

## Antigenic Phenotypes and Complementation Groups of Temperature-Sensitive Mutants of Simian Virus 40

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The antigenic phenotypes of several temperature-sensitive mutants of simian virus 40 were determined by an immunofluorescence microtechnique that allowed a very high degree of internal control for the conditions of virus infection and antigenic staining. The tumor (T), U, capsid protein (C), and virion (V) antigens were investigated. Productive infection in monkey cells and abortive infection in mouse cells were simultaneously monitored for antigen production at both permissive and restrictive temperatures. Complementation analyses of the mutants demonstrated two complementing groups (A and B) and one noncomplementing group (\*). One of the complementing groups could be subdivided into two subgroups having very different antigenic phenotypes. The following phenotypes were observed at the restrictive temperature in monkey cells. (i) The noncomplementing group produced none of the antigens. (ii) Group A induced T antigen in moderately but consistently reduced numbers of cells. Other antigens were markedly reduced or absent. (iii) Some of the group B mutants produced T antigen but little or no U and V antigens. The C antigen appeared in the nucleolus and cytoplasm of this subgroup. (iv) In the other group B mutants, antigen synthesis was not altered. Similar phenotypes were observed in mouse cells, except that U, C, and V antigens could not be detected during either the mutant or wild-type virus infections at any temperature.

Simian virus 40 (SV40) has a productive infectious cycle in monkey kidney cells and an abortive or transforming cycle in mouse cells. Because of the small size of the SV40 genome, the analysis of all essential viral functions may be possible. Such an analysis would provide specific information about the molecular nature of several important biological processes known to occur during infection. Early viral functions include the induction of cellular DNA synthesis (7), initiation of each round of viral DNA replication (20), integration of viral DNA into cellular DNA (8), neoplastic transformation of animal cells (2), and the control of late viral transcription (3). These early viral functions have not yet been associated with specific viral-coded proteins. However, they may be related to the SV40-specific tumor (T) and U intranuclear antigens that appear early within infected cells. Late viral genes code for structural proteins which react with antisera prepared against purified virions or their components (11). The present investigation was undertaken to standardize the complementation

grouping of temperature-sensitive mutants isolated in different laboratories and to relate these complementation groups to the production of different SV40-specific antigens by using an immunofluorescent microtechnique.

### MATERIALS AND METHODS

Productive infections were performed in the African green monkey TC7 subline (15) derived from the CV-1 line (9). Abortive infections were performed in BALB/3T3/-clone A31 mouse cells (1; kindly provided by Helene Smith). The wild-type SV40-transformed BALB/3T3 cell line, SEA-45, was also provided by Helene Smith. Powdered Dulbecco-Vogt modified Eagle medium (Flow Laboratories, Rockville, Md.), supplemented with 300 mg of arginine hydrochloride per liter, 1.5 g of glutamine per liter, 20 mg of histidine hydrochloride per liter, 1.5 g of glucose per liter, and 25  $\mu$ g of chlortetracycline per ml, was buffered at pH 7.4 in humidified air WEDCO incubators with 0.5 g of NaHCO<sub>3</sub> per liter and 80 mM *N*-tris(hydroxymethyl)methyl glycine (Tricine, Sigma Chemical Co., St. Louis, Mo.) for TC7 cells and 60 mM Tricine for BALB/3T3 and SEA-45 cells. Fetal bovine serum (Flow Laboratories) was added to a final concentra-

tion of 4.5% for TC7 cells and calf serum (Colorado Serum Co., Denver) was added to a final concentration of 9% for BALB/3T3 and SEA-45 cells.

All virus stocks were grown in TC7 cells at 33 C, by using a multiplicity of infection (MOI) of 0.005, and prepared for infection as previously described (16). The nomenclature for SV40 mutants has been described (19). Mutants 4, 8, 11, 28, and 30 were isolated by P. Tegtmeyer (20, 21), 101 was isolated by J. Robb and R. Martin (17), and 554 and 559 were isolated by A. Ishikawa (J. Gen. Virol., in press). Ad<sub>2</sub><sup>+</sup>ND<sub>1</sub> virus was provided by Andrew Lewis, National Institutes of Health.

