*Proc. Nat. Acad. Sci. USA*
Vol. 72, No. 2, pp. 673–677, February 1975

# Initiation and Maintenance of Cell Transformation by Simian Virus 40: A Viral Genetic Property

**(temperature-sensitive mutants of simian virus 40/gene III/reversion of transformed state/ 3Y1 fibroblast/DNA tumor virus)**

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*Communicated by Renato Dulbecco, November 26, 1974*

**ABSTRACT The transforming ability in 10% serum medium of the temperature-sensitive mutants of simian virus 40 in the complementation group III (*ts*640 type mutants) was greatly reduced when the infected rat 3Y1 cells were incubated at the restrictive temperature of 40° or incubated first at 40° for 3 days and then shifted to the permissive temperature of 33°. Transformation did occur efficiently after incubation at 33° or after an initial incubation at 33° for 5 days followed by a shift to 40°. When growth properties of 3Y1 cells transformed at 33° by the group III mutants were examined at 40°, several aspects of the transformed state were rendered temperature-sensitive. These aspects were the ability of cells to grow in low serum (1.5%) medium and to make colonies, in 10% serum medium, on monolayers of untransformed 3Y1 cells and in soft agar. It is concluded that a simian virus 40 gene (cistron III) controls the initiation, as well as at least some aspects of the maintenance, of transformation and that the initiation reaction is a more heat-labile event than the maintenance reaction(s) under the experimental conditions.**

Since the genome of one of the simplest oncogenic viruses, simian virus 40 (SV40), is a duplex DNA of only about 5500 nucleotide pairs, genetic analysis should clarify the role of viral genes in the rather complex biological phenomena induced in cells after infection and transformation by this virus. By use of temperature-sensitive (ts) mutants of SV40, three or four complementation groups have been distinguished (1–6). Virus particles from mutants of groups I (*B*) and II (*C*) are heat-labile, suggesting that ts mutations affect one or more of the structural proteins (1, 2, 5, 7). At nonpermissive temperature, mutants of these groups synthesize viral DNA in productive infection of monkey cells (1, 2, 4, 5, 7, 8) and transform rat cells normally in abortive infection (2). The group III (*A*) mutants are defective in the synthesis of viral DNA in productive infection (1, 3, 4, 8): more precisely, they are defective in the initiation of each round of the replication of viral DNA at 40° (8, 9). Tegtmeyer and Kimura and Dulbecco have shown that the group III (*A*) mutants cannot transform mouse and rat cells at 40° (3, 9), suggesting that the establishment of cell transformation is under the control of a viral gene.

In this paper we show that the function of gene III of SV40 controls not only the initiation of transformation but also at least some aspects of the maintenance of the transformed state in the SV40–rat 3Y1 cell system. That the transformed state in cells transformed by SV40 is under the control of a viral gene (gene *A*) is also being reported independently from other laboratories (10–12, ‡).

## MATERIALS AND METHODS

*Virus.* Wild-type (WT) SV40 strains were SV68C (3) and SV40-1 (2). Temperature-sensitive (ts) mutants of complementation group III, *ts*640 (3, 13) and *ts*641–644, were derived from SV68C after mutagenesis with ultraviolet light. The mutants of complementation groups I (*ts*660, 661, 662) and II (*ts*663) have been described (2, 3, 13). Properties of the SV40 ts mutants are summarized in Table 1.

*Cell Cultures.* Cells of a clonal derivative of 3Y1-B line of Fischer rat embryo fibroblast (3Y1-B clone 1) and a subclone derived from it (3Y1-B clone 1–6) were used as untransformed cells (ref. 2; Kimura, Itagaki, and Summers, in preparation). All the transformed lines used in the present study were derived from 3Y1-B clone 1 as described below. The 3Y1 cells have a highly regulated growth property (Kimura and Kaneto, unpublished data) and their chromosome number is within a diploid range (Matsui and Kimura, unpublished data). Infectious SV40 production is not detected in either cell extracts of 10 transformed 3Y1 lines tested or after infection of 3Y1 cells with virion or viral DNA, suggesting the nonpermissive nature of 3Y1 cells (Kimura, Itagaki, and Summers, in preparation), although most of the transformed lines produce virus upon fusion with permissive cells.

