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# Monomer molecular weight of T antigen from simian virus 40-infected and transformed cells

**(immunoprecipitation/*in vitro* protein synthesis/two-step cleavage/proteolysis inhibitors)**

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**ABSTRACT** T-antigens from simian virus 40 (SV 40)-transformed and lytically infected cells have been isolated by immunoprecipitation and their molecular weights estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. T-antigen from SV40-transformed mouse and hamster cells has an apparent molecular weight of 94,000 whereas that from several lines of SV40-infected monkey cells is 84,000. In a wheat germ cell-free system, mRNA from either transformed or productively infected cells is translated into a 94,000 species. Experiments with the protease inhibitors L-1-(tosylamide-2-phenyl)ethylchloromethyl ketone HCl and *N*-α-*p*-tosyl-L-lysylchloromethyl ketone HCl suggest that the 84,000 species of T-antigen found in infected cells is derived from the larger species by proteolytic cleavage. Further, the cleavage pathway probably involves a two-step reaction with an 89,000 intermediate. The biological significance of the two molecular weight forms of T-antigen is unknown, but the possibility that they have different physiological activities is discussed.

The T or tumor antigen of simian virus 40 (SV40) can be detected by complement fixation or immunofluorescence in cells lytically infected (1) or transformed (2) by SV40. The appearance of T-antigen in lytic infections occurs before the onset of viral DNA synthesis and is not inhibited if DNA synthesis is stopped by FdUrd or arabinosylcytosine (3–5). The function of T-antigen is not known and the protein must, at present, be assayed immunologically. However, some of the biochemical properties of T-antigen have been described. It is known to be labile to heat and protease treatments (3). It aggregates to form multiple sedimenting species (6, 7), and binds to double-stranded DNA (7–9).

T-antigen appears to be dependent on the viral *A* gene function because mutation of the gene leads to the production of an antigen with altered properties. At the nonpermissive temperature in cells infected with *A* gene mutants of SV40 the rate of synthesis of T-antigen is changed (10); the ability of the antigen to fix complement becomes thermolabile (11–13); the affinity for DNA decreases (14); and the aggregation of T-antigen to the larger of the multiple sedimenting forms is inhibited (11, 15). Thus, T-antigen probably mediates the biological activity of the *A* gene in the initiation of both viral (16, 17) and host (18) DNA synthesis in permissive cells and in initiation (16, 19–21) and maintenance (20–24) of transformation in nonpermissive cells. The binding of T-antigen to the origin of DNA replication on SV40 DNA (9) is consistent with the hypothesis that T-antigen directly controls the initiation of replication of viral DNA.

SV40 DNA codes for an early mRNA species, which is also found in transformed cells and which has a sedimentation coefficient of about 19 S (27–29). Such a messenger could only code for about 90–100,000 daltons of protein. In addition to T-antigen, the presence of the early region appears to be required for the induction of two other antigens, TSTA (a cell surface antigen) and U (a heat stable perinuclear antigen) (30). A precise measurement of the molecular weight of T-antigen is therefore required before (*a*) the proportion of the viral DNA required to code for such a protein can be calculated and (*b*) the additional sequences in the early region of SV40 DNA that might be available to code for the other early antigens can be estimated. To date, estimates of the molecular weight of the monomeric form of T-antigen have varied between 70,000–100,000 (6, 10, 31).

In this work we have used the "sandwich" immunoprecipitation technique first used in studying T-antigen by Gilden *et al.* (3) and first used in conjunction with sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis by Tegtmeyer (10). We have characterized T-antigen from SV40-infected and transformed cells and also that synthesized in a cell-free system from wheat germ primed with various mRNAs isolated from the same cells.

## MATERIALS AND METHODS

**Materials.** [$^{35}$S]L-Methionine (160–500 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. L-1-(tosylamide-2-phenyl)ethylchloromethyl ketone (TPCK) and *N*-α-*p*-tosyl-L-lysylchloromethyl ketone HCl (TLCK) were from Sigma. Hamster anti-T serum was prepared as previously reported (7). Normal hamster serum was obtained from golden hamsters, and rabbit anti-hamster IgG antiserum from Nordic Immunological Laboratories. Wheat germ was a generous gift from Prof. A. Kohn. The origin of all the other components used for cell-free protein synthesis has been described (34).

