Nonlytic Simian Virus 40-Specific 100K Phosphoprotein Is Associated with Anchorage-Independent Growth in Simian Virus 40-Transformed and Revertant Mouse Cell Lines

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Normal fibroblasts display two distinct growth controls which can be assayed as requirements for serum or for anchorage. Interaction of mouse 3T3 fibroblasts with simian virus 40 (SV40) thus generates four classes of transformed cells. We have examined viral gene expression in these four classes of cell lines. Immunoprecipitation of [^{35}S]methionine-labeled cell extracts with an antiserum obtained from tumor-bearing hamsters detected the SV40 large T and small t proteins (94,000 molecular weight [94K], 17K) and the nonviral host 54K protein in all cell lines tested. A tumor antigen with an apparent molecular weight of 100,000 was also found in some, but not all, lines. Similar "super T" molecules have been found by others in many rodent transformed lines. We carried out an analysis of the relation of phenotype to relative amounts of these proteins in cell lines of the four classes, using the Spearman rank correlation test. The amount of the 100K T antigen relative to the 94K T antigen or to total viral protein was well correlated with the ability to form colonies in semisolid medium. No significant correlation was found between quantities of labeled 94K T antigen, 54K host antigen, or 17K t antigen and either serum or anchorage independence. Mouse cells transformed with the small t SV40 deletion mutant 884 synthesized a 100K T antigen, suggesting that small t is not required for the production of this protein. The 100K T antigen migrated more slowly than lytic T. Since mixtures of extracts from cells expressing and lacking the 100K T antigen yielded the expected amount of this protein, it is unlikely that the 100K T derives from the 94K protein by a posttranslational modification.

Simian virus 40 (SV40) is a small deoxyribonucleic acid (DNA) tumor virus, which transforms mouse cells in the serum and anchorage assays (69). In the anchorage assay, transformation is accompanied by integration of that part of the SV40 genome (6, 33) which encodes two proteins of ~94,000 (94K) and ~17K molecular weight (11, 48). Thus, it is not surprising that these proteins can be detected as well in transformed mouse cells (for review see 62). Serum transformants need less commitment from the viral genome; many such cells even lack detectable SV40 genes (55) or gene products (50).

Many other proteins, some almost certainly of host origin, are also called out by SV40 transformation. These included a surface antigen (26), a tumor-specific transplantation antigen (63), a set of new proteins of various molecular weights (~100K, ~63K, ~54K) that can be immunoprecipitated either directly, or via their capacity to

complex with viral antigens (9, 12, 17, 19, 34–36, 39, 48, 53), and a set of secreted growth factors (32).

Causally linking this panoply of new proteins to either, both, or even neither of the transformations has not been one of the accomplishments of the popular strategy of amassing the most complete possible picture of viral gene expression in a rather small number of transformed cell lines. Indeed, few attempts have been made to select sets of cell lines that differ from each other only in their expression of one of the two transformations. We have isolated and characterized such a set of interrelated mouse cell lines, all positive for nuclear T antigen and all descended from Swiss 3T3 (Table 1). These lines discretely separate sensitivity to the two growth controls. In this study we have related the phenotypes of these lines to SV40 gene expression.

Multiple forms of large T have been reported for many SV40-transformed lines, especially those of mouse origin (9, 34, 35, 38–40, 49, 53, 57,

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TABLE 1. *Origins and properties of 3T3-derived cell lines*

Class[a]	Line	Origin[b]		Rank orders[c]			
		Parent	Selection	Serum[d]	Density[e]	Anchorage[f]	Tumorigenicity[g]
Fully transformed	SV101	3T3	Density	4	7	6	5
	SVR85	3T3	None		6	7	
Anchorage transformed	Aγ5	SV101	Reversion	1	3	5	4
Serum transformed	Fl101	SV101	Reversion	5	1.5	2	2
Fully reverted	LS₁	SV101	Reversion	2.5	1.5	1	2
	LS₂	SV101	Reversion	2.5	4	3	2
Intermediate	SVR18	3T3	None		5	4	

[a] All classes of lines are derived from 3T3 by SV40 infection or subsequent reversion of SV101. All classes are positive for SV40 T antigen by nuclear immunofluorescence.

[b] Lines derived from SV101 were cloned after negative selection to kill fully transformed cells, as reported earlier for Aγ4 and Aγ5 (70), Fl101 (45), and LS₁ and LS₂ (70).

[c] Rank orders of expression of a given phenotype were obtained from published data. In all cases rank order is descending; number 1 indicates the least transformed line in a given assay. Lines with identical phenotypes have been given the same rank order. See text for complete description.

[d] Ratio of doubling time in 1% serum to doubling time in 10% serum (50, 70).

[e] Saturation density in 10% serum (50).

[f] Percent of cells able to form colonies >0.3 mm in diameter in 3 weeks, in 10% serum on an agarose bed (50, 70).

[g] Fraction of nude mice injected with 2×10^6 cells succumbing to tumor by 6 months (21).

58). In those studies, the variant large T's have all been found to be largely virus coded. However, no previous reports to our knowledge have demonstrated a relationship between a nonlytic large T species and any specific phenotypic change. We find in this set of related cell lines that a 100K T antigen is specifically made only in the anchorage-independent transformed cells.

MATERIALS AND METHODS

Cell culture. 3T3 cells were established from Swiss mouse embryo fibroblasts (67). SV101 is a cloned transformant from SV40-infected 3T3 (68). SV101 was recloned twice in 1968, and Fl101 was then derived by 5-fluorodeoxyuridine-negative selection (45). The serum revertants LS₁, LS₂, Aγ4, and Aγ5 were derived in 1970 from SV101 by bromodeoxyuridine-negative selection (70). The revertant cells used in these studies were thawed from freezings made within a few months of their isolation. For this study, growth properties were obtained from the initial publications describing these lines. However, recent thawings of these cell lines reveal that they have all retained plating efficiencies on plastic of at least 10% and have retained their reported growth phenotypes (G. Blanck, manuscript in preparation).

