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Characterization of T Antigen in Cells Infected with a Temperature-Sensitive Mutant of Simian Virus 40

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T antigen induced in African green monkey kidney cells by a temperature-sensitive mutant of simian virus 40, defective in a function required for cell transformation, was characterized. The number of T antigen-positive cells estimated by an immunofluorescent techniques was almost equal at permissive (32.5 C) and restrictive (38.5 C) temperatures, but was slightly reduced when the infected cells were incubated at a higher temperature (40.5 C). However, a complement fixation test indicated that the amount of T antigen induced by the mutant is not significantly different from that induced by wild-type virus at 40.5 C. These results suggest that the T antigen-inducing ability of the mutant is not defective. Two distinct molecular species of T antigen were induced by the mutant at the permissive temperature, whereas only one form was observed at the restrictive temperature. The larger molecular form (14 to 15*S*) induced by the mutant at the permissive temperature was more heat labile than that induced by wild-type virus, suggesting that the mutated gene product is a component of the larger molecular form.

T antigens of simian virus 40 (SV40) and polyoma virus are detectable not only in the nuclei of tumors and transformed cells initially induced by these viruses, but also in acutely infected cells; yet they are not structural proteins of the virions. The viruses induce immunologically distinct T antigens, and yet each virus induces antigenically identical T antigens irrespective of the species of cells used for infection and transformation (see review 21). The induction of the antigen after infection depends on the expression of some part of the viral genome, since some temperature-sensitive (*ts*) mutants of polyoma virus and SV40 fail to induce T antigen at restrictive temperature (15, 19, 20). The same conclusion can be drawn from the observations that the ability of polyoma virus or SV40 to induce T antigen is inactivated by gamma- or UV-irradiation, or by beta-propiolactone (2, 3, 5, 6, 30), and that a deletion mutant of SV40, whose genome is 13% shorter than that of the wild-type (WT) virus, fails to induce T antigen (31). However, none of these findings unravels whether T antigen is a virus-coded protein or a cellular protein specifically derepressed by the virus.

The present study demonstrates that a molecular form of T antigen induced by *ts* 900, a transformation-defective *ts* mutant of SV40 (29), is more heat labile than that induced by WT virus, indicating that the mutated gene product may be T antigen or another component of the molecular form of the antigen.

MATERIALS AND METHODS

Virus. The WT SV40 was derived from the small-plaque 777 strain by cloning and a mutant, *ts* 900, was derived from the WT virus mutagenized with UV irradiation (29).

Cell culture. The secondary cultures of African green monkey kidney (AGMK) cells were used to prepare T antigen. The GC7, a clone derived from a AGMK cell, was used to prepare virus stocks and for plaque assay as described in the preceding paper (29). An SV40-transformed AGMK cell line, clone T22 which contains SV40 T antigen but not virion (V) antigen (24), was provided by K. Shiroki and used to prepare T antigen free from V antigen. The cells were cultivated in Eagle minimal essential medium supplemented with 10% calf serum.

Preparation of T antigen. T antigen was prepared from confluent cultures of either T22 or virus-infected AGMK cells. Monolayer cultures of AGMK cells were infected with the virus at a multiplicity of 1 or 10 PFU/cell. After adsorption for 2 h at 37 C, and then addition of maintainance medium (Eagle medium supplemented with 2% calf serum) with or without cytosine arabinoside (20 μg/ml, Sigma Chemical Co.), the cultures were incubated at either 40.5 C for 28 h or at 35 C for 48 h. The monolayer cells were then washed twice with isotonic gelatin Veronal-NaCl buffer containing 0.15 mM Ca^{2+} and 0.5

mM Mg^{2+} (pH 7.5, ionic strength 0.147) (14), scraped off with a rubber policeman, and centrifuged at 2,500 rpm for 10 min. The cell pellet was suspended in the gelatin Veronal-NaCl buffer to give about 20% (vol/vol) suspension, frozen and thawed six times, and centrifuged at 2,500 rpm for 10 min. The supernatant fluid was stored at −80 C and used as T antigen. T antigen from T22 cells was extracted similarly.