**Cell Cultures and Viral Infections.** As permissive cell lines, we used African green monkey cells (AGMK), both secondary AGMK and various established cell lines of CV1s: the TC7 clone (32), a clone obtained from L. Crawford, and another from M. Fried. The nontransformed nonpermissive cells used were Balb/c 3T3. Two established SV40-transformed cell lines were used: the hamster cell line SV28 (33) and a line of Balb/c 3T3 cells which had been freshly transformed with the SV40-SP1 strain by Dr. Y. Ito of this laboratory.

**Labeling and Extraction of Proteins.** Cells were grown in 90 mm Nunc petri dishes and were labeled for 24 hr at 37° with 12.5–25 μCi of [$^{35}$S]methionine/ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cells were harvested by detaching them with EDTA, pelleted, and extracted with pH 8.0 extraction buffer (20 mM Tris·HCl at pH 8.0, 80 mM NaCl, 20 mM EDTA, 1 mM dithiothreitol, and 0.3 mg/ml of phenylmethylsulfonyl fluoride) at a ratio of 1 part packed cells to 2.5 parts buffer. The suspension was twice frozen

Abbreviations: AGMK, African green monkey cells; $NaDodSO_4$, sodium dodecyl sulfate; *TPCK*, L-1-(tosylamide-2-phenyl)ethylchloromethyl ketone; *TLCK*, α-*p*-tosyl-L-lysylchloromethyl ketone.

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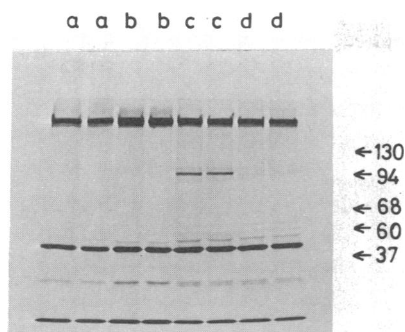


FIG. 1. T-antigen from transformed cells. The electrophoretic mobilities of proteins immunoprecipitated from Balb/c 3T3 and SV3T3 cell extracts. The [$^{35}$S]methionine-labeled soluble proteins were immunoprecipitated, subjected to NaDodSO$_4$-polyacrylamide gel electrophoresis, and visualized by autoradiography as outlined in *Materials and Methods*. The gel was 8.5% in acrylamide. (a) Balb/c 3T3 proteins immunoprecipitated with normal hamster serum. (b) Balb/c 3T3 proteins immunoprecipitated with anti-T serum. (c) SV3T3 proteins immunoprecipitated with anti-T serum. (d) SV3T3 proteins immunoprecipitated with normal hamster serum.

and thawed and then centrifuged for 30 min at 20,000 × *g*. The cellular debris was discarded and the supernatant used for immunoprecipitation.

**Cell-Free Protein Synthesis.** Most of the procedures used for cell-free protein synthesis have been described previously (34, 35). To summarize, mRNA was isolated from lytically infected monkey cells (CV1) 40 hr after infection with SV40 (strain SP1) and from transformed hamster cells (SV28) by lysing them in a buffer containing naphthalenedisulfonic acid (1%) and Nonidet 40 (NP40) (0.5% vol/vol) followed by extensive phenol: chloroform extraction of the post nuclear supernatant. Poly(A) containing RNA was purified by chromatography on poly(U)-Sepharose and translated in the wheat germ cell-free system essentially as described by Roberts and Paterson (36) except that incubations also included 50 $\mu$M spermine and 250 $\mu$M spermidine. The $Mg^{++}$ concentration for cell-free synthesis was 1.5 mM and each 50 $\mu$l incubation contained 1–3 $\mu$g of mRNA. After 3 hr incubation at 22° the reaction mixture was centrifuged at 10,000 × *g* for 10 min, a small aliquot of the supernatant taken to determine the incorporation of [$^{35}$S]methionine in TCA insoluble material, and the remainder used for immunoprecipitation.

