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Extraction and Fingerprint Analysis of Simian Virus 40 Large and Small T-Antigens

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A study of simian virus 40 (SV40) T-antigens isolated from productively infected CV1 cells using a variety of different extraction procedures showed that under some conditions the highest molecular weight form of T-Ag (large-T) isolated comigrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with large-T from SV40-transformed H65-90B cells. Other faster-migrating forms of large-T are probably generated during the extraction procedure by a protease which is active at low pH, and such forms are probably experimental artifacts. After extraction under conditions which minimize proteolytic degradation of large-T, a further form of T-antigen was isolated; this has an apparent molecular weight in the range 15,000 to 20,000 and is referred to as small-t. Fingerprint analysis of [^{35}S]methionine-labeled SV40 proteins showed that small-t has 10 to 12 methionine peptides whereas large-T has 15 to 18 methionine peptides. All but two of the methionine tryptic peptides present in small-t are also present in large-T. The fingerprint data also showed that T-antigens have no peptides in common with SV40 VP1. Experiments using reagents which inhibit posttranslational cleavage of encephalomyocarditis virus polyproteins showed that these reagents do not affect the synthesis of small-t and suggest that it is not made by proteolytic cleavage of large-T in vivo. An alternative model, which proposes that large-T and small-t are synthesized independently, is discussed in terms of the fingerprint data and the number of methionine tryptic peptides predicted from the primary sequence of SV40 DNA.

Simian virus 40 (SV40) T-antigen (T-Ag) can be detected, using antiserum from hamsters bearing SV40-induced tumors, in extracts of cells that are either productively infected with or transformed by SV40 virus (3, 30). The antigen is produced early in the productive infection cycle and is, at least in part, the product of the early region of SV40 DNA (26-28, 33). T-Ag has been implicated as an important control element in the synthesis and transcription of SV40 DNA during the productive infection cycle (2, 8, 37, 40), and it is also probably involved in both the initiation and maintenance of the SV40-induced transformed state (4, 20, 23, 38). T-Ag binds to DNA, and appears to bind specifically to SV40 DNA at or near the origin of DNA replication (6, 14, 31). This property may well be the basis of its biological activity. However, until recently, experiments to characterize T-Ag and to determine more precisely its function at the biochemical level have been hampered by difficulties in isolating and purifying the antigen. These problems have been partly solved by using immunoprecipitation techniques.

T-Ag can be isolated from labeled cell extracts by forming an antibody-antigen complex with anti-T serum and collecting the complex either by using immunoprecipitation with a second antibody (40) or, more recently, by adsorption to protein A present on the surface of *Staphylococcus aureus* (16). The antigen can be examined after disrupting the antibody-antigen complex by polyacrylamide gel electrophoresis followed by autoradiography. By using such methods several proteins have been detected, and these have apparent molecular weights ranging from 15,000 to almost 100,000 (1, 7, 26-29, 32, 39, 40). Here we report experiments which examined the parameters involved in the generation of some of these different forms of T-Ag and which analyzed the different forms by peptide mapping. We conclude that all the different forms of T-Ag examined share common methionine tryptic peptides, that some forms are generated by proteolytic cleavage during the extraction procedure, and that two forms of T-Ag, the so-called large-T and small-t, are synthesized independently.

MATERIALS AND METHODS

Production of antisera. Anti-T serum was produced by injecting SV40-transformed hamster cells

into golden hamsters. Cell lines used were H65-90B and FLSV (10). Initial screening tests indicated that, by using these cell lines, the serum from all the tumor-bearing animals was positive in the immunoprecipitation reaction described below. Subsequently, serum from up to 50 animals was taken without screening, pooled, and stored frozen in aliquots.

Growth of cells and virus. Stocks of SV40 seed virus were grown in CV1 P1 cells using low multiplicities of infection of plaque-purified SV40 of the SVS strain obtained from Paul Berg (10). Productive infection was studied in CV1 cells, which were maintained at 37°C in 30-mm or 90-mm Nunc plastic dishes. At confluence, the dishes contained approximately 10^6 and 10^7 cells, respectively. The cells were infected at a multiplicity of 20 to 50 PFU/cell just before they reached confluence and were maintained in Dulbecco-modified Eagle medium (E4) containing 5% fetal calf serum (7, 10).

Encephalomyocarditis (EMC) virus was grown in 3T6 cells using a multiplicity of infection of 5 to 10 as previously described (5a).

Labeling and extraction of cells. SV40-infected CV1 cells were labeled at 24 to 72 h postinfection using 0.5 to 1.0 ml (30-mm dish) or 1.5 to 2.0 ml (90-mm dish) of E4-methionine containing 5% fetal calf serum and 80 to 400 μCi of [^{35}S]methionine (Radiochemical Centre, Amersham; specific activity, 500 to 1,000 Ci/mmol). During the labeling period (30 min to 4 h), the dishes were gently rocked at 37°C (Denly Rocker model A610). After labeling, the cell layer was rinsed with phosphate-buffered saline, and the cells were removed with Versene. After washing twice in Tris-buffered saline, the cell pellet was lysed by vigorous suspension in a buffer (0.25 ml per 10^6 cells) containing 50 mM Tris-hydrochloride (pH 8.0), 120 mM NaCl, and 0.5% Nonidet P-40. Other buffers tested are mentioned in the legend to Fig. 1; freeze-thawing as described (7) was also tested as an extraction method. After extraction, the labeled cell lysate was centrifuged at 12,000 × *g* for 5 min, and the supernatant was fast-frozen and stored at −70°C. [^{35}S]methionine-labeled SV40 virus was grown and purified as described by Ozer (24).

Immunoprecipitation. Before immunoprecipitation, the cell extracts and antisera were centrifuged at 8,000 × *g* for 5 min. To 80 μl of extract, 20 μl of NET buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 7.5], and 0.05% Nonidet P-40, sometimes also containing 1 mg of bovine serum albumin per ml) and 20 μl of normal sheep serum were added, and incubation was continued at 22°C for 1 h. A 20-μl volume of a 10% suspension of protein A-bearing *S. aureus* bacteria (16) were then added. After 15 min at 22°C, the bacteria were removed by centrifugation at 12,000 × *g* for 30 s. The supernatant was removed and divided into two samples (~60 μl) to which 10 μl of either hamster anti-T or normal hamster serum was added. After incubation for 60 min at 22°C, 10 μl of *S. aureus* was added, and incubation was continued for 15 min at 22°C. The bacteria were recovered by centrifugation (12,000 × *g*, 30 s) and washed twice in NET buffer, and finally the labeled antigens were recovered by elution with 20 to 25 μl of gel electrophoresis starting buffer (7, 10, 42).

Polyacrylamide gel electrophoresis. The conditions for polyacrylamide gel electrophoresis using the method of Laemmli (18), drying the gels, and autoradiography have all been described in detail (7, 10, 27, 42). Gels from which labeled proteins were recovered for fingerprinting were wrapped in Saran wrap and exposed wet for 6 to 24 h. ^{14}C-labeled marker proteins were prepared by reacting standard proteins (β-galactosidase [Worthington], [molecular weight 130,000]; phosphorylase *a*, 94,000; fructose 6-phosphate kinase, 81,000; catalase, 60,000; glutamate dehydrogenase, 53,000; creatine phosphokinase, 40,000; and lysozyme, 14,000; all purchased from Boehringer except where noted) with ^{14}C-labeled iodoacetic acid (Radiochemical Centre; specific activity, 57 mCi/mmol) by the method of Gibbons and Perham (11). The molecular weights assumed for the markers after carboxymethylation are given above.

