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Characterization of a 54K Dalton Cellular SV40 Tumor Antigen Present in SV40-Transformed Cells and Uninfected Embryonal Carcinoma Cells

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Summary

SV40 infection or transformation of murine cells stimulated the production of a 54K dalton protein that was specifically immunoprecipitated, along with SV40 large T and small t antigens, with sera from mice or hamsters bearing SV40-induced tumors. The same SV40 anti-T sera immunoprecipitated a 54K dalton protein from two different, uninfected murine embryonal carcinoma cell lines. These 54K proteins from SV40-transformed mouse cells and the uninfected embryonal carcinoma cells had identical partial peptide maps which were completely different from the partial peptide map of SV40 large T antigen. An Ad2$^+$ND4-transformed hamster cell line also expressed a 54K protein that was specifically immunoprecipitated by SV40 T sera. The partial peptide maps of the mouse and hamster 54K protein were different, showing the host cell species specificity of these proteins. The 54K hamster protein was also unrelated to the Ad2$^+$ND4 SV40 T antigen. Analogous proteins immunoprecipitated by SV40 T sera, ranging in molecular weight from 44K to 60K, were detected in human and monkey SV40-infected or -transformed cells. A wide variety of sera from hamsters and mice bearing SV40-induced tumors immunoprecipitated the 54K protein of SV40-transformed cells and murine embryonal carcinoma cells. Antibody produced by somatic cell hybrids between a B cell and a myeloma cell (hybridoma) against SV40 large T antigen also immunoprecipitated the 54K protein in virus-infected and -transformed cells, but did not do so in the embryonal carcinoma cell lines. We conclude that SV40 infection or transformation of mouse cells stimulates the synthesis or enhances the stability of a 54K protein. This protein appears to be associated with SV40 T antigen in SV40-infected and -transformed cells, and is co-immunoprecipitated by hybridoma sera to SV40 large T antigen. The 54K protein either shares antigenic determinants with SV40 T antigen or is itself immunogenic when in association with SV40 large T antigen. The protein varies with host cell species, and analogous proteins were observed in hamster, monkey and human cells. The role of this protein in transformation is unclear at present.

Introduction

Simian virus 40 (SV40) contains the genetic information for two known gene products expressed at early times after productive infection or in transformed nonpermissive cells. The gene A product, or large tumor antigen (T antigen), has an apparent molecular weight of about 94K in SDS-polyacrylamide gels (Tegtmeyer, 1974). The second, or small t antigen, has an estimated molecular weight of 17K (Prives et al., 1977; Crawford et al., 1978) and shares common N terminal amino acid sequences (Paucha et al., 1978a) and antigenic determinants (Lane and Robbins, 1978; Carroll, Goldfine and Melero, 1978) with the large T antigen. Two messenger RNAs that code for these proteins have been isolated from infected cells (Paucha et al., 1978b). The larger RNA is about 2500 nucleotides long and codes for the small t antigen. The polynucleotide sequence that contains the information for the amino acid sequence of small t antigen is found between 0.67 and 0.54 map units on the viral genome (Berk and Sharp, 1978). The polynucleotide sequence containing the coding information for large T antigen is located in two noncontiguous segments of the SV40 genome between 0.67–0.59 and 0.54–0.14 map units (Berk and Sharp, 1978). Temperature-sensitive A gene mutants affect the stability of large T antigen (Tegtmeyer et al., 1975), and A gene deletion mutants (0.43–0.32 map units deleted) affect the size of this protein (Rundell et al., 1977). Deletion mutants in the 0.54–0.59 map unit region of the genome (Shenk, Carbon and Berg, 1976) affect only the size of the small t (17K dalton) protein (Crawford et al., 1978). These mutants have been used to demonstrate that both the large T antigen and small t antigen are required for viral transformation (Tegtmeyer, 1975; Brugge and Butel, 1975; Kimura and Itagaki, 1975; Martin and Chou, 1975; Osborn and Weber, 1975; Sleigh et al., 1978).

Animals bearing SV40-induced tumors produce antibody to both the large T and small t antigens (Black et al., 1963; Tegtmeyer, 1974; Prives et al., 1977; Crawford et al., 1978). Using antibody from tumor-bearing animals, it has been possible to immunoprecipitate ^{35}S-methionine-labeled large and small T antigens from infected or transformed cells (Tegtmeyer et al., 1975; Crawford et al., 1978). In all of these experiments, it has been common to immunoprecipitate additional proteins from infected or transformed cells specifically with molecular weights of 44K–88K, depending upon the species of the host cell or particular cell line used for the study (Tegtmeyer et al., 1975; Griffin, Light and Livingston, 1978). The fact that these proteins were indeed specifically immunoprecipitated and not just contaminants was suggested by the fact that normal or preimmune sera did not detect these intermediate molecular weight proteins and they were found only in SV40-infected or -transformed cells (Tegtmeyer et al., 1975). The origin of these proteins, however, has not been clear. They could result (in whole or part) from the proteolytic breakdown of large T antigen (Mann et al., 1977), or

they might be viral or cellular proteins associated with large or small T antigen in solution and therefore co-precipitated with the viral tumor antigens. On the other hand, they may be cellular proteins that were antigenic in the SV40-induced tumor tissue or transformed cells. In the related papovavirus, polyoma, these intermediate molecular weight antigens (36K–63K) were not immunoprecipitated when the polyoma small t antigen deletion mutant NG18 was used to infect cells, and they share common peptides with the large and small T antigens, indicating that they were either virus-encoded or regulated by the virus (Ito, Brocklehurst and Dulbecco, 1977; Smart and Ito, 1978; Hutchinson, Hunter and Eckhart, 1978; Schaffhausen, Silver and Benjamin, 1978).

The experiments presented here characterize one of these intermediate molecular weight (54K) SV40 tumor antigens of murine cells. The results demonstrate that this protein is of cellular origin and is expressed in several uninfected murine embryonal carcinoma cell lines. Analogous but clearly different proteins were detected in hamster, monkey and human cells infected or transformed with SV40, showing a distinct species distribution of the host cell rather than virus specificity. The available evidence indicates that SV40 large T antigen and the murine 54K middle T antigen are physically associated in solution, and that either these proteins share antigenic determinants or the cellular protein is itself immunogenic when complexed or associated with SV40 T antigen.

