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Transformation by polyoma virus alters expression of a cell mutation affecting cycle traverse

(temperature-sensitive BHK 21 cells/G1 arrest/induction of DNA synthesis/unbalanced growth)

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ABSTRACT **A temperature-sensitive mutant of hamster BHK 21/13 cells, tsAF8, which at 39° becomes arrested in the G1 (G0) phase of the cell cycle, is phenotypically altered with respect to temperature sensitivity after transformation with polyoma virus. Polyoma transformation does not produce reversion to a non-temperature-sensitive phenotype but causes increased entry into S and increased rate of cell death at the nonpermissive temperature, compared to untransformed tsAF8 cells. The increased frequency of cells synthesizing DNA is not accompanied by an increased frequency of mitosis, since most of the polyoma-transformed tsAF8 cells that synthesize DNA at the nonpermissive temperature do not divide. At the permissive temperature, polyoma-transformed tsAF8 cells, unlike tsAF8, also lose viability when exposed to other methods of arresting cells in G1. The most likely explanation for this phenomenon is that polyoma virus transformation interferes with the cellular response to this mutation as well as to other conditions that cause cell cycle arrest in G1.**

Cellular mutants can be used to characterize the phenotype of virus-transformed cells, and to study the effects of viral transformation on cellular physiology. In a previous paper (1), we described the characterization of a temperature-sensitive (ts) mutant of the BHK 21 hamster cell line, tsAF8. tsAF8 cells are incapable of growing at 39°, but remain viable for long periods of time. Experiments designed to investigate the effect of this mutation on the cell cycle, including cell synchronization–shift up experiments, show that tsAF8 cells at 39° are blocked in cell cycle traverse and accumulate in G1 (1).

It was considered of interest to determine the effect of polyoma virus on the tsAF8 phenotype, since infection with this DNA oncogenic virus has been shown to overcome a variety of G1 blocks (reviewed in refs. 2 and 3). BHK cells are not permissive for polyoma multiplication, but can be transformed by the virus. Infection with polyoma virus will induce DNA synthesis in resting BHK cells (4).

The results described in this paper show that infection or transformation of tsAF8 cells does not overcome the ts block, since polyoma-transformed tsAF8 are still incapable of growth at 39°. However, polyoma-transformed tsAF8 display a number of phenotypic differences with respect to their untransformed parent. Transformation by hamster sarcoma virus (HSV), an oncogenic RNA virus, does not cause these changes.

Abbreviations: ts, temperature-sensitive; wt, wild-type; BHK, baby hamster kidney; HSV, hamster sarcoma virus; SV40, simian virus 40.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The cells were grown on plastic petri dishes in Dulbecco's modification of Eagle's medium containing 10% calf serum, with medium changes at least every 3 days. tsAF8 is a temperature-sensitive growth mutant (1) derived from BHK 21/13, an established line of Syrian hamster fibroblasts (5). PyAF8 and HSVAF8 are polyoma- or hamster-sarcoma-transformed derivatives of tsAF8, respectively. The permissive temperature used in these experiments was 33° and the nonpermissive temperature, 39° or 39.5°.

Viral Infection and Transformation. Polyoma-transformed tsAF8 cells were derived from tsAF8 cells infected with polyoma virus (small plaque) and plated in suspension in medium containing 0.34% agar. Transformed colonies were picked directly from the soft agar and were then serially propagated. In those experiments in which the induction of DNA synthesis was to be measured, tsAF8 cells were infected in monolayers. HSV transformation was performed as described by Zavada and Macpherson (6), using DEAE-dextran treatment of cells prior to infection. Titration was performed on hamster NIL cells (6). Transformed foci were counted after fixing the cells and staining with Giemsa, at the appropriate time after infection (8 days at 37°, 15 days at 33°).

Polyoma virus infectivity was assayed by plaque assay on mouse 3T3 cells as previously described (7).

Radioactive Precursor Labeling and Radioautography. The methods used have been previously described (1).

Determination of Mitotic Index and Labeled Mitoses. Metaphase cells were prepared after incubation with Velban and processing as previously described (1). Labeled mitoses were evaluated using mitotic preparations dipped in Kodak nuclear track emulsion and processed for radioautography.