Cells were cultivated in Vogt and Dulbecco's modification of Eagle's medium (14) with a glucose concentration of 1 g/liter supplemented with, unless otherwise stated, 10% fetal bovine serum in a humidified incubator flushed with a $CO_2$–air mixture. The permissive temperature was 33 ± 1° and the restrictive one was 40 ± 0.5° (3, 13).

*Isolation of Transformed 3Y1 Clones.* All the transformed 3Y1 lines used in the present study were produced and car-

Abbreviations: SV40, simian virus 40; WT, wild type; ts, temperature-sensitive.

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‡ Martin, R. G. & Chou, J. Y. (1975) "SV40 functions required for the establishment and maintenance of malignant transformation," *J. Virol.*, in press; Osborn, M. & Weber, K. (1975) "The SV40 *A* gene and the maintenance of transformation," *J. Virol.*, in press.

TABLE 1. *Properties of the SV40 temperature-sensitive mutants*

| Complementation group | I | II | III |
|---|---|---|---|
| Mutant | *ts*662 | *ts*663 | *ts*640 |
| Time of expression* | Very late | Not very late | Early |
| Adsorption and uncoating | + | + | + |
| Synthesis of T antigen | + | + | ND |
| Synthesis of viral DNA | + | + | − |
| Synthesis of virion antigen | + | − | −* |
| Infectious virus | − | − | − |
| Heat stability of virions produced at permissive temperature | Reduced | Reduced | ND |
| Ability to help adenovirus | + | + | − |
| Transformation of rat 3Y1 cells | | | |
| Initiation | + | + | − |
| Maintenance (in certain conditions) | + | + | −† |

Data are taken from the results of the previous studies of Kimura and Dulbecco (2, 3) and Kimura (13), unless otherwise specified.

+, Normal at nonpermissive temperature (39–40°); −, restricted at nonpermissive temperature; ND, not determined.

* Our unpublished results.

† This study.

ried in exactly the same manner in parallel cultures at 33° except for the lines transformed by SV40-1, which were incubated at 37° according to a different isolation protocol. 3Y1-B clone 1 cells were infected with SV68C, SV40-1, *ts*640, or *ts*641 as described below, with multiplicities of infection of 258, about 500, 131, or 1380 plaque-forming units (PFU) per cell, respectively. Independent cell lines were established from well-isolated transformed colonies. The cell lines were stored at −70° at their early passages to avoid possible secondary alterations and used within not more than a month after thawing. All transformed 3Y1 lines used in the present study were positive for SV40 T antigen, as revealed by complement fixation. The cell lines were designated by the viral strain that induced the line followed by parent cells from which the line was induced plus an isolation number: e.g., SV-3Y1-3 is the third independent 3Y1 line transformed by the WT strain SV40-1; SV68-3Y1-4 is the fourth 3Y1 line transformed by the WT strain SV68C; 640-3Y1-2 is the second 3Y1 line transformed by SV40 mutant *ts*640.

*Assay for Transformation of 3Y1 Cells* has been described (2, 3, 14). Briefly, rapidly growing 3Y1 cells at 37° in 50-mm plastic dishes (Nunc, Denmark) were inoculated with virus after medium was removed. After virus adsorption at 37° for 3 hr, cells were dispersed with an EDTA–trypsin mixture, diluted, and inoculated into 50-mm dishes in fresh medium at varying cell densities less than $2 \times 10^4$ cells. Cultures were incubated at desired temperatures. Medium was replaced once or twice a week. Transformed colonies were identifiable at 10–14 days at 40° and at 18–28 days at 33° after fixation with 75% ethanol and staining with Giemsa as deeply stained, multilayered foci against the background of lightly stained untransformed monolayer in the cultures plated at higher cell densities, or as thick, multilayered, isolated colonies among the flat untransformed colonies in sparsely plated cultures. The number of transformed colonies is proportional to the concentration of virus used for infection and to the number of cells plated per dish (ref. 2; Kimura, Itagaki, and Summers, in preparation).

