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Identification of simian virus 40 tumor and U antigens*†

(immunoprecipitation/*tsA* mutants/polyacrylamide slab gel electrophoresis)

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ABSTRACT **The synthesis and identity of the tumor and U antigens of simian virus 40 (SV40) have been examined during productive infection in monkey cells, abortive infection in mouse cells, and in SV40-transformed mouse cells by using sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis to analyze [^{35}S]methionine-labeled radioimmune precipitates. The following observations were made: (*i*) the tumor and U antigenic sites are on the same 94,000, 89,000, and 84,000 molecular weight species detected during productive infection; a 94,000 species made during abortive infection; and a 94,000 species found in transformed cells. (*ii*) The 94,000 species is relatively unstable compared to the relatively stable 89,000 and 84,000 species produced during productive infection. (*iii*) The stable 89,000 and 84,000 molecular weight species are differentially extracted from productively infected cells, which suggests an intracellular compartmentation and/or different affinities of these species for cellular substrates. (*iv*) The 94,000 species synthesized during abortive infection is more stable than the comparable 94,000 species synthesized in transformed cells. (*v*) Three *tsA* group mutants overproduce several unstable species of tumor antigen at restrictive temperature.**

Simian virus 40 (SV40) tumor (T) antigen is required for the initiation and at least partial maintenance of the SV40 transformed phenotype in animal cells (2–7). The T antigen is the SV40 "*A*" gene product as defined by the *tsA* group of SV40 mutants, and T antigen is encoded in the SV40 genome (8–12). This "transformation" protein does not require the replication of SV40 DNA for its synthesis (13) and is termed an "early" antigen or protein. Lewis and coworkers (14) described another early SV40 antigen, "U" antigen, after examining monkey cells infected with an adenovirus-SV40-hybrid virus, Ad2$^+$ND$_1$. The U antigen had some properties different from T antigen, but U antigen itself was not identified.

The portion of the SV40 genome present in the Ad2$^+$ND$_1$ hybrid corresponds to about 17% of the SV40 genome and maps from 0.11 to 0.28 fractional units on the SV40 map (see ref. 15 for a review). This region includes the carboxyl termini of both T antigen and the major capsid protein, VP1 (16). The protein carrying the U antigenic site(s) in Ad2$^+$ND$_1$ infected cells has a molecular weight of 28,000 to 30,000 (17, 18). This protein is too large to be encoded by either the T antigen or the VP1 regions which are on opposite strands of the SV40 genome sequences. This protein probably is a hybrid of adenovirus and SV40-coded polypeptides.

SV40-specific cytoplasmic RNA has been found in Ad2$^+$ND$_1$ infected cells. This RNA is transcribed from the early strand of SV40 DNA (19, 20), the same strand that encodes the information for T antigen (9, 10). SV40 late-strand RNA has not been detected in Ad2$^+$ND$_1$ infected cells. The U antigenic site(s) should, therefore, be located on the T antigen molecule somewhere in the region between 0.17 and 0.28 fractional units.

I have investigated this prediction using sodium dodecyl sulfate (NaDodSO$_4$)/polyacrylamide slab gel electrophoresis to analyze [^{35}S]methionine-labeled radioimmune precipitates of T, U, and VP1 from SV40 infected monkey and mouse cells and from SV40-transformed mouse cells. The antigenic site(s) for U antigen is only located on the T antigen molecule which indicates that U antigen is encoded in the SV40 genome. A large species of T antigen is "processed" to two smaller stable species during productive infection in monkey cells. This "processing" is not detected in abortively infected or transformed mouse cells. Three *tsA* group mutants do not form stable species of T antigen during productive infection at restrictive temperature.