Viability. Viability was determined by plating cells and allowing them to remain at the nonpermissive temperature, followed by shift to 33°. The plates were then incubated at the permissive temperature for about 2 weeks and the number of colonies per plate was determined.

RESULTS

Infection of tsAF8 Cells with Polyoma Virus. At the nonpermissive temperature, the frequency of DNA-synthesizing cells in the tsAF8 population is greatly reduced. This reduction is accompanied by a decrease in the mitotic index (ref. 1; see also Fig. 3). To test whether polyoma infection would induce DNA synthesis or mitosis in tsAF8 cell, we infected cells with polyoma virus (1000 PFU/cell) at 24 hr

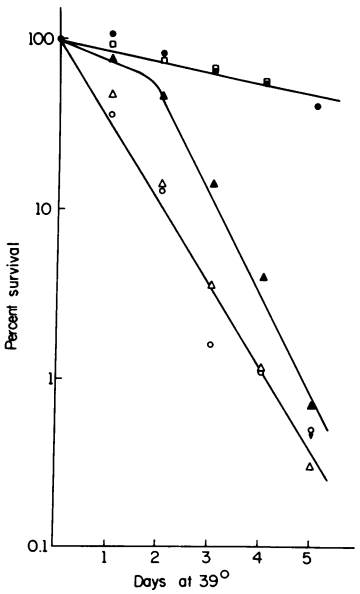

FIG. 1. Survival of tsAF8 and polyoma-transformed tsAF8 cells at 39°. Cells were plated at 33° at a concentration of 500 or 5000 cells per 100 mm petri dish. After 4 hr they were shifted to 39°. At the times indicated, some cultures were shifted to 33° and incubated for an additional 14 days. Survival is expressed as the percent of colonies formed in plates exposed to 39° relative to the plates kept at 33° throughout the experiment. ●, tsAF8; ▲, PyAF8-7; △, PyAF8-2; ○, PyAF8-8; □ HSV-AF8.

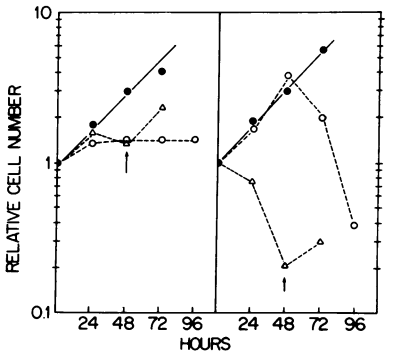

FIG. 2. Growth of tsAF8 and PyAF8 cells in medium containing low serum or deprived of isoleucine, at 33°. Cultures of cells growing at 33° were washed two times with serum-free, ileu$^-$ medium, and then received either complete medium with 10% calf serum (●), complete medium with 0.3% calf serum (○), or ileu$^-$ medium with 10% dialyzed calf serum (△). At the times indicated the number of cells attached to the plate was determined. The data are expressed as number of cells/culture relative to the number determined at time 0. After 48 hr (arrow), the cultures incubated in ileu$^-$ medium received complete medium. left panel, tsAF8; right panel, PyAF8-7

after shift up. The frequency of DNA-synthesizing cells was then determined at intervals thereafter. A small but insignificant induction of DNA synthesis was observed, and the cells did not increase in number (results not shown).

Transformation of tsAF8 Cells with Polyoma Virus. The failure to observe an induction of cellular DNA synthesis upon infection of tsAF8 at 39° could be due to a lack of effect of polyoma virus on the tsAF8 phenotype, but could also be ascribed to the fact that the number of infected cells in the population might have been too low to produce a significant increase in overall DNA synthesis. In order to obtain a pure population of cells under the influence of the virus, we infected tsAF8 cells with polyoma virus and plated them in soft agar to allow selective growth of transformed cells. Large colonies were picked from the plates incubated at 33°; all displayed typical transformed morphology. Incubation at 39° did not result in any colony formation.

Several polyoma-transformed tsAF8 clones (hereafter referred to as PyAF8) were tested for ability to multiply at the nonpermissive temperature. All were found to be ts in growth. It was, however, noticed that at 39° cultures of PyAF8 cells tended to shed a large number of dead cells into the medium. We, therefore, tested the survival of PyAF8 cells at 39°. As already described, tsAF8 cells remain viable at 39° for long periods of time, presumably because their growth is arrested in a balanced way. The results of several experiments showed that when PyAF8 cells were exposed to 39° for variable lengths of time and tested for colony-forming ability at 33° they lost viability at a much faster rate than did tsAF8 cells (Fig. 1). Six independent clones of PyAF8, obtained from two separate transformation experiments, were tested and all displayed a similar behavior. On the other hand, five sub-clones of tsAF8 cells exhibited the high survival of the parental population when exposed to the nonpermissive temperature.