Fingerprinting of labeled proteins. Labeled proteins to be fingerprinted were eluted from the polyacrylamide gel slices by vigorous shaking at 37°C overnight in a buffer containing 50 mM ammonium bicarbonate, 0.1% sodium dodecyl sulfate, and 0.1% β-mercaptoethanol. The eluate (5 ml) was passed through a glass fiber filter, 50 to 200 μg of rabbit immunoglobulin G (Miles) was added as carrier, and 100% trichloroacetic acid was added to 20%. After 45 min at 0°C, the precipitate was collected by filtration through a siliconized glass fiber filter, washed with 20% trichloroacetic acid (100 ml), chloroform (100 ml), and ether (10 ml), and eluted twice with 1 ml of formic acid as described (27). The eluted proteins were lyophilized, and, after redissolving in 100 μl of formic acid, 25 μl of methanol and 40 μl of performic acid were added. After 2 h at −5°C, 3 ml of water was added, and the sample was lyophilized overnight. The oxidized protein was digested with 1:20 (wt/wt) L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) for 5 h at 37°C. The digests were then lyophilized three times.

Fingerprints were prepared on Kodak plastic-backed cellulose thin-layer plates. Electrophoresis was at pH 2.1, 4.5, or 6.5, and chromatography used butanol-acetic acid-water-pyridine (30:6:24:20). Details of all these procedures have been described (42).

Partial proteolysis fingerprints were prepared using a modification of the method of Cleveland et al. (9). Strips of polyacrylamide gel containing the protein of interest were cut from undried gels and ground in a small homogenizer in a buffer containing 10 mM sodium bicarbonate, 0.1% sodium dodecyl sulfate, 0.1% β-mercaptoethanol, and 20% sucrose. The sample was divided into aliquots (25-μl), and 5 μl of *Staphylococcus* protease V8 (Miles) at different concentrations was added. After 60 min at 37°C, 10 μl of gel electrophoresis starting buffer was added, and the gel suspension was loaded directly into the slots of a polyacrylamide gel and subjected to electrophoresis as described above.

Treatment of cells with protease inhibitors. EMC virus-infected 3T6 cells were labeled 4 h postinfection, and SV40-infected CV1 cells were labeled 48 h postinfection. The protease inhibitors used were TPCK (20 μg/ml), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (50 μg/ml), *N*-CBZ-L-phenylalanine

chloromethyl ketone (ZPCK) (25 μg/ml), canavanine (33 mM), acetidine (5.4 mM), fluorophenylalanine (2.5 mM), and $ZnCl_2$ (0.1, 0.5, and 1.0 mM) (5, 13, 36). These were added 10 min prior to addition of labeled methionine. After the labeling period, EMC-infected cells were lysed and prepared for electrophoresis as described (5a), and SV40-infected CV1 cells were extracted for subsequent immunoprecipitation as described above.

RESULTS

Extraction of SV40 large-T from productively infected monkey cells. We and other authors have previously shown that, using some extraction conditions, SV40 T-Ag isolated from transformed hamster cells has a greater apparent molecular weight than T-Ag isolated from productively infected monkey cells (1, 7, 32, 39). We also concluded that the smaller form of T-Ag from the monkey cells, which has an apparent molecular weight of 84,000, was generated by a two-step proteolytic cleavage of a larger form similar to the 94,000-dalton T-Ag from transformed cells (7). However, at that time we were unable to establish whether the putative cleavage of T-Ag occurred physiologically inside productively infected monkey cells, in which case it might have been significant, or whether it occurred only during or after extraction from the cells. The interpretation of our earlier results was also hindered by a variable background of proteins, such as actin and myosin, which precipitated nonspecifically during the immunoprecipitation procedure.

We have modified our immunoprecipitation regime to include a preabsorption step using normal sheep serum to remove proteins likely to precipitate nonspecifically. In addition, we now use the protein A method to collect the antibody-antigen complexes (16). Figure 1 shows an autoradiograph of a 7.5% polyacrylamide gel of SV40 T-Ag extracted from [^{35}S]methionine-labeled, infected CV1 cells under a wide variety of conditions and precipitated using the new method. Various forms of T-Ag can be seen, the largest of which migrated on this gel system between the ^{14}C-labeled carboxy-methylated phosphorylase *a* and fructose 6-phosphate kinase markers (tracks 2, 3, 4, 9, and 13). At least two other forms of T-Ag were detected, depending on the extraction conditions, and these migrated with (track 8) or slightly faster than (track 1) the ^{14}C-labeled carboxy-methylated fructose 6-phosphate kinase marker.

The factor which most strongly influenced the mobility of the extracted T-Ag was the pH of the extraction buffer. In an extraction solution containing only 100 mM NaCl and 0.5% Nonidet P-40, the isolated T-Ag migrated as a single band, faster than fructose 6-phosphate kinase (track 1), and addition of 50 mM Tris-hydrochloride (pH 8.0) buffer resulted in the larger form (track 2). The presence of dithiothreitol appeared to reduce the yield of T-Ag (track 5), but this effect could be partially reversed by the addition of EDTA (track 6). Other factors tested, such as magnesium ions, calcium ions, glycerol, and EDTA in the absence of dithiothreitol, had little effect on the subsequent mobility of the isolated T-Ag.

Methods involving breakage of cells by freeze-thawing rather than detergent lysis often generated the smaller forms of T-Ag (tracks 10 to 13). This effect could be partially reversed by using a larger volume of buffer (2 ml per 10^7 cells) to extract the cells (compare tracks 10 and 11). Some previous freeze-thaw methods using only small volumes of buffer (250 μl per 10^7 cells) do not have sufficient buffering capacity to cope with the acid released from the cells upon breakage. The effective pH during extraction was much less than pH 8.0, and the smaller forms of T-Ag, in only low yield, resulted. Freeze-thawing per se is not detrimental, since freeze-thawing cells already lysed in a buffer containing Nonidet P-40 still resulted in the largest form of T-Ag (compare tracks 3 and 13).

By sequential extraction of cells, for example, using extraction at pH 8.0 followed by extraction at pH 6.0 or vice versa, we have found that reextraction does not release substantial amounts of additional T-Ag, either of the same mobility as that released during the first extraction or of a different mobility. It does not appear, therefore, that there are several populations of T-Ag in cells, each with a different mobility and each released preferentially by the different extraction procedures, but rather that there is one pool from which all the different forms are extracted. Tegtmeyer et al. (39) have reached the same conclusion. In other experiments we have shown that the effect of buffer on the subsequent mobility of T-Ag takes place during the extraction period after cell breakage and prior to removal of cell debris by centrifugation. After this step, incubation of the cell extract at pH 6.0 either before or during the immunoprecipitation reaction had little effect on T-Ag (data not shown). We conclude from these experiments that the agent causing the change in mobility of T-Ag is activated or released by low pH and dithiothreitol and is probably particulate.