MATERIALS AND METHODS

Cell Cultures and Virus Infections. Productive infections were performed in TC7 African green monkey kidney cells (21). Abortive infections were performed in BALB/3T3-A31 mouse cells from Richard Roblin. The wild-type SV40 (SV-S) transformed BALB/3T3-A31 line (SEA-45) has been previously described (22). Dulbecco-Vogt modified Eagle's medium, supplemented for TC7 cells as previously described (1), and for mouse cells with 2.0 g of glucose per liter, was buffered at pH 7.4 with 2.0 g of NaHCO$_3$ per liter and 15 mM each of Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), (4-morpholinepropanesulfonic acid), and 2-{[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid. The medium contained either 2% fetal bovine serum (TC7 cells) or 9% calf serum (mouse cells). All virus stocks were grown in TC7 cells at 33° with a multiplicity of infection of 0.001, prepared for infection, and titered as previously described (23). SV-S is the parent of *tsA209* (supplied by Robert Martin, ref. 24) and VA45-54 is the parent of *tsA30* and *tsA58* (supplied by Peter Tegtmeyer, see ref. 8). The cells were infected in suspension with virus (multiplicity of infection = 100–200) or uninfected cell lysate (mock) in a 37° water bath for 90 min with agitation every 15 min. After adsorption, the infected cells were plated into 60 mm petri dishes (Falcon) with 5 ml of medium and incubated at the appropriate temperature.

Radioactive Labeling and Extraction of Proteins. Productively infected TC7 cells, abortively infected BALB/3T3 cells, and the transformed SEA-45 cells were labeled with 100 μCi of [^{35}S]methionine (New England Nuclear Corp., 450–500 Ci/mmol) in 2 ml of methionine-and serum-free medium per 60 mm dish. The cells were rinsed twice with 5 ml of methionine- and serum-free medium prior to the addition of label. Chase experiments were performed by rinsing the dishes twice with 5 ml of complete medium and incubating the cells in an additional 5 ml of complete medium. The precise labeling conditions are indicated in each figure legend. The cells were extracted using 0.70 ml per dish of 1.0% Nonidet P-40 (NP-40)

Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; NaDodSO$_4$ sodium dodecyl sulfate; NP-40, Nonidet P-40.

* *This paper is dedicated to the memory of Gordon M. Tomkins, an inspiring teacher and remarkable man.*

† This is paper number VI in a series. The previous paper was ref. 1.

in Tris-buffered saline at 4° and pH 8.0 as described by Tegtmeyer *et al.* (8). The extraction buffer contained 0.3 mg/ml of freshly prepared phenylmethylsulfonyl fluoride to inhibit protease activity and 0.001 M dithioerythritol. The cells and cell extracts were kept in ice water. The extracted cells were scraped with a rubber policeman, agitated for 10 sec by using a Vortex Genie Mixer at setting no. 9, and centrifuged at 2000 × *g* for 10 min at 4°. The supernatant fluids were centrifuged at 100,000 × *g* for 20 min at 4°.

Immunoprecipitation. The supernatant fluids (0.3 ml) were thawed and/or kept in ice water until the appropriate immunoglobulin was added. The indirect immunoprecipitation technique (8) was employed by using 20 μl of the first serum or IgG and 40 μl of the second IgG per 0.3 ml of fluid. Both additions of immunoglobulin were incubated for 90 min at 25°. The precipitates were analyzed after an additional 18 hr incubation at 4°. Continuous incubation at 4° did not affect the stability or resolution of molecular species. The IgG was prepared by the method of Stulberg and Simpson (25). The antisera and purified IgG preparations used were as follows: *Hamster anti-T IgG (20 mg/ml)* was prepared from sera of adult female golden Syrian hamsters bearing 4- to 5-cm tumors composed of golden Syrian hamster cells transformed with wild-type SV40 (line H65/90B passage 43, supplied by David Porter). This anti-T IgG is U-negative when monitored by immunofluorescence on $Ad2^{+}ND_1$ infected TC7 cells. *Normal hamster IgG (25 mg/ml)* was prepared from sera of litter mates of the hamsters used to prepare the anti-T IgG. *Monkey anti-U serum* was the original serum used by Andrew Lewis to define U antigen (14) and supplied by Dr. Lewis. *Prebleed monkey serum* was the serum derived from the same monkey as the anti-U serum, but prior to the infection with $Ad2^{+}ND_1$ and supplied by A. Lewis. *Rabbit anti-C serum* was the serum previously described (26) and reacts with the SV40 capsid proteins VP1, VP2, and VP3. *Rabbit anti-VP1 serum* was the serum prepared against VP1 material eluted from $NaDodSO_4$/polyacrylamide gels and supplied by Harumi Kasamatsu. *Goat anti-hamster IgG (64/mg/ml), goat anti-monkey IgG (60 mg/ml),* and *goat anti-rabbit IgG (100 mg/ml)* were prepared from antisera obtained from Antibodies, Inc., Davis, Calif. All precipitations were performed at equivalent concentrations. The immunoprecipitates were washed 3 times with 1.0 ml buffer containing 0.06 M Tris·HCl, 15% (vol/vol) glycerol, 2% $NaDodSO_4$, 0.0025% phenol red (wt/vol), 5% 2-mercaptoethanol at pH 6.7, heated at 45° for 20 min, boiled for 10 min, and dialyzed overnight at 25° against two changes of buffer containing 0.06 M Tris·HCl, 0.001M sodium azide, 0.1% $NaDodSO_4$, and 0.1% 2-mercaptoethanol at pH 6.7.

