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Expression and Thermal Stability of Simian Virus 40 Tumor-Specific Transplantation Antigen and Tumor Antigen in Wild Type- and *tsA* Mutant-Transformed Cells

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We have explored aspects of a suggested relationship between the expression of simian virus 40 tumor-specific transplantation antigen (TSTA) and tumor antigen (TA). A unique rat embryo cell line transformed by a temperature-sensitive *A* mutant that loses TA during exposure to the nonpermissive temperature (A28-RE) was found to lose TSTA. On the other hand, a typical control *tsA*-transformed cell line (A239-MB) expressed both TA and TSTA at the nonpermissive temperature. TA in lysates obtained from A239-MB cells was found to be three to four times more thermolabile by complement fixation than TA obtained from wild-type-transformed cells (SVWT-MB) when incubated at either 33 or 40°C. These data complement previous reports using TA from lytic infection and are consistent with the suggestion that TA is virus encoded. In contrast to TA, which even in wild-type-transformed cells was completely destroyed in <10 min at 50°C, TSTA, assayed in vivo by tumor rejection, and tumor-specific surface antigen(s) (TSSA), defined by an in vitro cytolytic assay, were thermostabile. Even after 24 h of incubation of extracts at 50°C, high levels of TSTA and TSSA activity were present. Since these surface antigens when obtained from cells transformed by *tsA* mutants were also thermostabile, they could not be distinguished from the wild-type antigens. These results (i) indicate a coordinate expression of TA and TSTA in transformed cells; (ii) confirm that TA is virus encoded; and (iii) confirm that the antigenic and immunogenic determinants that characterize TA and TSTA activities are distinct. However, the possibility that TSTA, like TA, is of viral rather than cellular origin is not excluded.

Genetic studies with simian virus 40 (SV40) mutants indicate that the "early" gene *A* is essential for the initiation of viral replication in permissive infection and for the initiation and maintenance of transformation in nonpermissive infection (3, 6, 9, 14, 22, 23, 25, 26, 29, 30). The SV40-specific antigens T, TST, and U are expressions of this early region of the SV40 genome (17); however, relatively little is still known about their functions, origins, and possible interrelatedness.

T antigen (TA) appears to be the primary protein product of the *A* gene. The complement-fixing activity of TA extracted from cells lytically infected with *tsA* mutant viruses has been shown to be more thermolabile than that of TA produced during infection with wild-type virus (2, 15). TA binds to DNA (7), and, after incubation at 44°C, TA obtained from *tsA*-transformed cells loses binding activity more rapidly than TA from wild-type-transformed cells (32). We now show in addition that the TA from *tsA*-transformed cells is more thermolabile by complement fixation (CF).

Tumor-specific transplantation antigen (TSTA) is more difficult to assay and has been less thoroughly investigated than TA, although its expression also requires part of the early region of the SV40 genome (17, 19). Specifically, TSTA, but not TA, is expressed during infection with the adeno-SV40 hybrid Ad2+ND2, which contains only that segment of the early region of the genome of SV40 spanning the *hin*dII + III cleavage fragments H, I, and B (17, 19).

Several lines of evidence suggest that TA and TSTA are related polypeptides. First, the molecular weight of TA is sufficiently large that it would appear to require the entire coding capacity of the early region of the SV40 genome (25, 31). Second, all known *tsA* mutants synthesize defective TAs (2, 15, 32), but contain genetic lesions that map within the region re-

quired for TSTA expression (16, 17). Third, TA and TSTA are expressed coordinately after infection of monkey cells by SV40 (4). Fourth, nuclear fractions of an SV40-transformed cell line contain more TSTA activity than membrane fractions (5). Fifth, TSTA and TA copurify through several steps of purification (5). Sixth, anti-T serum immunoprecipitates an SV40-specific polypeptide after infection by the $Ad2^{+}ND2$ hybrid (11). We now demonstrate in addition that a *tsA* mutant-transformed rat embryo cell line (A28-RE) that loses TA expression in vivo after incubation at the nonpermissive temperature (26) also loses TSTA expression. As control, we demonstrate that a typical *tsA* mutant-transformed cell line that continues to express TA at the restrictive temperature (as do most *tsA*-transformed cell lines [3, 6, 14, 24, 29, 30]) also continues to express TSTA. In a separate communication we demonstrate that several different cell lines transformed by *tsA* mutants continue to express TSTA and tumor-specific surface antigen (TSSA) at the nonpermissive temperature (Chang, Anderson, Mora, and Martin, manuscript in preparation). We have already demonstrated that *tsA* mutants express TSTA upon lytic infection of monkey cells at the nonpermissive temperature (4).