**Immunoprecipitation.** Immunoprecipitation was carried out according to the method of R. Kurth (personal communication). Extracts (0.1–0.2 ml) of cells labeled *in vivo* were incubated in siliconized plastic tubes for 16 hr at 4° with 0.1 ml of hamster anti-T serum or normal hamster serum and 0.35 ml of bovine serum albumin (1 mg/ml in phosphate-buffered saline). Nordic rabbit anti-hamster IgG (0.7–0.8 ml) was added and the tubes incubated an additional 2–4 hr at 4°. The tubes were then centrifuged at 2500 × *g* at 4° for 5 min, and the resulting precipitates washed four times in 0.2 M Tris·HCl at pH 7.5, 50 mM NaCl, and 0.05% (wt/vol) deoxycholate. The immunoprecipitate was immediately treated with NaDodSO$_4$ electrophoresis sample buffer. Supernatants from the cell-free incubations were treated by a scaled down method, using only 20–30 $\mu$l aliquots.

**Gel Electrophoresis and Autoradiography.** Immunoprecipitates were boiled for 10 min in a solution containing 2% (wt/vol) NaDodSO$_4$, 2% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 0.08 M Tris·HCl at pH 6.8, 0.001% bromphenol blue, and 2 mM phenylmethylsulfonyl fluoride. Electrophoresis was carried out according to the method of Studier (37) in discontinuous slab gels. The gels were either 15, 10, 8.5, or 7.5% (wt/vol) in acrylamide (as indicated in the figure legends). The gels were stained with Coomassie blue, impregnated with 2,5-diphenyloxazole (PPO), dried, and exposed to Kodak RP/R540/X-Omat film at −70° in a clamp, according to the method of Bonner and Laskey (38). Exposure was for 1–7 days. The molecular weights assumed for the standards according to Osborn and Weber (39) were: $\beta$-galactosidase (130,000), phosphorylase *a* (94,000), bovine serum albumin (68,000), catalase (60,000), and yeast alcohol dehydrogenase (37,000). A value of 49,000 was assumed for the heavy chain of rabbit IgG.

## RESULTS

### The apparent molecular weight of SV40 T-antigen upon NaDodSO$_4$ polyacrylamide gel electrophoresis

The antiserum used in the experiments described here was first tested and found to have the properties expected of a serum specific for T-antigen. Thus, the antiserum reacted specifically with a nuclear antigen present in virus-infected cells when examined by immunofluorescence microscopy and the antigen with which it reacted was thermolabile in complement fixation tests.

The immunoprecipitation of [$^{35}$S]methionine-labeled proteins from an extract of SV40-transformed Balb/c 3T3 mouse cells with anti-T serum, yielded several proteins which were separated by NaDodSO$_4$ polyacrylamide gel electrophoresis as shown in Fig. 1. When extracts from nontransformed cells were treated in the same manner a similar pattern of proteins was obtained with one striking exception; a protein which migrated with the same mobility as phosphorylase *a* was present in the transformed cells but not in the nontransformed cells. This protein was not immunoprecipitated by normal hamster antisera whereas the other background proteins were. When the mobility of the protein from the transformed cells was compared with those of standard proteins an apparent molecular weight of 94,000 was obtained (data not shown). Since the 94,000-molecular-weight protein was the only one present in the immunoprecipitate with the expected properties of SV40 T-antigen, that is, it reacted specifically with anti-T serum and was present only in virus-transformed cells, we tentatively identified it as T-antigen and further studied its occurrence.

The same immunoprecipitation procedure was applied to extracts of monkey cells (CV1) lytically infected with SV40, and a protein was again specifically immunoprecipitated. Furthermore, this protein was not detected in uninfected cells, nor was it precipitated by normal hamster serum (Fig. 2). However, the protein from lytically infected cells with the properties expected of T-antigen migrated more rapidly than phosphorylase *a* on gel electrophoresis, and its molecular weight was estimated to be 84,000.