Results

SV40 Tumor Serum Contains Antibody to a 54K Protein Found in SV40-Infected Cells and Embryonal Carcinoma Cells

Infection of most mouse cells with SV40 results in the synthesis of two viral proteins with molecular weights of approximately 94K (T antigen) and 17K (t antigen) daltons. These proteins can be detected by immunoprecipitation of the labeled soluble infected cell proteins by serum from animals bearing SV40-induced tumors (Tegtmeyer, 1974; Crawford et al., 1978). Embryonal carcinoma cells, the undifferentiated murine stem cells of teratocarcinomas, are unusual in this regard in that they do not express these SV40 early viral proteins after virus infection (Swartzendruber and Lehman, 1975; Swartzendruber, Friedrich and Lehman, 1977; S. Segal, A. J. Levine and G. Khoury, manuscript submitted). This restriction on SV40 early virus gene expression in embryonal carcinoma cells is not at the level of adsorption, penetration, uncoating or transport to the nucleus (Swartzendruber et al., 1977). Indeed, apparently normal SV40 transcriptional intermediates detected in vitro can be isolated from SV40-infected embryonal carcinoma cells (F9 cell line) (S. Segal et al., manuscript submitted). In the course of studying the restriction of SV40 early gene expression in embryonal carcinoma cells, the following experiment was performed. Two culture dishes each of BALB/c3T3 cells, embryonal carcinoma cell line F9 and embryonal carcinoma cell line PCC-4aza-1 were prepared. One dish from each of these three sets was infected with SV40 and the remaining plate was mock-infected. All six cultures were labeled with ^{35}S–methionine 36–38 hr after infection and soluble extracts were prepared for immunoprecipitation. Infected and uninfected cell extracts from each of the three cell lines (3T3, F9 and PCC-4) were incubated with sera from SV40-induced tumor-bearing animals (T sera) or with normal sera from nontumor-bearing animals. The immunoprecipitates were collected and analyzed by SDS-polyacrylamide gel electrophoresis. Figure 1 shows the autoradiogram containing the labeled proteins immunoprecipitated from these cells. The 94K large T antigen was detected in SV40-infected 3T3 cells. This protein was not immunoprecipitated by normal serum from infected 3T3 cells, and neither normal nor immune sera detected this protein in uninfected 3T3 cells. Similarly, the 94K antigen was not found in either infected or

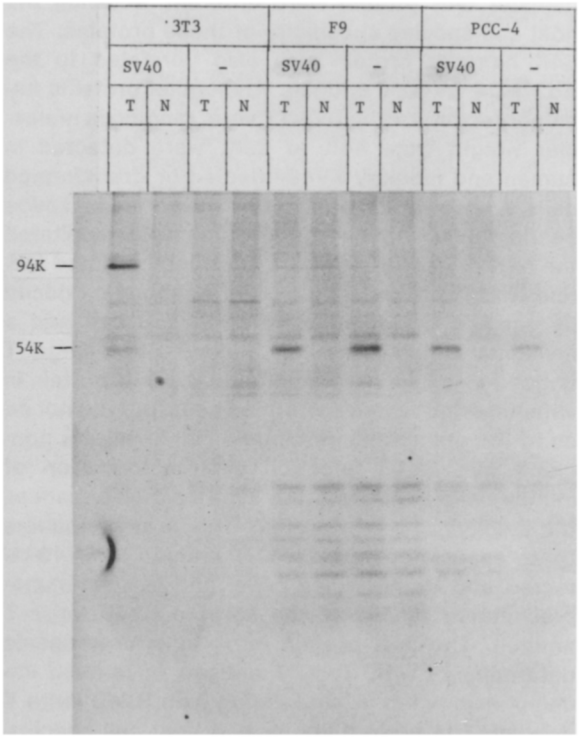

Figure 1. Immunoprecipitation of SV40- and Mock-Infected Mouse Cells

Labeled extracts (25 μCi/ml) of ^{35}S–methionine from 36–38 hr post-infection from SV40- or mock-infected (–) BALB/c3T3, F9 and PCC-4 cells were treated with hamster anti-SV40 tumor serum (T) or normal hamster serum (N). The immunoprecipitates were analyzed on a 15% polyacrylamide gel which was exposed to X-ray film for 1 week to give the autoradiograph.

uninfected F9 or PCC-4 cells with either immune or normal sera. The small t antigen of 17K daltons which is produced in SV40-infected BALB/c3T3 cells was not detected in this experiment because the amount produced in these cells is below the level of detection using this labeling protocol.

The SV40-infected BALB/c3T3 cells also synthesized a 54K dalton protein that was specifically immunoprecipitated by immune serum and not by normal serum. Comparable levels of this protein were not detected in uninfected cells using normal or immune sera. Perhaps more surprising was the observation that immune SV40 T sera immunoprecipitated a 54K protein from embryonal carcinoma cell lines F9 and PCC-4 regardless of whether these cells were infected with this virus. Normal sera failed to immunoprecipitate this 54K protein from F9 or PCC-4 cells.

These experiments demonstrate that SV40 infection of 3T3 cells stimulates the production of a 54K dalton antigen that can be specifically immunoprecipitated by serum from animals carrying SV40-induced tumors. A similar protein was not detected in uninfected 3T3 cells, but a protein of the same molecular weight and antigenic properties was constitutively expressed in two different embryonal carcinoma cell lines, F9 and PCC-4. It has previously been shown that antisera to mouse teratocarcinoma embryoid bodies made in rabbits (xenogeneic sera) contain antibodies that react with SV40-transformed fibroblastic cell lines (Edidin et al., 1971; Edidin, Gooding and Johnson, 1974; Gooding, Hsu and Edidin, 1976).

