JOURNAL OF VIROLOGY, June 1975, p. 1297-1301

Vol. 15, No. 6
Printed in U.S.A.

Temperature-Sensitive Mutants of Simian Virus 40 Selected by Transforming Ability

NOBUO YAMAGUCHI* AND TAKAKO KUCHINO
The Institute of Medical Science, The University of Tokyo, P. O. Takanawa, Tokyo, Japan 108

Received for publication 2 January 1975

Eight temperature-sensitive mutants of simian virus 40 which transform rat cells at 32.5 C but not at 38.5 C have been isolated. All the mutants were also temperature sensitive for replication in African green monkey kidney cells and five of them were classified into a single complementation group. No mutant incapable of transforming rat cells at either temperature was isolated.

Simian virus 40 (SV40) experiences productive infection in permissive monkey cells and brings about transformation of certain nonpermissive cells. Several temperature-sensitive (*ts*) mutants of SV40 have been isolated (1, 2, 6, 8-10, 13, 18-21), and some of them proved to be temperature sensitive for transformation of cells (6, 9, 14, 19). All of these were isolated as conditional-lethal *ts* mutants in productive infection. Although the size of SV40 genome, about 3.6×10^6 daltons (17), is small, it is not yet clear whether all the function(s) required for transformation of cells are necessary for replication of the virus. Transforming functions of avian tumor virus (3, 7, 24) are not required for virus replication.

Therefore, to analyze further the mechanism of transformation, we have tried to isolate mutants of SV40 (i) which are temperature sensitive in their ability to transform nonpermissive rat cells without prior selection for their temperature sensitivity in replication in permissive monkey cells, and (ii) which are defective in the transformation at permissive temperature for virus replication. The present communication describes the isolation and some of the characteristics of the mutants.

MATERIALS AND METHODS

Cell culture. Secondary cultures of African green monkey kidney (AGMK) cells and GC7 cells, a clonal line of an AGMK cell which was established by us, were used for propagation, plaque assay, and cloning of SV40. The plaquing efficiency of SV40 in GC7 cells was similar to that in AGMK cells. The GC7 cells showed no detectable mycoplasma contamination in direct culture (kindly performed by Hiroshi Chosa of our Institute). Rat cells were prepared by the method of Yamamoto (23) from baby rat brain tissue. Briefly, the brain tissues of baby rats (Donryu) were minced and placed into culture bottles with growth medium. After incubation at 37 C for several days, the masses of minced tissue were washed off by changing the medium, leaving the cells which had grown out from the masses on the glass. The confluent monolayers formed by further incubation were used for transformation assay. Culture media used were reinforced Eagle minimal essential medium (containing a twofold concentration of amino acids and vitamins) without serum (for growth of SV40 in AGMK cells), or supplemented with 10% (for growth of AGMK and GC7 cells) or 2% calf serum (for growth of SV40 in GC7 cells), and Ham F12 medium supplemented with 10% calf serum (for rat brain cells).

Virus. SV40 strain 777 was purified by two successive single plaque isolations at 39.5 C. The resulting parental clone formed plaques of small size and has been used as wild-type (WT) virus. *Ts* 911 is a mutant defective in replicating ability in AGMK cells which was isolated by the method similar to that of Suzuki and Shimojo (16) and used as a control *ts* mutant which has unimpaired ability to transform cells. Virus stocks were prepared by inoculating dilute suspension of virus (22) at 37 C for WT virus and at 32.5 C for the mutants. Medium and cells were frozen and thawed twice and sonically treated for 6 min at maximum power, using a Kubota sonicator (Kubota Seisakusho Inc., Tokyo) when the cultures showed marked cytopathic effect. After centrifugation at 3,000 rpm for 15 min, the supernatants were stored at -80 C.

Plaque assay of infectious virus. AGMK or GC7 monolayers were grown in 6-cm petri dishes. The virus inoculum of 0.2 ml per plate was allowed to adsorb for 2 h at 37 C. The plates were then overlaid with 4 ml of 0.9% agar in reinforced Eagle medium supplemented with 10% tryptose phosphate broth and 5% calf serum, and incubated at the indicated temperature. A recond agar overlay was carried out at the middle of incubation with 4 ml of the same medium but containing 1% calf serum. Plaques were counted on day 17 (32.5 C), 13 (35 C), 10 (38.5 C), and 12 (40.5 C). A third agar overlay of 4 ml containing neutral red (1:33,000) was added the day before the counting.