The protein seen in Fig. 1 migrating between ^{14}C-labeled carboxy-methylated glutamate dehydrogenase and creatine phosphokinase comigrates with virion protein VP1 (see below). This protein is often present in immunoprecipitates,

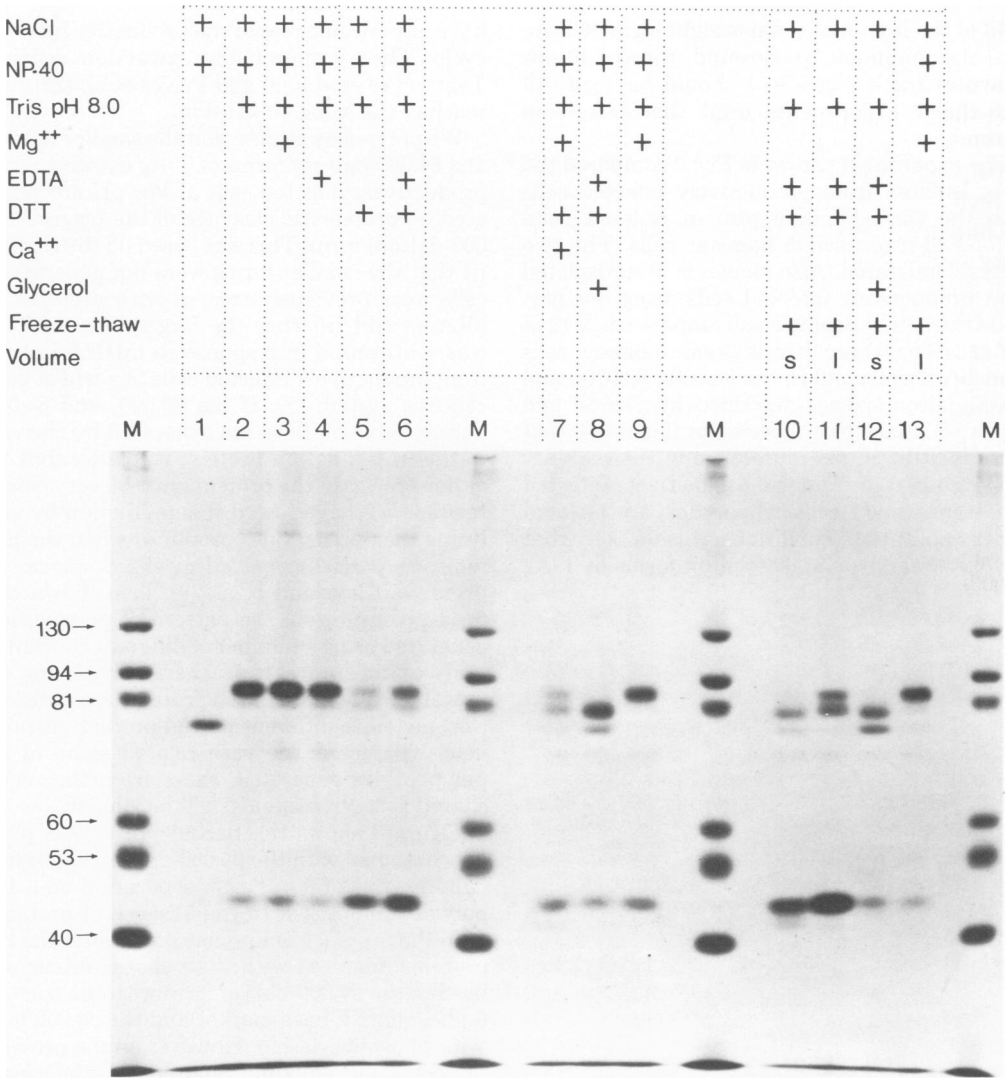

FIG. 1. *Isolation of T-Ag from CV1 cells infected with SV40 using different extraction buffers. Infected CV1 cells were extracted 48 h postinfection either with 2 ml of detergent-containing buffer per 10^6 cells (1 to 9) or by freeze-thawing (10 to 13). The composition of the various buffers is given in the figure, and the concentrations of the reagents were as follows: NaCl, 100 mM; Nonidet P-40 (NP-40), 0.5%; Tris-hydrochloride (pH 8.0), 50 mM (except tracks 7, 8, and 12, 20 mM); $MgCl_2$, 0.5 mM; EDTA, 20 mM; dithiothreitol (DTT), 1 mM (tracks 8 and 12) and 10 mM (tracks 5, 6, 7, 10, and 12); $CaCl_2$, 1 mM; glycerol, 10%. s indicates a small extraction volume (250 μl per 10^6 cells); l indicates a large extraction volume (2 ml per 10^6 cells). Samples of 10 and 50 μl were taken from extracts using small and large extraction volumes, respectively, and immunoprecipitated using hamster anti-T serum and the protein A method. The samples were separated on a 7.5% polyacrylamide gel, which after electrophoresis was dried and autoradiographed for 2 days. The numbers indicate the molecular weights (× 10^3) of the markers (M).*

even those using extracts previously absorbed against nonspecific serum of one species and subsequently precipitated with control serum of a second species. However, most of the other proteins (for example, actin and myosin) that sometimes nonspecifically precipitate during immunoprecipitation reactions are removed by the preabsorption step. Figure 1 shows that the amount of VP1-like material extracted and subsequently precipitated varied widely with the extraction conditions, and in some cases (tracks 6 and 11) resulted in a very high background of contaminating VP1-like protein. We conclude from the results shown in Fig. 1 that for the best

yield of the high-molecular-weight form of T-Ag with the minimum background, the conditions shown in track 2 of Fig. 1 should be used. All subsequent experiments used this extraction method.

The experiment shown in Fig. 2 compared the T-Ag isolated from productively infected cells with the corresponding protein isolated from H65 SV40-transformed hamster cells. The two T-Ags comigrated. Also shown is T-Ag isolated from productively infected cells using our previous extraction conditions (compare track 12 of Fig. 1). The three bands from monkey cells probably correspond to the 94,000-, 89,000-, and 84,000-dalton species described by Carroll and Smith (7). Since this experiment illustrates that there is little, if any, difference in the mobility of T-Ag isolated from the productively infected and transformed cells when they are isolated under appropriate conditions, it is unlikely that the 89,000- and/or 84,000-dalton forms of T-Ag have any specific role in the productive infection cycle. They are probably extraction artifacts. Tegtmeyer et al. (39) and Prives et al. (29) have reached the same conclusion.