SVR18, SVR85, and SVR63 are randomly picked clonal isolates of SV40-infected mouse 3T3 cells (50). The cell lines used here were all recloned from original clonal lines which had themselves been isolated from freezings made from 1 month to 1 year after the initial transformation (50). All cells used here have been passaged for less than 1 year since thawing. The clone of SV40 dl884-transformed 3T3 cells was isolated by the density assay in this laboratory. All mouse cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, catalog no. 430-2100) with pen-

icillin and streptomycin plus 10% calf serum or fetal calf serum. Mycoplasma tests by Hoechst stain (10) were negative on cells used in these experiments.

Selections of mock revertant cell lines. The procedures of bromodeoxyuridine or 5-fluorodeoxyuridine revertant selection were the same as previously described (45, 70). Ten clones were picked from each of the selection treatments, without regard to colony morphology. The growth properties of each of the clones are fully reported elsewhere (G. Blanck, in preparation). All clones used in this study grew without anchorage with a plating efficiency greater than 10^{-2}. For comparison, 3T3 and anchorage revertants grew without anchorage with plating efficiencies less than 10^{-4} (G. Blanck, in preparation).

Radiolabeling and extraction of cell cultures for immunoprecipitation. Exponentially growing cell cultures in 100-mm dishes (cell density, 2×10^4 to 4×10^4 cells per cm²) were labeled with 1 ml of methionine-free Dulbecco modified Eagle medium or phosphate-free Dulbecco modified Eagle medium containing 2% dialyzed fetal calf serum, and 100 µCi of [³⁵S]methionine (Amersham, >700 Ci/mmol) or 100 µCi of [³²P]phosphate (Amersham, >2,400 Ci/mmol) was added to each culture for 2 h at 37°C. After labeling, cells were lysed with 1 ml of extraction buffer [20 mM tris(hydroxymethyl)aminomethane (pH 8.4)–140 mM NaCl–1% Nonidet P-40–1 mM dithiothreitol–250 µg of phenylmethylsulfonyl fluoride per ml]. At the end of 30 min, the cells were scraped and centrifuged at 2,200 × *g* for 15 min. The supernatant was then centrifuged at 48,000 × *g* for 40 min. A sample of 10 µl was removed from the supernatant to determine trichloroacetic acid-precipitable counts.

Extracts (48,000 × *g* supernatants) containing the same amount of trichloroacetic acid-precipitable radioactive material were incubated with 10 µl of normal hamster serum or pooled hamster anti-SV40 tumor

serum (from the division of Cancer Cause and Prevention, National Cancer Institute) for 1.5 h at 4°C.

Thirty microliters of freshly washed, Formalin-fixed *Staphylococcus aureus* A (Calbiochem), 10% (vol/vol) in pH 7.4 buffer [10 mM tris(hydroxymethyl)aminomethane–5 mM ethylenediaminetetraacetic acid–0.5% Nonidet P-40], was then added to each reaction tube. After an additional 30 min of incubation, the immunocomplexes were washed three times with urea buffer [10 mM tris(hydroxymethyl)aminomethane (pH 8.0)–1 M NaCl–10 mM ethylenediaminetetraacetic acid–1% Nonidet P-40–2.5 M urea]. Finally, the absorbed immune complexes were eluted as described by Tegtmeyer (60). The eluted samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel electrophoresis system was essentially the same as that described by Tegtmeyer (60) with a 10 to 20% gradient polyacrylamide separating gel. The gels were fixed, treated with Enhance (New England Nuclear), vacuum dried, and autofluorographed at −80°C. SV40 lytic proteins from SV40-infected monkey cells were obtained from Peter Tegtmeyer and Carol Prives.

Computer reconstruction of relative contributions of different molecular weight T antigens. To quantitate the relative amounts of material in the bands of an immunoprecipitation autoradiogram, individual lanes were scanned on a Gilford 250 spectrophotometer equipped with a Gilford 2520 gel scanner. To subtract film background, a blank area of the film was scanned for the wavelength at which the absorbance was minimal. Typically, this wavelength was between 510 and 520 nm. The slit width was then adjusted to give an absorbance reading of 0.0, and the gel lane was scanned from bottom to top. For any particular piece of film, the wavelength was kept constant, and zero absorbance reading was maintained from lane to lane by adjusting the slit width, which was usually in the range of 0.14 to 0.15 mm. Analysis of the areas under the peaks corresponding to the 100K and 94K T antigens was complicated by the fact that these proteins were close together and their representative curves overlapped (Fig. 1A). We digitalized the spectrophotometric curves with a Numonics Electronic Graphics Calculator 1224 into x,y-coordinate pairs (Fig. 1B). Typical scans were digitalized by 300 to 400 data points. Peaks were then analyzed by a computer program which fit Gaussian curves to the data (42). These individual Gaussian curves accurately reflected the origins of individual radioactive protein bands on the original gel (Fig. 1C). The areas under these reconstructed Gaussian curves (in arbitrary units) were then used for calculation of ratios of the different immunoprecipitable proteins in the cells.

Spearman rank correlation test. The Spearman rank correlation test (41) was used to determine whether correlations exist between the relative amounts of immunoprecipitated proteins and the efficiencies of growth in various transformation assays. This test for correlations used both a calculated coefficient of correlation (41) and the number of pairs examined to determine the statistical significance of the correlation. Rank orders of expression of a given phenotype were derived for this study from published data. In all cases rank order is descending; number 1 indicates the least transformed line in a given assay.
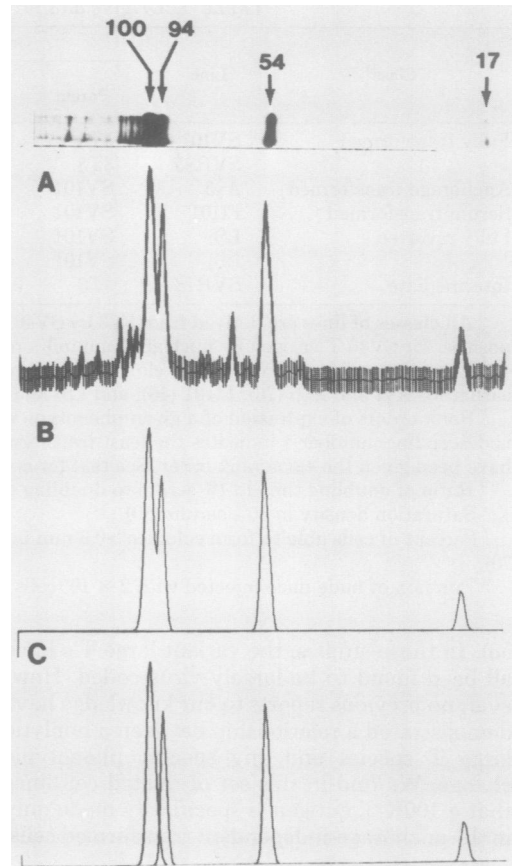