*Colony Formation on the Monolayer of Density-Inhibited Untransformed 3Y1 Cells (15).* Cells grown at 33° for 2 days and fed 1 day before the experiment were dispersed with EDTA–trypsin. Serial 10-fold dilutions were made in fresh medium containing 10% serum, and each diluted cell suspension (5 ml) was plated onto the already formed monolayer of 3Y1-B clone 1-6 cells in 50-mm plastic petri dishes (Falcon), which had reached confluency a week before the experiment at 37°. After incubation for 18 days at 33° with a medium change on day 13 and 10 days at 40° with a medium change on day 7, cultures were fixed with ethanol and stained with hematoxylin. Deeply stained colonies that overgrew the flat monolayer of 3Y1 cells were scored with the naked eye.

*Colony Formation in Soft Agar (16).* Aliquots of the diluted cell suspensions for the above-mentioned assay were further suspended in medium containing 0.3% Bacto agar (Difco) supplemented with 10% serum and plated (2 ml/60-mm glass petri dish) onto a preset base layer of 5 ml of medium containing 0.9% agar, 10% serum, and 20 units/ml of mycostatin (Squibb). Incubation was carried out at 33° and 40°. At 10 days, cultures were overlaid with 3 ml of soft agar medium so that they would not dry out. Cultures incubated at both temperatures were scored on day 28 for colonies (colonies larger than about 0.3 mm).

*Ability of Cells to Grow in 1.5% Serum Medium.* Cells incubated in 1.5% serum medium at 33° for 25 hr were dispersed with an EDTA–trypsin mixture, counted, and diluted in medium containing 1.5% serum. Cells ($2 \times 10^5$) were inoculated into 50-mm plastic petri dishes (Falcon) in 5 ml of medium. After incubation at 33° for 3 hr to allow cells to settle on the plastic surface (0 time), half of the cultures were fed with 10% serum medium and the other half were retained in 1.5% serum. The cultures were then incubated at 33° and 40°. Cells were counted in a hemocytometer after dispersion with EDTA–trypsin on days 6 and 10 at 33° and on days 3 and 6 at 40°. Accuracy of cell counting was ±10%. The growth rate is defined as the average number of population doublings per day during the first half of the incubation periods (0–3 days at 40° and 0–6 days at 33°).

## RESULTS

*Transforming Ability of ts Mutants of SV40.* It has already been shown that the group III mutant, *ts*640, fails to transform 3Y1 cells at 40° (3). Other recently isolated ts mutants of the complementation group III, i.e., *ts*641, *ts*642, and *ts*643, were found to be temperature-sensitive in their transforming ability (not shown), except for *ts*644, which exhibited a reduced transforming ability at both temperatures for unknown reasons. All these mutants, like *ts*640, were defective in the synthesis of infectious viral DNA (not shown). Members of group I (*ts*660, 661, and 662) and group II (*ts*663) were found to transform 3Y1 cells at 39° (2) and 40° (not shown). The results confirm previous observations (2, 3)