Microculture infections were carried out as previously described at an input MOI of 1 to 4 (13). A single pool of TC7 or BALB/3T3 cells derived from the same flask was used for all infections in a given experiment. The infection of cells and inoculation of microtest plates were carried out within a 2 h period at 25 C. Plates were placed at the appropriate temperature immediately after inoculation. Parallel plates were prepared for every six viruses tested (one virus per 10 well row). The plates were divided and placed at each of the following temperatures: TC7 at 33 C, 39 C, 41.5 C; BALB/3T3 at 33 C and 39 C. Infected cells at 39 and 41.5 C were fixed at 24 and 48 h after infection (HAI), and cells at 33 C were fixed at 48 and 96 HAI (17). To insure that only the first cycle of infection was being assayed in the productive TC7 cells, either cytosine arabinonucleoside (Ara-C, 20 µg/ml, Sigma Chemical Co.) or SV40 bovine-neutralizing antiserum (5%, Flow Laboratories) was added to the plates. The Ara-C was added at the beginning of infection, and the prewarmed (42 C) neutralizing antiserum was added at 20 HAI by using one plate at a time on a 42 C hot plate.

The fixed cells were stained for the appropriate antigens by using the indirect fluorescent-antibody technique adapted to microculture (13). Three wells for each virus were stained for each antigen, using three antisera per plate. The number of antigen-positive nuclei in all three well bottoms was counted for each antigen and each virus. Fluorescein-conjugated anti-SV40 T antigen hamster ascitic fluid and bovine SV40-neutralizing serum (anti-virion [V] antigen) were purchased from Flow Laboratories. The T antiserum (1:20 dilution) did not react with Ad<sub>2</sub><sup>+</sup>ND<sub>1</sub>-infected TC7 cells, indicating the absence of detectable antibody to U antigen because Ad<sub>2</sub><sup>+</sup>ND<sub>1</sub>-infected monkey cells do not form T antigen (10). Guinea pig antiserum to solubilized capsid proteins (anti-capsid [C] serum) has been previously described (11). It reacts by complement fixation (CF) with virus particles and with both the 25,000 and 45,000 dalton structural proteins. It was used at a 1:500 dilution. Monkey anti-U antigen serum (10) was kindly supplied by Andrew Lewis, National Institutes of Health, and did not react with T antigen. Fluorescein-conjugated anti-hamster globulin, anti-bovine globulin, anti-guinea pig globulin, and anti-monkey globulin were purchased from Sylvania Co., Millburn, N. J., and used at 1:20 dilutions.

Complementation assays were performed in replicate 32-mm petri dishes (Falcon) containing confluent TC7 cells (4 × 10<sup>6</sup> cells/dish) by singly or doubly

infecting the cells at an MOI of 0.5 to 1.0 for each virus as follows. Virus stocks were diluted in serum-free medium (SFM) to twice the desired MOI. For single infections, the stocks were mixed 1:1 with SFM. For double infections, two virus stocks were mixed 1:1. The virus mixtures were adsorbed by using 0.1 ml per dish for 2 h at 25 C with agitation every 15 min. The dishes were then rinsed twice with SFM, and 2 ml of growth medium was added. After 72 h at 41.5 C, the dishes were frozen at -40 C. The thawed viral lysates were treated with sonic oscillations (80%, 10 s, Biosonik IV sonifier), centrifuged at 5,000 rpm for 5 min in a RC-2 Sorvall refrigerated centrifuge, and filtered through a membrane filter (0.45 µm; Millipore Corp.). These viral preparations were end-point dilution titered (12) on TC7 cells at 33 and 41.5 C. Complementation assays were also performed by using TC7 cells with an MOI of 0.5 to 1.0 and titering by the plaque technique, as previously described (21). Complementation indexes were measured as the ratio  $(X + Y)_{33 C} - (X + Y)_{41.5 C} / (X)_{33 C} + (Y)_{33 C}$ , where X and Y were the yields of two mutant strains grown at 41.5 C and assayed at the temperatures indicated in the subscripts. Reversion and recombination frequencies were always less than 1%.

## RESULTS AND DISCUSSION

The complementation data derived from two different laboratories (J. R. and P. T.), using an MOI of 0.5 to 1.0, were similar. Table 1 indicates the existence of two complementing groups (A: 28, 30 and B: 11, 554, 4, 8, and 559) and one noncomplementing group (\*: 101). The B group contains two subgroups (11, 554 and 4, 8, 559) having different antigenic phenotypes as described below.