Transformation of cells. Primary monolayer cultures of rat brain cells were dispersed by trypsinization. The cells were suspended in Eagle medium containing 10% calf serum, but containing no bicarbonate, and buffered at pH 7.2 with 0.01 M Tris-hydrochloride buffer and inoculated with serially diluted virus in polyallomer tubes. Virus was adsorbed for 1 h at 37 C by shaking the tubes at intervals of 15 min. The cells were seeded into petri dishes (10^5 cells per 6-cm dish) and transformed foci were counted after 16 days at 38.5 C and 24 days at 32.5 C. The medium was changed at intervals of 4 days. The transformation efficiency of the cells by WT virus varied slightly among the batches of primary cultures and of virus stocks, but was reproducibly higher at 38.5 than 32.5 C. One transforming unit corresponded from 10^6 to 1.5×10^7 PFU at 38.5 C. A dose response curve of the transformation assay by WT virus is shown in Fig. 1. The number of transformed foci was proportional to virus concentration over a range of upto 2,400 PFU/cell; the curve then leveled off. Clones from nine foci in the dishes, which produced only two or three foci at 38.5 C, were examined for SV40-specific T antigen by immunofluorescence (11). All the clones contained T antigen-positive cells (more than 50% of the stained cells), and the cells grew in soft agar medium, suggesting that the foci were formed with transformed cells and the transformation was caused by SV40.

Mutagenesis. UV light (16) and hydroxylamine (5) were used as mutagens. WT virus stocks were irradiated with UV light from a germicidal lamp (Toshiba Electric Co., 10 W) at a distance of 24 cm for 21 min, or treated with 2 M hydroxylamine at 37 C for 48 h. The survival after either treatment was 0.1%.

Screening for mutants. Appropriate dilutions of the mutagenized virus were sonically treated and plated on confluent cultures of AGMK cells. Clones were isolated from the plaques after 3 to 4 weeks of incubation at 32.5 C. The clones were propagated in AGMK cells at 32.5 C and tested for their transforming ability at 38.5 C. Clones, which produced no or a very small number of foci, were then examined for their transforming ability both at 32.5 and 38.5 C. Those which formed no or a small number of foci at 38.5 C (less than one-fifth of the number of foci at 32.5 C) were selected as *ts* mutants, and those which did not produce discrete foci at either temperature were also looked for. For further studies each mutant was recloned by plaque purification and virus stocks were prepared in GC7 cells at 32.5 C and stored at −80 C.

Complementation. Complementation at 40.5 C was measured by infecting tube cultures of GC7 cells with a pair of mutants, each at about 10 PFU/cell. Singly infected control cultures received the same multiplicity of each mutant. After adsorption, washing, and incubation at 40.5 C for 48 h, the samples were frozen, thawed, and titrated on GC7 monolayers at 35 and 40.5 C. Complementation indexes were calculated as the ratio $(X + Y)_{35\ C} - (X + Y)_{40.5\ C} / (X)_{35\ C} + (Y)_{35\ C}$, where X and Y are yields of two mutants grown at 40.5 C and assayed at the temperatures indicated in the subscripts (21).

RESULTS

Isolation of mutants. Clones (253) of UV-irradiated virus and 505 clones surviving hydroxylamine treatment were tested for their ability to transform rat cells, and five (*ts* 900 to *ts* 904) from UV-irradiated and three (*ts* 905 to *ts* 907) from hydroxylamine-treated clones proved to have temperature-sensitive transforming ability (Table 1). WT virus and *ts* 911, which was taken as a transformation-plus control mutant, formed 1.1- to 2.2-fold more foci at 38.5 C than at 32.5 C, whereas eight transformation-defective mutants formed 9- to 125-fold smaller number of foci at 38.5 C than that at 32.5 C. Although most of the foci produced by the mutants at 38.5 C were smaller or less dense than those by WT virus, the size of the foci produced by mutants at 32.5 C was similar to that produced by WT virus (Fig. 2). *Ts* 911 produced foci indistinguishable from WT.