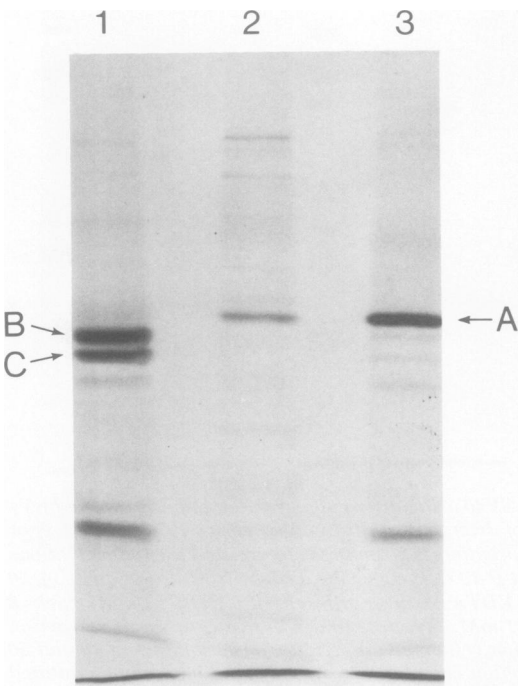

FIG. 2. *Isolation of SV40 T-Ag from productively infected monkey CV1 cells and H65-transformed hamster cells. T-Ag was extracted from infected CV1 cells (track 3) and H65 cells (track 2), using the conditions described for track 2 of Fig. 1, and from CV1 cells (track 1), using the conditions for track 12 of Fig. 1. The gel was 7.5% in polyacrylamide, and autoradiography was for 3 days. Band A corresponds to the 94,000-dalton form of T-Ag described by Carroll and Smith (7), and B and C correspond to the 89,000- and 84,000-dalton forms, respectively.*

We previously argued that the smaller (89,000- and 84,000-dalton) forms of T-Ag extracted from productively infected cells at low pH are generated by proteolytic cleavage of the biggest (94,000-dalton) form. This was based on the findings (i) that the smaller forms were not generated if cells were previously treated with protease inhibitors and (ii) that the larger form of T-Ag was synthesized in response to mRNA isolated from productively infected cells in a wheat germ cell-free system (7). If the 89,000- and 84,000-dalton forms of T-Ag are generated by cleavage of the 94,000-dalton protein, it follows that the various forms of the protein must be very closely related. We have tested this prediction by analyzing the polypeptides produced when the proteins are partially cleaved by the *S. aureus* V8 protease. Cleveland et al. (9) have shown that for a given protein the pattern of polypeptides generated using a number of different concentrations of the protease is characteristic for the protein in question. If two proteins are different, they generate different partial proteolysis products, whereas if they are related some of the polypeptides generated, those from the region shared by both proteins, will be similar.

Figure 3 shows the partial proteolysis products obtained with the 94,000-dalton and 84,000-dalton forms of T-Ag after separation on a 15% polyacrylamide gel. In the absence of protease, or in the presence of low concentrations, the two proteins migrated with their characteristic mobilities: the 84,000-dalton form with the fructose 6-phosphate kinase marker and the 94,000 form with phosphorylase *a*. However, in the presence of increasing amounts of protease, the partial digestion products became smaller, and those with an apparent molecular weight less than the ^{14}C-labeled carboxy-methyl carbonic anhydrase marker (29,000 daltons) were virtually identical. This indicates that the two proteins are closely related. This conclusion has been further supported by tryptic peptide fingerprinting (our unpublished data, and 33). It seems probable, therefore, that the agent responsible for the change in mobility of T-Ag is a protease; possibly it is a lysosomal-associated, sulfhydryl-requiring acid protease.

The molecular weight of T-Ag still remains unclear. From the data shown in Fig. 1, 3, and 4, using Tris-hydrochloride-buffered Studier-Laemmli gel conditions (18, 35), it is clear that the so-called 94,000-dalton form of T-Ag migrates anomalously, its mobility depending on the per-

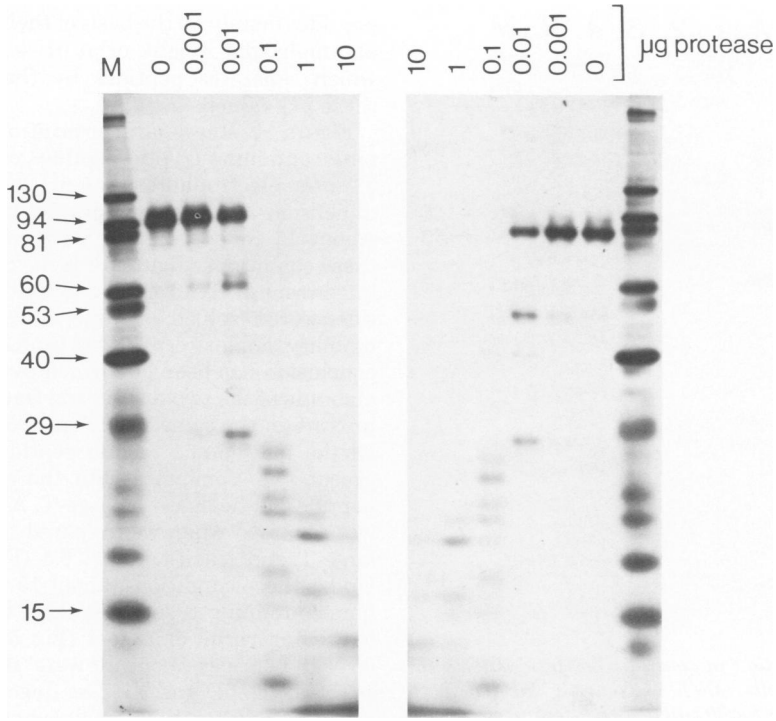

FIG. 3. *Partial proteolysis fingerprints of different forms of large T-antigen. Fingerprints were prepared using a modification of the method of Cleveland et al. (9). The left-hand panel shows the fingerprint of the 94,000-dalton form and the right-hand panel the 84,000-dalton form of SV40 T-Ag. The protease concentration used is given above each track. The gel was 15% polyacrylamide, and autoradiography was for 2 weeks.*

centage of acrylamide in the gel and the amount of cross-linking reagent. On the 7.5% gel shown in Fig. 1, it migrated faster than the phosphorylase *a* marker, whereas on the 15% gel in Fig. 3 and 4 it comigrated with phosphorylase *a*. We have also used the gel systems based on Tris-sulfate buffer developed by Neville (22) and on phosphate buffer used by Weber and Osborne (41) and have obtained apparent molecular weights ranging from 80,000 to 95,000 for the largest, undegraded form of T-Ag. When we refer to this species as 94,000-dalton T-Ag it is merely to distinguish it from the 89,000- and 84,000-dalton species. We do not imply that this is its true molecular weight. Perhaps the largest species of T-Ag is best referred to at present as large-T.

Isolation of SV40 small-t from productively infected cells. Having established conditions which (i) greatly reduce the background of nonspecific immunoprecipitation and (ii) prevent the proteolytic degradation of large-T during extraction, we searched for other forms of T-Ag. Figure 4 shows that extracts from productively infected cells also contain a low-molecular-weight protein that specifically immunoprecipitates with anti-T serum. This protein, which migrates slightly slower than the ^{14}C-labeled carboxy-methyl lysozyme marker, is not present in uninfected cells. The small form of T-Ag detected here probably corresponds to the 17,000-dalton protein described by Prives et al. (29). The immunoprecipitates shown in Fig. 4 of extracts of infected cells also contain a protein comigrating with SV40 virion protein VP1; however, as mentioned above, this protein also precipitates with control serum and represents nonspecific precipitation. The apparent molecular weight of the small form of T-Ag varies with gel conditions within the range 15,000 to 20,000. It is referred to here as small-t.