FIG. 1. *Quantitation of immunoprecipitated proteins from sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiograms. To quantitate the relative amounts of radioactive material in the bands of an immunoprecipitation autoradiogram, individual lanes were scanned on a Gilford 250 spectrophotometer equipped with a Gilford 2520 gel scanner. (A) Computer representation of the gel scan in vertical register with the gel. Curves were then generated with the computer which differed from the experimental curves by less than 10%, and usually by less than 5%. (B) Computer-generated curves for (A). (C) The computer was then used to break these "best-fit" curves into their several Gaussian components, which when summed gave the best-fit curve shown in (B).*

Lines with identical phenotypes have been given the same order.

A coefficient near +1 indicates a direct correlation between the two parameters being examined. A coefficient of correlation near −1 indicates an inverse correlation. A number near zero indicates no statistically significant correlation.

RESULTS

The mouse cell lines we studied are all derived from Swiss 3T3 (67). They have all been well characterized (Table 1). Their capacity to grow

in the various restrictive transformation assays and as tumors in nude mice varied from line to line. The ranked order of sensitivity to each growth control, based on published data, is given in Table 1. Recently thawed cultures of these lines have shown comparable phenotypes (G. Blanck, in preparation). Fully transformed lines such as SV101 and SVR85 grew well in all assays. These were ranked high (Table 1), whereas other lines such as the full revertant LS₁, unable to grow well in these assays, ranked low (Table 1). Not all lines linked their responses this way. Aγ5 was most sensitive in the serum assay, but showed little requirement for anchorage. Conversely, F1101 was quite anchorage dependent but lacked serum sensitivity (Table 1).

The Spearman rank coefficient (41) permits quantitation of the significance of correlations among rankings, and thereby helps to reveal the degree to which properties are related (Table 2). Clearly, results from the density, anchorage, and tumorigenicity assays were correlated with each other for these lines at a significance of 95%, whereas all three measurements were not correlated with serum sensitivity (Table 2).

Presence of 100K T-antigen in anchorage-transformed mouse cell lines. To measure the amount of immunoprecipitable stable proteins coded or induced by SV40 in this set of lines, we obtained a hamster serum directed against an SV40 tumor, labeled cells with [^{35}S]-methionine or [^{32}P]phosphate, and immunoprecipitated the antigenic proteins from cell extracts. All of the lines tested had been previously shown to be positive for nuclear T antigen by immunofluorescence, so the presence in them of the 94K T antigen (Fig. 2) was expected. All lines also had 17K t antigen, as well as the 54K host antigen which binds to 94K T antigen (Fig. 2) (35, 36). All lines showed 54K host antigen in amounts proportional to their large T. Apparently, reversion to anchorage sensitivity (F1101, LS₁) or retention of anchorage sensitivity despite SV40 integration (SVR18) occurred despite the persistent presence of the SV40-encoded small t and large T antigens.

Immunoprecipitates of these lines were, however, not all identical. The anchorage-transformed lines all had a second, larger SV40 T-antigen which immunoprecipitated with an apparent molecular weight of 100K (Fig. 2). Anchorage-requiring lines had little, if any, 100K T antigen. Other new proteins at ~130K and 110K were seen in some, but not all, anchorage transformants.

The presence of the 100K T antigen was not all-or-none. Indeed, both the degree of anchorage independence (Table 1) and the amount of 100K T antigen relative to other proteins (Fig.

TABLE 2. *Correlation of growth properties*

Comparison	Pairs[a]	Rank coefficient[b]	Correlation[c]
Anchorage vs serum	5	−0.10	None
Anchorage vs saturation density	7	0.85	Direct
Anchorage vs tumorigenicity	5	0.89	Direct
Saturation density vs serum	5	−0.08	None
Saturation density vs tumorigenicity	5	0.87	None[d]
Serum vs tumorigenicity	5	−0.11	None

[a] Number of observed cell lines in each assay.

[b] Rank orders of Table 1 are correlated by the Spearman rank correlation test (41). The higher the correlation coefficient, the more likely it is that two assays measure directly linked events. A negative number close to 1 means that the two assays measure inversely linked events. A number close to zero indicates no correlation.

[c] "Direct" indicates that the two sets of data were directly correlated at the 95% confidence level. "None" indicates that, at the 95% confidence level, no significant correlation was detected. The significance was determined from a table (41), using a combination of the absolute value of the Spearman rank coefficient and the number of pairs in the observation.

[d] A direct correlation existed at the 90% confidence level, but not at the 95% confidence level.

2) differed over a considerable range from line to line. To determine whether a correlation existed between 100K T and anchorage independence, as suggested by the results of Fig. 2, we determined the relative contributions of the 100K, 94K, 54K, and 17K bands from autoradiograms (Fig. 1). Various ratios of these bands were then ranked for the lines examined, and the rankings were tested for correlation with the rankings of expression of the various transformed phenotypes (Table 1).

Expression of 100K T antigen was strongly correlated with independence of the anchorage requirement (Table 3). This was the case for all four straightforward ways of expressing the amount of 100K T antigen relative to other immunoprecipitable bands. In contrast, neither any other viral gene product nor the 54K host antigen was correlated with anchorage independence at a significance of 95% (Table 3). In particular, the 17K t antigen, which had previously been reported to be necessary for anchorage-independent growth (52), was retained in anchorage revertants and had no apparent correlation with this growth control. Data on the 54K host antigen may reflect only our indirect assay for it, which depends on 54K host antigen binding large T.

