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# Mutants of Simian Virus 40 Differing in Plaque Size, Oncogenicity, and Heat Sensitivity

K. K. TAKEMOTO, R. L. KIRSCHSTEIN, AND K. HABEL

*Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, and Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland*

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## ABSTRACT

TAKEMOTO, K. K. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), R. L. KIRSCHSTEIN, AND K. HABEL. Mutants of simian virus 40 differing in plaque size, oncogenicity, and heat sensitivity. J. Bacteriol. 92:990–994. 1966.—Three mutants of simian virus 40 were isolated on the basis of the type of plaques produced in primary cultures of African green monkey kidney cells and designated as L (large), S (small), and M (minute) strains. Significant differences in oncogenicity for hamsters were observed, with the 50% oncogenic dose being $10^{4.5}$ for the L, $10^{5.2}$ for the S, and $10^{5.8}$ for the M strains. All three strains were capable of transforming human diploid cells (WI38 strain). At temperatures up to 41 C, the S and M mutants were capable of multiplying to titers almost equivalent to those obtained at 37 C. In contrast, infectious virus was not produced when cells were infected with the L mutant and were incubated at temperatures above 39 C, although complement-fixing viral and tumor antigens were formed. The temperature-sensitive phase of replication of the L strain was shown to be a late stage in viral maturation or assembly.

The biological and biophysical properties of plaque-size mutants of polyoma virus have been extensively studied in the past by numerous investigators. Large- and small-plaque mutants of polyoma virus have been reported to differ significantly in regard to oncogenicity (7, 12, 14), in vitro transformation, and lytic infection (2, 6, 10), and in hemagglutinin production, antigenicity, and ability to induce transplantation resistance (7). Detailed studies along similar lines of investigation with other oncogenic viruses have not been reported thus far. This report is concerned with the isolation and some of the properties of three plaque-size mutants of simian virus 40 (SV40).

## MATERIALS AND METHODS

*SV40.* An uncloned line of SV40 was received from Bernice Eddy. A stock of this virus was prepared by inoculating primary African green monkey kidney cells (AGMK) and harvesting fluid and cells at the time of maximal cell destruction. After clarification by slow-speed centrifugation, the virus was stored at −20 C.

*Cell culture and virus assays.* Trypsin-dispersed AGMK cells were grown in petri dishes or bottles in a medium of Earle's salt solution, 0.5% lactalbumin hydrolysate, and 5% fetal bovine serum. Virus assays were performed in these cells by the plaque technique with the use of an overlay consisting of Eagle's medium, 5% tryptose-phosphate broth, 5% fetal bovine serum, and 0.9% agar. A second overlay was added on the 5th day, and plates were stained on the 9th day by inclusion of neutral red at 1:40,000 in the medium. Plaque dishes were incubated in a humidified incubator with 5% $CO_2$ atmosphere.

The WI38 strain of human diploid cells used for in vitro transformation experiments were in the 24th through 27th passages (phase II) at the time they were used. These cells were grown in Eagle's medium with 10% fetal bovine serum.

*Complement-fixation (CF) tests.* Cell cultures to be tested for tumor antigens (T antigens) were washed twice with Veronal-buffered saline, *p*H 7.2 (VBS), and were scraped off the glass with a rubber policeman. After centrifugation, a 10% suspension in VBS was made, and was frozen and thawed twice before testing. SV40-tumor antibody was obtained from the ascitic fluid of tumor-bearing hamsters (15). For the CF test, 2 full units of complement were used during overnight fixation at 4 C. The tubes were incubated for 1 hr at 37 C, after the addition of the hemolytic system. End points were determined on the basis of 3+ fixation of complement.

## RESULTS

*Isolation and description of SV40 plaque mutants.* The original virus, received from Bernice Eddy, consisted predominantly of virus which

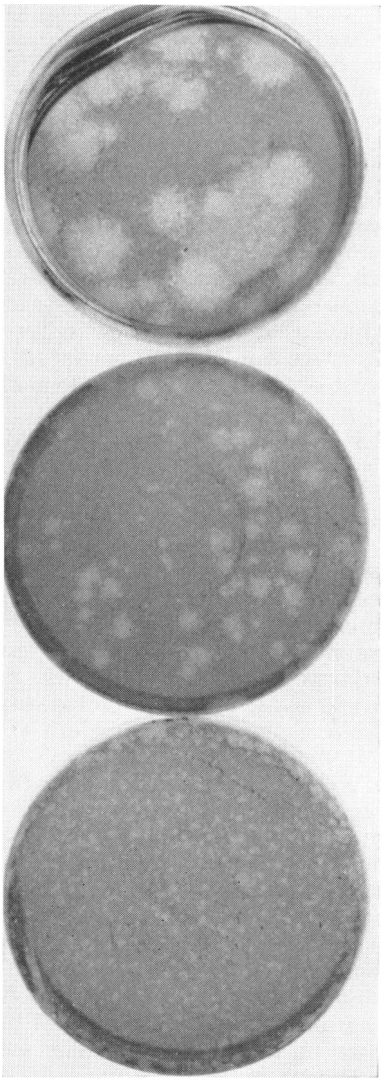

FIG. 1. *Plaques produced by three mutants of SV40 virus. Large (L), small (S) and minute (M) mutants (top to bottom) plated on primary African green monkey kidney cells photographed at 18 days.*

