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Association of a Murine 53,000-Dalton Phosphoprotein with Simian Virus 40 Large-T Antigen in Transformed Cells

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Serum raised against a mouse 53,000-dalton (53K) phosphoprotein precipitates both the 53K immunogen and simian virus 40 large-T from lysates of simian virus 40-transformed 3T3 cells. This serum, designated F5, does not recognize antigenic determinants on native or denatured large-T and precipitates large-T because the 53K phosphoprotein forms a stable complex with large-T. This complex sediments at 23S on sucrose density gradients, corresponding to a molecular weight of 600K to 1,000K, and appears to contain only 53K and large-T as major components. It is held together by noncovalent bonds and is located in the cell nucleus. All the 53K immunoprecipitated from cell lysates by F5 is present in the high-molecular-weight complex, but large-T can be separated into a complexed and a free form on sucrose density gradients. The complexed form of large-T is more readily phosphorylated than the free form. We have been unable to detect an association of large-T with comparable host cell proteins during productive infections with simian virus 40.

Serum from animals bearing simian virus 40 (SV40)-induced tumors specifically immunoprecipitate two major proteins from productively infected cells (5, 15, 17, 18). These two proteins, large-T (94,000 daltons [94K]) and small-t (20K), are coded by the early region of SV40 DNA and can be precipitated from SV40-transformed cells. However, lysates from these transformed cells contain a third class of antigens (2-4, 7, 9, 11, 13, 14, 19, 21a). These have been referred to as nonviral tumor (NVT) antigens (50K to 60K). Unlike large-T and small-t, these antigens appear to be host coded, as similar proteins can be immunoprecipitated from cells that do not contain SV40 DNA (13; L. V. Crawford, D. P. Lane, D. T. Denhardt, E. E. Harlow, K. Osborn, P. M. Nicklin, and D. C. Pim, Cold Spring Harbor Symp. Quant. Biol., in press). Consistent with this observation, the apparent molecular weights and tryptic fingerprints of NVTs vary with the species of transformed cell (9, 21a).

The precipitation of NVTs from cells not containing SV40 genomes demonstrates that some SV40 antitumor sera contain specific activities directed against these proteins. This may be because NVTs share antigenic determinants with SV40-coded antigens, or because they are antigenic in their own right. Lane and Crawford (11) have reported that serum raised against gel-purified large-T precipitates an NVT referred to as 53K from SV40-transformed mouse cells. The coordinate precipitation of large-T and 53K observed by these workers would at first suggest major cross-reactivity between antigenic determinants on these proteins, but it was found that their anti-large-T serum had no activity against gel-purified 53K. They concluded that these proteins do not share denaturation-resistant determinants and that 53K is probably precipitated through an association with large-T. Several other sera specific for large-T, including anti-U, anti-D2, and an anti-large-T hybridoma serum, precipitate large-T and 53K from transformed mouse cells (E. G. Gurney, personal communication; Crawford et al., in press). Furthermore, Linzer and Levine (13) have reported that sera from nine separate hybridomas, each capable of recognizing a single determinant on large-T, are able to precipitate both large-T and a 54K NVT from transformed mouse cells. These sera do not precipitate 54K NVT from embryonal carcinoma cells, showing that they are unable to recognize this protein directly. These results suggest an association between the 54K protein, which is probably identical with the 53K protein of Lane and Crawford, and large-T.

If the 53K mouse protein and large-T do indeed form a complex, we expect that a serum with activity only for 53K should precipitate large-T from SV40-transformed mouse cells. We have raised such a serum and find that it does immunoprecipitate both 53K and large-T from these cells, even though it fails to recognize antigenic determinants on large-T in the native

or denatured state. Moreover, this serum has proved to be a valuable immunochemical probe for characterizing the association of 53K and large-T. We report here that large-T in lysates from transformed cells is found in a free form, as well as in the 53K-associated form, and that large-T is more readily phosphorylated when bound to 53K. The 53K/large-T complex sediments at 23S on sucrose gradients, corresponding to a molecular weight of 600K to 1,000K. No other proteins can be detected in this complex, but it does contain most, if not all, of the 53K present in these cells. Immunofluorescent studies have localized the complex in the nucleus. We have been unable to find an analogous complex in lysates of productively infected cells.

MATERIALS AND METHODS

Cell lines and virus stocks. SV40-transformed mouse 3T3 A31 cells (SVA31E7) were isolated by Y. Ito and supplied by him. CV-1 African green monkey kidney cells were obtained from P. Berg. Both lines were grown in Dulbecco E4 medium supplemented with 10% fetal calf serum and were free from mycoplasma infection. Wild-type SV40 strain SV-S was used for infection of CV-1 cells.

Cell cultures and virus infections. Cells were routinely grown on 90-mm plastic dishes. For large-scale preparations, cells were grown on Sterilin Bulk Cell Culture Vessels (Sterilin Ltd., Teddington, England). These vessels contain a plastic film wrapped into a spiral, giving a surface area of 8,500 cm^2, which supports growth of up to 10^9 cells.

CV-1 cells were infected with SV40 at about 20 PFU/cell in 2 ml of medium. After 2 h of adsorption, a further 8 ml of medium was added.

Preparation of cell extracts. Subconfluent SVA31E7 cells were labeled with [^{35}S]methionine (ca. 1,000 Ci/mmol, 1 mCi/90-mm dish) in 2 ml of methionine-free medium, or with [^{32}P]phosphate (0.25 mCi/ml) in 4 ml of phosphate-free medium. Radiolabels were supplied by the Radiochemical Centre, Amersham, England. After 2 h of labeling, the cells were washed twice with phosphate-buffered saline and lysed at 4°C for 15 min in buffer containing 0.14 M NaCl, 10 mM Tris (pH 8.0), and 0.5% Nonidet P-40. The lysate was centrifuged for 1 min (microfuge B, Beckman Instruments, Inc., Fullerton, Calif.) and used immediately or stored at −70°C. Extracts from lytically infected CV-1 cells were prepared under identical conditions, at 48 h after infection.

For large-scale preparations, cells were scraped from the plastic films contained in the Bulk Culture Vessels and washed with phosphate-buffered saline, and the cell pellet (up to 25 ml packed volume) was lysed in 6 volumes of the extraction buffer used above. The lysate was centrifuged at 8,000 rpm in a Sorvall HB4 rotor before immunoprecipitation.