SV40-Transformed Cells and Embryonal Carcinoma Cells Contain a 54K Dalton Phosphoprotein Immunoprecipitated by SV40 T Serum

To determine whether a similar 54K protein was also present in SV40-transformed mouse cell lines, the following experiment was performed. Culture dishes of PCC-4 and SV40 BALB/cT2, an SV40-transformed BALB/c3T3 cell line, were labeled with ^{35}S-methionine for 4 hr. Soluble cell extracts were prepared, immunoprecipitated and analyzed on polyacrylamide gels as described in Experimental Procedures. Figure 2 shows the autoradiograms from this experiment. The SV40 T serum, but not the normal serum, immunoprecipitated a 54K protein from both PCC-4 and SVT2 cells. To determine whether the 54K protein found in these two cell lines was a phosphoprotein, the same experiment was repeated, except that $^{32}PO_4$ was used to label these cells for 4 hr instead of the ^{35}S-methionine. The autoradiograms of the $^{32}PO_4$-labeled proteins are shown in Figure 2. The 54K proteins from both PCC-4 and SVT2 were labeled with $^{32}PO_4$ added to the medium. The large T antigen, which is seen in these cells as a characteristic doublet at about 94K daltons, was detected only in SVT2 cells

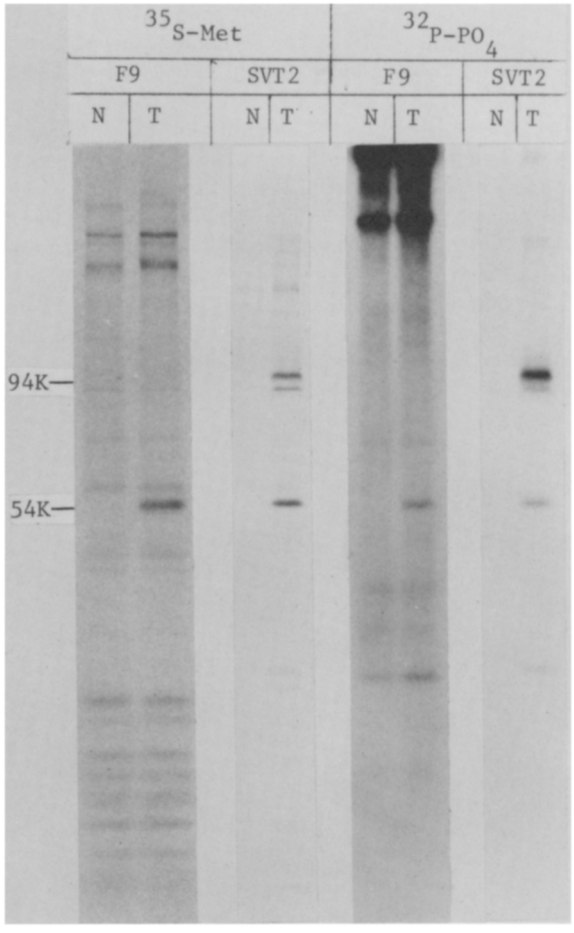

Figure 2. Phosphorylation of the 54K Protein

Uninfected F9 cells and SV40-transformed B3T3/c cells (SVT2) were labeled with either ^{35}S-methionine (25 μCi/ml) or ^{32}P-phosphate (25 μCi/ml) for 4 hr. Extracts were immunoprecipitated with hamster anti-SV40 tumor serum (T) or normal hamster serum (N) and run on a 15% polyacrylamide gel. The autoradiograph of the ^{32}P lanes was made with a Cronex Lightning-Plus intensifying screen (DuPont).

(immune serum), and was labeled with both ^{35}S-methionine and $^{32}PO_4$ as previously described by Tegtmeyer, Rundell and Collins (1977).

Partial Peptide Analysis of the 54K Dalton Protein from SV40-Transformed Cells and Embryonal Carcinoma Cells

The 54K proteins detected in SV40-infected or -transformed mouse cells and PCC-4 and F9 are not necessarily related proteins. Since sera from SV40-induced tumor-bearing animals contain antibodies of many different specificities, it was possible that antibody molecules directed against different antigenic determinants were responsible for immunoprecipitation of the SVT2 54K protein and the PCC-4 protein. The partial peptide mapping procedure of Cleveland et al. (1977) was used to examine the relationship between these two proteins. SVT2 and PCC-4 cells

were labeled with ^{35}S-methionine for 4 hr, and the 54K proteins from these two cell lines were obtained by immunoprecipitation and gel electrophoresis. The proteins from SVT2 and PCC-4 were removed from their respective gels and treated with the S. aureus V8 protease. After proteolysis, the partial peptide fragments were electrophoresed through a 20% polyacrylamide gel. Figure 3 shows the autoradiogram of the ^{35}S-methionine-containing partial peptides generated from the 54K proteins of PCC-4 and SVT2 cells. Nine distinct partial peptides were resolved with each of these proteins, and they were identical for the two 54K proteins derived from SVT2 and PCC-4.

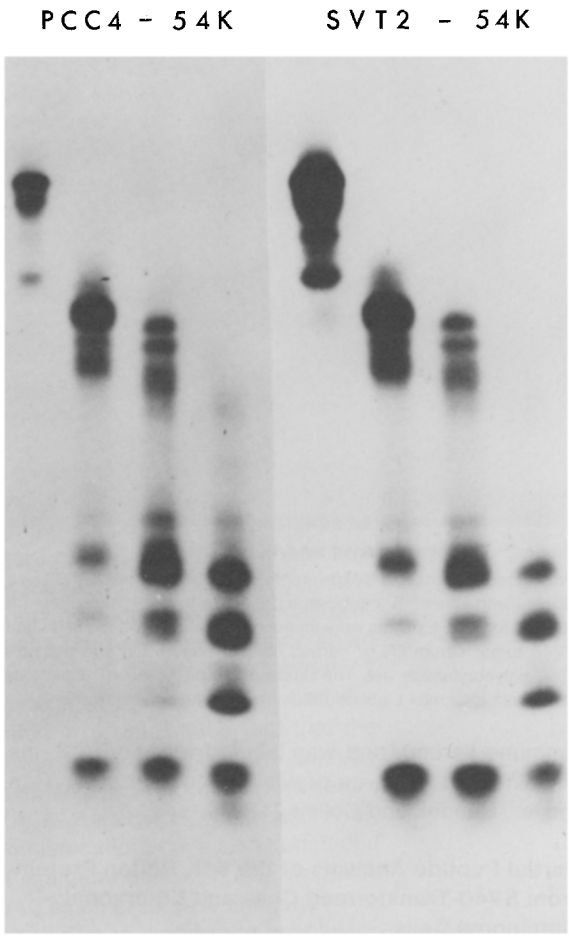

Figure 3. Limited Proteolysis Peptide Map of the 54K Protein

The 54K protein was prepared from PCC-4 and SVT2 cells which were labeled for 4 hr with 100 μCi/ml of ^{32}S-methionine. Immunoprecipitates with anti-SV40 tumor sera were run on a 10% polyacrylamide gel. Equal volumes were loaded into four separate wells for each of the two cell lines. After staining in 0.25% Coomassie brilliant blue (in water) and destaining in 0.125 M Tris–HCl (pH 6.8), the 54K protein bands were excised using protein markers as a guide. Each band was positioned in the bottom of a well in a 20% polyacrylamide gel and overlaid with 15 μl of 0.1% SDS-sample buffer containing 0, 18, 90 or 450 μg of S. aureus V8 protease (left to right). Electrophoresis at 30 V through a 1 in stacking gel was followed by electrophoresis at 140 V until completion. The gel was then fluorographed, dried and exposed at −70°C.