The antigenic phenotypes are summarized in Table 2, and the functional characteristics are summarized in Table 3. Four phenotypically different mutant classes were observed during productive infection in monkey TC7 cells, and three different classes were observed during abortive infection in mouse BALB/3T3 cells. Before describing the individual classes, the following general comments can be made about the experiments. (i) The control of internal variation was quite good, because all viruses were

TABLE 1. Complementation indexes between mutants at 41.5 C in TC7 cells

Complementation group	Virus	Virus							
		101	28	30	11	554	4	8	559
*	101	1.2	1.5	1.3	0.7	1.3	1.7	1.5	1.6
A	28		1.5	1.2	61	42	27	76	46
A	30			1.1	102	62	22	68	106
B	11 <sup>a</sup>				1.2	1.1	1.5	0.5	1.2
B	554 <sup>a</sup>					1.0	0.8	0.6	0.7
B	4						1.2	1.0	0.9
B	8							1.1	1.0
B	559								1.2

<sup>a</sup> Although 11 and 554 do not complement with 4, 8, and 559, their antigenic phenotypes are very different (see Table 2).

TABLE 2. Summary of antigenic characterization of SV40 temperature-sensitive mutants during productive infection in monkey TC7 cells and abortive infection in mouse BALB/3T3 cells

Complementation group	Mutant	TC7 cells (41.5 C/33 C × 100%)				BALB/3T3 cells (39 C/33 C × 100%)	
		T	U	C	V	T	U, C, V
WT	SV-S, VA 45-54	100 <sup>a</sup>	100	100	100	100	0 <sup>b</sup>
*	101	1	1	1	1	0	0
A	28, 30 (7) <sup>c</sup>	30	2	0	0	0.1	0
B	11, 554 (2) <sup>c</sup>	100	2	100 <sup>d</sup>	0	100	0
B	8, 559 (4) <sup>e</sup>	100	100	100	100	100	0

<sup>a</sup> These values are rounded off for the sake of clarity. The standard error of the mean was ±15%.

<sup>b</sup> 0, No antigen-positive nuclei in 2,000 cells at restrictive temperature.

<sup>c</sup> Mutants in parentheses are in the same group, but are not represented in these data due to their leakiness (2 and 7) or to not being tested (4).

<sup>d</sup> One-hundred percent of the C-positive cells had cytoplasmic and nucleolar staining, but only 20% had positive nuclei. At 39 C, 100% of the C-positive cells had cytoplasmic and nucleolar staining with no nuclear staining.

TABLE 3. Summary of characteristics of the presently identified classes of temperature-sensitive SV40 mutants at restrictive temperatures

Complementation group	Mutant	DNA synthesis		1st cycle		
		Cell <sup>a</sup>	Virus	Partic-les	DNA infec-tion <sup>b</sup>	Trans-formation <sup>c</sup>
*	101	0	0	0	WT	0
A	7, 28, 30	+	0	0	MUT	0
B	2, 11, 554	+	+ <sup>d</sup>	0	MUT	+
B	4, 8, 559	+	+	+	MUT	+

<sup>a</sup> Induction of cellular DNA synthesis during productive infection in African green monkey kidney cells.

<sup>b</sup> Infection of African green monkey kidney cells with purified viral DNA. WT, First-cycle infection similar to first-cycle wild-type virion infection. MUT, First-cycle infection similar to first-cycle mutant virion infection.

<sup>c</sup> Transformation of abortive BALB/3T3 cells at 39 C.

<sup>d</sup> 554 is moderately inhibited in viral DNA synthesis and is tentatively placed in this group due to its antigenic phenotype and complementation analysis.

tested simultaneously by using one pool of either TC7 or BALB/3T3 cells, all dishes were incubated together at the respective temperatures, and all dishes were stained for all antigens simultaneously. Three independent experiments gave similar results (ii) Only the first cycle of infection in the productive TC7 cells was being monitored, because there was no difference in the number of T or U antigen-positive nuclei in cells incubated with Ara-C or with SV40-neutralizing antiserum. The formation of C and V antigens was inhibited by Ara-C. (iii) Both wild-type viruses (SV-S, VA45-54) produced all antigens equally well in

TC7 cells and produced T equally well in BALB/3T3 cells. (iv) No mutants were inhibited due to slow accumulation of antigen as determined by early (24 h) and late (48 h) time points. (v) T and U antigens were not inhibited by Ara-C. These antigens did not require viral or cellular DNA replication for their expression and were early functions. The C and V antigens were inhibited by Ara-C. These antigens did require DNA replication for their expression and were late functions. (vi) The C and V antigens were not expressed during abortive infection in the BALB/3T3 cells. (vii) The inability to detect U antigen in the wild-type SV40-transformed BALB/3T3 cells (SEA-45) and the BALB/3T3 cells after acute infection was due to either the relatively low titer of the U antiserum or to deficient U antigen expression in mouse cells. Higher-titer U antisera than are presently available will be required to differentiate between these two possibilities.