Immunoprecipitations and gel electrophoresis. Serum was added to samples of cell lysates as described in the text, and after 1 h at 20°C, immune complexes were collected by addition of a 10% solution of Formalin-fixed *Staphylococcus aureus* Cowan 1 (8). A total of 3 volumes of this was added to every 1 volume of serum used. After 15 min at 20°C, adsorbed immune complexes were washed three times with a buffer containing 0.15 M NaCl, 0.05 M Tris (pH 7.4), 5 mM EDTA, and 0.05% Nonidet P-40. They were then eluted with buffer containing 2% sodium dodecyl sulfate (SDS) and 0.1 M dithiothreitol and subjected to gel electrophoresis, as described elsewhere (5).

Radioimmunoassay. The direct-binding radioimmunoassay of Lane and Robbins (12) was used to assay sera for activity against gel-purified large-T or 53K.

Immunofluorescence. SVA31E7 cells were grown on eight-well glass slides (Flow Laboratories, Rockville, Md.) in Dulbecco-modified E4 supplemented with 10% calf serum. The cells were washed twice in phosphate-buffered saline and fixed in acetone (−5°C) for 15 min. After fixation, the slides were rinsed in distilled water to remove phosphate-buffered saline and allowed to air dry. The slides were either used immediately or stored at −70°C for up to 1 month. Primary antisera were added to the wells of the glass slide and incubated for 1 h at 37°C in a humidified atmosphere. The slides were washed twice with phosphate-buffered saline, twice with distilled water, and air dried. The secondary antiserum was goat immunoglobulin G (IgG) raised against rabbit immunoglobulins conjugated with fluorescein isothiocyanate (Nordic) and was used at a dilution of 1:40. After a second series of washes, the cells were viewed with a Zeiss microscope equipped with epifluorescent illumination. Pictures were taken with a Plano 40× oil immersion objective.

Tryptic fingerprints. Two-dimensional tryptic peptide analysis was performed by the technique of Smart and Ito (21) as modified by Denhardt and Crawford (submitted for publication).

Sedimentation analysis of cell lysates. Samples of cell extracts (0.4 ml each) were centrifuged through 4.7-ml linear 5 to 20% sucrose gradients with a 0.4-ml 60% sucrose pad, under conditions similar to those described by Carroll et al. (1). The gradients contained 10 mM Tris-hydrochloride (pH 8.0), 10 mM dithiothreitol, and 0.14 M NaCl and were centrifuged for 15 h at 25,000 rpm in an SW50.1 Beckman rotor at 4°C.

RESULTS

Immunoprecipitation of large-T and 53K from SV40-transformed cells. Sera raised against SV40-induced tumors in hamsters or mice contain major activities against large-T and small-t. In addition, some SV40 antitumor sera are able to react directly with 53K and related proteins (11, 13; P. May, M. Kress, M. Lange, and E. May, Cold Spring Harbor Symp. Quant. Biol., in press). Figure 1 shows the result of immunoprecipitations using a mouse antitumor serum that binds directly to gel-purified 53K (designated 1211) and a hamster serum (designated 23) that has no detectable activity against 53K (E.H., unpublished data). In spite of their different activities, both sera were able to im-

munoprecipitate equivalent amounts of 53K, when used in serum excess. The lysates used in this immunoprecipitation were from SVA31E7 cells that had been labeled with [^{35}S]methionine or [^{32}P]phosphate. Like large-T, the 53K species from SVA31E7 cells incorporates both labels, as reported by others (13; May et al., in press; F. McCormick, F. Chaudry, R. Harvey, R. Smith, P. W. J. Rigby, E. Paucha, and A. E. Smith, Cold Spring Harbor Symp. Quant. Biol., in press). Small-t was synthesized at relatively low rates in these cells and was not detected in this experiment.

The phosphoprotein that we are referring to as 53K actually migrated with a mobility corresponding to 50K in some of our gel systems (e.g., see Fig. 2), but since it appears to be identical to the 53K protein described by Lane and Crawford (11) and by Smith et al. (21a), at least at the level of tryptic fingerprint analysis (unpublished data), we will continue to refer to it as 53K.

Purification of 53K by SDS-polyacrylamide gel electrophoresis. 53K was purified for use as an immunogen using the approach described by Lane and Robbins (12), which involves a large-scale immunoprecipitation followed by preparative SDS-polyacrylamide gel electrophoresis and elution of immunogen. This approach depends on obtaining a discrete, stainable band of immunogen on the gel, so that two criteria can be met: (i) the protein must be free of contaminating bands and (ii) the protein must be present in sufficient amounts to elicit an immune response (at least 10 μg).

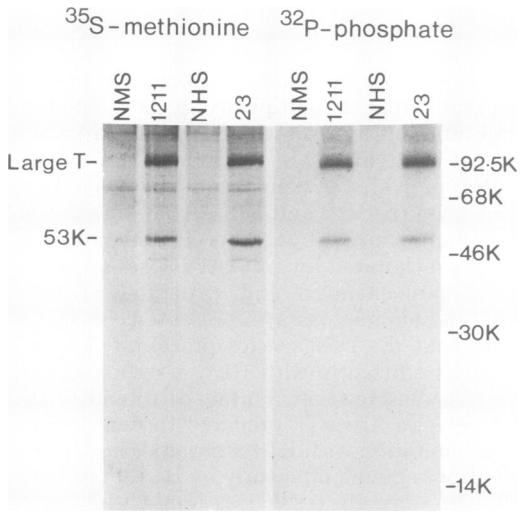

FIG. 1. *Immunoprecipitation of large-T and 53K from SVA31E7 lysates. Subconfluent monolayers of SVA31E7 cells were labeled for 2 h with [^{35}S]methionine (1.0 mCi/ml) or [^{32}P]phosphate (0.5 mCi/ml). Extracts were immunoprecipitated with normal mouse serum (NMS), mouse anti-SV40 tumor serum (1211), normal hamster serum (NHS), or hamster anti-SV40 tumor serum (23) and run on a 12% polyacrylamide gel. Autoradiography was for 2 days.*

To identify the 53K band, lysates from 10^9 to 10^{10} SVA31E7 cells were mixed with a small amount of ^{32}P-labeled lysate from these cells. After immunoprecipitation with hamster anti-tumor serum and preparative-scale SDS-polyacrylamide gel electrophoresis, the 53K band was located by autoradiography of the wet gel (Fig. 2).

