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Characterization of polyoma virus T antigen

(rat antiserum against tumor antigen/*Staphylococcus* protein A/ts A mutants/protein modification/cell transformation)

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ABSTRACT **High-titer antiserum raised in rats against the tumor (T) antigen of polyoma virus was used to purify the T antigen by the *Staphylococcus* protein A antibody adsorbent technique. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis allowed the identification of a protein with an apparent molecular weight of 100,000–108,000 as a major component induced in lytically infected mouse cells. In cells infected by ts A mutants this component was temperature sensitive. Several minor components were also observed. In pulse and chase experiments there was a slight decrease in electrophoretic mobility of T antigen during the chase period at the permissive temperature, suggesting that the T antigen is a modified protein. In two lines of transformed cells, the amount of T antigen seemed to be considerably less than in lytically infected cells, but the size of the antigen appeared to be equal.**

It is well established with both polyoma virus and simian virus 40 (SV40) that the part of the genome transcribed early in infection is involved in cell transformation (1). Among the early SV40 products is the tumor antigen (T Ag), which was originally demonstrated by complement fixation assay in SV40 tumors and transformed cells (2). A protein corresponding to T Ag was subsequently identified (3). Recent evidence suggests that at least a part of T Ag is coded for by the early part of the viral genome (4, 5). A similar antigen was detected by complement fixation in polyoma tumors or cells transformed by polyoma virus (6). This T Ag appears to be, at least in part, virus-coded (7). We now report on some of the properties of the polyoma T Ag, which we have studied using an improved methodology.

MATERIALS AND METHODS

Viruses and Cells. Polyoma virus A3 strain (wild type) was obtained from M. Fried (8). Early mutants of polyoma virus (ts A mutants), ts a (9) and ts 25 (10), were obtained from W. Eckhart. The mouse cell line 3T6 (11), and primary baby mouse kidney (BMK) cells were used as permissive hosts for lytic infection. The transformed clones TC1A and PyREWA5 were isolated from the Fischer rat cell line 3Y1 (12) and from secondary Wistar rat embryo cells, respectively, after infection with wild-type polyoma virus. They are both free of infectious polyoma virus. The PyREWA5/T1 line was obtained from a tumor in a Wistar rat injected with PyREWA5 cells. All cells were grown in Dulbecco's modified Eagle's medium containing either 5% fetal calf serum (3T6), 10% calf serum (PyREWA5, PyREWA5/T1, and TC1A), or 10% horse serum (BMK).

Infection. Subconfluent monolayers of 3T6 cells in 90 mm plastic petri dishes (approximately 5×10^6 cells per dish) were inoculated with 0.5 ml of a virus suspension in phosphate-buffered saline at a multiplicity of 10–20 plaque-forming units per cell. The virus was adsorbed to the cells for 90 min at 37° with occasional rocking. At the end of adsorption, 10 ml of Dulbecco's modified Eagle's medium containing 3% horse serum was added. This was considered to be 0 time of infection. Cells were incubated at 32° (permissive temperature), or 39° (nonpermissive temperature) in 10% CO_2. BMK cells were infected similarly at 50 plaque-forming units per cell.

Antiserum against T Ag (Anti-T Serum). The antiserum was prepared using the PyREWA5 line. This line, not tumorigenic during earlier passages, was found to be tumorigenic in Wistar rats when tested at the 30th culture passage (approximately 4.5 months). Cells (10^7) of the tumorigenic PyREWA5 line were inoculated subcutaneously in 6- to 8-week-old rats. Tumors became palpable in about 2 weeks; when they reached 2–3 cm in diameter (6–7 weeks after the inoculation), the animals were sacrificed. Sera from individual animals were collected separately and tested for anti-T activity, using nuclear immunofluorescence of primary BMK cells infected with polyoma virus in the presence of cytosine arabinonucleoside (ara C).