Gel Electrophoresis and Autoradiography. After dialysis, the samples were brought to 20% (vol/vol) glycerol and 0.0025% (wt/vol) phenol red and analyzed by discontinuous polyacrylamide slab gel electrophoresis according to the method of Laemmli and Favre (27). Each tract was loaded with 2–25 μl of sample containing 8,000–25,000 cpm per track. All tracks in any given experiment contained the same amount of radioactivity. The resolving gel contained 0.15 M Tris·HCl at pH 8.8, 10% acrylamide, and 0.13% bisacrylamide. The stacking gel contained 0.06 M. Tris·HCl at pH 6.8, 5% acrylamide, and 0.12% bisacrylamide, according to the method of Blattler *et al.* (28). The electrode buffer contained 0.025 M Tris·HCl, 0.19 M glycine, 1% $NaDodSO_4$ at pH 8.8. Electrophoresis was carried out at 25° for 6 hr at a constant voltage of 110 V. The gels were fixed in 10% trichloroacetic acid, stained with Coomassie brilliant blue G, destained, impregnated with 2,5-diphenyloxazole, dried, and exposed to pre-exposed Kodak RP/R2 Royal X-Omat x-ray film at −70° in a clamp according to the method of Laskey and Mills (29). The film was exposed for 2–6 days. The autofluorograms were scanned with an E-C Apparatus Corp. densitometer. At least one track of standard proteins was included in every slab gel. The standards were β-galactosidase (130,000), phosphorylase *a* (94,000), bovine serum albumin (68,000), catalase (60,000), L-glutamic dehydrogenase (53,000), ovalbumin (43,000), lactic dehydrogenase (36,000), myoglobin (17,000), and cytochrome *c* (13,400). Molecular weights of 49,000 and 43,000 were adopted for the heavy chain of IgG and actin, respectively.

RESULTS

Identification of SV40 U Antigen. The anti-U and anti-T immunoglobulins recognize the same molecular species. Fig. 1 demonstrates the identity of U and T antigens during productive infection in TC7 monkey cells. Both antiglobulins precipitate a 94,000, an 89,000, and an 84,000 molecular weight species. The amount of anti-U serum used in this experiment (20 μl) was not sufficient to precipitate all the available 89,000 and 84,000 species of T antigen. Thirty microliters is sufficient to precipitate all of the available species, and this amount was used in the remainder of the experiments. The appropriate prebleed and normal antiglobulins do not precipitate these species from infected cells nor do the anti-U and anti-T globulins precipitate these species from mock infected cells. Only the anti-C and anti-VP1 globulins specifically precipitate the major capsid protein, VP1. VP1 precipitates by itself, like actin, but at a level of about 5% of the amount specifically precipitated. Fig. 2 demonstrates that a 94,000 species is recognized by both anti-T and anti-U globulins during abortive infection in mouse BALB/3T3 cells and in SV40-transformed BALB/3T3 cells. The 89,000 and 84,000 species observed during productive infection are not detected during abortive infection or in transformed cells, although small amounts of these species would be obscured by the 87,000 cellular protein. Double reciprocal precipitations (Figs. 2 and 3) using first either anti-U or anti-T globulins followed by anti-T and anti-U globulins, respectively, demonstrate that either antiglobulin removes the molecular species recognized by the other and that the immunoprecipitation conditions are at saturation when 30 μl of anti-U serum is used.