This band was removed from the gel and used as a source of immunogen. To avoid contamination of the band with IgG heavy chain (molecular weight, ca. 50K) from the hamster serum used for the bulk immunoprecipitation (Fig. 2, track B), the samples were eluted from immune complexes under partial reducing conditions (0.1 M dithiothreitol, 4°C) and loaded onto the gel without boiling. Under these conditions, the heavy chain remained as a dimer with a molecular weight of about 100K, enabling 53K to be detected as a stained band free of contaminating proteins (Fig. 2, track E).

Recovery of 53K and large-T was reduced by about 20% by using partial reduction in preparing samples for electrophoresis.

Immunoprecipitation of 53K and large-T by anti-53K serum. Purified, denatured 53K was injected into a rabbit, and the immune response was monitored by testing samples of serum in a direct-binding radioimmunoassay and by immunoprecipitation. At 11 weeks after the initial injection, 53K binding activity was detected by the radioimmunoassay; after a further 3 weeks, during which time the binding activity doubled, 53K could be detected by immunoprecipitation reactions, using [^{32}P]phosphate-labeled SVA31E7 cells as a source of antigen. Figure 3 shows that anti-53K serum (designated F5) was able to immunoprecipitate two major polypeptides from the cells that were not precipitated by normal rabbit serum. These polypeptides comigrate with the major proteins immunoprecipitated by RL1, a serum raised against purified, denatured large-T (12). Subsequent analyses of these two proteins (described below) has confirmed that they are large-T and the same 53K protein described by Lane and Crawford (11) and Smith et al. (21a).

When the activity of F5 was tested in lysates of CV-1 cells infected with SV40, it was found that neither large-T nor small-t could be precipitated (Fig. 3). Thus, F5 has no detectable activity against large-T or small-t in their native states. We interpret the ability of F5 to immu-

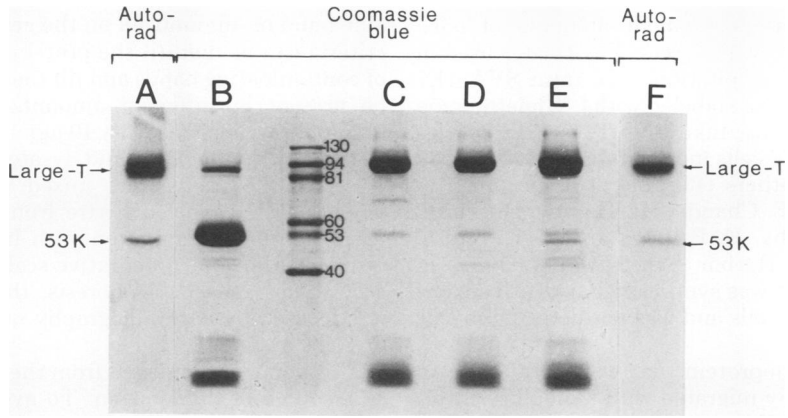

FIG. 2. *Purification of 53K by SDS-polyacrylamide gel electrophoresis. An extract from approximately 10^8 SVA31E7 cells was mixed with an extract from [^{32}P]phosphate-labeled cells and immunoprecipitated with normal hamster serum (track D), or hamster antitumor serum (tracks B and E). Track C was from a mock-immunoprecipitate in which lysate buffer replaced cell extract. Immune complexes were dissociated using a buffer containing 0.1 M dithiothreitol and 2% SDS, and samples were loaded onto a 10% polyacrylamide gel without boiling (tracks C, D, and E) or with 2 min of boiling (track B). The gel was stained with Coomassie brilliant blue and exposed for autoradiography. Tracks A and F are autoradiographs of B and E, respectively.*

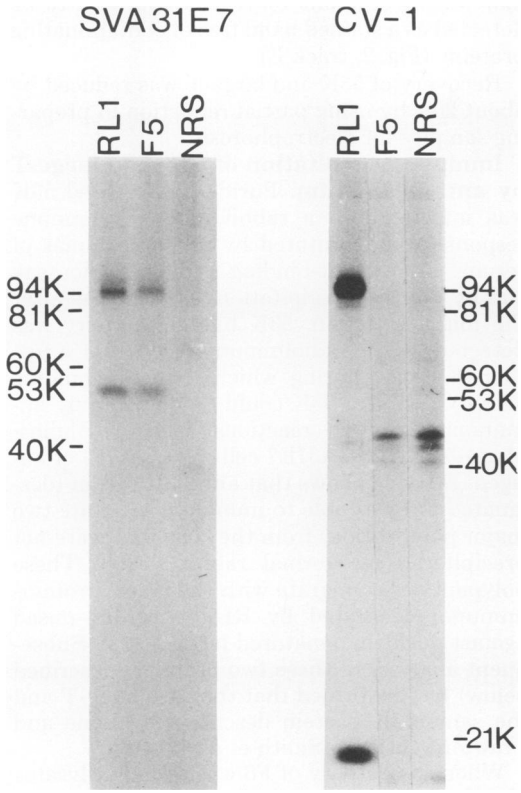

FIG. 3. *Immunoprecipitation of large-T and 53K by anti-53K serum. Extracts of ^{32}P-labeled SVA31E7 cells were reacted with 20 μl of anti-large-T serum (RL1), anti-53K serum (F5), or normal rabbit serum (NRS). Extracts of ^{35}S-labeled CV-1 cells that had been infected with 20 PFU/cell of SV40 for 48 h were reacted with 5 μl of RL1, 25 μl of F5, or 25 μl of NRS. Immunoprecipitates were collected and run on 15% polyacrylamide gels under full reducing conditions.*

noprecipitate large-T from SVA31E7 lysates to be a result of an association between large-T and 53K.

The failure of F5 serum to recognize a monkey protein that may be immunologically related to the 53K murine protein could be a result of the relatively low titer of this antiserum and of much lower levels of such a protein in these lysates. The possibility that such a protein would be detected under different conditions of infection (e.g., at a higher multiplicity, or at a different time after infection) or with a stronger antiserum cannot be excluded.