Although TA and TSTA appear to be related polypeptides, the antigenic determinants that specify TA antigenicity and TSTA immunogenicity appear to be different. Heating at 50°C inactivates partially purified TA from a human cell line transformed by SV40, but does not inactivate TSTA (5). Finally, we demonstrate here that at 50°C TSTA extracted from a wild-type-transformed line and a *tsA* mutant-transformed line is thermostable for 24 h, whereas TA activity is completely lost in less than 10 and 4 min, respectively.

MATERIALS AND METHODS

Cells. The murine cell lines A239-MB (*A239*-transformed mouse brain), A7-MB (*A7*-transformed mouse brain), C219-MB (*C219*-transformed mouse brain), and SVWT-MB (wild-type SV40, strain 776, small plaque-transformed mouse brain), and cloned sublines were derived from a secondary line of astrocytes originating from National Institutes of Health (NIH) Swiss mouse brain and have been previously described (3). A rat embryo line transformed by *A28* (A28-RE; Osborn and Weber [26]) was kindly supplied by R. Pollack and W. Topp for comparison studies.

The mKSA-ASC line is an SV40-transformed BALB/c tumor line carried by in vivo (intraperitoneal [i.p.]) passage and displays an ascites 50% lethal dose of $\leq 10^3$ cells (12). This cell line was kindly provided by L. Law. PY-3T3-4a (18), a polyoma-transformed Swiss 3T3 line (generously supplied by S. Aaronson and G. Todaro), and Meth-1-A, a methylcholanthrene-induced tumor line (obtained from L. Law; 21), were used in specificity experiments. A BALB/c 3T3 wild-type SV40-transformed cell line, SV-3T3-J, was also used (4).

Cells were grown in Dulbecco-Vogt medium (NIH media unit) supplemented with 10% fetal bovine serum and penicillin/streptomycin under humidified CO_2 at 33°C (the permissive temperature for *tsA* transformants) or, where noted, at 40.5 ± 0.5°C (the nonpermissive temperature).

Cell extraction for TA assays. TA was extracted from confluent cultures of transformed cells in a manner similar to that of Kuchino and Yamaguchi (15). Briefly, cell pellets were diluted in gelatin veronal-NaCl buffer (0.15 M NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.1% [grams/100 milliliters] Difco gelatin, 0.05 M sodium barbital, pH 7.5) to give about 20% (vol/vol) suspensions, sonicated at 0 to 4°C for 2 min, and stored at −70°C; these TA-containing lysates were used directly as TA or after clarification by centrifugation at either 1,000 × *g* for 10 min or 17,000 × *g* for 15 min.

CF test. The CF test for TA was performed by a standard microtiter assay as described by Casey (8). Briefly, 25 μl of anti-T antibody (Huntingdon Research Center, Brooklandville, Md.) containing 2 or 3 CF units and 50 μl of guinea pig complement (GIBCO, Grand Island, N.Y.) containing 5 CF units were added to microtiter wells containing serial dilutions of antigen in veronal-NaCl buffer. Duplicate wells were used for each experimental point. The wells were incubated overnight at 4°C, and 25 μl of 1.4% sheep erythrocytes, optimally sensitized with rabbit hemolysin (GIBCO), was added. Incubation was then carried out at 37°C for 1 h. After centrifugation, wells were assessed for percentage of hemolysis by comparison with standardized control wells.

Detergent extraction of surface antigens. Solubilization of surface antigens followed an extraction procedure described elsewhere (C. Chang, S. Pancake, S. Luborsky, and P. Mora, Fed. Proc. 35:545, 1976; Chang et al., manuscript in preparation.) Washed and scraped cells were extracted at 0 to 4°C for 30 min in 5 volumes of 0.5% Triton X-100 and 100 μM phenyl-methylsulfonyl fluoride in Tris-buffered saline (4). The membrane-cytosol extract was freed from nuclei and particulate matter by centrifugation at 27,000 × *g* for 15 min and filtered through a 0.22-μm membrane filter (Nalgene). Protein content of the extracts was determined by the method of Lowry et al. (20).