The "Processing" of SV40 T Antigen. The production of smaller molecular species of T antigen during productive infection as compared to abortive infection and transformed cells suggests that the 94,000 species is "processed" to the 89,000 and 84,000 stable species. The data in Fig. 3A support this suggestion. It is not known whether the 84,000 species is derived from the 89,000 species or directly from the 94,000 species. Pulses of 1 hr and 2 hr followed by either a 6 hr or 24 hr chase produced similar data.

This amount of "processing" of the 94,000 species does not occur in the abortive infection or in transformed cells (Fig. 2), although small amounts of the 89,000 and 84,000 species would be obscured by the 87,000 cellular protein. The stability of the 94,000 species, however, is different in the two types of infection. During the abortive infection, there is little change in the amount of 94,000 species relative to the amount of the adjacent cellular proteins after the 24 hr chase. The T antigen in the transformed cells, however, almost disappears after the 24 hr chase.

Quantitation of the "processing" of the 94,000 species, or the relative amounts of T antigen during abortive infection or in transformed cells is difficult. The amount of total T antigen precipitated is only 5–15% of the total radioactivity in the track.

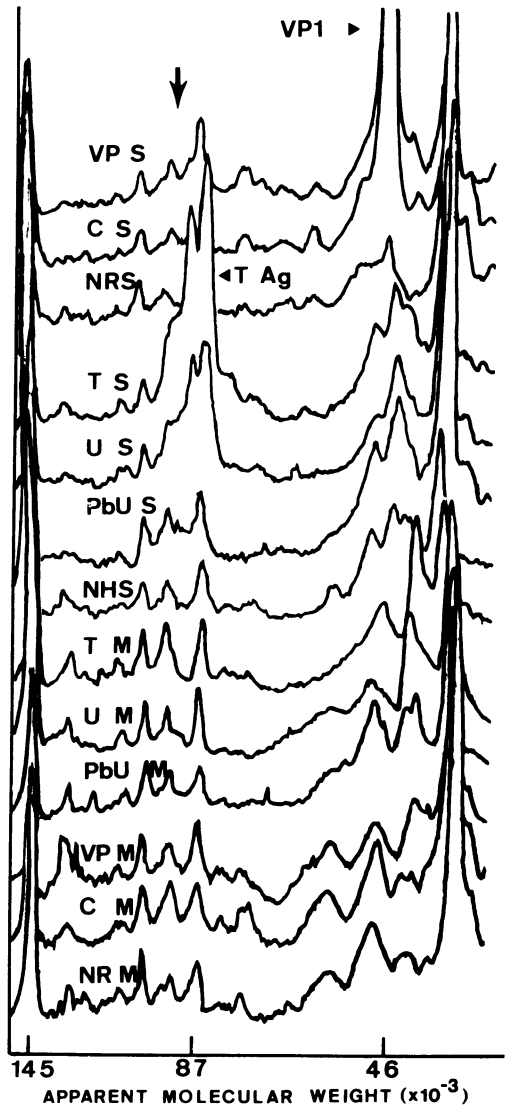

FIG. 1. The identification of SV40 U antigen during productive infection using SV-S wild-type SV40 in monkey TC7 cells at 37°. Cells were labeled with 50 μCi/ml of [^{35}S]methionine in methionine- and serum-free medium from 23 to 25 hr after infection after which the label was diluted to 30 μCi/ml with complete medium from 25 to 48 hr after infection. This labeling method was used to ensure the labeling of all polypeptides containing T, U, and VP1 antigenic sites. The gel was 10% in acrylamide, and tracings of the autofluorograms are presented. The cells were either infected with SV-S wild-type SV40 (S) or mock infected (M). VP, anti-VP1; C, anti-C; NR, normal rabbit; T, anti-T; U, anti-U; PbU, prebleed monkey, and NH, normal hamster. The arrow represents the location of the phosphorylase *a* marker (94,000 molecular weight). Ag, antigen.