Polypeptides precipitated by F5. We have confirmed that the anti-53K serum does indeed immunoprecipitate polypeptides identical to those precipitated by anti-large-T serum (RL1) by immunochemical and structural analysis. The radioimmunoassays presented in Fig. 4 show that the 94K species precipitated by F5 rebound efficiently with RL1, a serum specific for large-T. However, F5 failed to bind to the 94K species. Large-T prepared through immunoprecipitation with RL1 showed a similar profile. It was bound efficiently by RL1, but not by F5. The opposite result was found when comparing [^{35}S]methionine probes of 53K. Whether prepared by precipitation with RL1 or F5, 53K was recognized only by F5. Since both of these sera were prepared by similar methods against

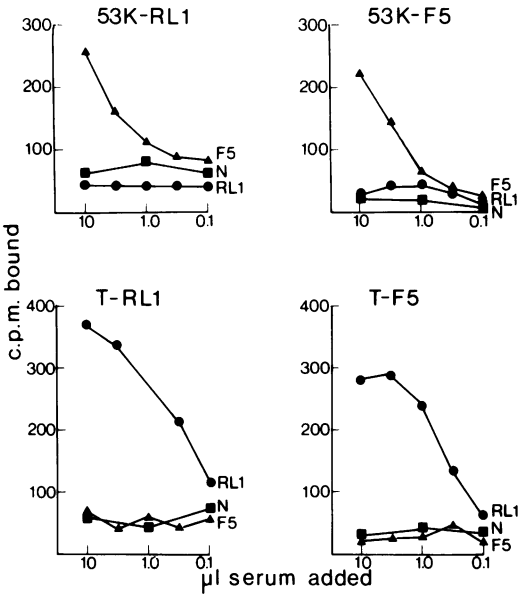

FIG. 4. *Radioimmunoassays of F5 and RL1 sera with 94K and 53K. [^{35}S]methionine-labeled polypeptides from SVA31E7 cells were precipitated with either F5 (anti-53K serum) or RL1 (anti-large-T serum), purified by SDS-polyacrylamide gel electrophoresis, and eluted from minced gel strips. These probes were then incubated with various dilutions of antisera. The antigen-antibody complexes were collected on S. aureus protein A, washed, and counted. Abbreviations: 53K-RL1, 53K probe prepared by immunoprecipitation with RL1; 53K-F5, 53K probe prepared by immunoprecipitation with F5; T-RL1, large-T probe prepared with RL1 serum; T-F5, large-T probe prepared with F5 serum.*

gel-purified polypeptides and this assay recognized only those determinants resistant to treatment with SDS and subsequent electrophoresis, we can conclude that at this level of analysis, these antisera are free from cross-reactivity. Figure 4 also shows that the titer of F5 was approximately 10-fold lower than RL1 in this assay.

Confirmation that the 53K species prepared by these antisera are closely related is presented in Fig. 5. The fingerprints of methionine-containing tryptic peptides are indistinguishable. Similar results are seen when the electrophoretic dimension was run at pH 2.1 or 6.5 (R. Smith, unpublished observations). Two-dimensional tryptic fingerprint analysis has also confirmed that the 94K species are identical and that their fingerprints are indistinguishable from those of large-T from infected CV-1 cells (data not shown). The relatively large number of methionine-containing tryptic peptides is the result of the partial oxidation of peptides used in this procedure (Crawford et al., in press).

Characterization of the complex between large-T and 53K. Lysates from SVA31E7 cells labeled with [^{35}S]methionine or [^{32}P]phosphate were centrifuged through 5 to 20% sucrose density gradients. Each fraction was immunoprecipitated by RL1, anti-large-T serum (Fig. 6a and c), or by F5, anti-53K serum (Fig. 6b and d). In each case, 53K was detected in the region of the gradient corresponding to high-molecular-weight forms, with a maximum yield in fraction 5. This represents a species sedimenting at 23S relative to rRNA markers, with an estimated molecular weight of between 600K and 1,000K.

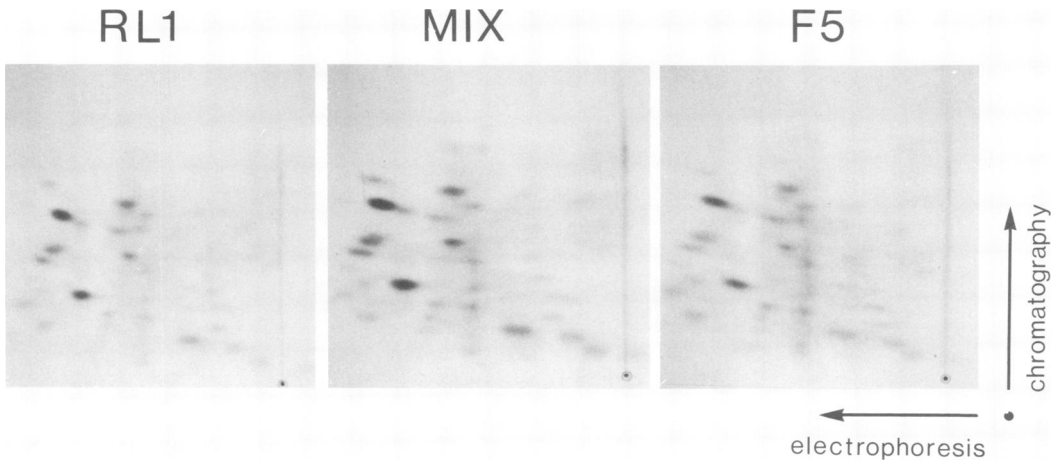

FIG. 5. *Tryptic fingerprints of 53K prepared by immunoprecipitation with F5 or RL1. [^{35}S]methionine-labeled 53K polypeptides were purified on SDS-polyacrylamide gel electrophoresis after immunoprecipitation with either F5 or RL1. Polypeptides were oxidized and digested with trypsin before separation in two dimensions in thin-layer plates. The first dimension was electrophoresis at pH 4.5 followed by chromatography in water-pyridine-butanol-acetic acid (4:4:5:1).*