produced small, ragged plaques visible only 12 to 13 days after infection. On continued incubation, these did not increase appreciably in size; they measured about 4 to 6 mm by the 20th day. After this prolonged incubation period, a few plaques were noted which were much larger, 10 to 12 mm in diameter. Both the large and the small plaques were purified, and stocks of both types were prepared in AGMK cells; these were designated as SV40-L (large plaque) and SV40-S (small plaque) strains. During these early studies, another plaque mutant was isolated from supernatant fluids of an SV40-transformed rhesus skin cell line described by Rabson et al. (11). This line, designated as SV40-M (minute plaque), produced extremely minute plaques visible only after an incubation period of 16 to 18 days. All three plaque strains were neutralized by SV40 antiserum. Figure 1 shows plaques produced by the three strains. The S mutant was found to be less stable than the L or M strains, and, despite repeated plaque purification procedures, when large pools of this virus were made, the S mutant always contained a small proportion of progeny which produced minute plaques.

The differences in plaque size were unrelated to sensitivity to the agar inhibitor, since plaque sizes were not influenced by plating virus in inhibitor-free, diethylaminoethyl (DEAE)-dextran treated overlay medium (9). Marked differences in yields of infectious virus have been consistently found for the three strains, and probably account for the differences in plaque sizes. Based on results obtained from a number of different experiments, the average yield per cell in primary AGMK cultures ranged from 100 to 500 plaque-forming units (PFU) for the L, 20 to 50 PFU for the S, and 5 to 10 PFU for the M mutants.

To demonstrate possible hemagglutinins associated with the three strains, heated (56 C for 30 min) and unheated virus preparations were tested with guinea pig erythrocytes in phosphate-buffered saline at *p*H 6.8, 7.2, and 7.6. The reaction mixtures were incubated at room temperature or at 4 C. All tests were negative for hemagglutinins.

*Comparative oncogenicity of SV40 strains in hamsters.* The L, S, and M strains of SV40 virus were tested for tumor production in hamsters. The three strains were adjusted to contain the same concentration of virus, and groups of newborn hamsters were inoculated subcutaneously with 10-fold dilutions of each virus. Animals were observed for tumor formation for a period of 11 months. The results are given in Table 1. The dose of virus causing tumors in 50% of inoculated animals ($OD_{50}$) was calculated by the method of Fisher and Yates (3), and was found to be $10^{4.2}$, $10^{5.2}$, and $10^{5.8}$ for the L, S, and M strains, respectively. These values were significant at the 0.01 level as calculated by the method of Cochran (1). Tumors appeared earlier in animals inoculated with the L strain. The weakly oncogenic M strain produced tumors in only five animals at the highest virus dose.

*Transformation of human diploid cells by SV40 mutants.* Human diploid cells, strain WI38 at passages 24 through 26 were infected with the L, S, and M strains at an input multiplicity of between 20 and 50 PFU of each virus. After an

TABLE 1. *Tumor production in hamsters by SV40 mutants*

| Virus | PFU inoculated ($\times 10^4$) | No. with tumors/ no. inoculated | $OD_{50}$[a] |
|---|---|---|---|
| SV40-L | 370 | 9/17 | |
| | 37 | 14/20 | $10^{4.2}$ |
| | 3.7 | 9/23 | |
| SV40-S | 370 | 15/25 | |
| | 37 | 5/23 | $10^{5.2}$ |
| | 3.7 | 0/22 | |
| SV40-M | 370 | 5/20 | |
| | 37 | 0/20 | $10^{5.8}$ |
| | 3.7 | 0/20 | |

[a] $OD_{50}$, dose of virus required to produce tumors in 50% of animals; calculated by the method of Fisher and Yates (3).

adsorption period of 2 hr, the inoculum was removed, and cultures were washed. Growth medium was then added, and the cultures were kept under observation for morphological evidence of transformation. With all three strains, cellular changes were observed between 4 and 6 weeks after initial infection, and were characterized by a morphological alteration from fibroblastic cells typical of human diploid lines to polygonal, epithelial-like cells. All the changes and manifestations of SV40 virus-induced transformation of human cells described in detail by Girardi et al. (5) were seen in cultures infected with the three mutants. SV40 virus was released for a prolonged period of time, and the plaque type corresponded to that of the virus used to initiate infection and transformation. All transformed cells contained the SV40 virus-specific CF T-antigen. The so-called "crisis" stage was also observed with massive cell lysis; permanent virus-free transformed lines were never obtained with cells transformed by the three SV40 strains. Although quantitation of transformation rates was not possible in these experiments, they provide evidence that the three strains are capable of transforming human cells to neoplastic cells in vitro.