No mutant incapable of transforming rat cells at either 32.5 or 38.5 C has been isolated from the same mutagenized virus clones.

Plaquing and growth characteristics of the mutants. All of the *ts* mutants except *ts* 901 failed to make clear plaques at 40.5 C, whereas the efficiency of plaque formation of the mutants was similar to that of WT virus at 35 C (Table 2). The efficiency of plaquing of *ts* 901 was lower at 40.5 C than that at 35 C only by a factor of 10^2, indicating a high degree of leakiness or reversion to WT. Two of the mutants, *ts*

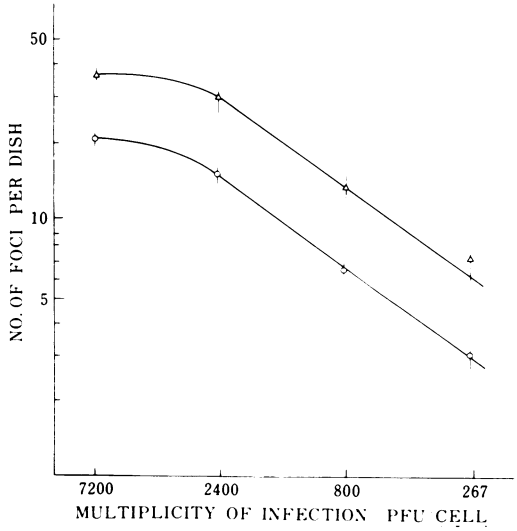

FIG. 1. *Relationship between concentration of WT virus and focus formation. Symbols for number of foci mean ± one standard error, at 38.5* (△) *and 32.5 C* (○).

TABLE 1. *Transformation of rat brain cells at 32.5 and 38.5 C*[a]

Expt. no.	Virus	No. of foci per plate[b]		Ratio 38.5 C/32.5 C
		32.5 C	38.5 C	
1	WT	15.0	30.7	2.0
	900[c]	37.6	0.3	0.008
2	WT	12.3	13.0	1.1
	901	20.0	0	<0.025
	902	9.0	0.5	0.056
	911	22.7	50.5	2.2
3	WT	36.0	55.5	1.5
	903	16.7	0	<0.03
	904	9.0	0.5	0.056
4	WT	19.0	24.0	1.3
	905	16.7	1.3	0.078
	906	20.0	0	<0.025
	907	12.0	1.3	0.11

[a] Transformation was performed as described in Materials and Methods. The input multiplicity of infection was 500 to 2,500 PFU/cell. No well-defined foci were detected in mock-infected control plates.

[b] Average of two to three plates.

[c] A block of mutant numbers has been assigned by J. A. Robb (15).

903 and 906, formed plaques almost equally well at 38.5 and 32.5 C, though they showed at least a 30-fold higher efficiency of transformation at 32.5 than 38.5 C (Table 1). Other transformation-defective mutants formed minute, poorly defined plaques at 38.5 C. The yields of virus after a single cycle of growth at 35 and 40.5 C after infection at an input multiplicity of 10 PFU/cell were examined (Table 2). The yield of WT virus was only threefold lower at 40.5 C than at 35 C. The ratio of the yield at 40.5 C to that at 35 C by *ts*-903 was only twofold less than that by WT virus after infection at an input multiplicity of 10 PFU/cell. At an input multiplicity of 1 PFU/cell, however, the ratio was about 10^{-3}, showing multiplicity-dependent leakiness. The other mutants produced yields which were 16- to 10,000-fold lower at 40.5 C than at 35 C.

Complementation studies in productive infection. Table 3 lists the complementation tests between five transformation-defective and one transformation-plus *ts* mutants in GC7 cells at 40.5 C. Each of the transformation-defective mutants clearly complemented transformation-plus mutant 911. No complementation between mutants 900, 902, 904, 905, and 907 could be detected under the same experimental conditions, providing evidence that these transformation-defective mutants are defective in the same function. Other transformation-defective mutants, 901, 903, and 906, were too leaky in productive infection to test for the complementation (Table 2).