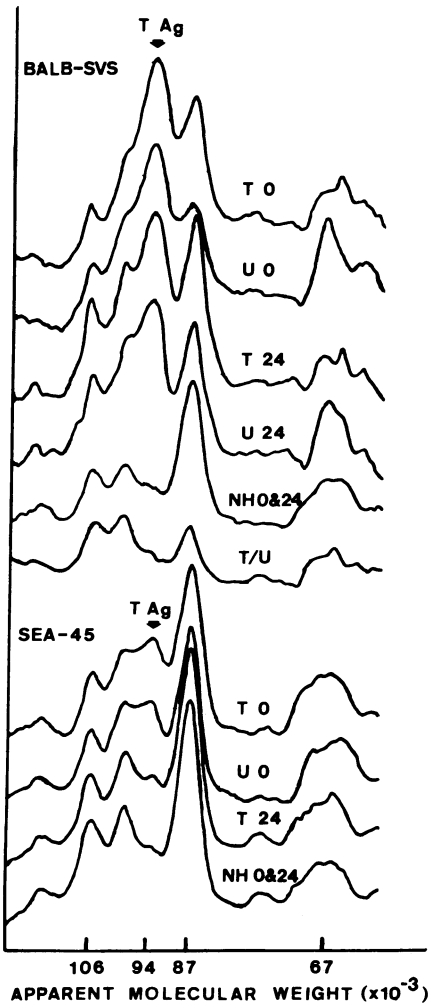

FIG. 2. The identification of SV40 U antigen during abortive infection using SV-S wild-type SV40 in mouse BALB/3T3 cells and in SV-S-transformed BALB/3T3 cells, (SEA-45). The acutely infected BALB/3T3 cells were labeled with 50 μCi/ml of [^{35}S]methionine in methionine- and serum-free medium from 23 to 25 hr at 33°, and then either extracted immediately (0, no chase) or treated for an additional 24 hr in complete medium with 10% calf serum (24, 24 hr chase). The transformed SEA-45 cells were grown to confluency at 33°, removed from the plastic, and replated at 50% of confluency. After 24 hr at 33°, the cells had reached 75% of confluency and were labeled and extracted in the same manner as the acutely infected BALB/3T3 cells. The gels were 10% in acrylamide. T, anti-T; U, anti-U; and NH, normal hamster. T/U is the double reciprocal immunoprecipitation, where the lysate was first precipitated with anti-U or anti-T globulins and then precipitated a second time with anti-T or anti-U globulins, respectively. The tracing is of the second precipitation, which was similar for both combinations. Ag, antigen.

The amount of T antigen relative to cellular protein bands and the amounts of the 94,000, 89,000, and 84,000 species relative to each other, are somewhat variable from experiment to experiment. This variability is probably due to the lability of the 94,000 species during extraction as discussed in the *Discussion*.

Possible Intracellular Compartmentation of the T Antigen Species. If the 89,000 and 84,000 stable species of T antigen are in different intracellular compartments and/or have different affinities for the cellular substrate(s) with which they are associated, then the two species might be differentially extracted from the infected cell. Fig. 3B demonstrates a differential extraction of the two species. The 84,000 species is selectively extracted when the cells are treated by the NP-40 method of Tegtmeyer (8). Freezing the cells in NP-40 does not change the extraction profile, and sonic disruption of the cells in NP-40 only slightly increases the amount of 89,000 species. Sonic disruption of cells frozen in NP-40, however, extracts equal amounts of both species. The fact that both the 89,000 and 84,000 species are stable and present in similar amounts is compatible with the suggestion that the two species have different intracellular locations and/or functions.

Inability of Three *tsA* Mutants to Produce Stable Species of T Antigen at Restrictive Temperature. The processing of T antigen during productive infection with three independent