The loss of viability of PyAF8 cells at 39° was not due to induction of virus production. We tested four PyAF8 clones; after 3 days of incubation at 39° we could not detect the presence of infectious polyoma virus (<1 PFU/2 × 10^5 cells).

At the *permissive* temperature, PyAF8 cells also lose viability when exposed to conditions that block untransformed tsAF8 cells in G1 (Fig. 2). When tsAF8 cells are incubated in 0.3% serum, or in isoleucine-deficient (ileu$^-$) medium at 33°, they become arrested in G1 and do not lose viability appreciably for periods up to 96 hr. On the other hand, PyAF8 cells lose viability and come off the plate if incubated in ileu$^-$ medium. In low serum they grow for about 2 days and then also come off the plate (Fig. 2). This behavior is in keeping with the low serum requirements of polyoma-transformed cells (3), and with the finding that they cannot respond to severe serum deprivation with a balanced growth arrest (8, 9).

DNA Synthesis in PyAF8 Cells at 39°. In a previous paper (1), we reported that DNA synthesis in PyAF8 cells at the nonpermissive temperature was impaired to the same extent as that of tsAF8 cells. However, those experiments had been performed at a temperature of 38.6°, at which the tsAF8 mutation displays some leakiness.

Fig. 3 shows the frequency of DNA-synthesizing cells in tsAF8 and PyAF8 at various times after shift to 39.5°, as determined by pulsing the cells with [^{3}H]thymidine for 1 hr. Two types of observations can be made: (*1*) The shut off of DNA synthesis in PyAF8 is delayed. Twenty-four hours after shift up the frequency of DNA-synthesizing cells in tsAF8 is already considerably reduced, while in PyAF8 it is still at wild-type (wt) levels. (2) Between 24 and 48 hr DNA synthesis seems to decrease at about the same rate in tsAF8 and PyAF8. However, while the frequency of DNA-synthesizing cells in the tsAF8 population reaches values of about 2–3%, in PyAF8 it levels off at much higher values (about 20%).