This analysis demonstrates that the 54K phosphoproteins from SV40-transformed mouse cells and uninfected embryonal carcinoma cells are very similar, if not identical. To determine the relationship, if any, between the 54K proteins and SV40 large T antigen (94K daltons), a partial peptide map analysis was carried out comparing the SV40 large T antigen from SV80 cells (SV40-transformed human cell line) and the SVT2 54K protein. The partial peptide mapping procedure has been useful in detecting the relationship between a protein and a proteolytic breakdown product of that protein (Levinson and Levine, 1977).

The SV40 large T antigen was labeled with ^{35}S-methionine in SV80 cells and isolated by immunoprecipitation and gel electrophoresis as described previously. The 54K protein from SVT2 cells labeled with ^{35}S-methionine was obtained as usual. Figure 4 shows the S. aureus protease partial peptide maps of these two proteins. The pattern of partial proteolysis of the SV80 large T antigen bears little or no resemblance to the 54K protein of SVT2 cells.

Murine Species Specificity of the 54K Dalton Protein

The experiments described above demonstrate that sera from animals bearing SV40-induced tumors can specifically immunoprecipitate a 54K protein from SV40-infected (but not uninfected) and -transformed murine cells. The same or a very similar protein is constitutively expressed in two murine embryonal carcinoma cell lines. These results lead to the conclusion that the 54K protein is encoded for by the mouse genome and not by the virus. It was therefore of some interest to look for similar proteins expressed in SV40-infected or -transformed cells derived from animal species other than the mouse. Four different host cells were compared in this study: an Ad2$^+$ND4-transformed hamster cell line, ND4-HK-A1678; a human cell line transformed with SV40, SV80; a monkey cell line, BSC-1, productively infected with SV40; and a mouse cell line, SVTER14, which is an SV40-transformed cell line (from 129Sv/S1 mice) derived from differentiated teratoma cells in culture (Topp et al., 1977). Cell cultures from these four cell lines were labeled for 2 hr with ^{35}S-methionine. SV40-infected and mock-infected BSC-1 cells were labeled at 30–32 hr after infection. In all cases, soluble protein extracts were prepared from the labeled cells. Anti-SV40 T sera from two independent sources—SV40 virus-induced tumors in hamsters and Ad2$^+$ND4-transformed cell-induced tumors in hamsters—were used to immunoprecipitate the SV40 tumor antigens; normal hamster serum was used as a control. The immunoprecipitates were analyzed on SDS-polyacrylamide gels, and Figure 5 shows the autoradiogram produced from this experiment. Both SV40 T sera from tumor-bearing hamsters, but not normal sera, immunoprecipitated SV40 large T antigen from

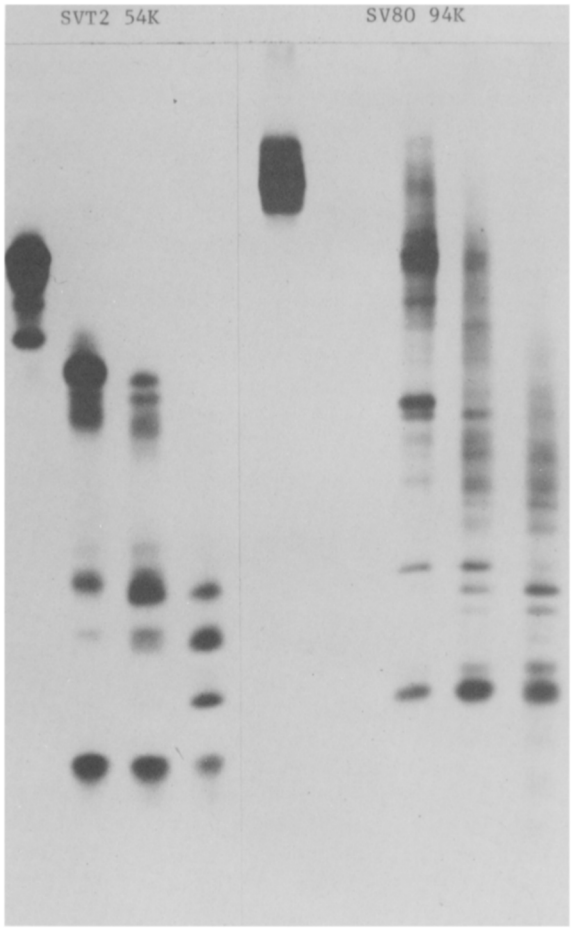

Figure 4. Comparison of the 54K Protein with SV40 T Antigen

Peptide mapping of the ^{35}S-methionine (100 μCi/ml)-labeled SVT2 54K and SV80 94K (T antigen) proteins was performed as described in the legend to Figure 3. The final 20% polyacrylamide gel was fluorographed prior to exposure at −70°C.

Ad2$^+$ND4-transformed hamster cells, the SVTER14-transformed mouse cells, the SV80-transformed human cells and the infected (but not uninfected) monkey BSC-1 cells. The 54K protein was also detected by both immune sera but not by normal serum in the SVTER14 mouse cells and Ad2$^+$ND4-transformed hamster cells. In the human and monkey cells transformed or infected by SV40, no indication of a 54K protein was obtained. Instead, the immune sera precipitated a variety of labeled proteins in the 44K–60K dalton range (44K, 48K, 58K and 60K daltons). One of the immune sera was consistently better at detecting these middle molecular weight tumor antigens, and the levels of these proteins were generally lower than that of the 54K protein detected in mouse and hamster transformed cells with immune hamster sera. The immune T sera used here did not detect SV40 T antigens and 54K proteins in uninfected BSC-1 cells, uninfected hamster cells (BHK21) or human cells (HeLa cells). A strong doublet at 17K daltons was also detected with the SVTER14 cells, while longer exposures of the autoradiogram were required to observe similar proteins in the SV80 cells and SV40-infected BSC-1 cells.