Heat inactivation of antigens. Cell lysates in buffer or detergent extracts obtained as described above were heated in equal-volume aliquots in plastic vials in a water bath for various times, quenched on ice, and stored at −70°C if not used immediately.

In vitro assays for TSSA. SV40-associated surface antigens determined by a modified in vitro cytolytic-antibody test are designated TSSA. Details of the assay are described elsewhere (27; Chang et al., manuscript in preparation). In brief, soluble antigen extracts were first absorbed with Biobeads SM2 (Bio-Rad Laboratories, Richmond, Calif.) to remove detergent (13) and then reacted with anti-TSSA an-

tibody at 4°C. The residual antibody activity in diluted aliquots was determined by measuring the amount of chromium-51 released in a microcytolytic assay from target SV-AL/N mouse cells reacted with antibody in the presence of rabbit complement at 37°C for 3 h.

In vivo assays for TSTA. Female BALB/c mice, 6 to 8 weeks old, were obtained from the NIH breeding colony. Immunization consisted of two i.p. injections of the detergent extract, 1 week apart, followed 10 days later by an i.p. tumor challenge of 10^4 mKSA-ASC cells. Later experiments (see table legends) were done with a single immunizing dose after it was observed that protection against tumor challenge was not substantially improved by a second injection. Mice were followed for at least 5 weeks post challenge for ascites tumor development and death.

RESULTS

To determine whether a correlation existed between TA and TSTA expression and/or stability, various transformed cell lines were grown under defined conditions and assayed for each activity. Although TA expression and thermolability studies have been reported (2, 3, 15, 25, 26, 32), we have repeated them with different cell lines and under slightly different conditions as controls for the TSTA studies.

Expression of TA. The content of TA in lysates of mouse brain cells is presented in Table 1A. When normalized to soluble protein, the cell line transformed by wild-type SV40, SVWT-MB, contained about twice as much antigen at both the permissive (33°C) and nonpermissive (40.5°C) temperatures as the *tsA*-transformed cell line, A239-MB. The ratio of TA in nonpermissively versus permissively grown cells was about unity for each cell line. We have previously reported that A239-MB cells, like most *tsA*-transformed cell lines (3, 6, 14, 24, 29, 30), retain TA at the nonpermissive temperature as detected by immunofluorescence (3).

TABLE 1. *TA content[a] of several cell lines at 33 or 40.5°C*

Cells	Temp (°C) of culture		Ratio 40.5/33°C
	33	40.5 for 5 days	
(A) SVWT-MB	19	24	1.3
A239-MB	9.2	10	1.1
Ratio wild-type TA/ *tsA* TA	2.1	2.4	
(B) A28-RE	36	1.4	25

[a] Assayed by CF; results are presented in CF units (as defined in reference 8) per milligram of protein in the cell lysates.

On the other hand, a rat embryo transformed cell line, A28-RE, showed a dramatic drop in CF titer for TA after incubation at the nonpermissive temperature (Table 1B), as originally reported by Osborn and Weber (26). After 5 days at 40.5°C, TA content fell from 36 to 1.4 CF units per mg of soluble protein in the cell lysates, a drop of 25-fold. It has been previously reported that the percentage of A28-RE cells staining for TA by immunofluorescence also drops dramatically under these conditions (25, 26).

Thermostabilities of TA. TA from cells transformed by the *A* mutant (*A239*) was more thermolabile than TA from cells transformed by the wild-type virus. Figures 1 and 2 demonstrate the loss of CF activity for TA after incubation of lysates at 40 (Fig. 1) and 33°C (Fig. 2). At both temperatures the inactivation of TA from cells transformed by *A239* was three- to fourfold more rapid than the inactivation of TA from wild-type-transformed cells. The inactivation experiment at 40°C was performed three times with similar results: TA from A239-MB cells inactivated with a half-life of 4 ± 1 min, whereas the half-life for wild-type TA was 14 ± 3 min, a ratio of 1:3.5. As noted, 40°C is the nonpermissive temperature for the *tsA*-transformed cells. During incubation of cell lysates at 33°C, both TAs were relatively more stable (by 8- to 10-fold) than at 40°C, but again the *tsA* antigen inactivated approximately four times more rapidly. *tsA* TA displayed a half-life of 35