Labeling and Extraction of T Ag. Infected 3T6 cells were labeled with 50 μCi/ml of L-[^{35}S]methionine (275–460 Ci/mmol, The Radiochemical Centre, Amersham) in methionine-free Dulbecco's modified Eagle's medium in the absence of serum, usually for 1 hr at 32°. Chase experiments were performed in the presence of 1100- to 1900-fold excess of unlabeled methionine. The experiments were stopped by adding ice-cold phosphate-buffered saline to the cultures. The cells were washed, scraped off the dish with a rubber policeman, and pelleted. T Ag was extracted from these cells essentially as described by Carroll and Smith (13). Briefly, 150 μl of the following extraction buffer was added per 5×10^6 cells: 20 mM Tris·HCl at pH 8.0, 80 mM NaCl, 20 mM EDTA, 1 mM dithiothreitol, and phenylmethylsulfonyl fluoride at 0.3 mg/ml. After freezing and thawing three times, cell debris were spun down at $12,000 \times g$ for 15 min. The supernatant was used as a source of T Ag.

Protein A Antibody Adsorbent (PAA) Technique. The PAA technique (14) was used for isolating the T Ag. Although a substantial proportion of the anti-T activity did not bind to protein A from *Staphylococcus aureus* there was no qualitative difference in results between the PAA method and the conventional indirect immunoprecipitation. Cell extracts were first treated with a pool of serum from nonimmunized rats (control serum) for 16–20 hr at 0° at the ratio of 50 μl of serum per 150 μl of extract in order to reduce the background. IgG molecules of this serum were removed by adding 200 μl of fixed *Staphylococcus* suspension and pelleting after incubation. The supernatant was then mixed with the specific antiserum. Twenty-five microliters of the anti-T serum with an immunofluorescence titer of 1:400 was sufficient to complex the T Ag in an extract from 2.5×10^6 cells. Routinely, the extract and the anti-T serum were incubated together for 5 min and then for further 15 min at 0° with bacteria [4 volumes of 10% (vol/vol) suspension to one volume of serum]. Increasing the incu-

Abbreviations: T Ag, tumor antigen; PAA, protein A antibody adsorbent; $NaDodSO_4$, sodium dodecyl sulfate; SV40, simian virus 40; BMK, baby mouse kidney; ara C, cytosine arabinonucleoside; M_r, molecular weight.

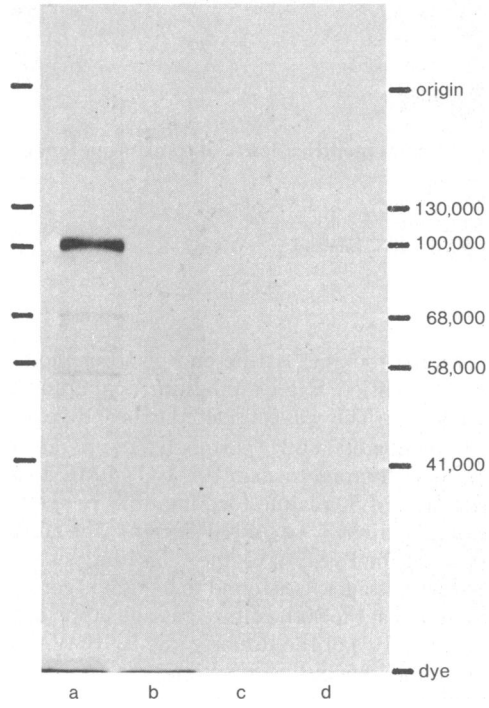

FIG. 1. Identification of polyoma virus T Ag in $NaDodSO_4$/polyacrylamide gel by the PAA technique. 3T6 cells were infected with wild-type polyoma virus and incubated for 42 hr at 32°. Cells were labeled with [^{35}S]methionine for 1 hr and cell extracts were prepared and pretreated with control serum as described in *Materials and Methods*. The extracts were divided into two parts, which were treated with anti-T serum or control serum. Each part was processed by the PAA method as described in *Materials and Methods* followed by electrophoresis in $NaDodSO_4$/polyacrylamide slab gels and fluorography. (*a*) Infected cells, treated with anti-T serum; (*b*) infected cells, treated with control serum; (*c*) mock-infected cells, treated with anti-T serum; (*d*) mock-infected cells, treated with control serum. The migration of molecular weight markers is indicated.

bation time did not increase the amount of T Ag recovered. After incubation, the bacteria were pelleted at 2000 × *g* for 15 min, and washed three times. Then the bacteria were resuspended in electrophoresis sample buffer containing 2% sodium dodecyl sulfate ($NaDodSO_4$), 10% (vol/vol) glycerol, 0.08 M Tris·HCl at pH 6.8, and 2 mM phenylmethylsulfonyl fluoride and the T Ag was eluted for 15 min at room temperature. The eluted sample obtained from approximately 1.5×10^6 cells was applied in a slot of a polyacrylamide slab gel.