DISCUSSION

The results we have presented in this paper showed the following: (i) all the eight *ts* mutants selected for their inability to transform nonpermissive rat cells at 38.5 C were also temperature sensitive for replication in permissive AGMK or GC7 cells, and (ii) no mutant incapable of transforming rat cells but capable of replicating in permissive cells both at 38.5 and 32.5 C was isolated. The results indicate that at least a viral function required for the formation of dense foci of rat cells is also necessary for the replication of SV40 in monkey cells. The temperature sensitivity of the *ts* gene function, however, was differently expressed in the transformation and in the viral replication. For example, the efficiency of plaque formation of *ts* 903 in permissive GC7 cells was equal both at 38.5 and 32.5 C (Table 2), whereas the efficiency of transformation of the mutant was more than 30-fold lower at 38.5 C than that at 32.5 C (Table 1). Even in this case, the same *ts* gene product may be required both for the transformation and for the viral replication, because the plaque-forming capacity of *ts* 903 was 50% less than that of WT virus at 38.5 C, and the mutant produced a yield which was about 1,000-fold lower at 40.5 than 35 C at a low input multiplicity of infection (Table 2). Tegtmeyer (19) observed that some of his A group *ts* mutants are more defective in transformation than in replication at 39 C. The difference in the temperature sensitivity between the transformation and the replication may reflect a difference in the amount of the *ts* gene product in the permissive and the nonpermissive cells or may reflect a difference in receptor sites for the *ts* gene product in the two different kinds of cells. A *ts* character of transformed cells infected with a *ts* mutant of Rous sarcoma virus also expressed differently in permissive and restrictive cells (4). In rat cells, restrictive for replication of Rous sarcoma virus, 37 C is a nonpermissive temperature, whereas in permissive chicken cells, 37 C is a permissive and 41 C is a nonpermissive temperature for the expression of transformed phenotype induced by the *ts* mutant.

In our present study we used a transformation assay which monitors multilayered foci. Re-