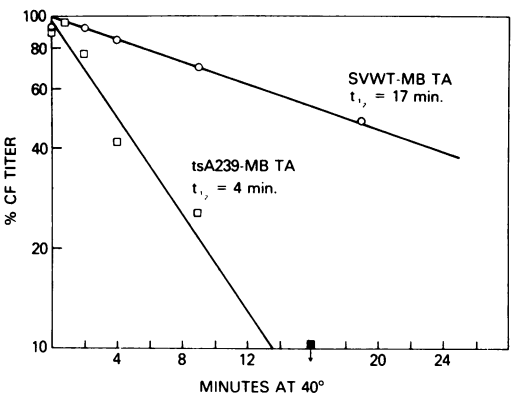

FIG. 1. *Decay of TA during in vitro incubation at 40°C. Whole-cell extracts were incubated in 0.2-ml aliquots and assayed in duplicate at the indicated times. The $t_{1/2}$ of inactivation is given in the figure, and the average values of three separate experiments are presented in the text. Before heating, all extracts contained 64 to 128 CF units per 25 μl of lysate. Results are plotted as percentage of CF titer at zero time of incubation. Solid square at 16 min represents 6% CF titer.*

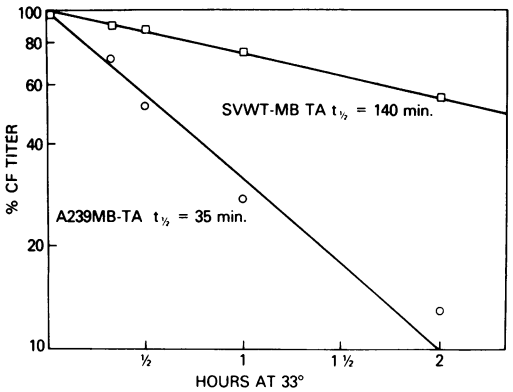

FIG. 2. *Decay of TA during incubation at 33°C. Similar to Fig. 1.*

± 5 min, whereas the half-life of wild-type TA extrapolated to 140 ± 20 min (average of two independent experiments). In this regard it is known that five of six *A* mutants examined (including *A239*), function somewhat defectively even at 33°C, the permissive temperature in culture (10). At both temperatures, the TA thermal stabilities were the same whether crude sonicates or clarified supernatants (17,000 × *g* for 15 min) were used.

A mixing experiment was done to exclude the possibility that lysates of wild-type-transformed cells stabilize TA, or that lysates of *tsA*-transformed cells render TA more labile to thermal inactivation. Equal amounts of TA from A239-MB and SVWT-MB lysates were mixed and then incubated at 40°C (Fig. 3). As can be seen, the half-life of inactivation was 7 min, intermediate between that of wild-type and *tsA* TA, and approximately coincident with the decay line expected for mixtures of equal amounts of antigen decaying with half-lives of ~4 and ~14 min.

TA preparations were highly unstable during incubation at 50°C. Supernatants of lysates centrifuged at 1,000 × *g* for 10 min were incubated in 0.25-ml aliquots and assayed at 0.5- to 1-min intervals. All antigen activity was lost by 10 min in wild-type samples and by 3 to 4 min in the *tsA* samples. The half lives of inactivation were, respectively, about 1.8 and 0.5 min.

These data demonstrate a greater thermal instability for TA activity from the cell line transformed by *A* mutant virus than from the cell line transformed by wild-type SV40, as assayed by CF. This finding is similar to and complements data already reported for antigen obtained from cells lytically infected with *tsA* mutant or wild-type virus (2, 15).

Thermostability of TSSA. TSSA is defined by an in vitro cytolytic microassay that depends on the lysis of target (SV-AL/N) cells by complement and serum hyperimmune to SV40-transformed cells (27, 28). When measured by the ability of transformed cells to inhibit target cell lysis, TSSA shows a high degree of specificity to SV40 and correlates with TSTA activity (27, 28; Chang et al., manuscript in preparation). The TSSA assay has the advantage of being more rapid and considerably less expensive than that for TSTA. TSSA solubilized by detergent extraction of cell membranes and cytosol proved considerably more resistant to inactivation at 50°C than did TA (Fig. 4). Furthermore, in contrast to TA, no significant difference in stability was evident (within the limits of the assay) on comparing the inactivation curves of TSSA from *tsA*- and wild-type-transformed cells. Assays having a precision of ±15% were performed in duplicate or triplicate on each of four separate occasions with similar results: in each case there was an initial rapid drop of activity within 5 to 15 min to 50 to 75% of the starting activity. Further incubation resulted in only a gradual decline in activity over several hours. Significant activity was retained