We have detected small-t in cells infected with all the strains of SV40 we have tested and in a number of SV40-transformed cells (10, 26). Small-t is not present in uninfected monkey cells or in the parents of the SV40-transformed cells (10). It is readily detected in productively infected cells pulse-labeled at any time during the period 16 to 96 h postinfection. Different batches of antisera raised in hamsters against transformed cells can have different anti-small-t activities, and some sera which react strongly with

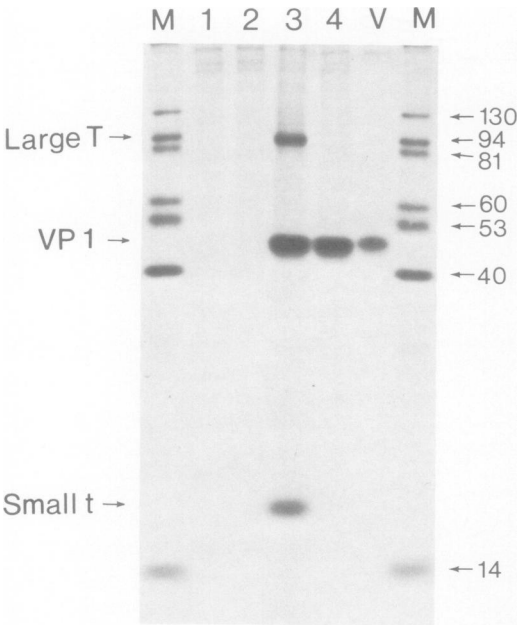

FIG. 4. *Isolation of small-t from productively infected CV1 cells. CV1 cells were labeled with [^{35}S]methionine for 30 min at 72 h after infection with SV40 (20 PFU/cell). Extracts of the cells were reacted with control hamster (track 1 and 4) or hamster anti-T serum (tracks 2 and 3). Tracks 1 and 2 were from uninfected cells; tracks 3 and 4 were from infected cells. The gel was 15% polyacrylamide; autoradiography was for 4 days; V is disrupted purified SV40 virions.*

large-T have virtually no anti-small-t activity. At present we have no explanation of this finding. Another problem we have encountered in the study of small-t is the presence of background proteins of a similar molecular weight which sometimes nonspecifically precipitate with both control and anti-T serum during the immunoprecipitation reaction. These proteins can be particularly troublesome when working with transformed cells.

Fingerprint analysis of SV40 T-antigens. To examine the relationship between large-T and small-t we compared their peptide fingerprints. To do this, cells were labeled with [^{35}S]methionine and T-Ags were isolated at pH 8.0. After separation on preparative scale slabs of polyacrylamide gel, the large-T and small-t as well as the VP1-like material were eluted, precipitated, oxidized with performic acid, and digested with trypsin. Fingerprints were prepared by electrophoresis on plastic-backed cellulose thin-layer sheets followed by chromatography in the second dimension (42). Electrophoresis was performed at either pH 2.1, which separates peptides mainly on the basis of their composition and molecular weight, or at pH 4.5 and pH 6.5, which separates peptides by their charge at these pH values.

Figure 5B shows a fingerprint of the methionine-containing tryptic peptides of SV40 large-T, after electrophoresis at pH 2.1 in the first dimension and chromatography in the second. About 16 to 18 peptides were resolved under these conditions. Figure 5A is the corresponding fingerprint of SV40 small-t. About 10 to 12 peptides were resolved; of these, all but two have a mobility similar to peptides from large-T. This conclusion has been confirmed by mixing equal amounts of the two digests and fractionating the mixture in the same way. Figure 5C shows that all the methionine tryptic peptides of small-t, except two, comigrate with the corresponding peptides in the digest of large-T. A similar result was obtained when we prepared fingerprints of large-T and small-t at pH 6.5 (Fig. 5D to F). Under these conditions, about 15 to 17 methionine-containing tryptic peptides were resolved in the fingerprint of large-T (Fig. 5E), and of the large-T peptides about 10 were also present in small-t (Fig. 5D and F). The fingerprints at pH 6.5 also indicate that small-t has two unique methionine peptides not contained in large-T. The positions of these two peptides at both pH 2.1 and pH 6.5 are marked with arrows in Fig. 5. At pH 2.1 one of the small-t unique peptides electrophoresed as a smear rather than a discrete spot. This effect was quite reproducible, but only occurred with some batches of cellulose thin-layer plates and only at pH 2.1.

Figure 6B shows the fingerprint of the band which nonspecifically coprecipitates with T-Ags and which comigrates with SV40 VP1. Five major methionine tryptic peptides were resolved by using pH 2.1 in the first dimension. Figure 6A shows the fingerprint of SV40 VP1 isolated from purified virion particles. It is clear that the protein coprecipitating with the T-Ags is closely related to authentic SV40 VP1 and has no peptides in common with T-Ag itself. This finding has been confirmed in experiments in which VP1 and T-Ag were mixed prior to fractionation and in experiments using separation at pH 6.5 rather than pH 2.1 (data not shown).

Biosynthesis of small-t. Since small-t shares most of its methionine peptides with large-T, it is likely that at least a part of the two proteins have a common amino acid sequence. Because small-t was extracted from productively infected cells under conditions which reduce degradation after cell lysis, we consider it unlikely that small-t is generated by breakdown of large-T during the extraction procedure. It remained possible, however, that small-t is pro-