Complement fixation (CF) test. The microcomplement fixation tests for T and V antigens were performed by the method of Sever (22) with minor modifications as previously described (10). Briefly, one drop of suitably diluted antiserum (2 CF units of T antibody or 4 CF units of V antibody) and one drop of guinea pig complement containing 5 CH50 units were added in a microtiter well containing serially diluted antigen. The microplate was placed overnight at 4 C. One drop of sheep erythrocytes sensitized with 4 units of rabbit hemolysin (2.5×10^8 cells/ml) was added and the plate was incubated at 37 C for 1 h. After centrifugation, the amount of hemolysis was graded on a scale of one through five. The CF titers are expressed as the reciprocal of the highest dilution yielding no more than 50% hemolysis. Diluent used was the gelatin Veronal-NaCl buffer, pH 7.5.

Antisera. Anti-T sera were obtained from hamsters bearing tumors induced by the SVH-93, a virus-free clone of SV40-induced hamster tumor cells. Each of the sera from individual animals was tested for antibody against "T antigen" using 4 CF units of soluble extract of WT virus-infected AGMK cells. To test for U antibody in the sera, the "T antigen" was heated at 50 C for 30 min and used as the antigen in CF test using 4 CF units of the serum. Sera which demonstrated a reduction of the "T antigen" titer by more than 64-fold were considered to contain no demonstrable amount of antibody against heat-stable U antigen (12) and were used as specific anti-T sera. None of the sera contained demonstrable V antibody against 4 CF units of purified WT virions. The antisera against SV40 V antigen were prepared by inoculating rabbits with a purified "empty" band of WT virus. The antisera had titers of 1:640 or more when tested by CF against 4 CF units of purified WT virions. A specific anti-V serum for CF test was selected from the antisera, which did not contain demonstrable amount of T antibody against 4 CF units of T antigen from T22 cells.

Immunofluorescence tests for T antigen. Monolayer cultures on cover slips were infected with serially diluted virus, which was allowed to adsorb at 37 C for 2 h. The cells were then washed and incubated at the indicated temperatures in medium (Eagle medium supplemented with 2% calf serum) containing cytosine arabinoside (15 μg/ml). The cultures were fixed with cold acetone at 44 h (38.5 and 40.5 C) and 68 h (32.5 and 35 C) postinfection, and stained with fluorescein-conjugated antibodies (23). The number of T antigen-positive nuclei were counted under a fluorescent microscope.

Heat inactivation of T antigen. Portions of 0.2 ml of the T antigen preparation in test tubes were heated at 50 C in a water bath for the times indicated, then cooled rapidly in an ice water bath, and tested for CF antigen titer.

Sucrose density gradient centrifugation. Linear sucrose gradients were prepared with the aid of a mixing device from 5 and 20% (wt/wt) solutions of sucrose in 0.01 M Tris-buffered saline (0.14 M NaCl, pH 7.4) containing 5 mM 2-mercaptoethanol (Wako Pure Chemical Co., Japan). The gradients were carefully overlaid with the T antigen preparations or with the marker SV40 DNA and centrifuged at 42,000 rpm at 4 C for 4 h in an SW50.1 rotor, or at 26,500 rpm at 4 C for 24 h in an SW27 rotor. After centrifugation, fractions were collected from the bottom and each fraction was assayed for T antigen in CF test, or for radioactivity in a liquid scintillation spectrometer (Beckman LS-233) after dissolving it in triton X toluene scintillator.

Marker SV40 DNA. SV40 DNA was used as a marker of known sedimentation coefficient, *S*, values. The WT virus was grown in the presence of 5 μCi of [^{3}H]thymidine per ml (25 Ci/mmol) and was purified by sedimentation onto a saturated KBr solution followed by two cycles of CsCl equilibrium density gradient centrifugation by the method of Uchida et al. (28). The virus DNA was extracted from the band of complete virions according to Pagano (17).

RESULTS

T antigen in infected cells. Table 1 shows that the number of T antigen-positive nuclei in GC7 cells infected with WT virus or *ts* 900 was almost equal at 38.5 and 32.5 C. Because the 38.5 C is the restrictive temperature for transformation or plaque formation by *ts* 900 (29), the results suggest that the mutation is not expressed at the stage of T antigen induction in the lytic cycle. However, the ratio of the number of T antigen-positive nuclei in *ts* 900-infected culture at 40.5 C to that at 35 C was about two-thirds, whereas the ratio in WT-infected cultures was 2.2 (Table 1). In contrast to the

TABLE 1. *SV40 T antigen-positive nuclei in infected GC7 cells*[a]

Virus	Ratio[b]	
	38.5 C/32.5 C	40.5 C/35 C
WT	1.4	2.2
ts 900	1.3	0.66

[a] SV40 T antigen-positive nuclei were scored by immunofluorescence at 44 h (38.5 and 40.5 C) and at 68 h (32.5 and 35 C) postinfection as described in Materials and Methods.