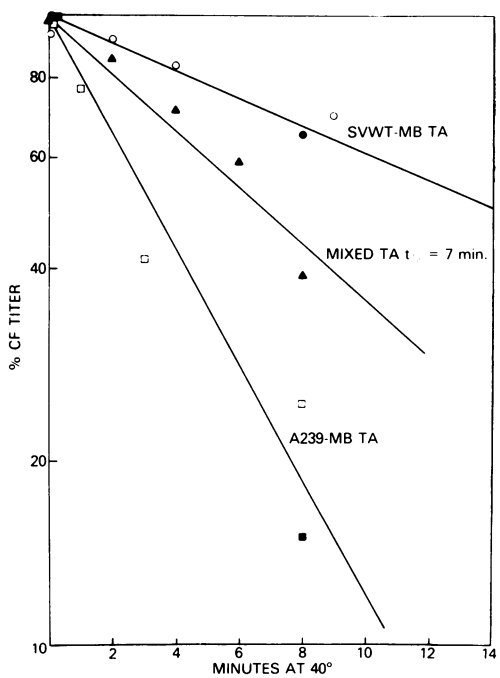

FIG. 3. *Decay of TA in mixed extracts incubated at 40°C. Extracts from the same TA batches as in Fig. 1 and 2 were incubated at 40°C either separately or mixed before incubation in a 1:1 ratio (by CF units) and assayed at various times for CF activity. SVWT-MB and A239-MB plots represented the average of two experiments (open and closed symbols).*

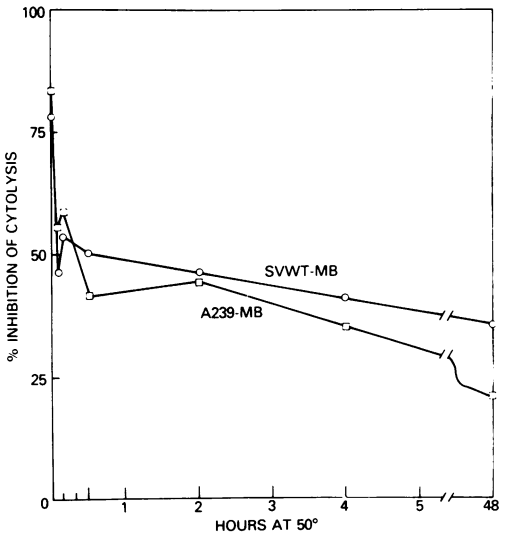

FIG. 4. *Stability of TSSA in detergent extracts. A239-MB and SVWT-MB cells grown at 33°C were extracted for TSSA by 0.5% Triton X-100, incubated at 50°C for various times, and assayed for TSSA as detailed in Materials and Methods. Each point was assayed in duplicate. TSSA is measured in the figure as percentage of inhibition of cytolysis of SV-AL/N target cells.*

in both preparations even at 24 to 48 h. The TSSA inactivation curve for extracts of mouse brain cells transformed by *A7*, which maps (16) in restriction fragment *hin* B (*A239* maps in fragment *hin* I), was similar to that shown in Fig. 4 (data not shown).

To eliminate the possibility that detergent extraction was responsible for stabilizing TSSA, assays were also performed on the same whole-cell lysates as used in the TA assays. Significant TSSA activity remained both in wild-type (not shown) and A239-MB lysates after 2 h at 50°C, whereas TA activity in these samples was completely lost by 15 min (Fig. 5).

In summary, each preparation tested for TSSA included, in addition to a rapidly inactivating component, large amounts of a thermoresistant component. The inactivation curves for *tsA* and wild-type TSSA activity were indistinguishable.