The messenger ribonucleic acid (mRNA) of the 100K T antigen seen in other mouse lines (49) is made from a subset of early-region SV40 transcripts that otherwise would have been made into mRNA for 94K T antigen or 17K t
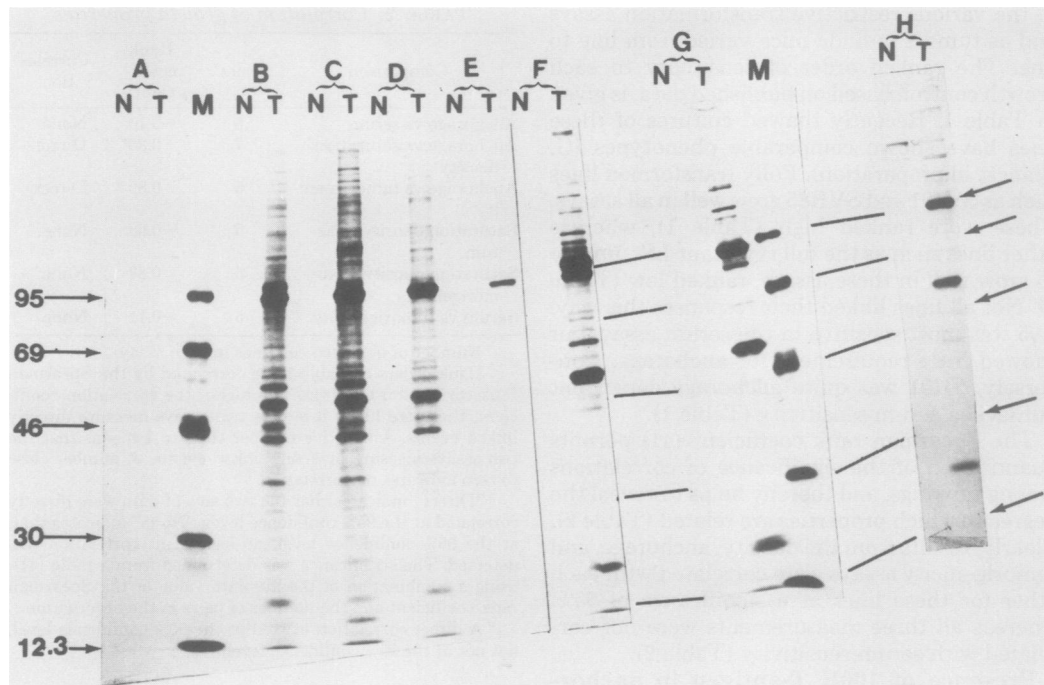
FIG. 2. *SV40 tumor antigens in mouse cell lines. The cell lines are described in Table 1. [^{35}S]methionine-labeled cell extracts were immunoprecipitated with normal hamster serum (N) or anti-SV40 tumor serum (T). Cell lines were as follows: (A) 3T3, (B) SV101, (C) Aγ5, (D) LS$_2$, (E) LS$_1$, (F) SVR85, (G) SVR18, and (H) Fl101. M, Markers; molecular weights (× 10^3). Horizontal lines indicate marker positions of different gels in this figure. Note the presence of a second larger (100K) T antigen in some lines, and the presence of a 94K T antigen, a 54K host antigen, and a 17K t antigen in all lines. In scans (Fig. 1) of similar gels, the T antigens were at least 95% of the total immunoprecipitated material. The ratio of 100K to 94K T antigens for LS$_2$ was not increased in other experiments in which more radioactivity was found in the immunoprecipitate (data not shown).*

antigen mRNA's (5, 12). We have not yet shown this for these lines. However, the significant inverse correlation of the ratio (17+94K/total viral protein) to anchorage independence (Table 3) is consistent with the 100K T antigen in these lines being of largely viral origin.

No significant correlation to viral gene expression was found for capacity to grow in low serum (Table 4) or for high saturation density (Table 5). Thus, the linkage of production of 100K T antigen to anchorage independence was the sole significant correlation of any immunoprecipitable protein with any in vitro growth assay for these lines.

100K T antigen is larger than lytic SV40 T. Proteolytic breakdown of T antigens is likely to be the reason behind at least some earlier reports of multiple species of large T in lytically infected and transformed cells (1). Molecular weight values are of little use in comparing our results to previous studies of large T, since different gel systems yield different molecular weights for lytic T, and since none display large T at its calculated molecular weight of 81K (69).

Therefore it was necessary to determine which of the two bands in the 90 to 100K region co-migrated in our gel system with bona fide lytic SV40 large T.

In Fig. 3, the large T doublet of line SVR63 (lane A) is compared with two different preparations of lytic large T antigen. In earlier work the lytic T of lane B was reported at 90K (49), and the lytic T of lane C was reported to be between "86K and 100K" (60). In our gel system, both of these lytic T's ran together at 94K, which is the mobility of the smaller of the two large T's in SVR63 (lane B). Thus, we conclude that the doublet seen in our anchorage-transformed lines is not due to breakdown of full-size 94K T antigen. This result is consistent with earlier work showing that mRNA from a mouse-transformed line bearing a regular and an extra large T can be translated into both proteins in a cell-free system (49).

To extend these observations, we carried out experiments in which extracts were made of a mixture of two different 3T3 transformed lines, one labeled with [^{35}S]methionine and the other

TABLE 3. Anchorage transformation: correlations with SV40-specific proteins[a]

Range[b] (fold)	Ratio[c]	Spearman rank[d] coefficient	Correlation[e]
10–50	100/viral	0.75	Direct
	100/94	0.75	Direct
	100/54	0.72	Direct
	100/17	0.71	Direct
	(100+94)/17	0.57	None
	94/17	0.25	None
≤6	(100+17)/viral	0.86	Direct
	54/viral	0.11	None
	(100+94)/viral	−0.49	None
	94/54	−0.57	None
	(100+94)/54	−0.58	None
	17/viral	−0.65	None
	(94+17)/viral	−0.75	Inverse
	94/viral	−0.86	Inverse

[a] Seven pairs were used for each of these correlations.