Immunofluorescence. The T Ag was stained by the indirect fluorescent antibody technique essentially as described by Fogel *et al.* (15). Fluorescein-isothiocyanate-conjugated rabbit antiserum to rat IgG, (Nordic Pharmaceuticals and Diagnostics) was used at a suitable dilution (up to 5-fold). Rabbit antiserum against virus capsid proteins (anti-V serum) conjugated with fluorescein isothiocyanate was kindly supplied by L. Crawford. The V Ag was stained by the direct method with 3-fold diluted serum for 1 hr at room temperature.

Gel Electrophoresis and Fluorography. Electrophoresis in $NaDodSO_4$/polyacrylamide slab gels was performed in 8.5% separating gel with 5% stacking gel (16) using a discontinuous buffer system (17). Samples were dissolved in sample buffer (see above) containing 5% (vol/vol) 2-mercaptoethanol and heated for 10 min in boiling water. After electrophoresis, gels were treated for fluorography as described by Bonner and Laskey (18). The film used was Kodak RP/R540/X-Omat. Molecular weight (M_r) markers used were (19): β-galactosidase (130,000), phosphorylase *a* (100,000), serum albumin (68,000), catalase (58,000), alcohol dehydrogenase (41,000), carbonic anhydrase (29,000), and myoglobin (17,200).

Table 1. *In vivo* stability of the T Ag at the permissive and the nonpermissive temperature

Virus used	Relative radioactivity: 32° pulse for 1 hr	32° pulse for 1 hr, 39° chase for 1.5 hr	32° pulse for 1 hr, 32° chase for 3 hr
Wild type	100	79	100
ts a	100	18	85
ts 25	100	10	88

T Ag in infected 3T6 cells was labeled 42 hr after infection, and after chase was analyzed in $NaDodSO_4$/polyacrylamide gel electrophoresis as described in *Materials and Methods*. The fluorograms were scanned in a Joyce-Loebl densitometer and the relative densities in T Ag bands were measured as percentage of the density obtained after a 32° pulse without chase.

RESULTS

Specificities of sera

Polyoma-infected BMK cells were used to define the specificity of sera by immunofluorescence. Nuclear fluorescence was detected with anti-V serum in cells infected in the absence of ara C but not in the presence of ara C (20). In contrast, with sera from tumor-bearing animals, cells infected in the presence of ara C gave a classical nuclear staining pattern for T Ag with no staining of nucleoli (21). Mock-infected cells in the presence or the absence of ara C showed no fluorescence with anti-V serum or anti-T serum.

Identification of T Ag induced in lytically infected cells

Extracts of 3T6 cells infected with polyoma virus and radioactively labeled with [^{35}S]methionine were reacted with anti-T serum as described in *Materials and Methods*. The antigen–antibody complexes were adsorbed on fixed *Staphylococcus*. The eluted samples were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. This yielded a rather thick single major band corresponding to a peptide of the apparent M_r of about 100,000 (i.e., equal to that of phosphorylase *a*) (Fig. 1*a*). This band was absent in extracts of uninfected cells (Fig. 1*c*) and did not react with control serum (Fig. 1*b*). This component was present in nuclei isolated from infected cells by the nitrogen cavitation method; however extracts used in this work were routinely made from whole cells. These results suggest that this peptide is the main component of the T Ag.