Expression of TSTA in transformed cells. Because it is not known whether TSSA activity is entirely analogous to TSTA activity, in vivo tests for tumor rejection were carried out. Detergent extracts of SV40-transformed mouse brain or Chinese hamster lung cells that are active in TSSA assays are also active for TSTA (Chang et al., manuscript in preparation). Experiments below quantitated TSTA content more precisely. Mice were immunized with graded amounts of these extracts and evaluated for their ability to reject an SV40 ascites tumor challenge of 10^4 mKSA-ASC cells (≥10× 50% lethal dose). Typical immunization results are presented in Table 2. Tumor rejection was complete at immunizing doses of 1 mg of protein, partial (25 to 62%) at 250- or 50-μg doses, and negligible at 10-μg doses of extracts obtained from permissively grown cells (Table 2A). This dose response was similar both in experiments where two (not shown) or one immunizing injection was used. Extracts from cells grown at the nonpermissive temperature contained comparable amounts of TSTA (Table 2B). The same

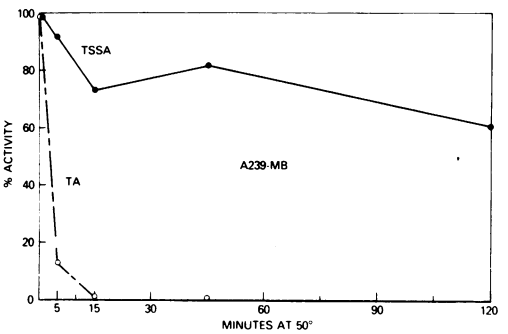

FIG. 5. *Stabilities of TSSA and TA in the same whole-cell sonicates. Whole-cell sonicates in NaCl-veronal buffer were prepared as described for TA in Materials and Methods (without centrifugation). After incubation for various times at 50°C, TA activity (given as percentage of CF titer at zero time) and TSSA activity (given as percentage of inhibition of lysis) were assayed from aliquots of the same samples. Each point represents the average of duplicate assays.*

TABLE 2. *TSTA activity of extracts of SV40-transformed mouse astrocytes*[a]

Source of extract	Malignant ascites after inoculation with cell extracts (tumor-bearing mice/total mice)				
	None	10 μg	50 μg	200 μg	1 mg
None (uninjected control)	15/15				
(A) Permissively grown cells (33°C)					
239-MB		8/8	6/8	5/7	0/7
SVWT-MB		8/8	3/8	5/7	0/7
(B) Nonpermissively grown cells (40.5°C for 5 days)					
239-MB		7/8	4/8	5/7	1/8
SVWT-MB		7/8	4/5	3/8	0/8

[a] Female BALB/c mice were inoculated i.p. with amounts of protein indicated and challenged i.p. 10 days later with 10^4 mKSA-ASC cells. Mean time to death in control mice was 21 days after challenge. Inoculated mice were followed for 5 weeks.

result has been found with several different *tsA*-transformed cell lines (Chang et al., manuscript in preparation). The continued expression of TSTA by A239-MB at 40.5°C paralleled the continued expression of TA at this temperature (cf. Table 1), although these cells show restricted growth at 40.5°C (3).

On the other hand, the rat embryo line, A28-RE, which lost most of its TA activity at the nonpermissive temperature (Table 1B; 26), also lost most of its extractable TSTA (Table 3) activity. Cells grown both at permissive and nonpermissive temperatures (for 5 days) were extracted and assayed for TSTA in mice. (These cells survive at 40°C although their growth rate slows. They become uniformly TA positive upon reincubation at 33°C for several days [W. Topp, personal communication].) As noted, extracts from cells grown at 33°C protected 50% of mice at immunizing doses of ~25-fold less protein than similar extracts from cells grown at 40.5°C. Results of in vitro TSSA assays (unpbulished data) support this in vivo TSTA result.

Thus, in both the mouse and rat SV40-transformed cell lines, TA and TSTA are expressed in parallel.