A direct comparison of T-antigen obtained from transformed cells with that obtained from infected cells (Fig. 3) confirmed that the two species had different electrophoretic mobilities, and consequently different apparent molecular weights. Further, the molecular weight of the protein specifically immunoprecipitated from the transformed cells was the same, 94,000, in both hamster and mouse. On the other hand, the 84,000 species which was immunoprecipitated from the infected cell extract (Fig. 3) has been detected in all the extracts of lytically infected monkey cells that we have examined. These include the TC7 clone and two other clones of CV1 cells from two different laboratories, and secondary AGMK cells.

We also examined T-antigen present in cells infected with two different virus strains and immunoprecipitated by different

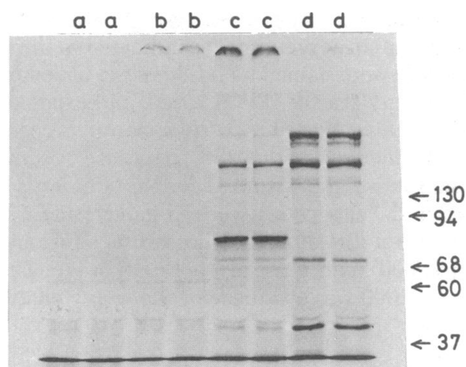


FIG. 2. T-antigen from infected cells. The electrophoretic mobilities of proteins immunoprecipitated from CV1 and SV40-infected CV1 cell extracts. The [$^{35}$S]methionine-labeled proteins were immunoprecipitated, subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis, and visualized by autoradiography as outlined in *Materials and Methods*. The gel was 7.5% in acrylamide. (a) CV1 proteins immunoprecipitated with normal hamster serum. (b) CV1 proteins immunoprecipitated with anti-T serum. (c) Proteins from SV40-infected CV1 cells immunoprecipitated with anti-T serum. (d) Proteins from SV40-infected CV1 cells immunoprecipitated with normal hamster serum.

antisera. The 84,000 species has been obtained from monkey cells infected with either the SP1 or S strains of SV40 virus. The same SV40 SP1 strain was used by Dr. Y. Ito to transform Balb/c 3T3 mouse cells to produce the SV3T3 line. Thus, the same virus strain induced the 94,000 species in the transformed mouse cell and 84,000 species in the lytically infected monkey cell (Fig. 3). Different batches of sera from hamsters bearing SV28 induced tumors have also been used and in every case have caused the immunoprecipitation of the 94,000 protein from transformed cells and the 84,000 proteins from infected cells.

## Cell-free synthesis of T-antigen

It is now possible to synthesize a wide variety of viral proteins *in vitro*. We have used a wheat germ cell-free system, which we have previously shown to synthesize accurately polyoma and SV40 virus capsid proteins (35), to make [$^{35}$S]methionine labeled proteins in response to mRNA isolated from both lytically infected (CV1) and transformed (SV28) cells. The electrophoretic mobility of the polypeptides immunoprecipitated by anti-T sera from the products made *in vitro* has been compared with the mobility of T-antigen immunoprecipitated from extracts of infected and transformed cells (Fig. 4). A very high background

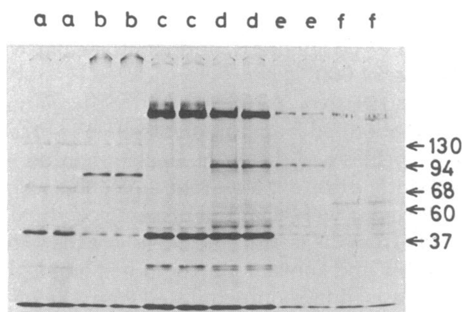


FIG. 3. Comparison of the electrophoretic mobilities of T-antigen from SV40-transformed and infected cells. The gel was 8.5% in acrylamide. (a) SV40-infected CV1 versus normal hamster serum. (b) SV40-infected CV1 versus anti-T serum. (c) SV3T3 versus normal hamster serum. (d) SV3T3 versus anti-T serum. (e) SV28 versus anti-T serum. (f) SV28 versus normal hamster serum.