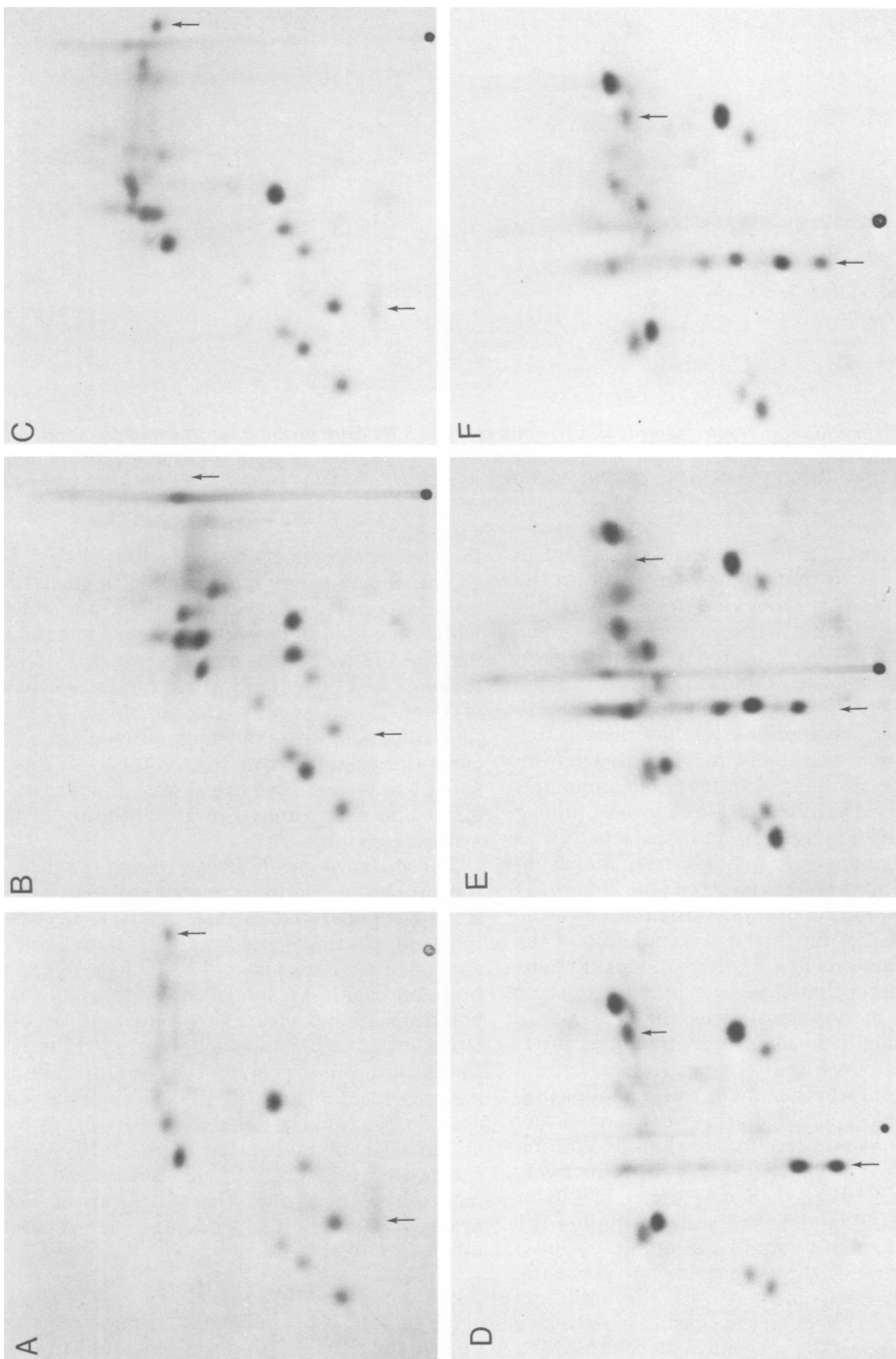

FIG. 5. *[^{35}S]methionine tryptic peptide fingerprints of SV40 large-T and small-t. Fingerprints of digests (~4 × 10^5 cpm) were prepared on cellulose thin-layer sheets, using electrophoresis at pH 2.1 (A to C) or pH 6.5 (D to F), followed by ascending chromatography. Autoradiography was for 5 days. (A and D) Small-t; (B and E) large-T; (C and F) mixtures of equal counts of small-t and large-T. Arrows indicate the position of the two methionine peptides unique to small-t.*

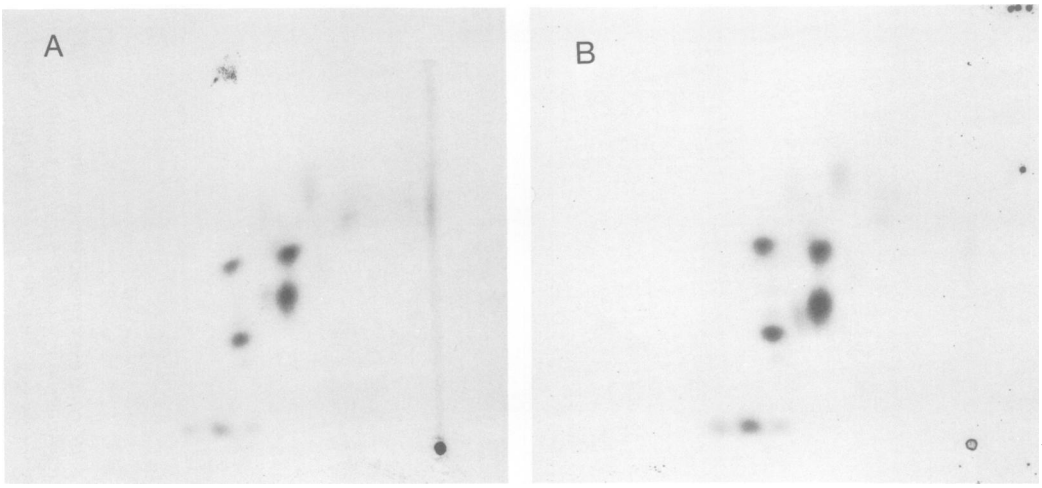

FIG. 6. *[^{35}S]methionine tryptic peptide fingerprints of SV40 VP1 from purified virions and from immunoprecipitates. Samples of 2.5 × 10^5 cpm of VP1 from purified virus (A) and from the immunoprecipitate of productively infected CV1 cells (B) were fingerprinted using pH 2.1 in the first dimension, then autoradiographed for 3 days.*

duced by a posttranslation cleavage mechanism in vivo similar to that which is involved in the production of many other viral proteins (17). For example, the genome of the picornaviruses polio, EMC, and Mengo is translated into a single giant precursor polypeptide, which is cleaved, in some cases before synthesis is complete, to give rise to three smaller polypeptides. Each of these is further processed eventually to yield the mature virus-specific proteins. A number of compounds have been used to inhibit these cleavages, among them TPCK, TLCK (36), and zinc ions (5), as well as the amino acid analogs azetidine, canavanine, and fluorophenylalanine (16). Figure 7A shows that addition of these inhibitors to EMC-infected cells results in the accumulation of the capsid protein precursor polypeptide A. Precursors even larger than A also accumulate in some cases, and in most instances there is also a corresponding decrease in the amount of intermediate proteins ϵ and γ.

To examine whether there was a similar precursor-product relationship between large-T and small-t, we tested the effect of the same inhibitors of proteolytic activity on SV40 productively infected cells and asked whether a precursor molecule could be detected and/or whether the synthesis of small-t could be inhibited by these agents. Figure 7B shows that, in contrast to the results with EMC-infected cells, the inhibitors had little or no effect on the synthesis of the two forms of T-Ag. Of the reagents tested with EMC-infected cells, a combination of three amino acid analogs was the most effective in preventing cleavage of polypeptide A, and virtually none of the cleavage products ϵ and γ were labeled. On the other hand, zinc ions resulted in the build-up of a number of very high molecular weight EMC precursor polypeptides, as well as inhibiting the production of smaller proteins. These reagents had little if any effect on the synthesis of large-T and small-t. No possible precursor molecules were detected, and, although in some cases (for example with TPCK) the synthesis of small-t was inhibited, this was a nonspecific effect and also resulted in an inhibition of the synthesis of large-T.

These results, while by no means conclusive, tend to argue against a model suggesting that small-t is generated by cleavage of a precursor molecule sharing sequences with both large-T and small-t but also having sequences unique to both, and against a model in which small-t is a posttranslational cleavage product of large-T. Other experiments, in which we studied the synthesis of both forms of T-Ag (i) in cell-free systems (25, 26) and (ii) in cells infected with specific deletion mutants of SV40 (10), as well as partial characterization of the mRNA's for the two proteins (25, 26), all support the alternative hypothesis that the two forms of T-Ag are synthesized independently on separate mRNA's (10, 27).