Results obtained with ts A mutants suggest that this component is specified by the *A* gene of the virus. In fact, as shown in Table 1, its *in vivo* stability in a high-temperature chase is reduced in cells infected by the ts a or ts 25 mutants. In contrast, the majority of the radioactivity of the main component induced by all three viruses persisted after a 3 hr low-temperature chase. These results agree with previous findings (7, 22). They indicate that the main components of the T antigens induced by the ts a and ts 25 mutants either lost their antigenicity at the nonpermissive temperature or degraded more quickly than wild-type T antigen. Essentially the same results were obtained when 20 μg/ml of ara C was present during the chase period (data not shown). Therefore, the difference was not caused by the cessation of viral DNA replication in cells infected by mu-

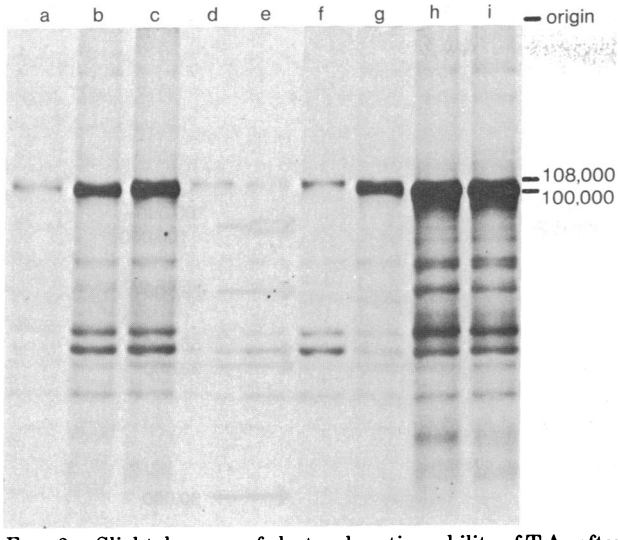

FIG. 2. Slight decrease of electrophoretic mobility of T Ag after chase. 3T6 cells were infected with polyoma virus wild type, ts a, and ts 25 for 42 hr at 32° and pulsed for 1 hr with [^{35}S]methionine at 32°; then they were processed as in Fig. 1. (*a*) Wild type, (*b*) ts a, and (*c*) ts 25. Cells were infected and pulsed as before and chased for 9 hr at 32°; (*d*) wild type, (*e*) ts a, and (*f*) ts 25. Cells were infected as before but at 43 hr after infection a set of cells was shifted to 39° and, 2.5 hr later, pulsed for 30 min; (*g*) wild type, (*h*) ts a, and (*i*) ts 25. The samples were collected from experiments performed at different times; therefore the relative radioactivities of T antigens in different slots were not comparable.

tant virus at the nonpermissive temperature. These results suggest that the main components of the T antigens induced by ts a and ts 25 viruses are altered in such a way as to become temperature sensitive.

Fluctuation of electrophoretic mobility of T Ag

In pulse-chase experiments, it was noted that there was a slight but reproducible difference in mobility of main T Ag component labeled under various conditions. These are shown in Fig. 2 for T Ag induced by wild type, ts a, and ts 25 labeled either for 1 hr at 32°, or for 30 min at 39° 2.5 hr after temperature shift up, or for 1 hr at 32° followed by chase for 9 hr at 32°. The fastest-moving component was that induced by ts a or ts 25 labeled at 39°. The slowest-moving component for all viruses was obtained after a chase of 9 hr at 32°. Components labeled under other conditions in Fig. 2 migrated at intermediate positions. These results indicated that the main T Ag component was being modified in some way during a chase at 32°. Since the T antigens of the mutants were likely to be denatured at high temperature, proper modification might not have occurred. The apparent M_r of the slowest-moving component was 108,000. SV40 T Ag produced in transformed 3T3 cells moves together with phosphorylase *a* (13) and it was confirmed by coelectrophoresis that the slowest-moving component of polyoma T Ag moved more slowly than SV40 T Ag extracted from SV40-transformed 3T3 cells.