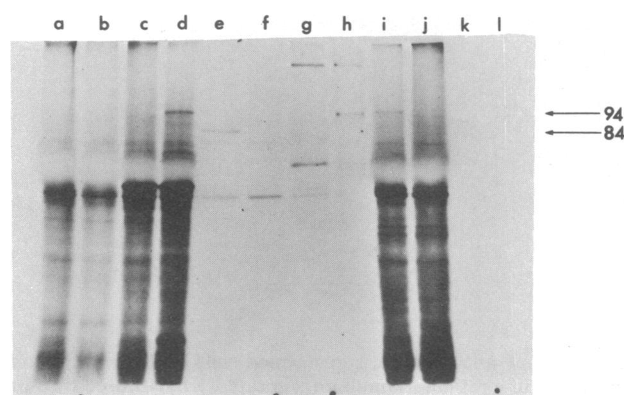


FIG. 4. Immunoprecipitation of T-antigen produced *in vivo* in SV40-infected and transformed cells and synthesized *in vitro* using messenger RNA from infected and transformed cells. In all cases the proteins were labeled with [$^{35}$S]methionine. (a) Mock infected CV1 mRNA *in vitro* product versus normal hamster serum. (b) Mock infected CV1 mRNA product versus anti-T serum. (c) SV40-infected CV1 mRNA product versus normal hamster serum. (d) SV40-infected CV1 mRNA product versus anti-T serum. (e) SV40-infected CV1 proteins labeled *in vivo* versus anti-T serum. (f) SV40-infected CV1 proteins labeled *in vivo* versus normal hamster serum. (g) SV28 proteins labeled *in vivo* versus normal hamster serum. (h) SV40 proteins labeled *in vivo* versus anti-T serum. (i) SV28 mRNA *in vitro* product versus anti-T serum. (j) SV28 mRNA *in vitro* product versus normal hamster serum. (k) No added mRNA *in vitro* product versus anti-T control. (l) No added mRNA *in vitro* product versus normal hamster serum control. The gel was 15% in acrylamide.

of labeled proteins was present in the immunoprecipitates of all the cell-free incubations containing added mRNA. However, one protein was specifically immunoprecipitated by anti-T sera from the proteins made *in vitro* on SV28 cell mRNA and this had a similar mobility as T-antigen from transformed cells. When the corresponding protein made *in vitro* on SV40-infected CV1 mRNA was compared with that made in lytically infected cells a clear difference in mobility was detected. As shown above, lytically infected monkey cell T-antigen has an apparent molecular weight of 84,000 whereas mRNA from the same cells directed the synthesis of a 94,000 protein species. Uninfected cell mRNA produced no detectable proteins that were specifically precipitated by anti-T sera.

We have not yet shown by peptide fingerprinting that the 94,000 presumed T-antigens made *in vitro* are identical to the corresponding species found in transformed cells. However, as they are specifically immunoprecipitated and have a similar electrophoretic mobility it would seem reasonable to suppose that the proteins made *in vitro* and *in vivo* are related, if not identical. This would imply that the mRNA for T-antigen from both transformed and infected cells is first translated as a 94,000 protein which, in lytically infected monkey cells, is subsequently modified to the faster migrating species. Presumably the modification does not occur in the wheat germ cell-free system, nor does it occur in transformed mouse or hamster cells.

## Evidence for proteolytic cleavage of T-antigen from SV40-infected monkey cells

Of the several possible modifications that could be responsible for the change in mobility of T-antigen from lytically infected cells, proteolytic cleavage seemed the most probable explanation. Labeling CV1 cells at different times after infection failed to reveal a larger precursor at any time between zero and 48 hr (data not shown). However, since it was necessary to use a 12 hr labeling period to accumulate sufficient labeled material,