Thermostability of TSTA. The relative stability of TSTA was tested by assaying heated extracts for TSTA activity in BALB/c mice. On the basis of the dose response for extracts of transformed mouse cells grown at 33°C (Table 2A), mice were immunized with 1-mg aliquots of these extracts, which had been incubated in vitro for 0, 2, or 24 h at 50°C. Results in Table 4 demonstrate the protection afforded against a subsequent (i.p.) challenge with 10^4 mKSA-ASC cells. Consistent with other results (Chang et al., manuscript in preparation), BALB/c mice were protected by 1-mg immunizing doses of unheated extracts of A239-MB and SVWT-MB cells, whereas control mice were unprotected against a challenge of 10^4 mKSA cells. This protection was not diminished when extracts were incubated for 2 or 24 h at 50°C. In contrast, TA was completely destroyed at this temperature by 15 min (cf. Fig. 5). Extrapolating the logarithmic loss of TA activity (cf. Fig. 1–3) and assuming ~2 min to represent the half-life of inactivation for wild-type TA at 50°C (see above), incubation for 24 h, which represents ~720 half-lives for TA, would result in a $2^{720} \cong 10^{216}$-fold decrease in TA activity. TSTA thus appears to be considerably more stable than TA, since a 5- to 20-fold drop in antigen failed to protect completely against tumor challenge, and a 100-fold drop afforded no significant protection (Table 2). The results of TSTA inactivation are consistent with those of TSSA inactivation; an active form is significantly stable at 50°C for at least 24 h.

TABLE 3. *TSTA activity of extracts of an A28-transformed rat embryo cell line*[a]

Group (source of extract)	Dose (μg of protein)	Malignant ascites (tumor-bearing mice/total mice)
Control (uninjected)		16/16
(A) Permissively grown A28-RE cells (33°C)	10	5/10
	50	1/10
	250	1/10
(B) Nonpermissively grown A28-RE cells (40.5°C for 5 days)	10	9/9
	50	6/6
	250	4/9

[a] Methods as described in Table 2 legend.

TABLE 4. *Thermostability of TSTA at 50°C*[a]

Source of extract	Time of incubation of extract at 50°C (h)	Malignant ascites (tumor deaths/total mice)
None (control)		9/9
Normal NIH Swiss Mouse (brain) (control)	0	8/8
A239-MB	0	0/10
	2	1/10
	24	0/9
SVWT-MB	0	1/9
	2	0/8
	24	0/5

[a] Female BALB/c mice were inoculated on days 0 and 7 with 1 mg of detergent extract of permissively grown cells (incubated at 50°C as above) and challenged i.p. on day 17 with 10^4 mKSA-ASC cells. Evidence of ascites tumor appeared by about 2 weeks in control mice, with a mean time to death after challenge of 20 days. Inoculated mice were followed for 5 weeks.

Specificity of TSTA. Detergent extracts of a polyoma-transformed murine line (PY-3T3-4a) were compared with extracts of an SV40-transformed line (SV-3T3-J). Whereas a single dose of 375 μg of protein from the SV-3T3 extract protected 8 of 10 mice from malignant ascites, a similar protein dose of PY-3T3 extract failed to protect 8 of 8 mice ($P < 10^{-3}$) when challenged with 10^4 mKSA tumor cells and followed for 5 weeks. Mice injected with polyoma extracts died of ascites tumor at a mean of 16 days.

Detergent extracts of A239-MB cells grown at 33°C did not induce significant protection against a tumor challenge with Meth-1-A, a methylcholanthrene-induced, non-SV40 tumor line (21). Two injections (1 mg each) of deter-

gent extracts of normal NIH Swiss mouse brain or A239-MB cells failed to protect 7 of 9 and 14 of 19 mice, respectively, from subcutaneous tumor development after a challenging subcutaneous dose of 10^4 Meth-1-A cells. There was no significant difference between these groups.

These results suggest specificity of the tumor transplant rejection response. Protection afforded by the detergent extracts of SV40-transformed cells (Table 2–4) was not provided by extracts of normal brain or by extracts of cells transformed by the closely related papovavirus, polyoma. Moreover, although extracts of SV40-transformed cells protected against mKSA, they did not protect against a non-SV40 tumor line, Meth-1-A, which possesses its own strong TSTA (21).

DISCUSSION

We have compared the expression of TSTA and TA in cells transformed by SV40 wild-type and *tsA* mutant virus. Cells transformed by *tsA* mutants of SV40 have been shown to behave like nontransformed cells with respect to several parameters of growth at the nonpermissive temperature (3, 6, 14, 23–26, 30). At this temperature, most of the lines retain TA expression although rat embryo transformants appear to lose TA expression (26). We therefore assayed a representative cell line that retains TA and one that loses TA at the nonpermissive temperature to see whether TSTA would parallel TA expression. The mouse astrocyte-transformed line, A239-MB, retained TA at nonpermissive temperatures. TSTA, when assayed, was present in equivalent amounts at both temperatures, thus paralleling the expression of TA. Similar results have been obtained with Chinese hamster lung cell lines transformed by several different *tsA* mutants (Chang et al., manuscript in preparation). The rat embryo line A28-RE lost TA activity when grown at the nonpermissive temperature (26; Table 1). When these cells were tested for TSTA, it was found that ~25-fold greater amounts of extract from cells grown at the nonpermissive temperature were required to protect 50% of the mice than of extract from cells grown at the permissive temperature. Thus, the loss of TA expression paralleled the loss of TSTA. These results suggest that TA and TSTA are coordinately expressed in transformed cells.