Minor components

Longer exposure of films to dried gels revealed other bands specifically recognized by anti-T serum. They retain 10–20% of the total radioactivity complexed by the antiserum. Fig. 3 (an experiment similar to that in Table 1) shows an overexposed film displaying bands of the T antigens induced by wild-type and ts a virus labeled at 32° or labeled at 32° and chased at 39°. TS 25 behaved like ts a. Two of the minor bands induced by the mutant virus (indicated by broken arrows in the figure) also were strongly reduced after the 39° chase. Their apparent M_rs were 67,000 and 63,000. They are probably related to the main T Ag component. In contrast, bands indicated by solid arrows remained essentially unchanged after chase at 39°. Their apparent M_rs were 72,000, 55,000, 52,000, and about 30,000. In some cases, a polypeptide of about 17,200–22,000 daltons was also present. Similar bands were absent when the extracts were complexed to normal serum.

T Ag in polyoma-transformed cells

In polyoma-transformed cells the amounts of T Ag appeared to be far less than in lytically infected cells, both by immunofluorescence and the PAA technique. With serially diluted anti-T serum the end point of immunofluorescent activity occurred at a 10- to 30-fold higher concentration with nuclei of transformed cells than with those of infected BMK cells (Table 2). In preliminary experiment with the PAA technique the two lines of transformed cells PyREWA5/T1 and TC1A each yielded a component that migrated very similarly in NaDodSO$_4$/polyacrylamide gel to T Ag produced in lytically infected cells (data not shown) but was much fainter. We did not observe the marked difference in size recently reported for SV40 T Ag produced in lytically infected cells and in transformed cells (13, 23).

DISCUSSION

We have shown that by using a rat antiserum we can study the polyoma virus T Ag without many of the difficulties that were encountered in the past. By using this antiserum in conjunction with the protein A adsorption technique we have identified the major component of this antigen both in lytic infection and in transformed rat cells as a protein with an apparent M_r of 100,000–108,000 in NaDodSO$_4$ gel electrophoresis, in close agreement with that of SV40 infection (100,000 without cleavage). The size is larger than that previously observed in extracts of polyoma-transformed mouse cells (24). The difference may be attributed to the species or may be related to the smaller component we have also observed. The marked lability of the major component *in vivo* (half life *ca* 30 min at 39°) in cells infected by ts A mutants suggests that at least part of the peptide is specified by the viral *A* gene.

We also observed a number of minor components complexed by the rat antiserum, which migrate more rapidly than the major component in the gel. They are of two groups: A-mutation sensitive, which, in cells infected by ts A mutants, decay like the major component at high temperature, and A-mutation insensitive, which do not appreciably decay under the same circumstances. Whereas those of the former class are probably virus-specified, those of the latter class may or may not be so. In fact, they may represent cellular proteins induced by the virus, or which became antigenic in the tumor. If they are virus-specified they may derive from parts of the genome unaffected by A mutations. It is not known how the minor components originate, whether in the cells or during extraction, and whether they have distinct biological functions.

The observed changes in electrophoretic mobility with time suggest that the main T Ag component undergoes modification. A preliminary experiment has shown that the main component can be labeled by [^{32}P]orthophosphate (data not shown). Therefore, the M_r of the primary gene product is not known and is probably smaller than that deduced from electrophoretic mobility. If the T Ag were to be specified entirely by the viral genome, it would have to be considerably less than 10^5 daltons. In fact, the main T Ag component induced by the polyoma mutant NG18 (25), which has a deletion of 140–150 base pairs