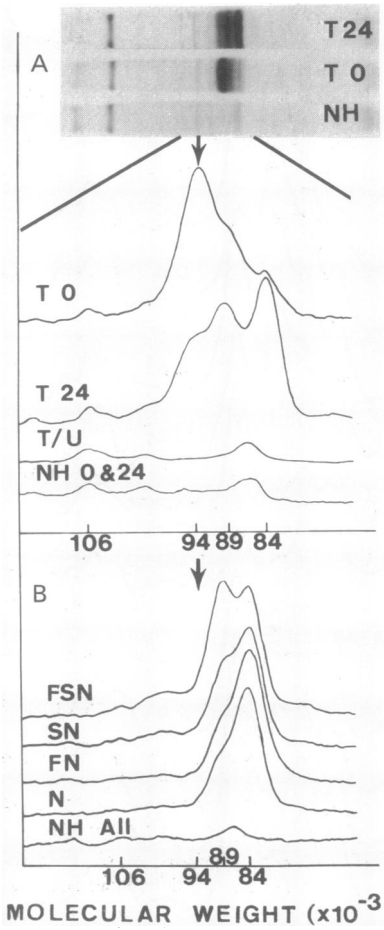

FIG. 3. The processing and differential extraction of T antigen during productive infection using SV–S wild-type SV40 in TC7 cells. Cells were labeled with 50 μCi/ml of [^{35}S]methionine in methionine- and serum-free medium from 23.5 to 24 hr after infection at 33°. This time of labeling and incubation temperature were selected to investigate the synthesis of T and U antigen during the "early" phase of SV40 infection before the synthesis of VP1 had begun. (A) Infected cells were either extracted immediately (0, no chase) or chased for 24 hr in complete medium with 2% fetal bovine serum (24, 24 hr chase). The abbreviations are the same as in Fig. 2. (B) Cells were labeled and chased for 24 hr as in Fig. 3A. After the chase, the cells were extracted by using four different methods and assayed for T antigen. N, the standard method of Tegtmeyer using NP-40 described in *Materials and Methods*. FN, the cells were frozen at −20° immediately after addition of the NP-40 extraction mixture. SN, the cells were sonically disrupted after being scraped in the NP-40 extraction mixture (75 W, 30 sec, in ice water with a cup probe in a Bronwill Biosonik IV sonicator). FSN, the cells were frozen in the NP-40 extraction mixture as in FN, scraped, and sonically disrupted as in SN. All frozen extracts were thawed in ice water. The FN, SN, and FSN extracts were then further processed in parallel with the N extracts. The arrows represent the location of the phosphorylase *a* marker (94,000 molecular weight). NH is normal hamster.

tsA group mutants that are structural mutants of T antigen, was examined at 41.5°. Fig. 4 demonstrates that the three mutants are not able to form stable species of T antigen after a 24 hr chase. At least six unstable species were observed after a 1 hr pulse. These species are similar for all three mutants except the 60,000 species which is missing in the *tsA209* infection. This observation may be explained by the recent finding of Rundell, Collins, and Tegtmeyer (12) that the 94,000 species of T antigen produced during productive infection by the SV-S (parent of *tsA209*) and VA45-54 (parent of *tsA30* and *tsA58*) wild-type strains of SV40 are not identical.

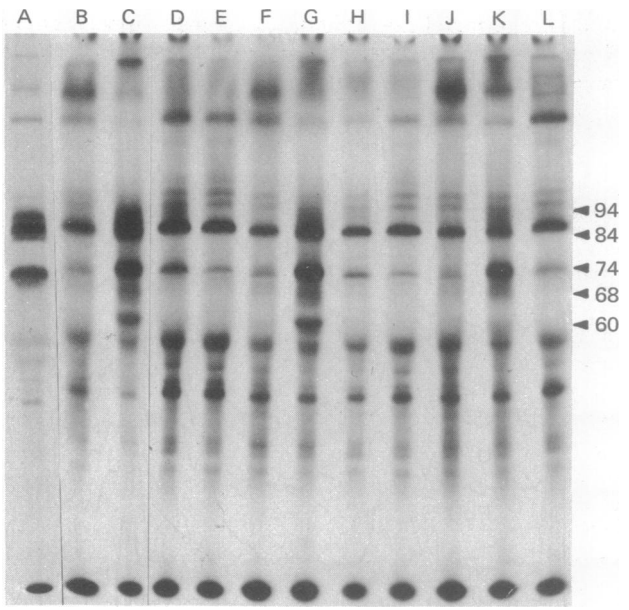

FIG. 4. The processing of T antigen in *tsA* mutant infected TC7 cells at 41.5°. Cells were labeled with 50 μCi/ml of [^{35}S]methionine in methionine- and serum-free medium from 23 to 24 hr after infection. Each dish was individually handled using medium and phosphate-buffered saline at 42° on a hot plate at 42°. The cells were either extracted immediately or chased for 24 hr at 41.5° in complete medium with 2% fetal bovine serum. The gels were 10% in acrylamide. The sample order is: (A) wild-type VA45-54, T antigen, no chase; (B) *tsA30*, NH, no chase; (C) *tsA30*, T antigen, no chase; (D) *tsA30*, T antigen, 24 hr chase; (E) *tsA30*, NH, 24 hr chase; (F) *tsA58*, NH, no chase; (G) *tsA58*, T antigen, no chase; (H) *tsA58*, T antigen, 24 hr chase; (I) *tsA58*, NH, 24 hr chase; (J) *tsA209*, NH, no chase; (K) *tsA209*, T antigen, no chase; and (L) *tsA209*, T antigen, 24 hr chase. The apparent molecular weight is given for the unstable T antigen species (×10^{-3}). NH refers to normal hamster.