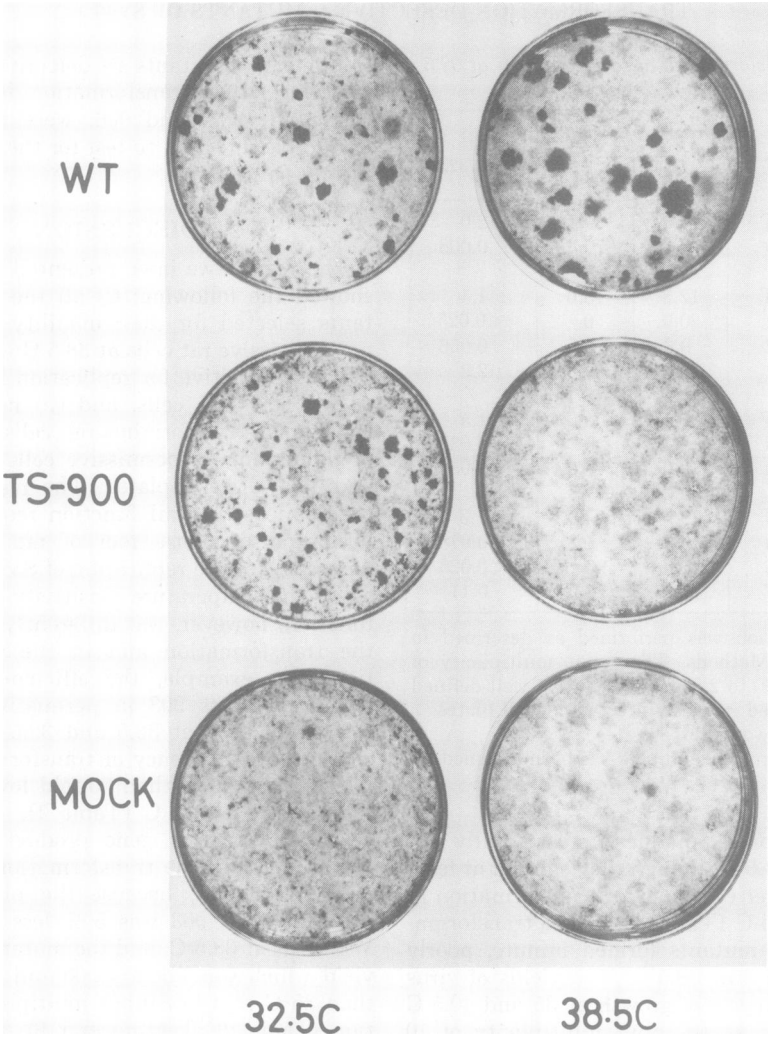

FIG. 2. *Transformation of rat brain cells by WT and ts 900 virus. The infected cultures were fixed with 10% formalin solution and stained with Giemsa solution after 24 days at 32.5 C and after 16 days at 38.5 C.*

TABLE 2. *Efficiency of plaque formation and virus yield in GC7 cells*

Virus	Ratio of plaquing efficiency[a]		Ratio of virus yield (40.5 C/35 C)[b]	
	38.5 C/32.5 C	40.5 C/35 C	MOI = 10[c]	MOI = 1
WT	2.5	9.3×10^{-1}	3.3×10^{-1}	2.2×10^{-1}
900	1.0×10^{-5}	2.5×10^{-6}	1.0×10^{-4}	
901	3.3×10^{-2}	7.3×10^{-3}	1.1×10^{-2}	
902	1.0×10^{-2}	6.7×10^{-6}	7.7×10^{-4}	
903	1.1	3.4×10^{-6}	1.7×10^{-1}	1.4×10^{-3}
904	1.3×10^{-2}	3.7×10^{-6}	6.3×10^{-3}	
905	4.0×10^{-3}	1.8×10^{-6}	2.8×10^{-3}	
906	5.6×10^{-1}	1.6×10^{-6}	6.0×10^{-2}	
907	1.8×10^{-2}	1.6×10^{-6}	2.5×10^{-3}	
911	$<6.6 \times 10^{-4}$	3.1×10^{-6}	1.8×10^{-4}	

[a] Plaques were counted on day 17 (32.5 C), 13 (35 C), 10 (38.5 C), and 12 (40.5 C).

[b] Infected cultures were harvested after 70 h of incubation at 35 C and after 48 h of incubation at 40.5 C and assayed for infectious virus production at 35 C.

[c] MOI, Multiplicity of infection; PFU per cell.

TABLE 3. *Complementation indexes between ts mutants at 40.5 C*[a]

Virus	911	900	902	904	905
900	327				
902	277	0.8			
904	12	0.5	0.8		
905	380	0.8	0.5	0.5	
907	46	0.9	0.6	0.7	2.2

[a] Complementation tests were performed as described in Materials and Methods. In all cases the frequency of reversion and recombination was less than 1%.

cently, Risser and Pollack (12) showed that wide variation in transformed properties are observed in 3T3 cells infected with SV40, suggesting that the process of cellular transformation by SV40 is the result of several complicated interactions of cellular and viral genes and not simply the result of a single viral gene acting directly to transform a cell. In their transformation test, only 17% of the transformed clones made dense colonies, when clones were first morphologically scored. Their study indicates that our transformation assay might score only one of several viral functions which affect transformed properties.

By complementation test, five of our mutants were grouped into one complementation group. They were defective in virion (V) antigen and viral DNA synthesis but not in induction of cellular DNA synthesis in permissive GC7 cells (unpublished data), and the number of T antigen-positive GC7 cells in cultures infected with the mutants is reduced slightly at 40.5 C compared to that at 35 C (11; unpublished data). These phenotypes of our mutants indicate that the complementation group of ours may correspond to Tegtmeyer's group A (19). J. A. Robb kindly examined synthesis of V, capsid, and T antigens in TC7 cells infected with *ts* 900 and grouped the mutant into group A.

ACKNOWLEDGMENTS

We are grateful to Hiroto Shimojo and Kinichiro Oda for their helpful discussions and critical review of this manuscript. We would like to thank James A. Robb for examining the antigenic phenotypes of *ts* 900.

The work was partially supported by a grant from the Ministry of Education of Japan.

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