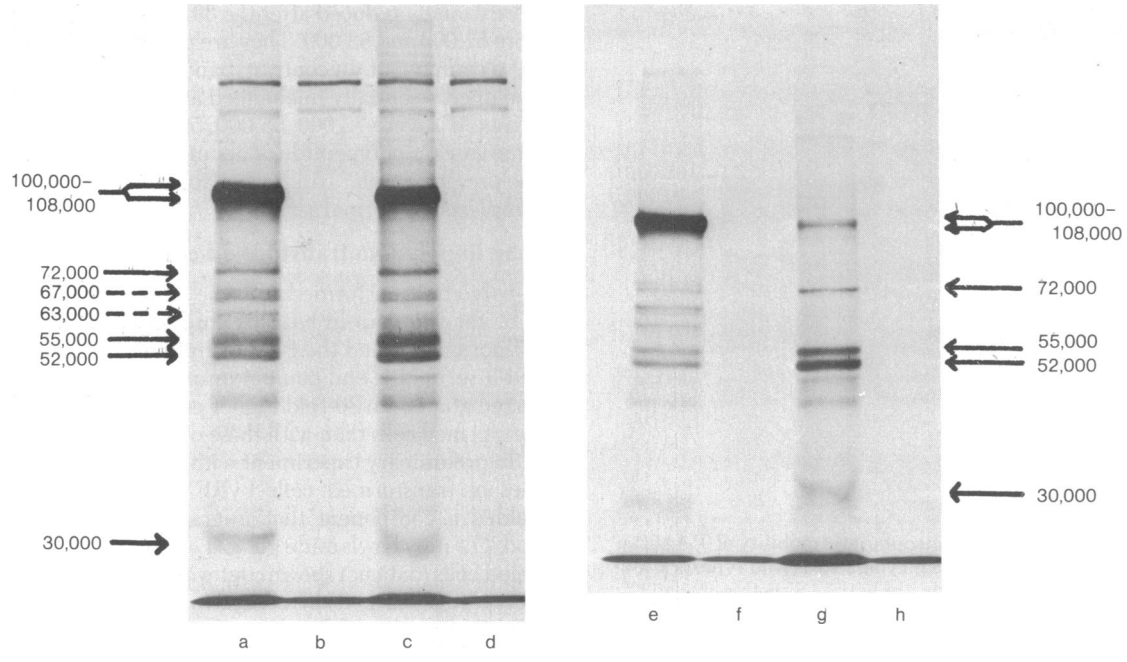

FIG. 3. Minor components complexed by anti-T serum. 3T6 cells were infected with wild type and ts a and incubated at 32° for 42 hr. Cells were pulsed for 1 hr at 32° with [^{35}S]methionine and chased at 39° for 2 hr. The cell extracts were processed as in Fig. 1. The film was exposed until minor components were clearly recognizable. (*a*) Wild type pulsed at 32°, treated with anti-T serum; (*b*) same as (*a*), treated with control serum; (*c*) ts a pulsed at 32°, treated with anti-T serum; (*d*) same as (*c*), treated with control serum; (*e*) wild type pulsed at 32°, chased at 39°, treated with anti-T serum; (*f*) same as (*e*), treated with control serum; (*g*) ts a pulsed at 32°, chased at 39°, treated with anti-T serum; (*h*) same as (*g*), treated with control serum.

in early part of the genome (1), is indistinguishable from that of wild-type virus, although a 5% difference in M_r should be easily detectable in the gels (Y. Ito and M. Fried, unpublished observations). The much reduced amount of T Ag in transformed cells suggests that the primary role of the T Ag is for lytic infection, probably viral DNA replication. The role it plays in transformation is more obscure. Some of the smaller components may be important—for instance, one might correspond to the surface tumor-specific transplantation antigen, which in SV40 is also specified by the early region of the viral genome (26).

Table 2. Immunofluorescence of T Ag of cells lytically infected or transformed by wild-type virus

	Cells			
	Polyoma-infected BMK			
Serum dilution	Without ara C	With ara C	PyREWA5/T1*	TC1A*
1:4	ND	ND	+	+
1:8	ND	ND	+	+
1:16	ND	ND	+	+
1:32	ND	ND	+	+
1:64	+++	+++	+	+
1:128	+++	+++	±	+
1:256	++	++	–	–
1:512	++	+	–	–
1:1024	+	+	ND	ND
1:2048	+	±	ND	ND
1:4096	–	–	ND	ND

The indirect immunofluorescence staining was performed as described in *Materials and Methods*. The anti-T serum was heated for 30 min at 56° before use. Infected BMK cells were incubated at 32° for 48 hr. ND: not done. Relative intensity of fluorescence: +++, strongly positive; ++, positive; +, weakly positive; –, negative.

* Cells were relatively homogenous in terms of the intensity of fluorescence. In some lines of transformed cells (not shown here), the intensity of fluorescence varied very much from cell to cell.

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