DISCUSSION

Large-T 84,000- and 89,000-dalton forms are proteolytic cleavage products generated after extraction of productively infected cells. The results shown here clearly show that the apparent molecular weight of the large-T isolated from

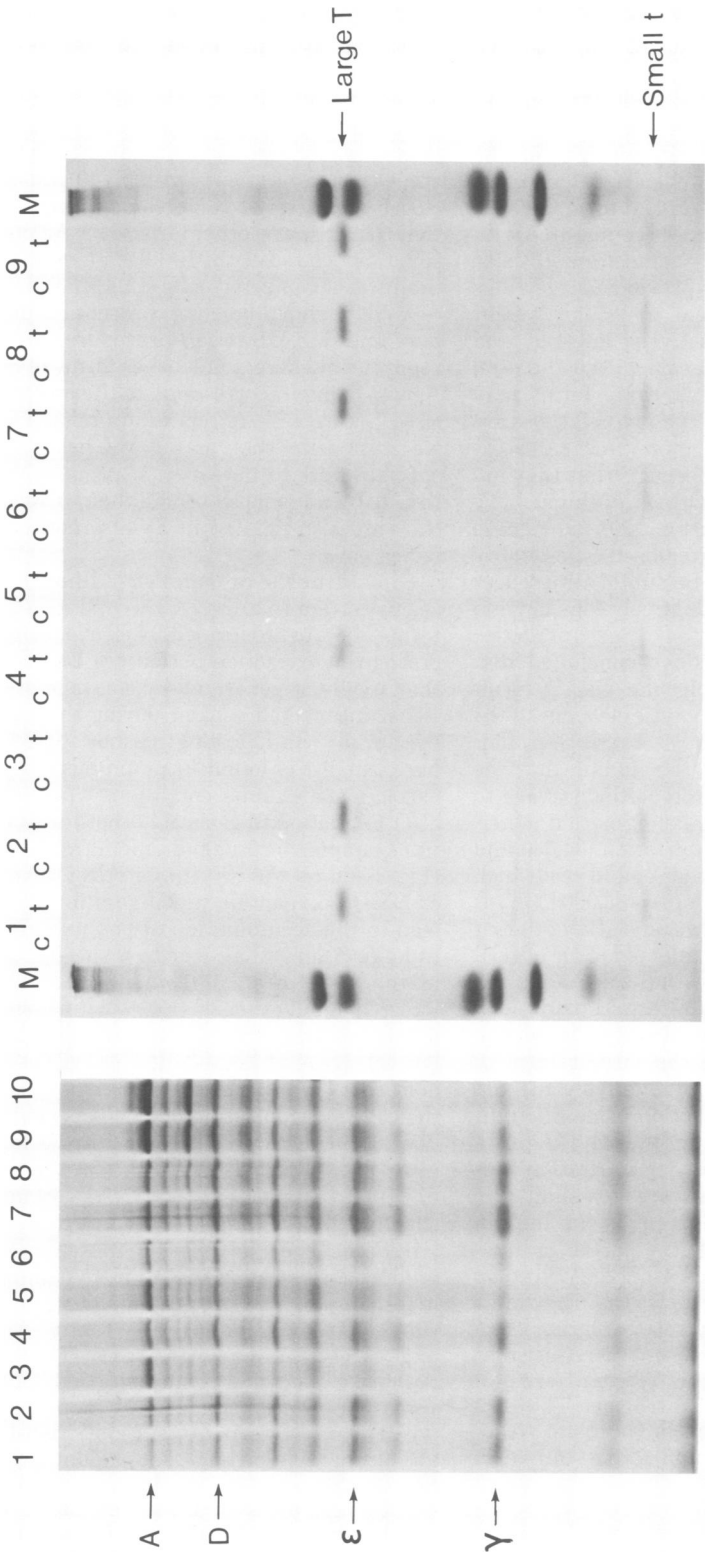

FIG. 7. *Effect of proteolysis inhibitors on the synthesis of EMC virus proteins and SV40 large-T and small-t. Left panel: Proteins labeled from 4 to 4.5 h after infection of 3T6 cells with EMC virus (10 PFU/cell) and following 10 min of pretreatment with (1) zero (control); (2) ethanol (0.5%); (3) azetidine, canavanine, and fluorophenyl alanine; (4) canavanine; (5) azetidine; (6) TPCK; (7) TLCK; (8) 0.1 mM $ZnCl_2$; (9) 0.5 mM $ZnCl_2$; (10) 1.0 mM $ZnCl_2$. The gel contained 15% polyacrylamide and was exposed for 16 h. Right panel: SV40 large-T and small-t labeled from 48 to 48.5 h after infection of CV1 cells with SV40 (25 PFU/cell) and following 10 min of pretreatment with (1) zero (control); (2) ethanol (0.5%); (3) TPCK; (4) TLCK; (5) ZPCK; (6) all three amino acid analogs; (7) 0.1 mM $ZnCl_2$; (8) 0.5 mM $ZnCl_2$; (9) 1.0 mM $ZnCl_2$. Immunoprecipitation was performed with both control (c) and anti-T (t) serum. The gel contained two layers consisting of 5 cm of 15% polyacrylamide and 5 cm of 7.5% polyacrylamide. Autoradiography was for 7 days.*

productively infected cells is strongly influenced by the conditions used to extract the cells. The form with the highest apparent molecular weight, previously referred to as 94,000 daltons, is extracted at pH 8.0, whereas at lower pH values the apparent molecular weight of the extracted large-T is less. When isolated under conditions which give the largest form, large-T from productively infected cells comigrated with large-T isolated from SV40-transformed cells. This result indicates that previous suggestions that there may be different molecular weight forms of T-Ag in productively infected and transformed cells (the so-called 84,000-dalton and 94,000-dalton) and that these forms may have different functions in the two cell types are unlikely to be true, and a more reasonable explanation of the different forms is that they are an extraction artifact. Other authors have reached the same conclusion (29, 39). These results, however, do not exclude the possibility that there are other, more subtle differences between large-T from productively infected and transformed cells.

We and other authors have concluded that the different apparent molecular weight forms of large-T result from proteolytic cleavage of the largest 94,000-dalton form (1, 7, 32, 39). This conclusion is based on (i) the finding that the addition of inhibitors of proteolytic cleavage to infected cells during the labeling period generated larger molecular weight forms (94,000 and 89,000) upon extraction under conditions which normally gave the smaller forms (84,000 [7]); (ii) the finding that the primary translation product of mRNA isolated from infected cells was a protein which comigrated with the 94,000-dalton form of T-Ag (7, 26); and (iii) experiments such as those described here showing that under appropriate conditions only the largest form of large-T was extracted from productively infected cells and that further extraction using different conditions does not yield additional T-Ag of a different form (29, 39). The demonstration that the different forms of large-T are very similar by both tryptic peptide analysis (33; our unpublished data) and analysis of the partial proteolysis intermediates generated using the *S. aureus* V8 enzyme (Fig. 3) clearly shows that the different forms are very closely related rather than different molecular species, and is consistent with the suggestion that the different forms of large-T are generated by proteolysis. The protease involved in the cleavage is possibly a lysosomal acid protease with an active sulfhydryl group.