*Comparative growth of SV40 mutants at different temperatures.* The possibility that the SV40 mutants differed in heat sensitivity or in the capacity to grow at high temperature was investigated in the following manner. Confluent monolayer cultures of AGMK cultures in 2-oz (50-ml) prescription bottles were infected with an input multiplicity of infection of 20 to 40 PFU of each virus. After a 3-hr adsorption period at 37 C, all cultures were washed five times to remove unadsorbed virus, and Eagle's medium with 1% fetal bovine serum was added. One culture infected with each virus was frozen at this time for determination of adsorbed virus. One set of cultures infected with each of the mutants was then completely immersed in a water bath at 41 C. Another set of infected cultures was similarly incubated at 37 C. At 72 hr after infection when cellular degeneration at both temperatures was generalized in all virus-infected cultures, they were frozen and thawed three times to disrupt cells. A number of experiments gave essentially similar results. Although the yields of S and M mutants at 41 C were slightly lower than at 37 C, in the case of the L mutant, there was no increase of infectivity over that found at 3 hr at 41 C.

Since cultures infected with the SV40-L virus incubated at 41 C showed typical cellular degeneration without apparent production of infectious virus, it was of interest to determine whether virus-induced antigens (viral or tumor CF antigens) were produced under these conditions. Cultures were infected and incubated at the two temperatures as in previous experiments. Infected cells were harvested at 72 hr and were tested for viral and tumor antigens by the CF test; infectivity assays were also performed on the same samples. The results are given in Table 2. With the S and M mutants, comparable levels of both types of antigens were produced at the two temperatures, and infectivity titers were only slightly lower at the higher temperature. In the case of the L mutant, while the T antigen titers were the same at both temperatures, a fourfold difference in CF viral antigen titer was found in cultures at 41 C. It should be pointed out here that the viral and T antigen titers of cells harvested at the end of the adsorption period were either low (1:2) or negative with all three strains; the CF antigen titers given in Table 2 therefore represent new antigen synthesis.

There was no detectable increase in infectivity

TABLE 2. *Comparative titers of complement-fixing viral and tumor antigens and infectivity of SV40 mutants at 37 and 41 C*

| Virus | Temp | Viral antigen | Tumor antigen | PFU/ml |
|---|---|---|---|---|
| | C | | | |
| L | 37 | 256 | 64 | $3.0 \times 10^9$ |
| | 41 | 64 | 64 | $6.0 \times 10^5$ |
| S | 37 | 64 | 64 | $5.0 \times 10^8$ |
| | 41 | 32 | 32 | $1.2 \times 10^8$ |
| M | 37 | 32 | 32 | $7.5 \times 10^6$ |
| | 41 | 16 | 32 | $2.0 \times 10^6$ |

of the L mutant over that found after three hr of adsorption, which indicated lack of infectious virus synthesis. Viral antigen was being synthesized, however, but this antigen was apparently not associated with infectivity. Fluorescent-antibody studies by the indirect procedure also confirmed that both tumor and viral antigens were formed at 41 C.

*Effect of various incubation temperatures on yield of SV40-L virus.* Viral yields of the SV40-L virus at various incubation temperatures below 41 C were next determined. Procedures for infection, incubation, and harvesting were the same as in the previous experiment. The results shown in Table 3 reveal a sharp limit to the incubation temperature permitting complete viral replication. Whereas the L mutant grew to high titer at temperatures up to 38 C, temperatures of 39 C or higher resulted in complete suppression of production of infectious virus.

*Determination of thermosensitive period in SV40-L viral growth cycle.* Two types of experiments were performed to determine the temperature-sensitive period of SV40-L viral growth. In the first experiment, replicate cultures of AGMK cells were infected as described above and were incubated at 37 C. At various times thereafter, cultures were removed and placed at 41 C. All cultures were incubated for a total of 72 hr after initial infection. Results of this experiment, presented in Table 4, show that incubation up to 30 hr at 37 C followed by transfer to 41 C resulted in complete suppression of infectious virus synthesis. An effect was still noted in cultures kept at 37 C for as long as 48 hr, with final infectivity titers decreased by greater than 1 $\log_{10}$. It may be noted here that single cycle growth at 37 C showed new virus synthesis appearing after 30 hr.