[b] Amounts of various immunoprecipitated radioactive proteins were determined as in Fig. 1. Ratios of these amounts varied from line to line over a greater or lesser range.

[c] "100/viral" represents ratio of area under 100K peak to areas of 100K, 94K, and 17K peaks. Other ratios are calculated from the areas of the molecular weight peaks as stated. Background peaks represent less than 5% of the peaks measured here.

[d] See text for explanation of the derivation of this parameter.

[e] See Table 2, footnote c. "Inverse" indicates that the two sets of data were inversely correlated at the 95% confidence level.

not. When the labeled line contained 100K T antigen (Fig. 3, lane D), and the unlabeled one lacked it, an immunoprecipitate of the mixed extract still (Fig. 3, lane E) revealed the 100K band. When the labeled line lacked the 100K band (Fig. 3, lane F), so did the mixture (Fig. 3, lane G). Thus, it is unlikely that the absence or presence of the 100K T antigen in a given mouse line of this set was the consequence of a post-translational activity capable of acting in a mixed extract.

17K t antigen is not needed for production of 100K T or for anchorage-independent growth. 17K t antigen is coded for by a proximal portion of the early region of SV40. Deletions in the region of 0.54 to 0.59 map units remove a portion of the small t coding sequence. Certain deletions in this region, such as dl2006 and dl884, remove a splice junction and totally eliminate the capacity of the virus to code for even a stable t fragment (69). Mutant dl884 nevertheless can transform cells in the anchorage assay, although under certain restrictive culture conditions this transformation is reduced in efficiency (7, 51, 52, 59). We examined such a

dl884 anchorage-transformed mouse line for large T antigen production. Figure 3, lane H, shows that this line produces 100K T antigen as well as 94K T antigen. It is interesting to compare these results with an earlier study (39). In this report the 100K T antigen was seen in three different wild-type transformants, whereas an anchorage-requiring dl2006 mouse transformant lacked it.

We conclude from these data, and from the

TABLE 4. Serum transformation: correlations with SV40-specific proteins[a]

Range[b] (fold)	Ratio[c]	Spearman rank[d] coefficient	Correlations[e]
10–50	100/54	−0.21	None
	100/94	−0.36	None
	100/17	−0.36	None
	100/viral[c]	−0.36	None
	94/17	−0.36	None
	(100+94)/17	−0.36	None
≤6	(100+94)/54	0.46	None
	(94+17)/viral	0.36	None
	17/viral	0.36	None
	94/54	0.21	None
	94/viral	0.03	None
	(100+17)/viral	−0.21	None
	54/viral	−0.21	None
	(100+94)/viral	−0.36	None

[a] Five pairs were used in each correlation.
[b] See Table 3, footnote b.
[c] See Table 3, footnote c.
[d] See Table 3, footnote d.
[e] See Table 2, footnote c.

TABLE 5. Saturation density: correlations with SV40-specific proteins[a]

Range[b] (fold)	Ratio[c]	Spearman rank[d] coefficient	Correlation[e]
10–50	100/54	0.66	None
	100/17	0.59	None
	100/94	0.56	None
	100/viral[d]	0.56	None
	(100+94)/17	0.54	None
	94/17	0.34	None
≤6	(100+17)/viral	0.71	Direct
	(100+94)/viral	0.58	None
	94/54	−0.14	None
	(100+94)/54	−0.24	None
	54/viral	−0.27	None
	(94+17)/viral	−0.56	None
	17/viral	−0.58	None
	94/viral	−0.71	Inverse

[a] Seven pairs were used in each correlation.
[b] See Table 3, footnote b.
[c] See Table 3, footnote c.
[d] See Table 3, footnote d.
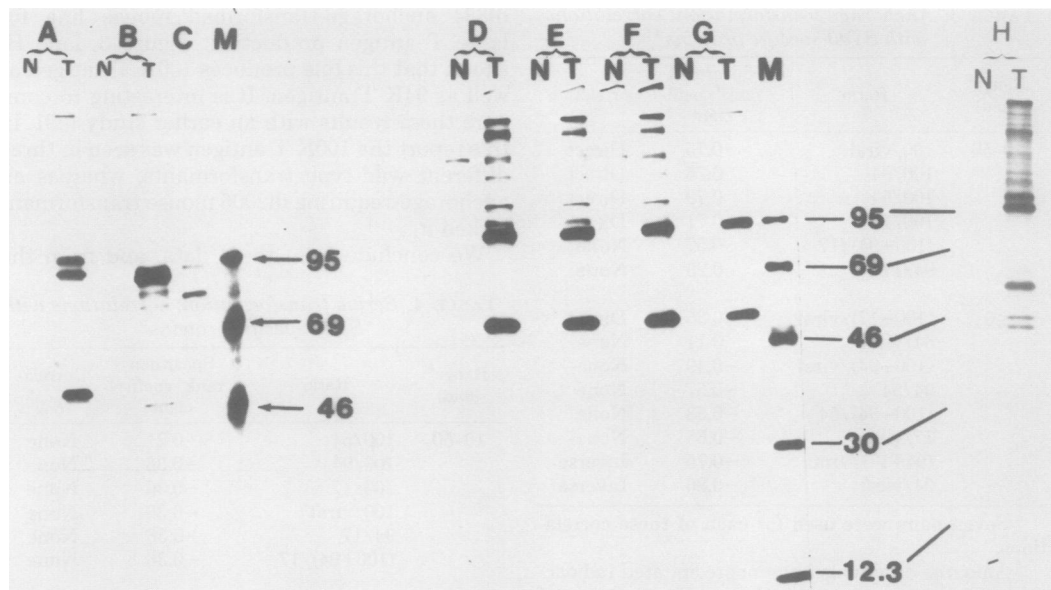[e] See Table 2, footnote c.