[b] The number of T antigen-positive nuclei per culture was counted and the ratio of the number of positive nuclei at the indicated temperatures was calculated.

results obtained by the immunofluorescence test, no difference between the ratio of the amount of the T antigen induced at 40.5 C to that at 35 C by WT virus and *ts* 900 was demonstrated by complement fixation (Table 2).

Heat-inactivation of T antigen. To investigate whether the T antigen induced by *ts* 900 was identical in its properties to that induced by WT virus, a heat-inactivation test for the T antigen was performed (Fig. 1). The T antigen induced by *ts* 900 at 35 C was inactivated much more rapidly at 50 C than that induced by WT virus. The slopes of the inactivation curves both of WT T antigen and of *ts* 900 were steep at the beginning of the inactivation. The rate of inactivation of the WT T antigen slowed down after 5 min of incubation at 50 C, whereas that of the T antigen induced by *ts* 900 proceeded almost linearly. The results suggest the presence of relatively heat-stable fraction in addition to heat-labile fraction in the WT T antigen preparation, and the lack of the relatively heat-stable fraction in the T antigen induced by *ts* 900. The titer of V antigen in the T antigen preparation from mutant-infected cells was not affected by the heating at 50 C for 30 min.

Heat-inactivation of T antigen fractionated by sucrose gradient centrifugation. The two main molecular weight classes of T antigen reported previously (18) have been observed repeatedly when the T antigen induced by WT virus or *ts* 900 at 35 C was centrifuged through sucrose gradient and assayed by CF (Fig. 2, peaks 1 and 2). However, the larger molecular weight class, peak 2, which sediments with an *S* value of 14 to 15*S*, was not detected in the T antigen extracted from cells infected with *ts* 900

TABLE 2. *Amounts of SV40 T antigen in infected AGMK cells*[a]

Virus	MOI[b]	CF units[c]		Ratio (40.5 C/35 C)
		40.5 C	35 C	
WT	1	32	90.5	0.35
	10	128	362	0.35
ts 900	1	32	90.5	0.35
	10	64	181	0.35

[a] Infected cells were harvested after 28 h of incubation at 40.5 C and after 48 h at 35 C in the presence of cytosine arabinoside (20 μg/ml). The amount of T antigen in the cells was determined in CF test as described in Materials and Methods.

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

[c] The CF titers of the 20% cell extracts are the averages of duplicate titrations

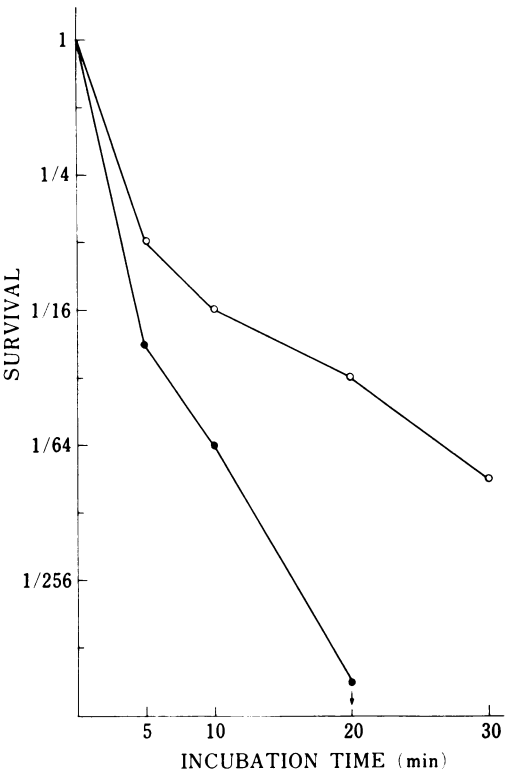

FIG. 1. *Heat inactivation of SV40 T antigen induced by WT virus and ts 900 at 35 C. T antigen was extracted from AGMK cells infected with WT virus* (○) *or ts 900* (●) *and incubated for 48 h at 35 C. Portions of 0.2 ml of the extract in test tubes were heated at 50 C for the times indicated and then cooled rapidly at 0 C. Survival was assayed by CF test.*

at 40.5 C (Fig. 2B). The T antigen species of peaks 1 and 2 in extracts of cells infected with WT virus or *ts* 900 at 35 C were tested for heat lability at 50 C (Fig. 3). No difference in heat stability was noted between the smaller forms (peak 1) of T antigen induced by WT virus or *ts* 900. However, in the case of the peak 2, T antigen obtained from cells infected with *ts* 900 was considerably more heat labile than that obtained from WT-infected cells.