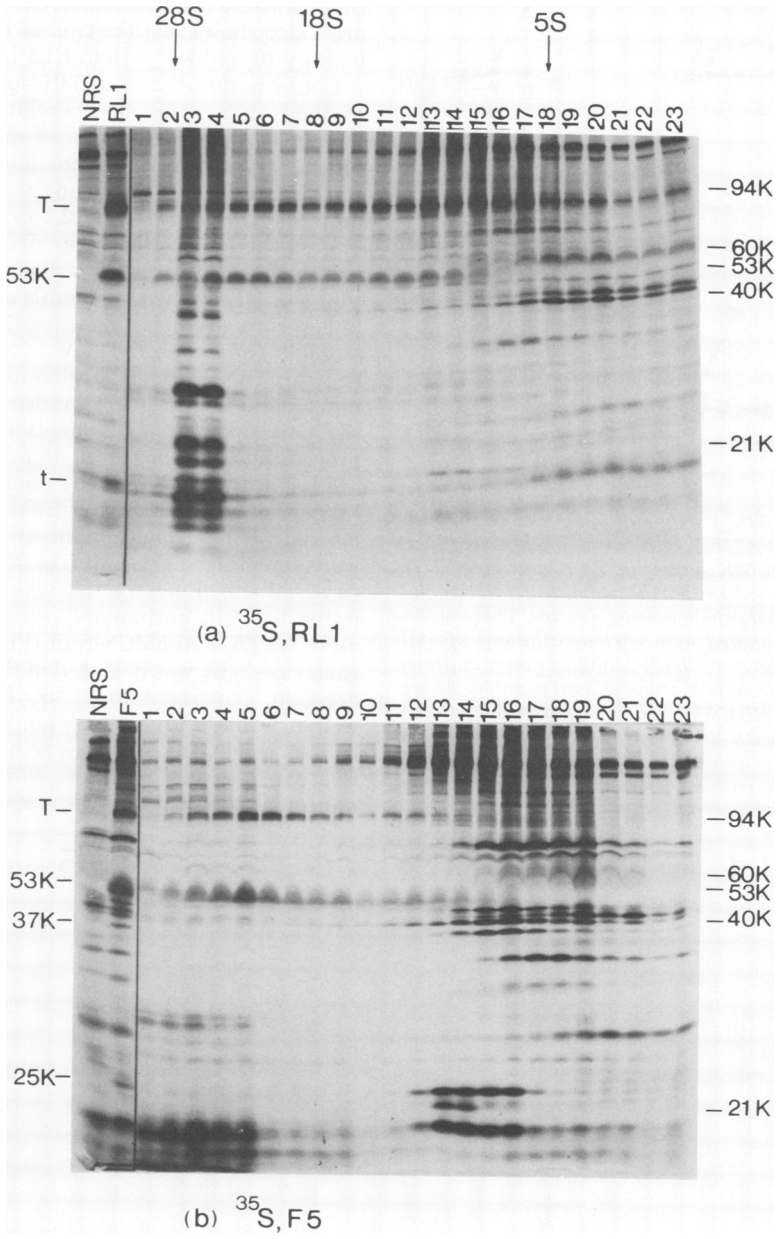
28S
18S
5S
NRS
RL1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
T–
53K–
t–
–94K
–60K
–53K
–40K
–21K
(a) ^{35}S, RL1
NRS
F5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
T–
53K–
37K–
25K–
–94K
–60K
–53K
–40K
–21K
(b) ^{35}S, F5

FIG. 6 a, b

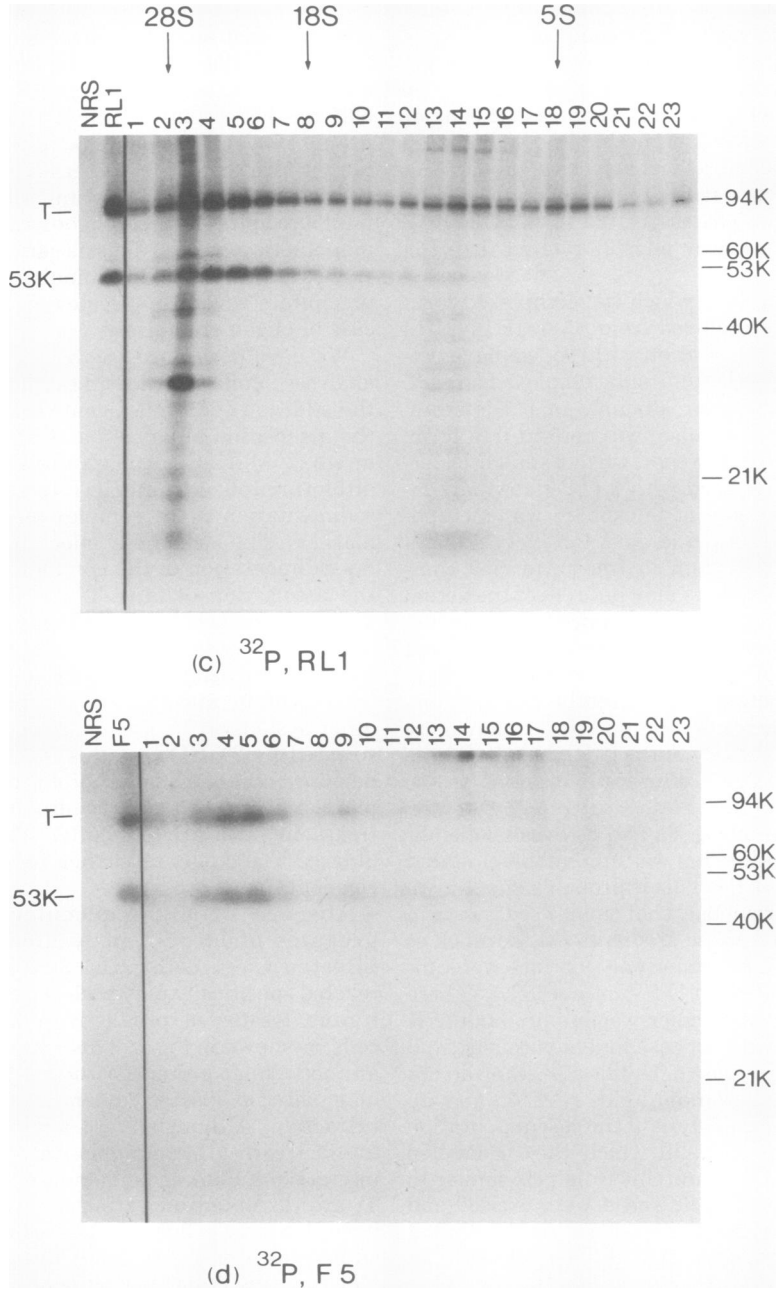

FIG. 6. *Sedimentation of the large-T-53K complex. Lysates of SVA31E7 cells labeled with [^{35}S]methionine (a and b) or [^{32}P]phosphate (c and d) were centrifuged through sucrose gradients (5 to 20% linear gradients with 0.4 ml of 60% sucrose cushion) as described in the experimental section. Each fraction was immunoprecipitated with 5 μl of RL1 (anti-large-T) or 25 μl of F5 (anti-53K) and analyzed by SDS-polyacrylamide gel electrophoresis. S values were obtained from ^{32}P-labeled rRNA markers run in parallel. The two tracks on the left end of each gel represent immunoprecipitations of the unfractionated lysate by normal rabbit serum (NRS), RL1, or F5.*

Large-T was immunoprecipitated by RL1 from all fractions of the gradients, with peaks at 23S and 5 to 6S. However, only the 23S, high-molecular-weight form was immunoprecipitated by F5. With this serum, no large-T was immunoprecipitated from fractions that do not contain 53K, although these fractions contain substantial amounts of large-T (e.g., fractions 18 to

23). This again argues that these proteins exist in a high-molecular-weight complex.