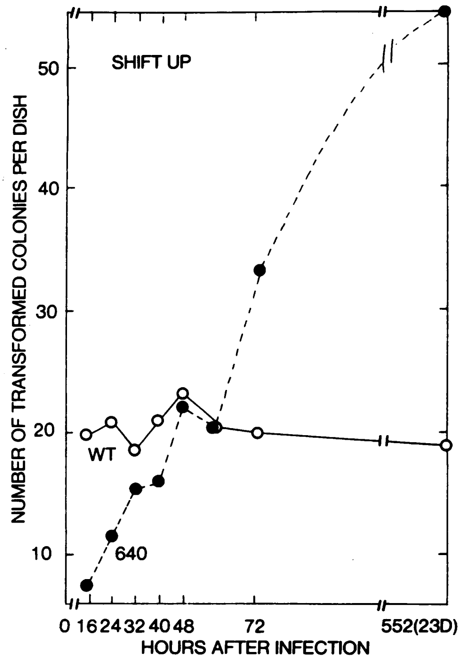


FIG. 1. Temperature shift-up transformation experiment. 3Y1-B clone 1-5 cells (3) were infected and plated as described in *Materials and Methods*. Multiplicities of infection (plaque-forming units per cell) were about 100 for *ts*640 and 400 for SV68C (WT). Cells were incubated at 33°, and at indicated times a part of the cultures was shifted to 40° and incubated until 23 days. Each experimental point represents the number of transformed colonies per dish, which was determined by using eight petri dishes each.

and suggest that the function of cistron III but not of cistrons I and II is involved in the establishment of transformation of 3Y1 cells under the experimental conditions.

*Initiation of Transformation by Group III Mutants ts640 and ts641*. To determine whether the group III mutants affect initiation or maintenance of transformation, we examined the effect of the shift of temperature of incubation after infection of 3Y1 cells with *ts*640 on the final yield of transformation in medium containing 10% serum. In the experiment shown in Fig. 1, 3Y-1 cells were infected with WT virus or *ts*640 and incubated at 33°. At indicated times after infection, a group of cultures was shifted to 40°. All cultures were scored for transformed colonies at 23 days. The results show that in cultures infected with *ts*640, the longer the incubation at 33° before the shift to 40°, the more transformed colonies appeared, while with WT cells the temperature shift-up at any time after infection did not affect the final yield of transformation. To further determine the role of gene III in the process of establishment of transformation, a temperature shift experiment shown in Fig. 2 was carried out. 3Y1 cells were infected and divided into four equal groups that were each incubated in one of the following fashions before they were scored for transformation: (*i*) incubation for 13 days at 40°; (*ii*) incubation first for 5 days at 33° and then for 9 days at 40°; (*iii*) incubation first for 3 days at 40° and then for 20 days at 33°; (*iv*) incubation for 25 days at 33°. The result shows that transformation was markedly inhibited when cultures infected with *ts*640 were incubated either at 40°, or first for 3 days at 40° and then at 33°, while high rates of transformation were observed after

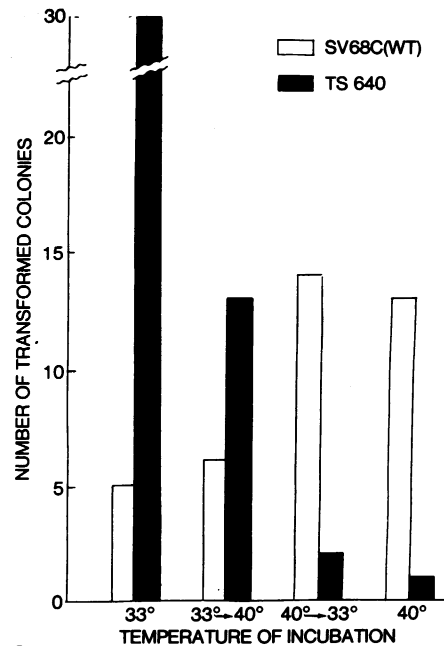


FIG. 2. Effect of temperature shift-up and shift-down after infection of 3Y1 cells on the appearance of transformed colonies. 3Y1-B clone 1 cells were infected and plated as described in *Materials and Methods*. Multiplicities of infection (plaque-forming units per cell) were 106 for *ts*640 and 209 for SV68C (WT). Incubation was carried out as described in the *text*. Each experimental point represents the number of transformed colonies per dish, which was determined by using eight petri dishes each.

incubation either at 33° or first for 5 days at 33° and then at 40° (Fig. 2). Similar results were obtained with another group III mutant, *ts*641. There was approximately a 2-fold increase in the colony-forming efficiency of dilute single cells infected with WT virus and *ts*640 as with uninfected cells. This higher plating efficiency was almost the same in the four incubation conditions in this (data not shown) and in other experiments (3). This result excludes cell death as the primary cause of transformation inhibition. We conclude that gene III of SV40 controls initiation of transformation. The gene affected does not apparently control the maintenance of transformed state under the conditions used, but the same gene might well be involved in determining the transformed state under other conditions. This possibility was tested. Results are given in the following section. A viral gene with a similar function has been known for polyoma virus (*ts*-a gene) (17, 18).