FIG. 3. *Analysis of origin of 100K T antigen in mouse anchorage transformants. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis comparison of [^{35}S]methionine-labeled immunoprecipitable proteins with normal hamster serum (N) and anti-SV40 tumor serum (T) from (A) SV40-transformed mouse cells SVR63, (B) SV40-infected monkey CV1 cells from C. Prives, and (C) lytic T immunoprecipitate from P. Tegtmeyer. Although the latter T antigen preparation has undergone significant breakdown, the 94K band remains distinct. Note the presence of a band of SV40 T antigen at 94K in all three extracts. (D) [^{35}S]methionine-labeled Aγ5; (E) mixture of [^{35}S]methionine-labeled Aγ5 and unlabeled Fl101; (F) [^{35}S]methionine-labeled Fl101; (G) mixture of [^{35}S]methionine-labeled Fl101 and unlabeled Aγ5. Note the presence of 100K T antigen in lanes D and E, but not in F or G, indicating that mixtures of cell extracts from Aγ5 or Fl101 were not able either to convert the 94K to the 100K form of T antigen, or to convert the 100K to the 94K form. (H) [^{35}S]-methionine-labeled 3T3 mouse cells transformed by SV40 small t deletion mutant d1884. Note the presence of 100K T antigen in these cells, despite the absence of small t antigen.*

absence of correlation of 17K with any phenotype in our set of cell lines (Tables 3 to 5), that 17K is not necessary or sufficient for the maintenance of anchorage independence.

100K T antigen is retained in mock revertants. Many of the lines lacking the 100K protein are derived by reversion from the 100K-positive line SV101 (Fig. 2). Enrichment of SV101 subcultures for rare revertants involved exposure of the transformed line to a killing agent such as 5-fluorodeoxyuridine (44) or bromodeoxyuridine plus light (46, 70) while in restrictive culture conditions. Since these agents can be mutagenic, we next considered the possibility that the 100K T antigen is lost from revertant cells as an inadvertant consequence of these reversion selection procedures. Mock revertants provide a necessary control to test this.

Several clones of SV101 were isolated after reversion treatment. Only 1 of 50 clones was revertant in any assay (45, 70). Six of these, when assayed, were found to have retained the 100K protein (Fig. 4), as well as the anchorage-independent phenotype. Thus, we can conclude

that the anchorage dependence of our revertants, and not the selection procedure per se, was responsible for their failure to express the 100K T antigen made by SV101 (Fig. 2 and 4).

Phosphorylation of the 100K T antigen. Protein kinases transfer phosphate from adenosine 5'-triphosphate to the serine, threonine, and tyrosine residues of their substrate proteins. At least some tumor virus-transforming proteins are tyrosine-specific protein kinases (16, 18, 19, 31). The 94K T antigen is apparently an adenosine triphosphatase, and it has a closely associated protein kinase (24, 65, 66). 54K host antigen and 94K T antigen are both phosphorylated (40), but neither protein has any detectable phosphotyrosine (G. Khoury, personal communication). The 100K antigen in our cells also was phosphorylated (Fig. 5).

When the ratio of 100K to 94K was examined for [^{32}P]phosphate-labeled cell lines, the range of values for these lines was not as great as the range seen with [^{35}S]methionine labeling (Table 6). This suggests that differences may exist in the relative phosphorylation of the two large T
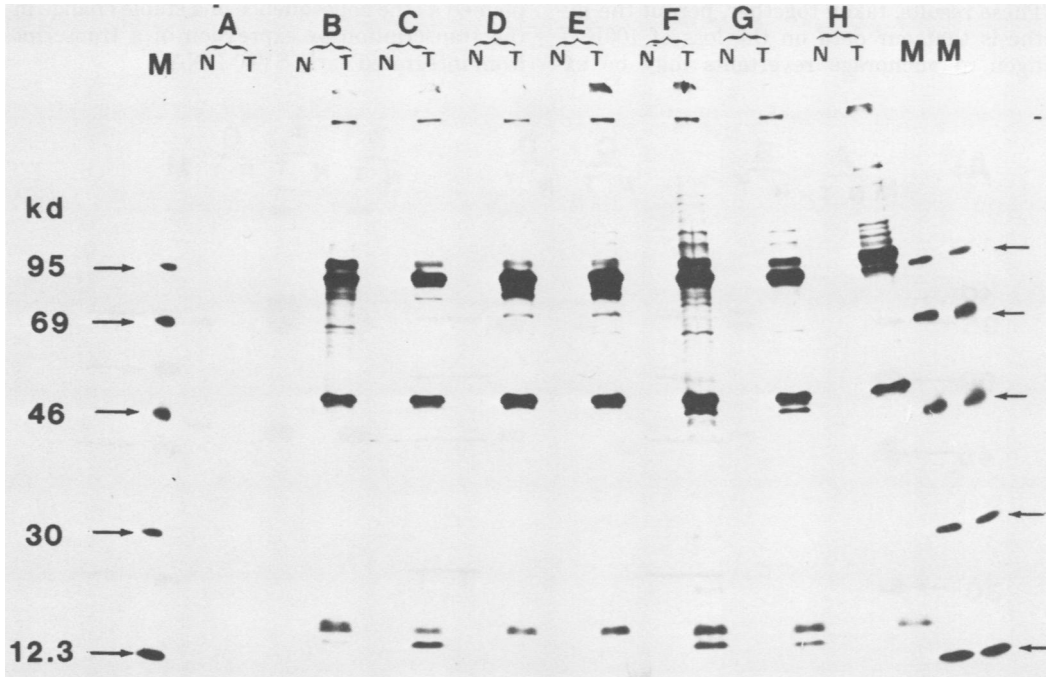
FIG. 4. *100K T antigen in mock-revertant subclones of SV101. Subclones of SV101 obtained after passage through reversion-selection protocol that retain their anchorage-independence are called mock revertants. These lines were labeled with [35S]methionine, extracted, immunoprecipitated, and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The lines were 3T3 (lane A); SV101 (lane B); three 5-fluorodeoxyuridine-selected mock revertants (lanes C, D, and E); and three bromodeoxyuridine-selected mock revertants (lanes F, G, and H). Note the presence of 100K T antigen in all mock revertants.*

antigens. When the ^{32}P/^{35}S ratio for 94K was examined (Table 6), the range was small, approximately threefold. When the ^{32}P/^{35}S ratio of the 100K protein was examined for the same set of lines, however, the range was approximately 10-fold. This suggests that 100K protein was more phosphorylated in lines with low amounts of 100K.