DISCUSSION

The present study was undertaken to identify the mutated gene product of an "early" SV40 mutant, *ts* 900. The results indicated that the antigen extracted from AGMK cells infected with *ts* 900 was more heat labile than that extracted from the cells infected with WT virus, as assayed by CF test using hamster antisera. SV40 can induce V, capsid (C), T, U, and surface antigens in infected AGMK cells (16,

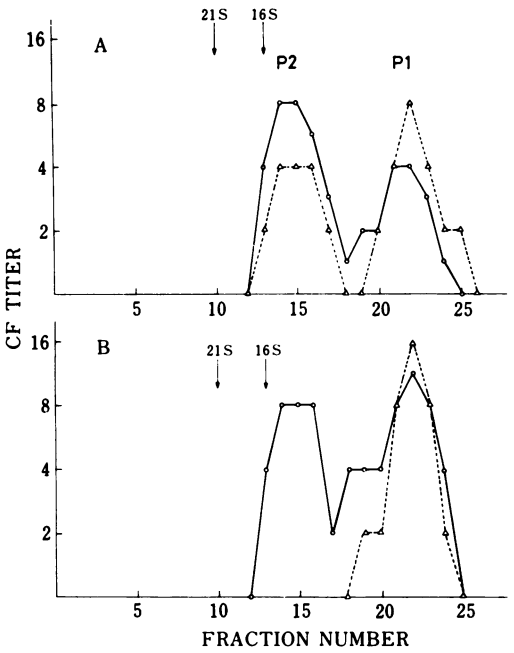

FIG. 2. *Rate zonal centrifugation of SV40 T antigen induced by WT virus and ts 900. T antigen was extracted from AGMK cells infected with WT virus (A) and ts 900 (B) and incubated for 28 h at 40.5 C (Δ) or for 48 h at 35 C (O). The 4.5-ml sucrose gradients (5 to 20%), each with 0.2 ml of the extract or marker SV40 DNA, were centrifuged at 42,000 rpm for 4 h at 4 C in an SW50.1 rotor. Fractions were collected from the bottom and assayed for T antigen or for radioactivity. The peaks of the radioactivity of the marker DNA are shown by the arrows.*

21). The antisera used in the present study were obtained from tumor-bearing hamsters, which had been transplanted with virus-free tumor cells. The sera did not contain detectable amount of antibody against V antigen. Antibody against C antigen was not examined. But C antibody is reported to be produced in animals immunized with disrupted SV40 virions (16), and it is unlikely, therefore, that the sera used in this study contained C antibody. The presence of antibody against U antigen was examined by using as antigen in CF tests the extract of virus-infected AGMK cells which had been heated at 50 C for 30 min. In extract of SV40-infected AGMK cells, U antigen can be demonstrated by a T^+U^+ hamster serum and the titer of U antigen in the extract is usually about one-fourth the titer of T antigen (12). The sera used in this study were those which demonstrated the reduction of at least 64-fold in CF antigen titer after the extract had been heated. Therefore, it is unlikely that the antisera contained antibody against heat-stable U antigen. This evidence may provide further proof that the antisera used did not contain antibody against heat-stable V and C antigens (1, 16). Antibody against surface antigens is present in hamsters which have rejected transplantation of transformed cells, or have been immunized with SV40 virus, as well as in pregnant animals, but it is absent from tumor-bearing animals (8, 26, 27). Because our antisera were obtained from tumor-bearing hamsters, the surface antigens are unlikely to be involved in the CF reaction in the present study. Based on these observations, we are convinced that the extracts of SV40-infected AGMK cells reacted with antibody against T antigen alone.