In a second experiment, AGMK cultures were infected and, after 3 hr of adsorption at 37 C, were incubated at 41 C. At various times thereafter, cultures were transferred to 37 C and incubated further until 72 hr postinfection. As shown in Table 5, cultures could be incubated up to 24 hr at the high temperature without any effect on final infectivity titer. Incubation for 32 and 48 hr at 41 C before transfer to low temperature resulted in about 1 $\log_{10}$ decrease in virus yield. Incubation at 41 C beyond 48 hr resulted in suppression of infectious virus production.

These two types of experiments indicated that the temperature-sensitive phase of the viral replicative cycle is a late event probably involved in maturation of the infectious viral particle.

TABLE 3. *Effect of temperature on yield of SV40-L virus*

| Incubation temp | PFU/ml |
|---|---|
| C | |
| 37 | $3 \times 10^9$ |
| 38 | $1.3 \times 10^9$ |
| 39 | $5 \times 10^5$ |
| 40 | $8.5 \times 10^5$ |
| 41 | $2.5 \times 10^5$ |

TABLE 4. *Titers of SV40-L virus after varying periods of incubation at 37 C, before transfer to 41 C*

| Hr at 37 C before transfer to 41 C | Hr at 41 C | PFU/ml |
|---|---|---|
| 3[a] | 0 | $1.2 \times 10^5$ |
| 24 | 48 | $9.5 \times 10^4$ |
| 30 | 42 | $9.0 \times 10^4$ |
| 48 | 24 | $1.3 \times 10^7$ |
| 72 | 0 | $5.5 \times 10^8$ |

[a] Control culture for assay of amount of cell-associated virus after adsorption period.

## DISCUSSION

Although investigations on mutants of polyoma virus have been extensive, up to the present time, published work on SV40 mutants has been scanty. Riggs and Lennette (13) described two plaque types of SV40, of which one was distinguished by the production of "red plaques." The relationship between the plaque types isolated by these workers and those described in this report is unknown. The so-called "red plaques" have not been observed with any of the three mutants we have studied.

Of the various properties of the SV40 mutants described here, the most interesting is the difference in growth at high temperature. The SV40-L virus did not produce infectious virus at temperatures above 39 C, whereas growth of the S and M mutants was unimpaired at the higher tem-

TABLE 5. *Titers of SV40-L virus after varying period of incubation at 41 C before transfer to 37 C*

| Hr at 41 C before transfer to 37 C | Hr at 37 C | PFU/ml |
|---|---|---|
| 0 | 72 | $1.2 \times 10^9$ |
| 24 | 48 | $1.9 \times 10^9$ |
| 32 | 40 | $1.2 \times 10^8$ |
| 48 | 24 | $1.7 \times 10^8$ |
| 56 | 16 | $5 \times 10^6$ |
| 72 | 0 | $3.1 \times 10^6$ |
| 0[a] | 3 | $1.8 \times 10^6$ |

[a] Sample taken for determination of amount of cell-associated virus after adsorption period.

perature. With all three strains, T antigen production was not inhibited at high temperature. Kitahara and Melnick (8) showed that both viral and tumor CF antigens of SV40 virus were formed at supraoptimal temperatures; however infectivity measurements were not reported. In the experiments described here with the L mutant, CF viral antigen was not associated with infectivity. The nature of the noninfectious CF viral antigen produced in cells infected with the L mutant and incubated at high temperature is currently under investigation.

It has recently been reported by Uchida et al. (16) that serial, undiluted passages of SV40 virus in AGMK cultures resulted in decreased yields of infectious virus which resembled the so-called "von Magnus phenomenon" described with certain myxoviruses (17). These investigators reported the presence of an interfering factor which was neutralized by anti-SV40 serum and, thus, postulated that it was an incomplete form of SV40 virus.

It does not appear likely that a similar phenomenon of "interference" is the explanation for the SV40-L mutant's inability to grow at high temperature, since high yields of virus were obtained when cultures were incubated at 37 C.

The inability of deoxyribonucleic acid (DNA) but not ribonucleic acid viruses to replicate in BHK-21 hamster cells which had been heated briefly at high temperatures has recently been reported by Gharpure (4). In these experiments, there was a progressive loss in the ability of DNA viruses to replicate, when cells were preheated at temperatures above 41 C. The relationship of this finding to our present results remains to be assessed.

The SV40-L mutant possesses two genetic markers which make it well suited for investigations on tumor virus-cell relationships, especially those concerned with "rescue" experiments of the integrated viral genome. Plaque size and heat sensitivity are genetic attributes which can be readily tested in vitro.

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