On the other hand, the mitotic index declines at about the

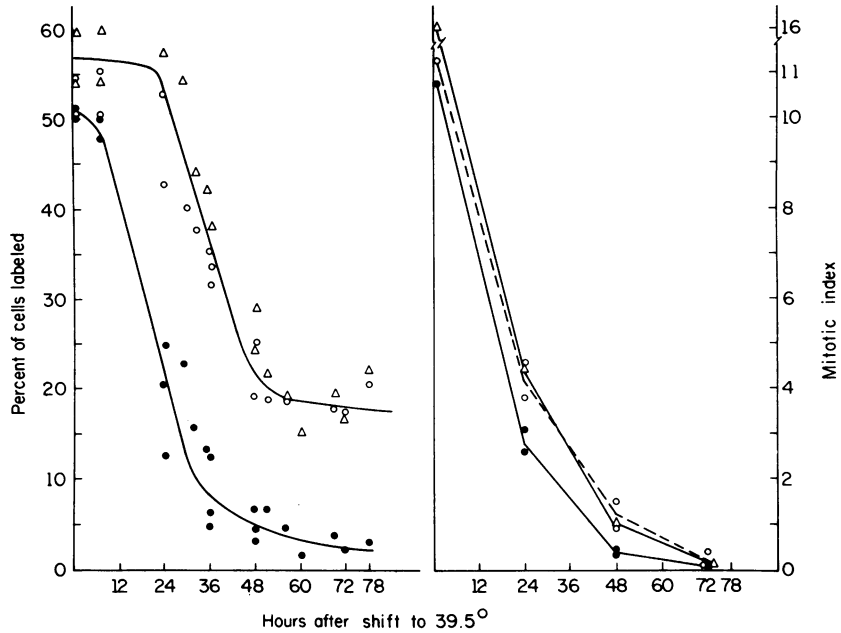

FIG. 3. DNA synthesis and mitosis in cultures of tsAF8 and PyAF8 cells after shift to 39.5°. Exponentially growing cultures were shifted to 39.5° after receiving a fresh medium change. At the times indicated the cultures were labeled with [^{3}H]thymidine (3 μCi, 0.1 μg/ml) for 1 hr and then fixed and processed for autoradiography. The mitotic index was determined after 3 hr of incubation with Velban. Left, percent of [^{3}H]thymidine-labeled cells, as determined autoradiographically; right, mitotic index (%). ●, tsAF8; ○, PyAF8-7; △, PyAF8-2. The figure is a compilation of data from 3 independent experiments.

same rate in tsAF8 and PyAF8, suggesting that most of the PyAF8 cells that synthesize DNA do not progress through the cycle and enter mitosis (Fig. 3).

We then determined the rate of entry of PyAF8 cells into S at 39.5°. Cells were shifted to 39.5° and after 36 hr they were continuously labeled with [^{3}H]thymidine, the frequency of DNA-synthesizing cells being determined at various times after addition of label. The results are seen in Fig. 4. As already described (1), the rate of entry into S of tsAF8 cells compared to BHK is markedly reduced. The results for

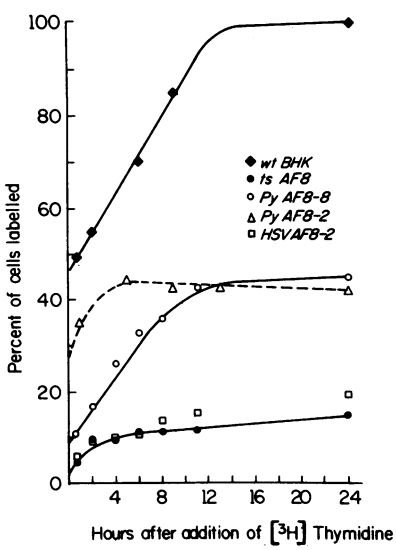

FIG. 4. The rate of entry of cells into the S phase at 39.5°. Cells growing at 33° were shifted to 39.5° after receiving a fresh medium change. Thirty-six hr later [^{3}H]thymidine (4 μCi, 0.5 μg/ml) was added to the medium. At the times indicated the cells were fixed for autoradiography.

PyAF8 show that, although in this experiment the initial frequency of DNA-synthesizing cells in one of the PyAF8 clones was somewhat lower than usual, they can enter S with a much higher probability than tsAF8, so that after 24 hr about 50% of the cells have become labeled. This rate of entry is, however, still clearly reduced with respect to wt cells.

Cell Cycle Progression of PyAF8 Cells at 39°. It can be asked whether the relatively high rate of entry into S is the direct cause of cell death in PyAF8, or whether these two phenomena are independent. To test whether PyAF8 cells entering DNA synthesis would have a higher probability of dying and coming off the plate than the rest of the population, we shifted tsAF8 and PyAF8 cells to 39.5°. Thirty-five hours later they were labeled for 1 hr with [^{3}H]thymidine. The label was washed off, and the cells were incubated in the presence of a large excess of unlabeled thymidine. The

Table 1. Loss of cells that have synthesized DNA from cultures of PyAF8 at 39.5° *

Hours after [^{3}H]thymidine pulse	Frequency of labeled cells (%)	
	tsAF8	PyAF8-7
0	12.9	31.9
4	12.3	20.3
8	16.8	21.5
23	15.0	10.4

*tsAF8 and PyAF8 cells were shifted to 39.5°. Thirty-five hours later they were labeled for 1 hr with [^{3}H]thymidine (3 μCi, 0.1 μg/ml). At the end of the pulse, the medium was removed and replaced with fresh medium containing 15 μg/ml of unlabeled thymidine. Cells were fixed and processed for autoradiography at the times indicated.