TABLE 2. *Colony formation by 3Y1 cells at 40° in 10% serum on untransformed 3Y1 monolayers and in soft agar after transformation at 33°*

| Cell line | Colony-forming ability (ratio: 40°/33°) | |
|---|---|---|
| | On 3Y1 monolayer | In soft agar |
| 3Y1-B clone 1 | 0/0 | 0/0 |
| SV68-3Y1-1 | 0.35 | 0.049 |
| SV68-3Y1-4 | 0.96 | 0.049 |
| 640-3Y1-2 | 0.014 | 0.003 |
| 640-3Y1-4 | <0.0002 | <0.0001 |
| 640-3Y1-7 | Not done | <0.0022 |

TABLE 3. *Growth rate in different serum concentrations of 3Y1 cells at 40° after transformation at 33°*

| Cell line | Transformed by | Number of population doublings/day | | | |
|---|---|---|---|---|---|
| | | 1.5% serum | | 10% serum | |
| | | 40° | 33° | 40° | 33° |
| 3Y1-B clone 1-6 | None | 0.38 | 0.04 | 0.77 | 0.45 |
| | WT | | | | |
| SV68-3Y1-1 | SV68C | 1.0 | 0.76 | 1.5 | 0.93 |
| SV-3Y1-3 | SV40-1 | 1.1 | 0.76 | 1.5 | 0.84 |
| SV-3Y1-9 | SV40-1 | 1.2 | 0.95 | 1.5 | 0.98 |
| | group III | | | | |
| 640-3Y1-2 | *ts*640 | 0.70 | 0.87 | 1.4 | 1.2 |
| 640-3Y1-4 | *ts*640 | 0.52 | 0.78 | 1.4 | 0.89 |
| 640-3Y1-5 | *ts*640 | 0.34 | 0.52 | 0.91 | 0.72 |

*Maintenance of Transformed State in 3Y1 Cells Transformed by Group III Mutants, ts640 and ts641.* The possibility that cistron III of SV40 controls the transformed state was tested under three different conditions, as described in *Materials and Methods*, using 3Y1 clones transformed independently at 33° by *ts*640. As can be seen from Table 2, 640-3Y1 lines failed to maintain some of the transformed phenotypes at 40°, as shown by the greatly reduced cloning efficiency at 40° on confluent 3Y1 monolayer and in soft agar as compared to that at 33° and to that of WT-transformants at 33° and 40°. Untransformed 3Y1 cells did not form colonies on 3Y1 monolayer and in soft agar at both temperatures. Since the ability of 640-3Y1 lines as well as that of WT-3Y1 lines and of 3Y1-B clone 1 cells to form colonies on plastic were not inhibited at 40° in 10% serum in parallel experiments, it is unlikely that cell death is the cause of the inhibition of colony formation on 3Y1-monolayers and in soft agar.

Like other untransformed fibroblastic cells widely used in transformation experiments (19, 20), 3Y1 cells require a high concentration of serum for growth and can grow only poorly in medium containing low concentrations of serum, whereas 3Y1 cells transformed by SV40 can grow well in medium containing low concentrations of serum (Kimura and Kaneto, unpublished data). When the growth rates of three 640-3Y1 clones were examined in 10% and 1.5% serum at 33° and 40° as described in *Materials and Methods* (Table 3), their growth was inhibited much more greatly in 1.5% serum medium at 40°. The effect of the mutation was largely suppressed in 10% serum at 33° and 40° and in 1.5% serum at 33°. The growth rate of 640-3Y1 lines at 40° in 1.5% serum was almost the same between 0–3 days and 3–6 days. This result shows that neither cell death nor a possible lag for cells to adapt to the high temperature is the cause of the observed growth inhibition. The results suggest that the viral gene under study determined the low serum requirement of transformed cells, one of the most fundamental aspects of transformation of fibroblasts. This result can explain the successful occurrence of transformed colonies under the temperature shift-up condition in the transformation experiment shown in Fig. 2. Growth of 641-3Y1 lines (three lines tested) in 1.5% serum was inhibited less markedly but significantly at 40°, reflecting the observed leakiness of *ts*641 in productive infection (not shown).