A viral origin for TA (2, 15, 25, 32) is again suggested by the results of thermal inactivation tests. When assayed by CF, TA from the *tsA* transformant, A239-MB, was more thermolabile than that from the wild-type transformant. This finding complements observations made on TA from lytic infection. Alwine et al. (2) found that lytic TAs were inactivated at 41°C with half-lives of 1.8 to 3.2 min when obtained from infections with various wild-type viruses and late mutants, and with half-lives of 0.5 to 0.75 min when obtained from infections with various *tsA* mutants of SV40. TA from transformed cells was somewhat more stable than this in our assays at 40°C. TA from A239-MB inactivated with a half-life of 4 min, as compared with 14 min for SVWT-MB cells. It is not clear whether this is due to differences in assay conditions or to differences in lytic versus transformed cell TA (1). However, the two sets of data are consistent in two regards: (i) the half-life of *tsA*-TA was consistently shorter than that of wild-type TA (by ratios of 1:2–6); and (ii) the same ratios also occurred at 32 to 33°C, even though individual half-lives were about eightfold longer. At 50°C, where transformed cell TA was rapidly inactivated, we could still distinguish wild-type from *tsA* mutant TA. Kuchino and Yamaguchi (15) similarly noted rapid inactivation for lytic TA during 50°C incubation and could distinguish wild-type from *tsA* mutant TA. The data presented here for TA from transformed cells thus further support the suggestion that TA is virus encoded.

Whereas TSSA (Chang et al., manuscript in preparation), TSTA (Tables 2 and 3), and TA (Table 1) expression is parallel, their thermal stabilities are distinct. The in vitro assay for TSSA represents a great savings in time and effort over classical in vivo studies for TSTA. Several types of controls indicate that the assay under appropriate conditions is quite specific to SV40-induced surface antigens (27, 28, 33). It therefore seemed reasonable to expect that changes in TSTA activity would be reflected in changes in TSSA activity. The TSSA assay allowed generation of kinetic curves for thermal inactivation of the TSSAs, which showed considerable amounts of a thermostabile component in extracts of both wild-type- and *tsA*-transformed cells (Fig. 4). We thus were led to predict that TSTA might be similarly heat stable.

Complete protection against SV40 tumor challenge was afforded by extracts incubated for 24 h at 50°C, suggesting that TSTA is relatively heat stable. Differences between TA and TSTA heat stabilities are not explained by a "threshold" effect in the in vivo assay of heated lysates (Table 4), since as little as a 5- to 20-fold decrease in the amounts of injected TSTA led to incomplete protection against challenge with SV40 tumor cells (Table 2). Curiously, the other

known early antigen, U, requires a smaller segment of the distal portion of the early region of the SV40 genome for expression than TSTA and is heat stable when incubated at 56°C for 30 min (17, 19).

In summary, TA and TSTA expression have been quantitated in representative rodent lines transformed with *tsA* mutants. Expression of the antigens was coordinate. When TA was retained at nonpermissive temperatures, TSTA was retained, and when TA was lost, TSTA activity fell. The TA from A239-MB was more heat labile during in vitro incubation than the TA from SVWT-MB, consistent with a viral origin of transformed-cell TA. In contrast to TA, TSTA (defined by in vivo assay) and TSSA (defined by in vitro assay) were relatively stabile during incubation at 50°C for 24 h or more. This held whether the latter antigens were extracted from cells transformed by wild-type or by *tsA* mutant virus. These results do not exclude the possibility that the TSTA of *tsA* transformants is actually "mutant" (i.e., more thermolabile than wild type) or that TSTA is, like TA, a virus-encoded protein. The results presented here do indicate, however, that the structural determinants necessary for TSTA activity differ from those for TA activity, confirming earlier studies with the nondefective adeno-SV40 hybrids (17).

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