**Noncomplementing group \* (ts\*101).** This mutant has been previously described as being inhibited in the initiation of T, U, and V antigens during productive infection (17) and of T antigen during abortive infection (18). The data indicate that *ts\*101* was also inhibited in initiating C antigen in monkey cells.

**Group A (tsA28 and tsA30).** These mutants have been previously described (20) and were less leaky at 41.5 C than the *tsA7* mutant originally described (21). Our data indicate that this group was partially inhibited in forming detectable T antigen and markedly inhibited in forming U antigen during productive infection. This group was markedly inhibited in initiating T antigen during abortive infection. No formation of C or V antigens by these mutants was detectable in the TC7 cells.

**Group B (tsB11, tsB554, tsB8, tsB559).** During productive infection by *tsB11* and *tsB554*, T antigen synthesis was normal, whereas U antigen was markedly inhibited. Unexpectedly, staining by the anti-C serum appeared in the nucleolus and cytoplasm as well as in the nucleus at restrictive temperature in TC7 cells. However, these cells had no reaction with V antisera at 39 or 41 C. There was no cytoplasmic or nucleolar staining with T, U, or V antisera at any temperature in either the TC7 or BALB/3T3 cells or with C antiserum in TC7 cells at 33 C. The other subgroup, *tsB8* and *tsB559*, did not differ from the wild-type infection for any of the antigens at restrictive temperatures in TC7 or BALB/3T3 cells.

Some of the antigens monitored have known functions. The V antiserum assayed the intact capsid (empty or filled with DNA). The capsid proteins could only be monitored with the C antiserum in V-negative cells (e.g., *tsB11*- and *tsB554*-infected TC7 cells). We do not know which of the capsid proteins are being monitored in the 11- and 554-infected TC7 cells with the C antiserum because monospecific antisera for the individual capsid proteins are not presently available. There is no direct evidence available to determine whether T and U antigens are viral-coded proteins or whether they are specifically induced cellular proteins. T antigen is a 70,000-dalton protein that is neither a virion protein nor a precursor to a virion protein (4), but may be involved in SV40 DNA

replication (20). The size, structure, and function of U antigen are presently unknown.

The noncomplementing group represented by *ts\*101* is inhibited in initiating all antigens at the restrictive temperature during both productive and abortive infections. The mutant protein is probably a virion protein that has to be removed or activated before any SV40 genome activity can occur (14, 17, 18). This protein may also have a role in regulating T antigen in *ts\*101*-transformed mouse 3T3 cells (14). The *ts3* mutant of polyoma has some similar properties. For example, it is not complemented in mixed infections, and the temperature-sensitive phenotype is not observed after *ts3* DNA infections (W. Eckhart and R. Dulbecco, manuscript in preparation and personal communication).

The group A mutants represented by *tsA7*, *tsA28*, and *tsA30* are moderately inhibited in the formation of immunologically detectable T antigen during productive infection and markedly inhibited during abortive infection at restrictive temperatures. The T antigen-positive nuclei that do appear have as great an intensity as do those initiated by wild-type viruses. Whether the mutation occurs at a step prior to T antigen initiation or is in the T antigen itself is presently not known. The group A protein is required for the initiation of SV40 DNA replication (20). This group is analogous to the polyoma group II mutants represented by *ts-a* (5, 6).

The B subgroup represented by *tsB2*, *tsB11*, and *tsB554* is very complex. During productive infection, the mutants are inhibited in the formation of both U (an early antigen) and V (a late antigen). The apparent deficiency in U antigen must be confirmed by complement fixation assays when higher-titer U antiserum becomes available. The inability of this group to form capsids (21) physiologically separates them from the other B group mutants (*tsB4*, *tsB8*, and *tsB559*) that do form capsids at restrictive temperatures in monkey cells. We do not yet know whether these two B subgroups are affected in the same gene. The cytoplasmic and nucleolar distribution of the C antigen at restrictive temperatures in 11- and 554-infected TC7 cells is unusual and further differentiates these two subgroups. The capsids made by *tsB8* at 41.5 C are heat labile, and empty-shelled structures accumulate intracellularly (11). Some late mutants of polyoma (e.g., *ts10* in group I) also produce heat-labile virus (5).

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