonic cell surface antigens and antigens expressed on carcinogen-induced tumors of rats and SV40-induced tumors of hamsters (Coggin and Anderson, 1974; Baldwin *et al.*, 1974). Such observations and the data of Fig. 1 may direct attention to embryonic antigens as being of interest in studies of the process of SV40 transformation.

It is important to note that the transformability of even 16-day cells is quite low compared to established 3T3 cells. This level of susceptibility is comparable to that seen with adult human fibroblasts (Aaronson and Todaro, 1970). Furthermore, the intrinsic susceptibility to transformation of embryonic cells is not particularly stable and increases with passage *in vitro*. An increased susceptibility of passaged rat cells has also been observed in studies of chemical carcinogenesis (Lasne *et al.*, 1974). It is not unreasonable to think that several interactions of virus and host are necessary to produce a transformant. Thus transformation is dependent on successful virus–cell interactions at the level of virus adsorption, events prior to T-antigen induction yet after adsorption (Aaronson and Todaro, 1970; Aaronson, 1970) and events after T-antigen induction (Figs. 1 and 2).

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