DISCUSSION

Cells transformed by SV40 have often been observed to contain immunoprecipitable proteins whose apparent molecular weights are not identical to either SV40-encoded early protein (9, 12, 25, 34, 38, 49, 53, 58). In the past, these variant antigens have usually been merely noted. We have found that a 100K T antigen variant, in addition to the 94K T antigen, is present as a major immunoprecipitable protein specifically in anchorage-transformed 3T3 cells (Fig. 2). It is reduced or absent from their anchorage-requiring revertants and from lines isolated as serum transformants (Fig. 2). Mixed extract immunoprecipitations and comparison of molecular weights to that of lytic 94K T antigen (Fig. 3) render remote the possibility that the 100K T

antigen is a posttranslational by-product of a virus-encoded 94K T antigen.

A phosphoprotein similar or identical to ' 'is 100K T antigen has already been described in SV40-transformed mouse cells by many groups. McCormick et al. (39) observed a 100K-94K doublet of large T in many mouse lines and showed that these molecules shared a virus-encoded N-terminal amino acid sequence (40). May et al. (38) showed that a 115K phosphoprotein in one mouse line shares the same methionine tryptic peptide map with the 94K T antigen, and that it is the product of a tandem repeat (0.33 to 0.28 map units) of integrated early viral DNA. Repeated passage, which may increase anchorage independence, also increased the 115K T antigen in this line (38). Smith et al. (53) also found both 100K T antigen and 94K T antigen in a mouse cell line and recorded no large differences in their peptide maps. Finally, using cell-free translation, both Prives et al. (49) and Kress et al. (34) found the 100K T antigen of a mouse-transformed line to be the product of a unique mRNA, distinct from the mRNA for 94K T antigen. This mRNA is never detected in the lytically infected cells.

These results, taken together, permit the hypothesis that our data on the loss of 100K T antigen in anchorage revertants may be explained as the consequence of a stable change in the transcription or expression of a transcript from integrated early SV40 DNA.
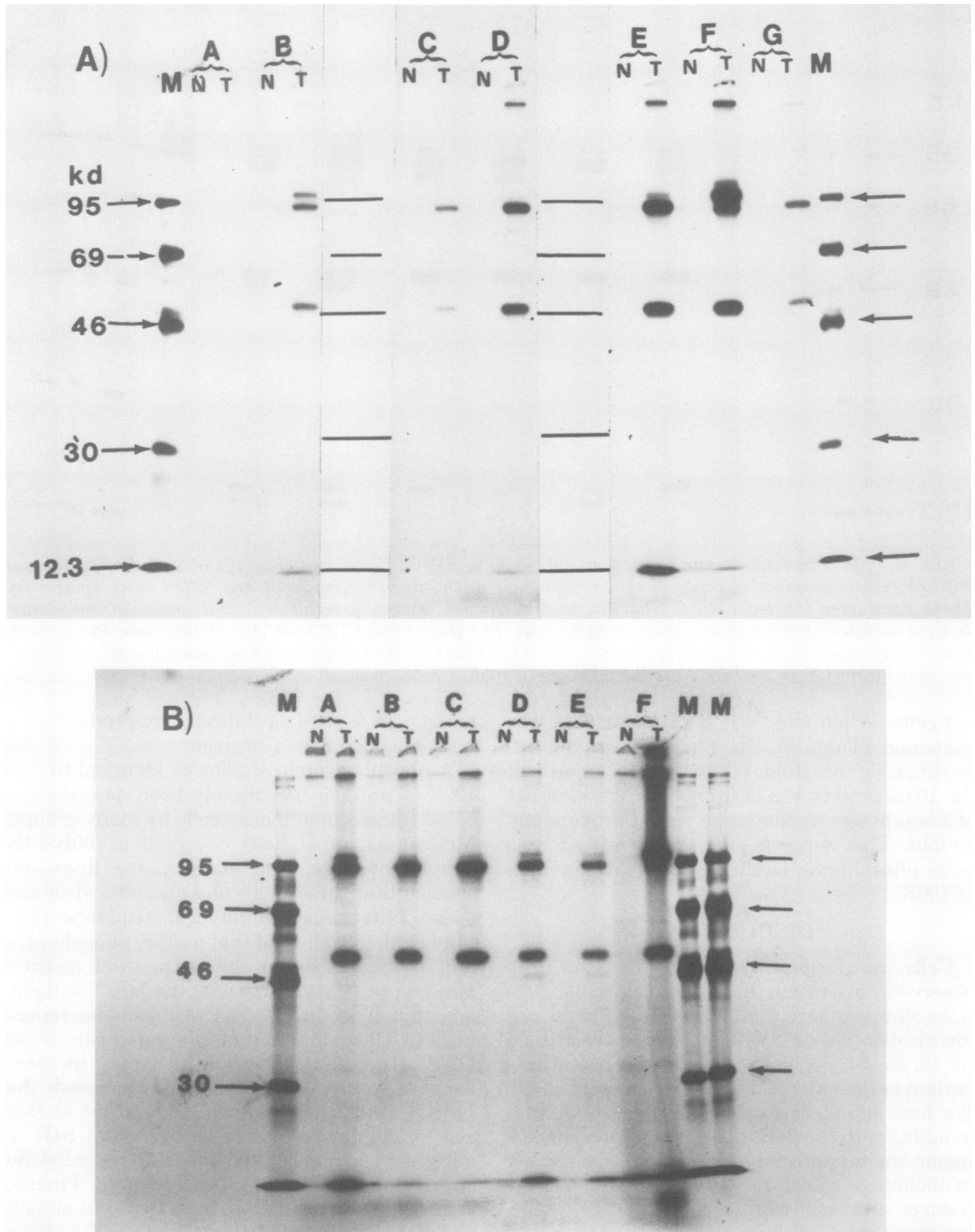

FIG. 5. [^{32}P]phosphate incorporation into 100K T antigen. (A) cells labeled with [^{32}P]phosphate were immunoprecipitated with normal (N) or antitumor (T) hamster serum. Lines shown are 3T3 (lane A), SV101 (lane B), LS$_1$ (lane C), LS$_2$ (lane D), Aγ5 (lane E), SVR85 (lane F), and SVR18 (lane G). (B) [^{32}P]phosphate-labeled immunoprecipitates of mock-revertant lines isolated after 5-fluorodeoxyuridine treatment (lanes A, B, C) or bromodeoxyuridine treatment (lanes D, E, F).