Table 2. DNA synthesis and mitosis in tsAF8 and PyAF8 cells at 39.5°*

Cells	A, % Cells synthesizing DNA	B, Mitotic index	A/B
BHK	42.0	7.1	5.9
tsAF8	3.4	0.4	8.5
PyAF8-2	29.5	0.5	59
PyAF8-7	35.5	0.6	59.2

*The data are from the experiment described in Figure 5. A is the frequency of DNA-synthesizing cells measured by autoradiography at the end of the pulse. B is the average of the mitotic indices determined at the times in which the highest percentage of labeled metaphases was observed.

frequency of DNA-synthesizing cells was determined by autoradiography at the end of the pulse and at various times thereafter. The results are seen in Table 1. In tsAF8 cells the frequency of labeled cells increased with the time of chase, consistent with the hypothesis that the few cells entering S at 39.5° are capable of completing S and mitosis, giving rise to two labeled cells. On the other hand, in PyAF8 cells the frequency of labeled cells decreased with time: after 24 hr of chase, the frequency of labeled cells had decreased by about ⅔, instead of showing the expected increase. These data show that in PyAF8 the cells entering DNA synthesis at 39.5° have a higher probability of dying than the rest of the population, and are consistent with the hypothesis that the loss of viability of PyAF8 cells at high temperature is due to their entering S in an unbalanced way.

To better investigate this point, we exposed PyAF8 and tsAF8 to 39.5° for 36 hr, labeled them with [^{3}H]thymidine for 30 min, and determined the appearance of labeled mitoses in the population. This type of experiment allows measurement of the phases of the cell cycle, and also can be used to monitor how many of the cells entering S will eventually reach mitosis. The results are shown in Fig. 5. The few tsAF8 cells that enter S under these conditions are capable of reaching mitosis. The duration of S appears to be somewhat elongated, as already reported (1). The data for PyAF8 show that, although the number of cells synthesizing DNA is much higher than in tsAF8, a large proportion of these do not reach mitosis (Table 2). The few cells which reach mitosis appear to have traversed an S phase which is extremely elongated, and some elongation of G2 is also apparent. These data therefore support the hypothesis that PyAF8 cells enter S in an abnormal fashion, leading to elongation of both S and G2, and causing, in most cases, cell death prior to mitosis.

Transformation of tsAF8 Cells with Hamster Sarcoma Virus. It was considered of interest to test whether transformation by an oncorna virus would produce in tsAF8 the same type of changes produced by polyoma transformation. tsAF8 cells were transformed by hamster sarcoma virus (HSV). Transformed foci were isolated, and these cells were examined for survival and entry into S at the nonpermissive temperature. The results are shown in Figs. 1 and 4. HSV-transformed AF8 do not lose viability at 39° at a faster rate than tsAF8. An experiment was performed in which tsAF8 cells were infected with HSV, allowed to grow at 33° for 5 days, plated at 39°, and shifted down at various times. The number of transformed foci in the plate was then determined after the appropriate time of growth at 33°. Also, this

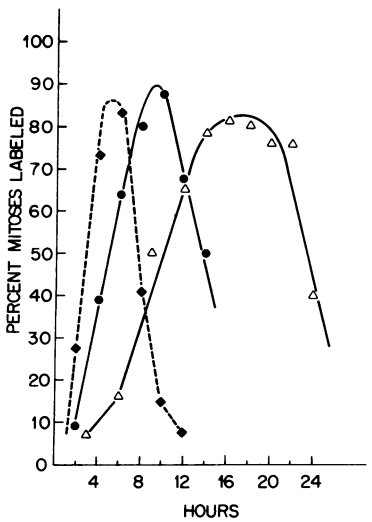

FIG. 5. Entry into mitosis of tsAF8 and PyAF8 cells at 39.5°. Cells growing at 33° were shifted to 39.5° for 35 hr, at which time they were labeled for 30 min with [^{3}H]thymidine (5 μCi, 0.15 μg/ml). After the pulse, the cultures were washed once and then received fresh medium containing 10 μg/ml unlabeled thymidine. Mitotic cells were collected at the times indicated after 2 hr of incubation with Velban. Mitotic preparations were made as described. The percentage of labeled metaphases and/or labeled cells (see Table 2) was then determined autoradiographically. For each point, at least 50 metaphases were counted. ♦, BHK; ●, tsAF8; △, PyAF8-2.

experiment failed to show any difference between the survival of HSVAF8 and that of the general population. The rate of entry into S of HSVAF8 at 39° is not significantly higher than that of untransformed tsAF8 cells. In addition, HSVAF8 exposed to low serum or ileu$^-$ medium at 33° do not come off the plate like PyAF8, but arrest their growth as do tsAF8.