These experiments demonstrate that both SV40-transformed hamster and mouse cell lines contain a 54K protein. The middle molecular weight T antigens from human and monkey cells differed in size from the 54K protein and were detected in lower quantities by immunoprecipitation with hamster immune T sera. This result could be explained by a number of factors, including the possibility that some of the middle molecular weight T antigens from human or monkey cells result from proteolysis of large T antigen.

Partial Peptide Maps of Mouse and Hamster 54K Dalton Proteins

The fact that both mouse and hamster transformed cell lines express a 54K protein immunoprecipitable by hamster SV40 T sera suggested the possibility that these proteins might be related, as is the case with the murine embryonal carcinoma and murine SV40-transformed cell proteins. To test this hypothesis, the 54K proteins from murine SVT2 cells and hamster Ad2$^+$ND4-transformed cells were labeled with ^{35}S-methionine, immunoprecipitated and isolated from SDS-polyacrylamide gels as described in Experimental Procedures. These two proteins were then treated with S. aureus V8 protease to generate a series of partial peptides. Figure 6 shows the autoradiogram of the partial peptides from the mouse and hamster 54K proteins. No apparent relationship can be discerned between these two proteins, indicating that the host cell species specificity has generated different 54K proteins in SV40-transformed hamster and mouse cells. Furthermore, the 54K T antigen from the Ad2$^+$ND4-transformed cells does not have an S. aureus partial peptide map that resembles that of the Ad2$^+$ND4 SV40 large T antigen, indicating that the 54K protein is not related (by proteolysis) to the SV40 large T antigen (results not shown).

Survey of SV40 T Sera with Anti-54K Protein Activity

The 54K protein in SV40-infected and -transformed mouse cells is specifically immunoprecipitated by sera from hamsters carrying SV40-induced tumors. Sera from sources other than hamster were also tested to determine whether mice would produce antibody to the 54K protein in SV40-transformed cells or embryonal carcinoma cells. Using immunoprecipitation of ^{35}S-methionine-labeled proteins from BALB/c3T3 cells, BALB/c3T3 cells infected with SV40, SVT2-transformed BALB/c3T3 cells and F9 or PCC-4 embryonal carcinoma cells, sera from six different sources were tested for anti-54K protein activity. These sera were derived from hamsters bearing SV40 virus-induced tumors, hamsters bearing Ad2$^+$ND4-in-

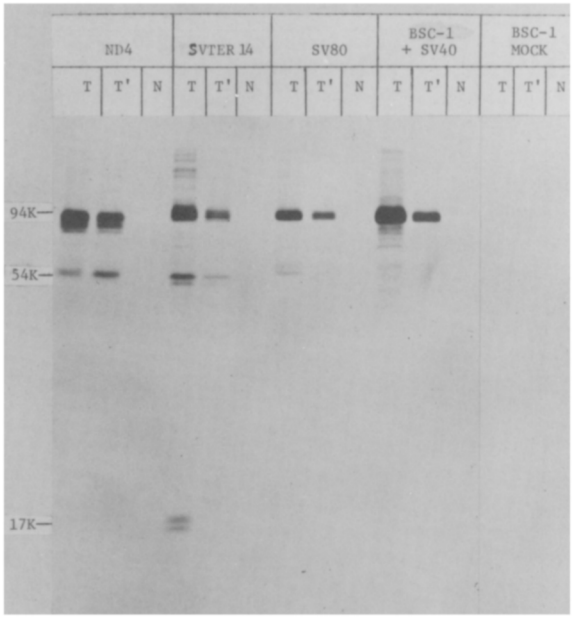

Figure 5. Presence of Middle Molecular Weight Antigens in Other Species

SV40- or mock-infected BSC-1 monkey cells were labeled with 50 μCi/ml of ^{35}S-methionine from 30–32 hr post-infection. The Ad2^{+}ND4-transformed hamster line (ND4), the SV40-transformed teratocarcinoma cell line (SVTER14) and the SV40-transformed human line (SV80) were also labeled for 2 hr with 50 μCi/ml of ^{35}S-methionine. Extracts were treated with hamster anti-SV40 serum (T), hamster anti-ND4 serum (T') or normal hamster serum (N). Samples were run out on a 20% polyacrylamide gel which was fluorographed prior to a 1 day exposure at −70°C.

duced tumors, BALB/c mice bearing SVT2-induced tumors, 129Sv/S1 mice hyperimmunized with SV40-transformed 129Sv/S1 cell lines SVTER14 and SVTER104, 129Sv/S1 mice bearing tumors induced by an injection of F9 or PCC-4 cells, and anti-SV40 T sera produced by nine hybridoma cell lines against SV40-transformed mouse cells (Martinis and Croce, 1978).

Table 1 reviews the results of these experiments. None of the six different sera tested detected the 54K protein in uninfected BALB/c3T3 cells. Both hamster and mouse sera from either tumor-bearing or hyperimmunized isogeneic mice (BALB/c and 129Sv/S1 mice) contained anti-54K protein activity in SV40-infected and -transformed mouse cells as well as in embryonal carcinoma cells (F9 and PCC-4). Normal sera did not contain any detectable antibody to this protein. On the other hand, 129Sv/S1 mice bearing F9- or PCC-4-induced tumors failed to make detectable levels of antibody to the 54K protein. A wide variety of other embryonal carcinoma cell lines (twelve were tested in all) which produced tumors in several inbred strains of mice also failed to produce detectable levels of anti-54K antibody. Anti-large T antigen antibodies produced by nine independent hybridoma cell lines immunoprecipitated both SV40 large T antigen and the 54K protein from SV40-infected and -transformed 3T3 cells. The same hybridoma sera, however, did not immunoprecipitate detectable levels of the 54K protein from the PCC-4 or F9 cells. While SV40 T sera from all sources tested specifically immunoprecipitated the 54K protein from F9 and PCC-4 cells, the hybridoma sera consistently failed to immunoprecipitate the same detectable levels of 54K protein from these cells.