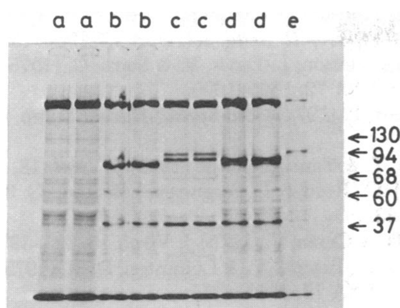


FIG. 5. The effect of treatment with TPCK and TLCK upon the electrophoretic mobility of T-antigen from SV40-infected CV1 cells. CV1 cells were infected with SV40 strains SVS at multiplicity of infection = 10. At 24 hr post infection, either TPCK (20 $\mu$g/ml) or TLCK (50 $\mu$g/ml) was added to some of the cultures. Thirty min later, [$^{35}$S]methionine was added (12.5 $\mu$Ci/ml) and the cells were exposed to the inhibitors and labeled for 24 hr. Harvesting, immunoprecipitation, electrophoresis, and autoradiography were carried out as described in *Materials and Methods*. The gel was 8.5% in acrylamide. (a) Uninfected CV1 versus anti-T serum. (b) Infected CV1 versus anti-T serum. (c) TPCK-treated infected CV1 versus anti-T serum. (d) TLCK-treated infected CV1 versus anti-T serum. (e) SV28 versus anti-T serum.

a rapidly metabolized precursor would not be detected. We therefore examined the effects of inhibitors of proteolysis on the production of presumed T-antigen. SV40-infected CV1 monkey cells were treated with TPCK or TLCK and soon afterward labeled with [$^{35}$S]methionine for 24 hr after which cellular extracts were prepared and immunoprecipitated. Treatment with TLCK led to the production of the 84,000 dalton protein plus an intermediate 89,000 daltons species (Fig. 5). Treatment with TPCK led to the appearance of a similar 89,000 species and the 94,000 daltons species characteristic of transformed cells. These data strongly suggest that the smaller forms of T-antigen are derived from the larger by proteolytic cleavage, probably in a two-step reaction.

This conclusion is further supported by our failure to detect other possible modifications that might otherwise be responsible for the apparent difference in molecular weights. Thus far, we have no evidence to suggest that T-antigen is glycosylated, and although T-antigen is phosphorylated (R. B. Carroll, manuscript in preparation; Tegtmeyer, personal communication), both the transformed cell and infected cell species are so modified.

## DISCUSSION

In this work we have used an immunological precipitation method to identify those proteins from SV40-infected and transformed cells that react with antibody formed by hamsters carrying SV40-induced tumors. Only one protein which was specifically immunoprecipitated from SV40-transformed hamster and mouse cells by anti-T sera and was not present in untransformed cells was detected and this protein we refer to as T-antigen. T-antigen from transformed hamster and mouse cells has an apparent molecular weight of 94,000. By contrast T-antigen isolated by the same method from monkey cells lytically infected with SV40 virus has an apparent molecular weight of 84,000.

We suggest that the smaller molecular weight species of T-antigen found in lytically infected cells is derived from the larger molecular weight form, and that the conversion between the two forms is probably caused by proteolytic cleavage. T-antigen made *in vitro* in a wheat germ extract primed with mRNA from either SV40-infected monkey cells or SV40-transformed hamster cells has a similar electrophoretic mobility to T-antigen from transformed cells. Furthermore, the addition of inhibitors of proteolytic enzymes to infected cells during the labeling period leads to the production of the 94,000 and 89,000 forms of T-antigen. Thus the primary significance of the results reported here is that the molecular weight of T-antigen made in both SV40-infected and SV40-transformed cells is 94,000. Other more rapidly sedimenting species of T-antigen (3, 6, 7, 11, 15) are probably aggregates either through self-association or association with some other molecular species, and smaller species found in infected cells are probably derived from the primary translation product, by a two-step proteolytic cleavage.