DISCUSSION

The SV40 T and U antigenic sites are in the same molecular species during productive infection in monkey cells, abortive infection in mouse cells, and in SV40-transformed mouse cells. The U antigen is, therefore, encoded in the early region of the SV40 genome, because T antigen is encoded in this region (8–12). No U antigenic sites are detectable in VP1. Thus, the prediction made earlier that the U antigenic sites(s) would be in the same molecular species as the T antigenic site(s) is confirmed. The possibility that I have observed a low level of anti-T activity in the anti-U serum rather than the major anti-U activity is remote for the following reason: I have not detected any other U-specific polypeptide when the precipitates were analyzed on 7.5%, 12.5%, 15%, 20%, 25%, or 7.5–25% gradient slab gels and film exposures of 1–20 days. A small amount of anti-T activity is not likely to precipitate such a large amount of specific polypeptides, while the major anti-U activity precipitates no detectable polypeptide.

A relative thermal lability of T antigen is the only difference between T and U antigens during productive infection in monkey cells or in transformed cells. The two antigens are intranuclear and morphologically identical when examined by immunofluorescence during SV40 productive infection. During Ad2^{+}ND$_1$ productive infection, however, U antigen is present in a perinuclear location, and T antigen is not detectable by immunofluorescence. One explanation for the difference between T and U antigens in SV40 infected cells is that the U antigenic site(s) is located in the portion of the molecule from 0.17 to 0.28 map units, while the T antigenic site(s) is located

in another portion of the molecule. Separable subpopulations of T and U species are unlikely to exist because anti-T globulin removes all the detectable U-reactive species from cell extracts, and vice versa (Figs. 2 and 3).

A 94,000 species of T antigen is present during productive and abortive infection and in transformed cells, but only in the productive infection is this species "processed" to 89,000 and 84,000 stable species (Fig. 3). Although the 94,000 species is relatively unstable, some is still present after a 24 hr chase. At least two explanations for this "processing" of the 94,000 species exist that are not mutually exclusive. (*i*) The large species is proteolytically degraded during the extraction process to form the 89,000 and 84,000 species which do not have any functional importance during infection. (*ii*) The 94,000 species is a precursor for the 89,000 and 84,000 species, which do have a functional importance during infection. Tegtmeyer *et al.* and Rundell *et al.* (11, 12) have recently provided evidence that the majority of the "processing" of the 94,000 species to the 89,000 and 84,000 species is probably an artifact of extraction. They showed that when the cytoplasm is removed before extraction, the 94,000 species within nuclei of productively infected TC7 cells remains relatively intact during extraction. One or more cytoplasmic factors are probably responsible for the "processing". Norkin and Ouellette (30) have shown that lysosomal enzymes "leak" into the cytoplasm within the first 24 hr after SV40 infection at 37° in monkey cells. The pulse-chase data in Fig. 3A can be explained by the relative absence of lysosomal proteases at 24 hr (post-infection) at 33° during the pulse, and the presence of proteases at 48 hr (post-infection) during the chase extraction. Ahmad-Zadeh *et al.* (31) and Carroll and Smith (10) have observed a similar "processing" of the 94,000 species.

A small amount of the 89,000 and 84,000 stable species may have a functional role during productive infection. This possibility has not been rigorously eliminated, and the stability of the 89,000 and 84,000 species is intriguing. This hypothesis is still attractive because of the developing evidence for the intracellular compartmentation of T antigen: probable presence in the plasma membrane (32–34); in ribosomes as the adenovirus helper function (33, 35); and in the cellular chromatin as a regulator of cellular DNA synthesis (36, 37). Multiple functional species of T antigen, a transformation protein, might help explain the "pleiotypic response" of cellular metabolism to infection by SV40 described by Tomkins and coworkers (38).

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