Figure 7 shows examples of the autoradiograms of the SDS-polyacrylamide gels displaying the SV40 tumor antigens detected by these different sets of sera and used to construct the data presented in Table 1. It is clear from this survey that a wide variety of mouse and hamster sera from SV40-induced tumor-bearing animals produce antibody that immunoprecipitates the 54K dalton proteins.

Discussion

When the soluble proteins from SV40-infected or -transformed cells were analyzed by immunoprecipitation with sera from animals bearing SV40-induced tumors, a number of proteins were specifically immunoprecipitated (Tegtmeyer, 1974). In addition to the known viral-encoded proteins, large T antigen of 94K and small t antigen of 17K daltons (Tegtmeyer, 1974; Crawford et al., 1978), several investigators have described immunoprecipitable proteins in the 36K–66K molecular weight range (Tegtmeyer, 1974; Mann et al., 1977; Griffin et al., 1978; Melero et al., 1979), depending upon the species of host cells used or other factors. The origin of this so-called middle T antigen species has been obscure. They could be virus-encoded proteins which have mRNAs distinct from those for large and small T antigens (Berk and Sharp, 1978), or even proteolytic breakdown products, perhaps artifactual, of large T antigen; cellular proteins which are complexed with large or small T antigen and co-immunoprecipitate with these viral proteins; or cellular proteins that either share antigenic determinants with large or small T antigens or are immunogenic themselves in SV40-induced tumors.

The experiments presented here appear to eliminate the first possibility. SV40-infected and -transformed mouse cells contain a 54K phosphoprotein that is specifically immunoprecipitated by sera from mice or hamsters carrying SV40-induced tumors. This protein was not detected in uninfected 3T3 cells at the levels observed in SV40-infected or -transformed cells. Very small amounts of a 54K protein (about 1–5% of the levels found in SV40-transformed 3T3 cells) have been detected in uninfected 3T3 cells by immunoprecipitation with immune but not normal sera. Whether this protein is similar or identical to the 54K protein expressed in SV40-infected or -transformed

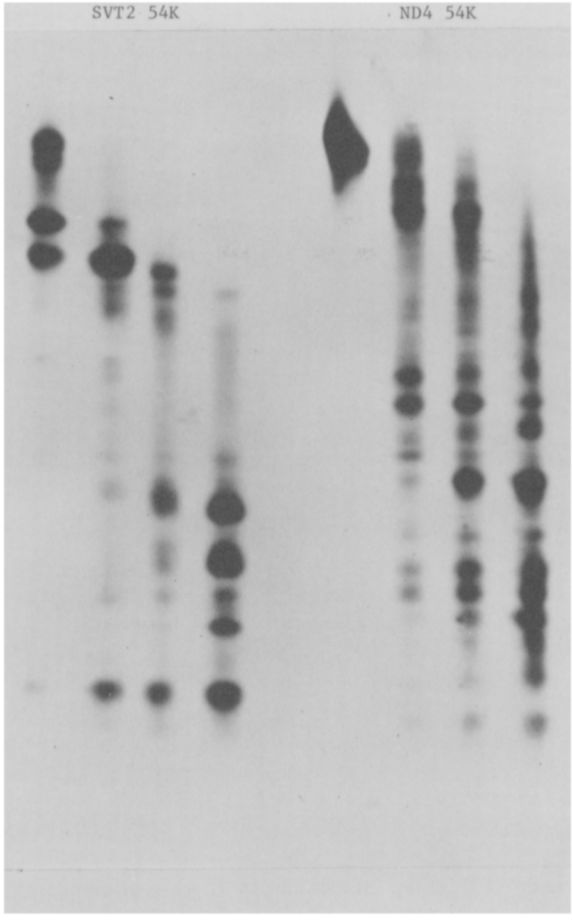

Figure 6. Comparison of the Murine and Hamster 54K Proteins

The 54K proteins from SVT2 and ND4 were prepared and analyzed as described in the legend to Figure 3. The final gel was fluorographed before exposure at −70°C.

cells remains to be determined. It is clear, however, that SV40 infection or transformation specifically stimulates the synthesis or stabilizes the turnover (prevents the degradation) of this 54K protein. A protein of the same molecular weight, immunoprecipitated by sera from animals bearing SV40-induced tumors, is constitutively expressed in two different embryonal carcinoma cell lines, F9 and PCC-4. These two cell lines are refractory to SV40 infection. They do not express SV40 early proteins because of a defect in the processing of viral early RNA (S. Segal et al., manuscript submitted), and thus it is highly improbable that these cell lines would be contaminated with SV40 as a carrier culture. The 54K proteins from SV40-transformed cells and PCC-4 embryonal carcinoma cells have identical partial peptide maps when analyzed with the S. aureus protease (specific for aspartic and glutamic acid residues), demonstrating that these proteins are very similar or identical. Partial proteolysis tends to maximize differences between proteins where one difference in a glutamic acid residue between two similar proteins, for example, could lead to several different partial peptides. Since, however, only methionine-containing partial peptides were analyzed in this study, it remains possible that some small differences exist in the SVT2 and PCC-4 54K proteins. The constitutive expression of the 54K protein in F9 and PCC-4 cells in the absence of SV40 virus eliminates the possibility that this protein is a proteolytic breakdown product of large T antigen or is encoded by an SV40 mRNA. This is consistent with the observation that the mRNA coding for the in vitro synthesis of middle molecular weight SV40 T antigens does not hybridize to SV40 DNA-cellulose (E. Paucha, R. Smith and A. E. Smith, personal communication). These experiments also eliminate the possibility that the SV40 T serum does not have antibody to the 54K protein itself and that this protein is co-precipitated only because of its association with large T antigens. While a physical association between 54K protein and SV40 T or t antigens remains possible in virus-infected or -transformed cells, this cannot be the case in embryonal carcinoma cells that express this protein in the absence of SV40 large and small T antigens. Thus it would seem that SV40 specifically induces or stabilizes a 54K cellular protein that either shares antigenic determinants with large T antigen or is itself antigenic. Melero et al. (1979) have concluded that the complex of 48K–55K middle T antigens observed in SV40-infected or -transformed cells does in fact share antigenic determinants with SV40 large T antigen.