DISCUSSION

The results presented in this paper show that transformation by polyoma virus of a ts BHK mutant, tsAF8, causes a set of specific changes which result in an altered ts phenotype at the nonpermissive temperature. The effect of polyoma transformation consists of decreasing drastically the viability of these cells at high temperature, and causing them to enter S with a much higher probability than the parental cells. This does not result in cell multiplication, probably because PyAF8 cells at 39° traverse the S phase of the cycle in an "unbalanced" way, leading to cell death.

The mechanism by which tsAF8 cells become arrested in G1 at the nonpermissive temperature is unknown. Although cell synchronization–shift up experiments place the cycle arrest point of the tsAF8 mutation in early G1, the gene product affected may also be required during the S phase (1). According to this hypothesis, the few tsAF8 cells that enter S at the nonpermissive temperature would do so because they have accumulated enough of the defective gene product to allow them to pass the cycle arrest point, and to slowly complete S. In the case of PyAF8 cells, it is possible that polyoma transformation would lower the requirements for such a gene product for the cell's progression through G1 into S, or totally circumvent it. This stimulation of DNA synthesis might force the cells into S while the tsAF8 function is still defective; this would lead to an abnormal DNA synthetic phase causing cell death in most cases.

In interpreting the effect of polyoma transformation on tsAF8, it is important to attempt to correlate it with other changes produced by this virus. The loss of viability of PyAF8 cells at 39° is reminiscent of the behavior of other Py- or simian virus 40 (SV40)-transformed cells when exposed to conditions such as serum starvation and isoleucine or glutamine deprivation. These conditions, which generally arrest normal cells in a viable state in G1 (8–11), have a different effect on the growth of virally transformed cells (9–12). When the condition is severe, transformed cells die rapidly (9–12). In the case of PyAF8, we have been able to show that these cells at 33° do not withstand serum starvation or isoleucine deprivation. On the other hand, HSV-transformed AF8 cells can be arrested in growth by serum starvation or isoleucine deprivation at 33°, and they do not lose viability at 39°. Therefore, we feel that the behavior of PyAF8 cells at 39° is an expression of a general property of polyoma-transformed cells; namely their inability to respond to a G1 block with a balanced growth arrest.

Not only do polyoma-transformed cells have an altered response to conditions that normally arrest cells in G1, but it is known that infection with polyoma and SV40 overcomes a number of blocks leading to G1 arrest in normal cells (2, 3). It is pertinent to mention here that Scheffler and Buttin (13) reported that infection by SV40 of a ts mutant of Chinese hamster ovary (CHO) cells also resulted in a small, temporary induction of DNA synthesis at the nonpermissive temperature. This ts CHO mutant does not represent the same mutation as tsAF8, as the two mutants complement (H. Meiss, unpublished). It is likely that the ability of these viruses to overcome, at least partially, many G1 blocks in untransformed cells is also responsible for the behavior of transformed cells when exposed to conditions that otherwise would cause G1 arrest.

It is possible that many conditions can cause G1 arrest in normal cells. When some condition necessary for ordinate progression through G1, or for entry into S becomes limiting, the cells will enter a state in which they are no longer traversing the cell cycle. This implies a cellular system for detecting and reacting to unfavorable growth conditions with a coordinated response involving control of multiple cell functions. This response would lead to a balanced growth arrest in G1, a condition often defined as G0. This quiescent state could be entered from several points in the G1 phase (1). In transformed cells the central coordination system may be altered. Therefore, in these cells most conditions causing an impairment of cycle progression would not lead to a G0 state. According to this hypothesis, it could be thought that PyAF8 cells express the tsAF8 mutation in an unmodified state. In untransformed tsAF8 cells the lack of the tsAF8 function leads to the activation of a cellular response that is beneficial for cell survival, and in this case actually amplifies the phenotypic effect of the mutation. In polyoma-transformed tsAF8 cells, the failure of the coordination system would permit unbalanced growth and cell death.

Finally, it is worth mentioning here the fact that transformation of tsAF8 cells by HSV does not cause the same type of changes effected by polyoma virus, or if it does, it is to a much lesser degree. This suggests that cell transformation by DNA or RNA oncogenic viruses does not cause identical changes in growth regulation.

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