Figure 6c shows that the major form of large-T that was labeled with [^{32}P]phosphate is the 23S, 53K-associated form. In contrast, the major form labeled with [^{35}S]methionine sedimented as a low-molecular-weight species (about 6S). This difference suggests that under our labeling conditions, the 53K-associated form of large-T becomes more highly phosphorylated than the free form.

Using an assay in which the complexed form of large-T was removed from SVA31E7 lysates by repeated precipitation with F5, we have calculated that free large-T and complexed large-T label with equimolar amounts of [^{35}S]methionine during a 3-h pulse (870 cpm in free form, 890 complexed). However, when a similar experiment was performed with a [^{32}P]phosphate-labeled lysate, 2 mol of phosphate was incorporated into complexed large-T for every mole in free large-T (161 cpm in free form, 369 complexed). These figures may not reflect the actual molar ratios of these forms, since we do not yet know the rates of turnover or association and dissociation of the complex during labeling. These parameters are currently being determined. These results cannot be explained by phosphatase activity since [^{32}P]phosphate was not lost from large-T after incubations of lysates for 10 days at 4°C.

Comparison of Fig. 6a and b reveals an additional polypeptide that was immunoprecipitated by RL1 but not F5. This is probably small-t; we have shown previously that small-t sediments in this position on these gradients (McCormick et al., in press). Two minor polypeptides were immunoprecipitated by F5 but not RL1. These have apparent molecular weights of about 37K and 25K. They do not cosediment with 53K, and their origin is unknown. Unlike 53K, they do not appear to be phosphoproteins (Fig. 6b and d).

A quantitative analysis of the cosedimentation of large-T and 53K, in which the ^{32}P-labeled bands of these two proteins from gels similar to those shown in Fig. 6c and d were excised and counted, shows that the two proteins cosediment as one major species at 23S. The ratio of ^{32}P-labeled 53K to large-T was identical whether the complex was immunoprecipitated by RL1 or by F5. This suggests that there are little, if any, homopolymeric forms of phosphorylated 53K or large-T in this region of the gradient, since an unbound species would clearly alter the ratio of the two proteins immunoprecipitated by the two specific antisera. Similar results have been obtained with [^{35}S]methionine-labeled cells.

Since the top fractions of all these gradients contained no detectable levels of 53K and there did not appear to be homopolymers of 53K, we conclude that all the detectable 53K present in SVA31E7 cells is complexed to large-T. The absence of substantial levels of uncomplexed 53K has been confirmed by Crawford et al. (in press). However, it should be noted that uncomplexed forms may be less easily detected since equivalent amounts of serum would favor immunoprecipitations of oligomeric rather than monomeric species. This is simply because a single antigen-antibody binding event would precipitate several molecules of antigen in the case of oligomeric forms.

We have performed sucrose density gradient analyses similar to those described above, with the addition of various agents that might affect the association of large-T and 53K. Addition of up to 2 M NaCl, 0.5% Nonidet P-40, 0.1 M dithiothreitol, or 5 mM EDTA did not affect the sedimentation of the complex relative to rRNA markers. The lack of any effect of Nonidet P-40 on sedimentation of the complex indicates that the association of large-T and 53K is not an artifact generated by centrifuging these proteins out of detergent-containing solution into a nondetergent environment. Such an association to form protein micelles has been described in other systems (20). The complex is clearly dissociated by 2% SDS, as this reagent is used to denature and dissociate proteins for gel electrophoresis. The complex is also dissociated by treatment with pH 10.0 buffer. The association of large-T and 53K must therefore involve noncovalent bonds.

Absence of high-molecular-weight aggregates of large-T in lysates of lytically infected CV-1 cells. RL1 anti-large-T serum reacted specifically only with large-T and small-t from lysates of productively infected CV-1 cells, as shown in Fig. 3. This suggests that there are no cellular proteins associated with large-T or small-t in lysates under our conditions of extraction. Although these antigens may well interact with other proteins within cells, these interactions, unlike that of mouse 53K and large-T, are disrupted on extraction. Other authors have detected cellular proteins in immunoprecipitations from productively infected cells (14). However, the possibility remains that under our conditions of extraction and immunoprecipitation, large-T or small-t or both form aggregates, as reported by other workers (e.g., C. Prives, personal communication). To test this and to confirm that large-T does not associate with cellular proteins in lysates from productively infected CV-1 cells, we sedimented lysates from these cells under conditions identical to those used above. Figure 7 shows that all the large-T extracted from cells labeled with either [^{35}S]me-