The fact that nine different monoclonal hybridoma sera immunoprecipitate or co-immunoprecipitate both SV40 large T antigen and the 54K protein from virus-infected and -transformed cells, but do not immunoprecipitate the 54K protein from F9 or PCC-4 cells, indicates the following: first, the hybridoma series of antibody does not detect cross-reacting antigens, by this immunoprecipitation procedure, between large T antigen and the 54K protein; and second, the hybridoma sera most probably co-immunoprecipitate the 54K proteins in virus-infected and -transformed cells by virtue of an association or complex of large T antigen and the 54K protein in these cells. It should be pointed out that while a series of nine monoclonal antibodies to large T antigen fail to detect a cross-reacting antigenic determinant with the 54K protein, one cannot yet conclude that such a determinant does not exist.

The hybridoma sera do indicate that the 54K protein in virus-infected and -transformed cells is associated with the SV40 large T antigen. There is some evidence that this complex might render the 54K protein immunogenic, as if it behaved like a haptene, with SV40 large T antigen as the carrier protein. This would explain the presence of anti-54K protein activity in

Table 1. Survey of SV40 T Sera with Anti-54K Protein Activity

Serum Source	BALB/c3T3	BALB/c3T3 + SV40-Infected	SVT2-Transformed	Embryonal Carcinoma F9, PCC-4
Hamster, SV40-induced tumor	–	+	+	+
Hamster, Ad2+ND4-transformed cell tumor	–	+	+	+
Mouse, SVT2-transformed cell tumor	–	+	+	+
Mouse, SVTER14, 104 hyperimmunized	–	+	+	+
Mouse, embryonal carcinoma cell tumor F9, PCC-4	–	–	–	–
Mouse, SV40 T hybridoma sera	–	+	+	–

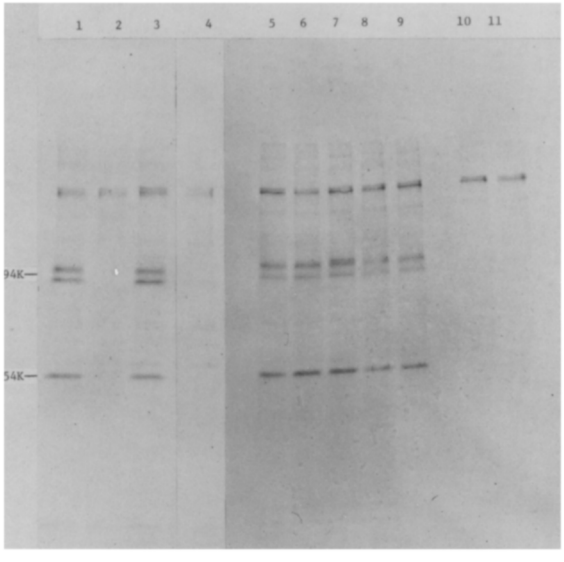

Figure 7. Survey of Various Sera for Anti-54K Protein Activity

A labeled SVT2 extract (40 μCi/ml of ^{35}S–methionine) was immunoprecipitated with the following: (1) BALB/c anti-SV40 tumor serum; (2) BALB/c normal serum; (3) hybridoma B16.1 #1B6 C15 fluid; (4) ascites fluid from the parental myeloma, P3 × 638Ag, which was used for the hybridoma line; (5–7) three independent sera from 129Sv/S1 mice hyperimmunized with SVTER14; (8–9) two independent sera from 129Sv/S1 mice hyperimmunized with SVTER104; (10–11) two independent 129Sv/S1 teratocarcinoma tumor sera. Samples were analyzed on a 15% polyacrylamide gel.

SV40 tumor sera and the failure of mice bearing F9 or PCC-4 tumors to produce detectable levels of antibody to the 54K protein. Alternate explanations, however, may account for the lack of immunogenicity of the 54K protein in F9- or PCC-4-induced tumors.

Another line of evidence that the 54K protein is in fact encoded by the mouse genome derives from a comparison of the 54K proteins immunoprecipitated from mouse and hamster transformed cells. While antibodies to both hamster and mouse 54K proteins were present in a large number of SV40 T sera, the partial peptide maps of these two proteins are clearly dissimilar. An analysis of the methionine-containing complete tryptic peptides of the mouse and hamster 54K proteins has indicated that between 1 and 4 peptides were common to these two proteins, while there were also a large number of unique peptides (W. Maltzman, unpublished observations). The human and monkey middle T antigens have a set of molecular weights different from the mouse-hamster proteins and appear to be immunoprecipitated less efficiently by mouse or hamster SV40 anti-T sera. This could be due to the more distant relationship of these proteins to the mouse-hamster 54K protein or even to SV40 large T antigen itself. It certainly remains possible that the human-monkey set of middle T antigens does indeed consist of proteolytic breakdown products of large T antigen which are not analogous to the hamster-mouse 54K proteins. It is clear, however, that the S. aureus partial peptide maps of SV40 large T antigen are not similar to the same peptide maps of mouse or hamster 54K protein. If the 54K protein contained a reasonably sized subset of peptides in common with SV40 large T antigen, this technique should detect it, as was the case with the adenovirus 72K protein and its proteolytic fragment of 44K daltons (Levinson and Levine, 1977). Thus the fact that mouse and hamster transformed cells contain an immunoprecipitable protein that differs with the host cell species once again points out the cellular origin of the SV40 middle T antigen. The comparison of the mouse and hamster 54K proteins is limited in scope by the fact that the mouse cell is transformed by SV40 and the hamster cell is transformed by Ad2+ND4. Other investigators (Melero et al., 1979; E. Paucha, R. Smith and A. E. Smith, personal communication) have also concluded that SV40 middle T antigen has few, if any, peptides in common with SV40 small or large T antigen.

The studies presented here lead to the conclusion that SV40 induces the synthesis of or stabilizes a specific cellular protein after infection or transformation of murine cells. This protein is either immunogenic itself or shares antigenic determinants with SV40 T antigens. It appears to form a complex with SV40 T antigen which could allow it to be immunogenic. The fact that a similar or identical protein is expressed in some embryonal carcinoma cell lines in the absence of SV40 infection raises the possibility that the 54K protein is an antigen expressed in fetal cells of the

mouse and hamster. Antigenic specificities shared between teratocarcinomas and SV40-transformed cells have been previously recognized (Edidin et al., 1971; Edidin et al., 1974; Gooding et al., 1976), and embryonal carcinoma cells are known to share antigenic determinants with fetal mouse tissue (Artzt et al., 1973). In addition, several lines of evidence have suggested that SV40 tumors can express fetal antigens of the hamster (Coggin, Ambrose and Anderson, 1970; Coggin et al., 1971). A cellular antigen expressed in a fetus prior to the development of immune self-tolerance might well be antigenic when it is also expressed in a tumor. In this case, the 54K protein itself might be immunogenic in the absence of SV40 T antigen, but the available evidence does not support this contention.