On extraction under our new conditions, we still extract at least two forms of T-Ag, the so-called large-T and small-t. These are commonly referred to as having molecular weights of 94,000 and 17,000, respectively, but since both proteins appear to migrate anomalously on polyacrylamide gel electrophoresis we emphasize that these numbers are almost undoubtedly inaccurate. Other experiments with SV40 and with the closely related virus polyoma have indicated that there may be other forms of T-Ag, including some associated with the plasma membrane of productively infected cells (12, 19, 34). Using the relatively short labeling procedure described here and our extraction procedure, we did not reproducibly detect any other forms of SV40 T-Ag which might correspond to the intermediate-sized putative T-Ag related proteins. However, we emphasize that as yet we have made no special effort to detect such proteins, and our results by no means exclude the possibility that they are also present in SV40 productively infected cells.

Biosynthesis of small-t. Although it now seems certain that the 84,000- and 89,000-dalton forms of T-Ag are formed by cleavage of large-T during the extraction period, we do not believe that small-t is generated by cleavage of large-T either in infected cells or during the extraction period, for the following reasons. (i) As shown here, small-t is found in undiminished yield in extracts made under conditions where cleavage after extraction to generate other forms of T-Ag (for example, the 84,000-dalton form) is inhibited (Fig. 4 and ref. 29). (ii) Inhibitors of the cleavage of EMC polypeptide precursors in vivo do not lead to the accumulation of possible precursors to large-T and small-t or to a specific decrease in the yield of small-t when added to SV40-infected monkey cells (Fig. 7). (iii) Small-t is found in transformed cells, in which the proteolytic cleavage of large-T during extraction does not occur (10). (iv) Small-t is a major product of the cell-free translation of SV40-specific mRNA (25, 26, 29), and degradation in vitro is relatively rare (15). (v) Small-t is synthesized in response to SV40 complementary RNA, conditions in which complete large-T is not formed (25, 26). (vi) Some specific deletion mutants of SV40 produce altered small-t both in vivo and in vitro in response to mRNA and complementary RNA, whereas they produce apparently wild-type large-T (10; M. J. Sleigh, W. C. Topp, and J. Sambrook, personal communication; E. Paucha and A. E. Smith, manuscript in preparation). (vii) The mRNA's for large-T and small-t appear to be separable on sucrose gradient centrifugation (25, 26).

Although none of these results in itself is conclusive, they all tend to argue against a model

in which small-t is produced by proteolytic cleavage of large-T. Instead, the data favor a model which predicts that the two proteins are synthesized independently, and such a model has been described in detail elsewhere (10, 27). For the purposes of this discussion, the only details of the model needed are the proposed locations of the sequences coding for both large-T and small-t. We have shown that the amino terminus of small-t is coded at about 0.65 map units, and we proposed that its carboxyl terminus is coded at about 0.55 map units. The amino terminus of large-T is also coded at about 0.65 map units, and its carboxyl terminus is coded at or near 0.17 map units (10, 10a, 27, 30a). The most novel feature of the model is the proposal that the region between about 0.59 and 0.54 map units does not contain information coding for large-T and that at some stage during or after transcription of the E strand of SV40 DNA, this region is "spliced out" to generate the mRNA for large-T (10, 27).

Fingerprint analysis of large and small T-Ags. In our fingerprints (Fig. 5A to F) we have resolved 10 to 12 methionine peptides from small-t and 15 to 18 peptides from large-T. All but two of the small-t methionine peptides are also present in large-T. These numbers are somewhat greater than those reported by Prives et al. (29) for large-T and small-t and by Tegtmeyer et al. (39) for large-T but similar to those reported by Simmons and Martin (34a).

Since the entire nucleotide sequence of SV40 DNA has been established (10a, 30a), it is possible to predict the number of methionine residues coded for by the early region of SV40 DNA (see Fig. 4 of reference 10a). The region from 0.65 to 0.59 map units (predicted to be the common amino terminus of both large-T and small-t) contains 7 AUG codons; the region from 0.59 to 0.55 map units (predicted to be the region unique to small-t) contains 3 AUG codons; and the region from 0.54 to 0.17 map units (predicted to be unique to large-T) contains 17 AUG codons. From the predicted amino acid sequences it can be deduced that these AUG residues would generate 7, 3, and 14 methionine-containing tryptic peptides, respectively, including one Met-Lys sequence in each. Assuming that the splice sites in large-T mRNA are positioned as outlined above, this would predict (i) that small-t would have nine methionine tryptic peptides, including two which are unique to small-t, and (ii) that large-T would have 20 methionine tryptic peptides, of which 13 would not be present in small-t.

It should be emphasized that peptide fingerprinting cannot be regarded as a quantitative method; the extent of enzymatic cleavage at any particular site, the degree of side-chain destruction during oxidation, and the different solubility of various peptides are all unknown and may result in unequal yields of the various peptides. Furthermore, we do not know exactly which sequences are spliced from large-T mRNA, or how many AUG codons, if any, are removed in this operation. Nevertheless, it is interesting to compare our estimates for the number of methionine peptides with those predicted from the proposed DNA sequences for both large and small T-Ag.

The number of peptides that we have found to be shared between small-t and large-T is somewhat higher than expected (8 to 10, compared with 7). In addition to the inaccuracies mentioned above, this finding may result from a rather unusual feature of the predicted amino acid sequence of small-t (10a, 40a); i.e., it contains many adjacent basic amino acids (e.g., Lys-Lys, Arg-Lys, etc). Five of the trypsin cleavages generating methionine peptides involve two such residues, and it is possible that trypsin will cleave to the carboxyl side of either one of these residues but perhaps not both (21). If this were the case, a single methionine residue might give rise to multiple tryptic peptides, differing at either end by only a single basic amino acid. Further, the predicted sequence of small-t contains an AsN-Pro sequence in a methionine tryptic peptide; such bonds are known to be labile (43). Small-t also contains a high proportion of sulfur-containing amino acids (21/174); some of these could be subject to incomplete conversion during performic acid oxidation. Our finding of two methionine peptides unique to small-t agrees with the DNA sequence data (assuming the position of the splice given above is correct); however, we have no evidence to show that the two peptides detected correspond to the two predicted peptides.

Our estimate for the number of methionine peptides unique to large-T is somewhat lower than predicted (7 to 9, compared with 13). This may be partly explained in that four predicted methionine tryptic peptides from the region unique to large-T have greater than 20 amino acids. These may well not fractionate in our separation systems, and indeed we always find some methionine-containing material present at the origin in fingerprints of large-T and material which streaks from the origin on chromatography.

Thus, although the agreement between the predicted and observed numbers of methionine-containing tryptic peptides present in large and small T-Ags is far from perfect, we believe that

our data are not greatly at variance with the DNA sequence data. It remains to be seen whether the differences detected merely reflect inaccuracies of the fingerprinting method, as we have suggested, or whether they indicate that the model for the location of sequences coding for large-T and small-t is not completely accurate. We emphasize that the methionine tryptic fingerprints presented here only show that large-T and small-t share some tryptic peptides and both contain some unique peptides. Amino-terminal sequence data on large-T and small-t strongly suggest that the shared region is at the amino terminus of both proteins (27). To obtain a more extensive, direct correlation between the amino acid sequences of both large-T and small-t and SV40 DNA, and particularly to establish details of the regions around the splice sites, requires a great deal more amino acid sequence analysis of the two forms of T-Ag.

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