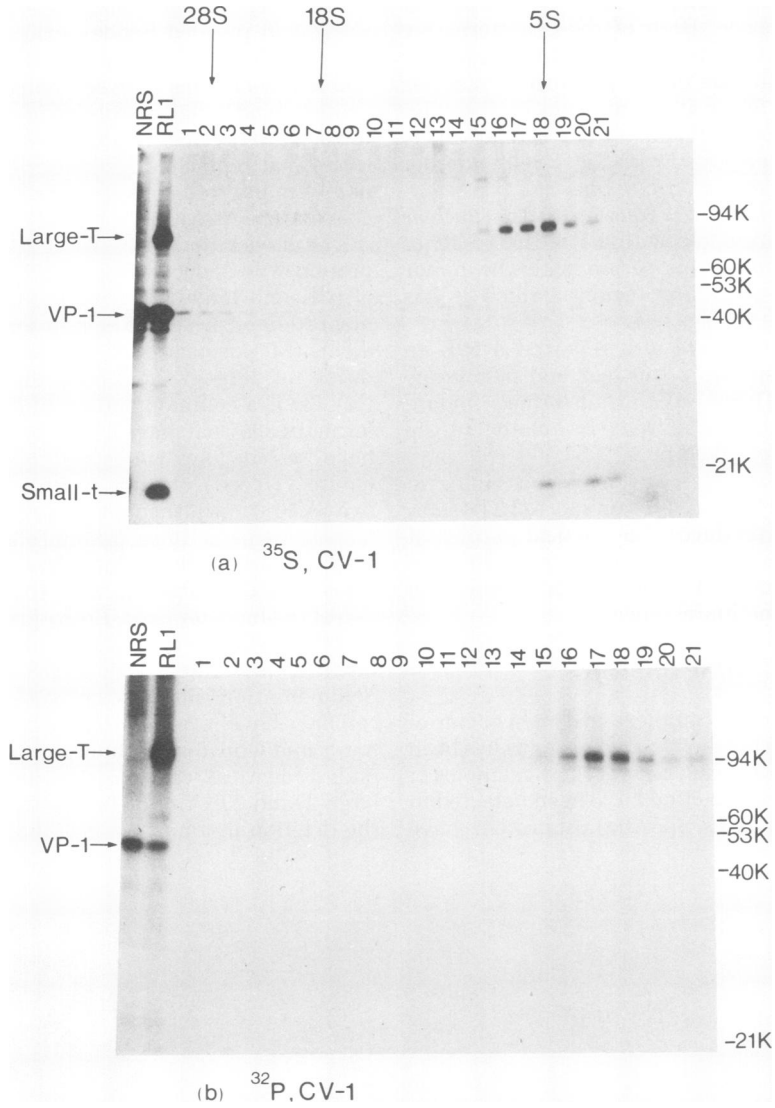

FIG. 7. *Sedimentation analysis of large-T from SV40-infected CV-1 cells. Lysates of SV40-infected CV-1 cells labeled with [^{35}S]methionine (a) or [^{32}P]phosphate (b) were centrifuged through sucrose gradients (5 to 20% linear gradients with 0.4 ml of 60% sucrose cushion) as described in the experimental section. Each fraction was immunoprecipitated with 5 μl of RL1 and analyzed by SDS-polyacrylamide gel electrophoresis. S values were obtained from ^{32}P-labeled rRNA markers run in parallel. The two tracks on the left end of each gel represent immunoprecipitations of the unfractionated lysate by normal rabbit serum (NRS) or RL1.*

thionine or [^{32}P]phosphate sediments at 5 to 6S, corresponding to a species with a molecular weight of about 100K. When similar gradients were immunoprecipitated with F5, no polypeptides could be detected, apart from VP-1, which is also precipitated by normal rabbit serum.

These experiments do not exclude the possibility that under different conditions of infection (e.g., higher multiplicity or at a different time after infection), it may be possible to detect interactions with cellular proteins or aggregates of the tumor antigens themselves.

Location of the large-T-53K complex in the cell nucleus. Since no free 53K can be detected in SVA31E7 cells (Fig. 6), the subcellular localization of 53K will also demonstrate the location of the complex. Figure 8 shows that the complex, detected by indirect immunofluorescence, is located in the cell nucleus. Fluorescent staining with F5 shows the classical nuclear

T-antigen pattern and, in some cells, a perinuclear fluorescence. Both of these patterns are also seen in cells stained with RL1 serum (Fig. 8). The significance of the perinuclear staining pattern is not clear at the moment but has also been seen in low frequency when similar experiments were performed with SVT2 cells, another SV40-transformed BALB/c line.

It is possible that a fraction of the nuclear fluorescence observed with F5 is the result of the interaction of this serum with the minor species (37K and 25K), against which it has weak activity.

Other authors have also reported a 50K to 60K protein species in nuclear and membrane preparations from SV40-transformed mouse cells (22). This protein may be related to the 53K protein described here.

F5 fails to produce any nuclear staining in SV40-infected CV-1 cells or uninfected 3T3 cells, whereas RL1 produces the classical pattern of nuclear fluorescence (data not shown). In all of these assays, normal rabbit serum produces no specific immunofluorescence.

DISCUSSION

The formation of complexes between bacteriophage proteins and host-coded proteins has been known for many years, but very few examples of these types of interaction have been detected in animal virus systems. Specific host proteins have been detected in virus preparations (e.g., actin is present in purified Sendai virions [10]), but to our knowledge there are no other instances of stable associations between nonstructural viral proteins and cellular proteins in eucaryotic systems. Even the high-molecular-weight DNA factories that replicate and transcribe vaccinia genomes in infected cells appear to be free of host enzymatic activities (16).

The association of SV40 large-T and a 53K protein was deduced from the co-precipitation of 53K and large-T (11) and from the co-sedimentation of these two proteins on density gradients (McCormick et al., in press). Co-sedimentation of large-T and 50K to 60K proteins (NVTs) is a common feature of all SV40-transformed cells that have been tested so far. We have tested four wild-type transformants of mouse 3T3 cells, one line transformed by *tsA*30, two SV40-transformed rat lines, and three transformed hamster lines (unpublished data). Lysates from all of these lines showed similar patterns of sedimentation, with the exception of two of the hamster lines. Both of these, CHLA30 and FLSV, showed low levels of NVTs cosedimenting with large-T at 10 to 15S instead of 23S. Sedimentation analyses on other transformed cell lines have recently been performed by Denhardt and Crawford (submitted for publication) under identical conditions. They found that large-T and 53K from 3T3 cells transformed by the deletion mutants *dl* 1263 and *dl* 1265 cose-