The mobility on polyacrylamide gel electrophoresis of T-antigen made *in vitro* is virtually the same as that isolated from transformed cells, and no larger forms of T-antigen were isolated from cells treated with inhibitors of proteolytic enzymes. Thus we have no evidence for higher molecular weight precursors to the 94,000 form of T-antigen, such as those described for other viral proteins (40). Furthermore, we have shown that the mRNA which directs the synthesis of T-antigen *in vitro* can be purified by preparative hybridization to a restriction enzyme fragment of SV40 DNA coming from the early region (*Hae*III fragment A), and that the active mRNA species sediments on denaturing sucrose gradients containing 85% formamide at about 18 S (Smith, Wheeler, and Carroll unpublished results). This argues that SV40 T-antigen is a virus-coded molecule of molecular weight 94,000 that is the product of the early region of the viral DNA. This is in agreement with the conclusions of Tegtmeyer (10) based on the analysis of the T-antigen present in cells infected with SV40 containing a temperature-sensitive mutation in the *A* gene. If the molecular weight of T-antigen is 94,000, then almost the entire coding capacity of the DNA in the early region would be used up in coding for this one protein. This suggests that the TSTA and U antigens are (*a*) antigenic determinants on the same polypeptide as T-antigen, (*b*) cleavage products of the 94,000 species, or (*c*) host-coded species which are induced by the virus.

We do not yet know the biological significance of the difference in molecular weight between T-antigen from transformed and lytically infected cells. It could be that the two forms are necessary for lytic infection and the failure by transformed cells to cleave at least a proportion of the T-antigen molecules is responsible for their being nonpermissive. Such a model might predict that the early stages of lytic infection and the initiation and maintenance of transformation require the 94,000 form, whereas the switch to the late phase of replication requires cleavage. It should be noted, however, that we have not detected any change in the mobility of T-antigen at different times during lytic infection.

On the other hand, the two forms of T-antigen may be an artefact of the extraction method. For example, the cleavage of T-antigen from infected cells may occur only after cell lysis. We have attempted to test this directly by adding TPCK to cells just prior to harvesting in order to prevent only those cleavages that might occur after lysis. However, this procedure inhibited the subsequent immunoprecipitation reaction. Presumably the TPCK used in the experiment described in Fig. 5 entered the cells during the labeling period and irreversibly modified those proteases with chymotrypsin-like substrate specificity but was itself exhausted before the cells were harvested. This would explain both why no 84,000 dalton T-antigen was detected in the TPCK-treated lytically-infected cells and why immunoprecipitation could occur, but it does not enable us to decide if the enzyme which cleaves T-antigen and which was inhibited by this procedure, acts in the cell or only after cell lysis. Other experiments in which labeled extracts from transformed cells

were mixed with equal volumes of unlabeled extracts from lytically infected cells prior to immunoprecipitation showed that some cleavage of the transformed cell 94,000 daltons protein took place. Presumably the cleavage was catalyzed by a protease present in the infected cell extract. But again this experiment is difficult to interpret, because it is possible that although cleavage normally occurs in whole cells the proteolytic enzymes may remain active after extraction.

Again we do not know if the significant difference between cells yielding the different forms of T-antigen is the fact they are either transformed or lytically infected. It is possible that quite by chance monkey cells contain a protease or lack a protease inhibitor, but that its presence or absence is quite irrelevant to the activity of T-antigen in whole cells. Perhaps its presence only manifests itself upon cell lysis. It is difficult to test whether the cleavage is specific to monkey cells or lytic infection because of the limited host range of SV40. Another trivial explanation is that since T-antigen binds to viral DNA and that such DNA is only made in permissive cells perhaps only T-antigen bound to DNA is susceptible to cleavage. The experiments described here and others not mentioned in detail which were intended to decide between these various alternative models for the cleavage of T-antigen have so far proved ambiguous.

We are indebted to the Cell Production Department of the Imperial Cancer Research Fund for the production of SV28 and SV3T3 cells. We thank Kit Osborn for supplying CV1 cells, Cynthia Dixon for performing immunofluorescence tests on the sera, and Tricia Wheeler for excellent technical assistance. We are indebted to Dr. R. Dulbecco and many other colleagues, especially Dr. W. Mangel, for frequent and fruitful discussions throughout the course of this work. When the work began R.B.C. was a Special Fellow of the Leukemia Society of America.

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