## DISCUSSION

The results presented in this paper demonstrate that the gene identified in SV40 mutants of complementation group III is responsible for both the initiation of transformation of, and at least some aspects of the maintenance of transformed state in, rat 3Y1 cells. The cloning efficiency of 3Y1 cells was not reduced after infection by group III mutants, excluding cell death as the primary cause of transformation inhibition at the nonpermissive temperature. The use of cloned cells as a parent for the transformed lines reduces the possibility that the group III mutants and WT virus are selectively transforming different variants that preexisted in the same cell population.

Under the standard culture conditions, where cells are cultivated on a plastic surface in medium containing 10% serum, the effect of the mutation in gene III is identifiable more readily in the initiation reaction than in the maintenance reaction. This permits the visualization of the presence of initiation reaction in the transformation process, as observed in Fig. 2. It appears that the initiation and maintenance reactions are dissociable events and that SV40 gene III has dual functions. The role of the maintenance reaction in the initiation of transformation remains to be determined. Also, it is not clear from the present study whether the initiation reaction or some aspect of the initiation reaction(s) plays a role in determining the maintenance of the transformed state. One of the technical difficulties at the moment is that there is a large difference in gene doses in the assays used, i.e., a difference in the quantity of infecting virus genome and that of the resident genome.

One of the phenotypes of the transformed state has been described as lowered serum requirement for cellular growth (19, 20). The present study suggests that this important aspect of transformation of fibroblasts is controlled by gene III of SV40, as shown by the fact that in 3Y1 lines transformed by the group III mutant *ts*640, the mutation is clearly demonstrable in medium containing a low concentration of serum but less clearly demonstrable in medium with a high serum concentration (Table 3). It has already been shown by Dulbecco and Eckhart that some aspects of serum requirements of cells transformed by polyoma virus are controlled by a viral gene(s) (21). Two other phenotypes of transformed states tested were also found to be temperature-sensitive in 3Y1 clones transformed by *ts*640. These are abilities of dilute cells to form colonies on the density-inhibited monolayer of untransformed cells and in soft agar. Our working hypothesis is that these apparently different phenotypes of the transformed state might well be a consequence of a common mechanism, the lowered serum requirement of transformed cells, which is under the control of SV40 gene III. The degree to which the ability of transformed cells to utilize serum for their growth might vary greatly depends, in some way, on physiological conditions of the culture, where cells are on the monolayer of untransformed cells or in soft agar or on a plastic surface.

It is known that the function of gene III is involved in the synthesis of viral DNA in permissive monkey cells (3). It is most likely that our gene III and the gene *A* described by others (1, 6, 9) represent the same gene. Tegtmeyer showed that *A* functions is required for the initiation but not for the propagation or completion of each round of the replication of viral DNA molecules (9). It can be postulated that,

since SV40 integrates its DNA into the host's chromosomal DNA during the establishment of transformation (22), it must introduce a new replicon for host DNA replication. The results could be the aberrant DNA synthesis signaled by the viral initiator (gene *A* or III) in transformed cells as compared with the regulated host DNA synthesis controlled by the host initiator in untransformed cells. Tegtmeyer has already predicted this possibility (9).

We thank Eiji Kaneto for his help with some of the experiments, Renato Dulbecco for his encouragement and stimulation during the course of this study and helpful advice in preparation of the manuscript, and Yoshiko Kimura for her dedicated assistance. We are indebted to Janet S. Butel, Robert G. Martin, and Peter Tegtmeyer for permitting us to read their manuscripts before publication. This work was supported by grants for cancer research from the Ministry of Education of Japan and a grant from the Asahi News and Co.

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