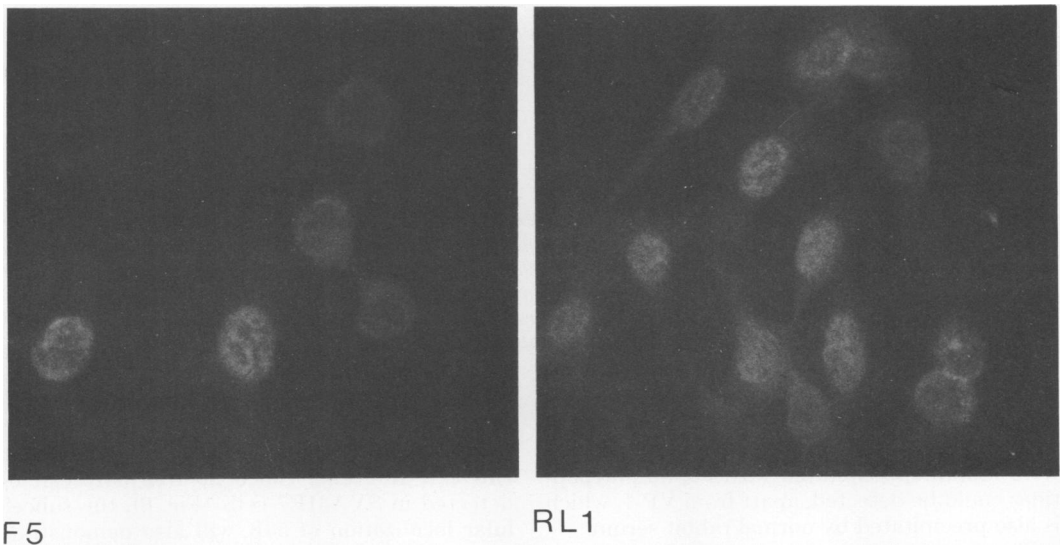

FIG. 8. *Immunofluorescence of SVA31E7 with F5 and RL1. Indirect immunofluorescence was performed on SVA31E7 monolayers fixed with acetone. The primary antiserum was F5 or RL1, followed by goat anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate. Photographed with 40× objective.*

diment as they do in wild-type transformants. Co-sedimentation is consistent with the hypothesis that NVTs and large-T are found in a complex, but could also have been explained by coincident sedimentation of uncomplexed proteins.

Here we have used immunochemical and physical approaches to investigate the possible association of these two cosedimenting proteins. The antiserum raised against 53K, a host-coded phosphoprotein, was able to precipitate large-T from SV40-transformed murine cells. We believe this is because 53K and large-T form a stable complex in these cells. This antiserum had no detectable activity against large-T in its native or denatured state and was unable to immunoprecipitate this protein in the absence of 53K. We have used SV40-infected CV-1 cells to assay for any activity against large-T since, in our hands at least, we were unable to detect any CV-1 proteins immunologically related to 53K. We have also observed that the unbound fraction of large-T found in transformed mouse cells is unable to react with anti-53K serum, and that large-T from transformed rat or hamster cells is not precipitated with this serum (data not shown). Presumably, this is because equivalent large-T binding proteins that cosediment with large-T found in these cells do not cross-react with serum raised against denatured 53K mouse protein.

The lack of shared antigenic determinants between denatured large-T and denatured 53K was originally demonstrated by Lane and Crawford (11). However, these workers were unable to test their anti-large-T serum for activity against native 53K because a source of native 53K free of large-T was not known at that time. Melero et al. (14) later reported the production of an antiserum raised against large-T that specifically recognized determinants on denatured 53K, but it now appears that these are separate activities (Melero, personal communication). The anti-53K serum, F5, described here has no detectable activity against native or denatured large-T and is therefore free of confusing cross-reactions.

The complex formed between large-T and 53K sediments at 23S relative to rRNA markers, giving an estimated molecular weight of 600K to 1,000K. A more accurate measure of the molecular weight must await further purification of the complex. This might also lead to an estimate of the number of subunits of large-T and 53K present in the complex. We have not yet been able to make such an estimate because the pulse-label conditions that we have used to label the proteins may not allow for equilibration of the specific activities of precursors before assembly into the complex. However, our results do show that the 53K-associated form of large-T is more readily phosphorylated than the free form during a pulse-label. This could mean that there are more sites for phosphorylation on the bound form, or that phosphate groups on this form turn over more rapidly and thus facilitate more rapid exchange of [^{32}P]phosphate with unlabeled phosphate during the pulse. A third possibility is that phosphate groups turn over rapidly and equally on both free and bound forms of large-T, so that the degree of incorporation of phosphate during a pulse-label reflects the actual level of free and bound forms, the latter being the major form. These possibilities are currently under investigation.

These sedimentation patterns have also allowed us to check for other proteins that might be present in the complex. No proteins detectable by immunoprecipitation cosediment with the large-T–53K complex. Nor can nucleic acids be found in the fast-sedimenting complex because extracts from cells labeled with ^{32}P that have been centrifuged, immunoprecipitated, and washed in extraction buffer show no bands other than 94K and 53K on SDS-polyacrylamide electrophoresis, even at the stacking region at the top of the gel.

There now seems to be ample reason to conclude that the complex is not an artifact of extraction. Attempts to dissociate the complex with reducing agents, chelating agents, and nonionic detergents have all proved unsuccessful. Only treatments with ionic detergents and strong denaturing solutions break the complex into its subunits. Similarly, the complex cannot be explained by micelle formation, because its sedimentation pattern is unaltered when centrifuged through gradients containing detergent. Numerous attempts to construct the complex by mixing free large-T and free 53K from separate sources have failed to date (unpublished data).

Because all the 53K recognized by F5 appears to be bound to large-T, we have been able to use the subcellular location of 53K to demonstrate that the complex is nuclear. Immunofluorescent staining of mouse transformants with F5 also shows a perinuclear pattern which is poorly understood at present.

Since only a portion of large-T is bound to 53K, it seems unlikely that a large-T function is effectively blocked by 53K unless the form of large-T that binds to 53K is the only active form. On the other hand, the "normal" function of 53K or the stability of this protein may well be modified by association with large-T.

Linzer and Levine (13) have suggested that

the 54K mouse NVT species they describe from SV40 transformants is absent, or present at low levels, in 3T3 cells, but infection or transformation by SV40 increases its synthesis or stability. Complexing with large-T may bring about this increased synthesis or stability. Interestingly, DeLeo and co-workers (6) have described a 53K mouse phosphoprotein that is detectable in all lines of transformed mouse cells that they have tested. This protein appears to be present in much lower levels in normal cells than in transformants and, like the 54K protein of Linzer and Levine, the 53K protein described here is immunogenic in some circumstances (11). The elevated production of a cellular protein in transformed cells is clearly of great interest and may be important in understanding the biochemical mechanism of transformation. Increased accumulations of numerous cellular proteins have been described as part of the process of transformation (*in* J. Tooze, ed., *Molecular Biology of Tumour Viruses,* in press), and the 53K protein described here may be another member of this class of proteins. In any event, 53K is distinguished from other host proteins in that it forms a stable complex with SV40 large-T. We have been unable to detect an analogous complex in productively infected cells, and the possibility that this association may be a necessary step in the process of transformation by SV40 must be considered.

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