Sedimentation velocity determinations showed that the extract from AGMK cells infected with WT virus or *ts* 900 at the permissive temperature contain two major molecular forms of T antigen (Fig. 2). This result agrees well with that reported by Potter et al. (18). However, the extract from AGMK cells infected with *ts* 900 at the restrictive temperature did not contain the larger molecular form of T antigen which sediments with an *S* value of 14 to 15*S* (Fig. 2B). Osborn and Weber have reported similar result using a *ts*A mutant of SV40 (M. Osborn and K. Weber, Abstr. Cold Spring Harbor Tumor Virus Meet. 1973, p. 21).

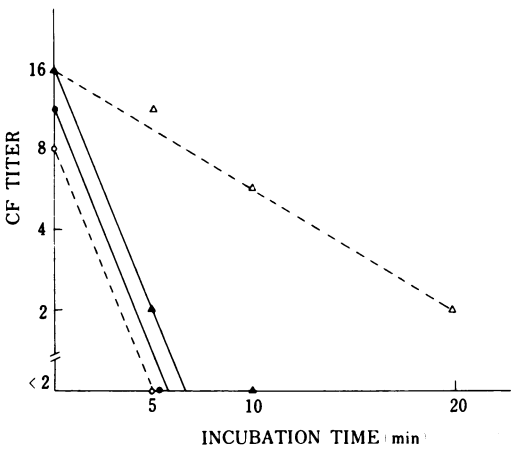

FIG. 3. *Heat inactivation of SV40 T antigen fractionated by sucrose gradient centrifugation. T antigen was extracted from AGMK cells infected with WT virus and ts 900 at 35 C as described in the legend of Fig. 2. The 36-ml gradient (5 to 20%), each with 1 ml of the extracts or marker SV40 DNA, were centrifuged at 26,500 rpm for 24 h at 4 C in an SW27 rotor. Fractions, 1 ml each, were collected from the bottom and assayed for T antigen or for radioactivity. A fraction from each of peak 1 (O, WT virus; ●, ts-900) and peak 2 (Δ, WT virus; ▲, ts-900) (see Fig. 2) was heat inactivated as described in the legend of Fig. 1.*

The 14 to 15S form corresponds to an estimated molecular weight of 340,000 to 390,000 (13). Since SV40 DNA cannot code for a protein of molecular weight larger than 200,000 (21), this species of antigen may be an antigen complex which is composed of an oligomer of the smaller molecular form, or an aggregate with another macromolecule(s). Recently Carrol et al. (4) showed the dissociation of larger molecular species of T antigen to the smallest molecular weight form in high salt concentrations. The 14 to 15S T antigen complex induced by *ts* 900 was more heat labile than that by WT virus (Fig. 3). The results suggest the *ts* gene product is a component of the 14 to 15S T antigen complex and may be best explained as follows: the mutated gene product affects the ability of the components of the antigen complex to make the more heat-stable, larger molecular form, thus the antigen complex induced by the mutant at the permissive temperature is easily converted by heating to the more heat-labile, smaller form. In infected cells incubated at the restrictive temperature, the ability is completely destroyed and the 14 to 15S antigen complex cannot be made. The results, however, do not answer the question whether the *ts* gene product is a protein which determines the antigenicity of T antigen or another protein which may exist in the antigen complex and interact with the antigen-determining protein. Further characterization of individual components of the T antigen complex will answer this question.

The function of T antigen seems to be closely correlated with its molecular forms, but not with its antigenicity because total amount of the T antigen induced by the mutant at the restrictive temperature was not significantly different from that induced at the permissive temperature (Table 2), whereas the 14 to 15S T antigen complex was not formed at the restrictive temperature. The defective function of *ts* 900 is not known. The function is required for transformation of nonpermissive cells and for viral replication in permissive cells (29). If *ts* 900 is a double-step mutant, the gene which affects the heat stability of the T antigen could be a different gene from that which affects both for the transformation and for the viral replication. But this is unlikely because T antigen induced by *ts* 904 and 907, which are grouped into the same complementation group as *ts* 900 (29), was also more heat labile than that induced by WT virus (data not shown). The gene product of *ts* A of SV40, to which group *ts* 900 may also belong (29), is suggested to be an initiator molecule for viral DNA replication itself or an essential component of the initiator (25). But the role of the gene product in transformation has not yet been studied extensively.

At present, attempts to purify T antigen have not been very successful (7, 9, 11, 18). Since the function of the T antigen induced by *ts* 900 is likely to be temperature sensitive, it may serve as a good material to purify and to identify the function of T antigen.

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