TABLE 6. *Relative phosphorylation of viral proteins*[a]

Line	^{35}S-100K/ ^{35}S-94K	^{32}P-100K/ ^{32}P-94K	^{32}P-94K/ ^{35}S-94K	^{32}P-100K/ ^{35}S-100K
SVR85	1.9	1.6	0.95	0.78
SV101	0.59	1.0	0.29	0.50
Aγ5	0.41	0.21	0.92	0.48
LS$_2$	0.35	0.16	0.62	0.29
LS$_1$	0.086	0.25	1.0	3.0
SVR18	0.038	0.38	0.31	3.1

[a] Ratios were obtained from area under the peaks of the curves generated as described in the text and in Fig. 1 for both [^{35}S]methionine and [^{32}P]phosphate-labeled immunoprecipitates.

Where does the 100K T antigen come from? Mark and Berg (37) have pointed out that the SV40 DNA sequence has a second open coding frame in the distal portion of the early transcript. If permitted, a splice into this frame would generate a T antigen with a very hydrophobic C-terminus. Presumably, the postulated hydrophobic T might migrate even more slowly than 94K T antigen in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The search in lytic cells for an mRNA with this splice has been difficult, due to small contaminating RNAs (2, 3). Transformed cells have not yet been examined.

An alternative means of producing 100K T antigen would be translation of a hybrid virus-cell mRNA, made perhaps by splicing a transcript out of the virus and into an adjacent host sequence. This event would probably demand some measure of host sequence specificity to SV40 integration in anchorage-transformed, 100K T antigen-containing mouse cells. Earlier studies on mouse cells have argued against such host specificity (6, 33), although partial transformants of adenovirus type 2 do make proteins which are of hybrid virus-host origin (14, 30).

We have examined the SV40 DNA integration patterns in this set of cells. Our observations are consistent with the hypothesis (28, 29) that specific integration sites are necessary for retention of the anchorage-transformed phenotype in these lines (G. Blanck, in preparation).

What is the 100K T doing? SV40-transformed cells differ from normal in many ways. A short list of changes that has been ascribed to the presence of SV40 include overgrowth in various restrictive conditions (Table 1), the secretion of growth factor(s) (32), the binding to and stabilization of a 54K T antigen (35, 36, 40, 53), the binding to SV40 and host DNA sequences (47), the appearance of new tumor-specific transplantation and surface antigens (12, 61–64), the activity of an adenosine triphosphatase (24, 65, 66), and the induction of cellular DNA and RNA

syntheses (56). Although ascribing a role for 100K T antigen in any of these assays will require study in each assay of sets of lines such as the one described in Table 1, earlier studies offer some enticing leads.

Soule et al. (57, 58) have fractionated transformed mouse cells and measured SV40 T antigens in various fractions. They found 100K T antigen as well as 94K T antigen in the plasma membrane fraction of a transformed mouse cell line, using [^{32}P]phosphate as label. Further, these plasma-membrane antigens were immunoprecipitated by antibodies both to nuclear T and to surface T antigen (58). Thus, the 100K T antigen variant may be at the cell surface, where, like the *src* gene product, it may have access to protein kinases and to the cytoskeleton.

The anchorage-transformed lines not only make 100K T antigen, but they also continue to make 94K T antigen. This suggests that the 100K T antigen variant might not fully substitute for the 94K T antigen in some activities, such as binding to SV40 and host DNA sequences (47), adenosine triphosphatase activity (22, 65, 66), and binding to 54K host antigen (35, 36, 39, 40, 53). The gene for large-T antigen has at least two complementation groups. One, affected by *tsA* mutations, maps in the region of 0.425 to 0.324 map units (8, 29, 44, 69). The other is defined by the mutant SV40 which originally transformed the monkey cell line C6 (23). The T antigen in this line cannot serve in the lytic cycle, nor can it bind SV40 origins (C. Prives, personal communication), but it can maintain the anchorage-transformed state in C6 monkey cells (23). We have no notion at this time about which of these two T functions (lytic A gene and monkey cell transformation) might be ascribed in mouse cells to the 100K T antigen.

In mouse cells carrying and expressing SV40 genes, serum transformation requires no particular viral gene product (Table 4). The biochemical correlates of serum transformation include enhanced capacity to pump glucose (15), the elevation of cyclic adenosine 5'-monophosphate in response to starvation (43), and the capacity to grow in serum-free medium without insulin (R. S. Powers, manuscript in preparation).

Conclusion. The transformation of mammalian fibroblasts by SV40 involves the intera·tion of one or perhaps two viral genes and their products with unknown cellular components. Although the two viral genes have been completely sequenced, and their products have been isolated, purified, and well characterized from lytically infected monkey cells (69), their actual role in maintaining the transformed state remains poorly defined. At the same time, the putative host cell molecules capable of specific

interactions with these viral proteins in the non-permissive cell are also poorly described. The impasse in understanding SV40 transformation may be based in part on the unproven assumptions that the viral genes and their products in a transformed cell are identical to their prototypes studied in a lytic cytotoxic interaction.

Few studies to date have seriously grappled with the possibility that the role of SV40 in maintaining transformation is dependent upon host DNA sequences as well as viral and host gene products. Yet precedent has recently been found for RNA tumor virus transformation requiring specific host integration sites (27), and for infection-dependent transformations which may require no viral gene products at all (50, 54, 55). This leads to the notion that SV40 might operate through specific alterations in host DNA sequences each time it stably perturbs either the serum or the anchorage growth requirement. A simple unifying hypothesis is that SV40 transforms mouse cells in these two assays by rearranging cellular sequences, presumably during abortive infection. Anchorage transformants would be lines in which SV40 has successfully integrated in a host sequence which permits synthesis of the 100K T protein. Serum transformants would be lines in which this host genomic rearrangement alone generated a set of host phenotypic alterations such as loss of insulin requirement (R. S. Powers, in preparation).

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