Whatever the relationship between the 54K protein and the viral T antigens, it is clear that virus infection or transformation specifically induces the synthesis or enhances the stability (perhaps through a complex with T antigen) of a cellular protein. Additional studies will be required to determine whether this cellular protein has an important role in virus infection or transformation.

Experimental Procedures

Cell Lines

BALB/c3T3 cells and the SV40-transformed BALB/c3T3 derivative, SV40-T2 (Aaronson and Todaro, 1968), were obtained from G. Todaro. The SV40-transformed human cell line SV80 (Todaro, Green and Swift, 1966) was obtained from D. Livingston. The Ad2$^+$ND4-transformed hamster cell line, ND4-HK-A1678, was a gift from A. Lewis. The BSC-1 monkey cell line (Garber, Seidman and Levine, 1978) was used for productive virus infections. The nullipotent embryonal carcinoma cell line F9 (Bernstine et al., 1973) and the pluripotent embryonal carcinoma cell line PCC-4aza-1 (Jakob et al., 1973) were from M. Sherman. The SVTER cell lines are SV40-transformed murine teratocarcinoma-derived cell lines described by Topp et al. (1977). The BHK21 cell line used in this study was from the American Type Culture Collection. All cell lines were tested monthly for PPLO and were free of contamination.

Viruses and SV40 Tumor Sera

The SV40 strain 776 (from G. Khoury) was used as the wild-type virus in all experiments. Hamster anti-SV40 tumor serum was obtained by two methods. With the first, newborn LAK (Lakeland, New Jersey) hamsters were injected with 10^8 pfu of SV40 2 days after birth. Tumors appeared in 5–9 months, and hamsters were bled 1–2 months after the tumor appeared. With the second method, 1–5 × 10^6 ND4-HK-A1678 cells were injected into hamsters 2–7 days after birth. Tumors appeared in 1–3 months, and the hamsters were bled 1–2 months after the tumor was first detected. Mouse anti-SV40 tumor serum was obtained by two methods: BALB/c mice were injected with 1–5 × 10^6 SV BALB/cT2 cells; tumors appeared in 2–3 weeks and mice were bled 2–3 weeks later; or, 129Sv/S1 mice were injected 4–6 times with 1–5 × 10^6 cells of either F9, PCC-4aza-1 or the SVTER cell lines 14 and 104 (Topp et al., 1977); the mice were bled 1–2 weeks after the last injection. The SVTER cell lines are SV40-transformed murine cells derived from differentiated embryonal carcinoma cells in vitro from the 129Sv/S1 mouse. The anti-SV40 T antigen hybridoma sera were a gift from C. Croce (Martinis and Croce, 1978).

Cell Cultures and Virus Infections

All cells were grown on 100 mm Falcon plastic dishes with Dulbecco's modified Eagle's medium (GIBCO) supplemented with 20 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal calf serum.

Virus infections were started in 2 ml of medium with 2% fetal calf serum at 37°C to allow efficient adsorption. After 2 hr, 8 ml of additional medium were added. The multiplicities of infection were typically 5–10 pfu per cell. Mock-infected cells were treated identically, except that no virus was added.

Preparation of Cell Extracts and Immunoprecipitation

Cells were labeled with ^{35}S-methionine (25–100 μCi/ml), (NEN) in methionine-free medium (DME, GIBCO), either directly after removing the spent medium or reincubation in methionine-free medium. Inorganic $^{32}PO_4$ labeling (25 μCi/ml, NEN) was carried out in phosphate-free medium (MEM, GIBCO) in a similar fashion. For labeling times longer than 4–6 hr, the medium was supplemented (1/10) with DMEM. At the end of a labeling period, the cells were washed with phosphate-buffered saline (PBS), harvested and centrifuged out of suspension. Cell pellets were either used immediately or stored at −20°C for future use. Upon thawing, 400–800 μl of extraction buffer [50 mM Tris-HCl (pH 8.0), 0.5% NP40, 5 mM EDTA, 600 mM NaCl, 0.3 mg/ml phenylmethylsulfonylfluoride (PMSF)] were added for each 100 mm culture dish of cells. After 20 min on ice, the suspension was sonicated for 10 sec with a Branson sonifier and the extract was centrifuged at 17,000 × g at 4°C in a Sorvall centrifuge. The supernatant was used as a source of antigen.

As a preliminary step to the immunoprecipitation, 200 μl of soluble antigen were mixed with 5 μl of normal rabbit or hamster serum for 15 min on ice, and then 100 μl of prepared S. aureus were added for 10 min. Following centrifugation, 5–10 μl of either immune or normal serum were added to the supernatant for 30 min, followed by the addition of 100 μl of S. aureus for 10 min (Kessler, 1975). After centrifugation, the pellet was washed repeatedly with 50 mM Tris-HCl (pH 7.4), 1% NP40, 5% sucrose, 0.5 M NaCl and 5 mM EDTA. The precipitate was then resuspended in 50–100 μl of SDS sample buffer (Laemmli, 1970) and placed in a boiling water bath for 3 min. The supernatants were collected after a final centrifugation and either analyzed immediately or stored at −20°C.

Gel Electrophoresis and Partial Peptide Maps

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). Gels were dried either after staining and destaining or following fluorography (Bonner and Laskey, 1974). High contrast X-ray film was used to detect the presence of the radioisotopes in each gel.

The partial proteolysis peptide mapping procedure of Cleveland et al. (1977) was used. The appropriate proteins were cut out of the stained gel and loaded in wells of a 20% polyacrylamide gel. The gel slices were overlaid with 15 μl of sample buffer containing 0.1% SDS and 18, 90 or 450 ng of S. aureus V8 protease. The gel was run at 30 V through a 1 in stacking gel and then run at 140 V until completion.

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