

## 94,000- and 100,000-Molecular-Weight Simian Virus 40 T-Antigens Are Associated with the Nuclear Matrix in Transformed and Revertant Mouse Cells

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A small fraction of the 94,000-molecular-weight multifunctional large T-antigen of simian virus 40 was associated with the nuclear protein matrix derived from simian virus 40-transformed mouse cells. The interaction between this fraction of T-antigen and the matrix was largely or entirely independent of nuclear DNA. Similar amounts of T-antigen were retained by the nuclei of transformed and revertant cell lines. A 100,000-molecular-weight variant of T-antigen, which has been found to correlate specifically with anchorage-independent growth, was present in the nuclear protein matrix of a transformed cell line. A T-antigen-containing revertant selected for the reacquisition of a high serum requirement and an anchorage requirement for growth retained T-antigen in association with its matrix.

The nucleus of eucaryotic cells contains a structural framework frequently referred to as the nuclear protein matrix (3, 7, 25). The nuclear matrix is resolved by the sequential extraction of whole nuclei with detergent, DNase, and 2 M sodium chloride buffer. Three components comprise the matrix after this extraction procedure: the outer pore-lamina complex, the fibrillar portion of the nucleolus, and the matrix network within the nucleus (3, 7). Polyacrylamide gel electrophoresis of rat liver nuclear matrices under denaturing conditions reveals three major polypeptides with molecular weights of 60,000 to 70,000, several minor polypeptides with molecular weights near 50,000, and a number of polypeptides with molecular weights greater than 150,000 (3).

The matrix spans the entire volume of the nucleus. In addition to serving as a skeletal framework, the matrix may have an important role in several nuclear functions, including the initiation and propagation of DNA replication (25). Electron micrograph studies by Comings and Okada have suggested that DNA appears to be attached to the nuclear matrix (7). It has also been shown that newly replicated DNA is preferentially associated with the nuclear matrix (2, 25).

Other matrix functions include the transcription, processing, and transportation of RNA in the nucleus (18). In addition to this, the outer lamina of the matrix, a protein assembly associated with the inner nuclear membrane, is appar-

ently involved in the disassembly of the nucleus during mitosis (11).

Simian virus 40 (SV40) is a small papovavirus that transforms mouse, rat, and hamster cells so that they no longer display growth requirements for high serum or for anchorage *in vitro* (36). Anchorage transformation generally requires the integration of the early region of the SV40 genome into the host cell and the expression of at least two virus-encoded proteins: large-tumor antigen (T-ag), with a molecular weight of approximately 94,000, and the small-tumor antigen (t-ag), with a molecular weight of approximately 17,000. Large T-ag stably complexes with at least one protein of cellular origin, the nonviral tumor (NVT) antigen, with a molecular weight of approximately 54,000 (22). The mechanism by which these proteins contribute to the maintenance of the transformed state has not yet been elucidated (for a review, see reference 15). In anchorage-transformed mouse cells, the large T-ag occurs in at least two forms: a 94,000-molecular-weight (94K) form and a 100K form. The electrophoretically slower form (100K) is expressed preferentially in transformed cell lines that have lost their anchorage requirement for growth (6).

Indirect immunofluorescence has demonstrated that T-ag accumulates primarily in the nucleus of SV40-transformed cells (27). The complete extraction of T-ag from the nucleus requires Nonidet P-40 detergent (NP-40) in a buffer of pH 8.0 or higher, since not all T-ag is removed at pH

6.0 or 7.0 (31). We wished to examine the possibility that the nonextractable fraction of T-ag might be specifically associated with the nuclear protein matrix.

The negative selection of T-ag-positive revertants from SV40-transformed mouse cells generates a set of cell lines which differ in their capacity to grow in low serum or without anchorage. For this set of cell lines, the presence of the SV40-related 100K protein is correlated specifically with anchorage-independent growth (6). We therefore assayed the presence of SV40-specific proteins in the nuclear matrix of three transformed mouse cell lines. One line (SV101) contains both the 94 and 100K T-ag, and one (B8) contains predominately the 100K T-ag. The third line (LS<sub>1</sub>) is a revertant which lacks the 100K T-ag and cannot grow in low serum or without anchorage.

#### MATERIALS AND METHODS

**Cell culture.** Cells were grown in Dulbecco modified Eagle medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% (vol/vol) fetal calf serum (Bioglass). 3T3 is a cell line established from Swiss mouse embryo fibroblasts (34). SV101 was cloned from 3T3 after infection with SV40 (35). Revertants FISV101 (26) and LS<sub>1</sub> (37) were derived by negative selection from SV101. B8 is a random subclone of SV101 picked in this laboratory. The growth properties of these cell lines are described in Table 1. Integrated SV40 DNA sequences in SV101 and in the descendants utilized in this study are described elsewhere (G. Blank, S. Chen, and R. Pollack, *Virology*, in press). All cell lines described are clonal descendants of Swiss 3T3 cells and therefore have a constant cell genetic background.

**Preparation of matrices.** Matrices were prepared by the protocol of Buckler-White et al. (4) with minor modifications. Subconfluent monolayers of cells were washed twice with cold phosphate-buffered saline (PBS) (pH 7.1) and then once with isotonic HEPES buffer (IH) (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5], 220 mM sucrose, 3 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>). IH buffer was made 0.5% (vol/vol) in NP-40 (Bethesda Research Laboratories), and phenylmethylsulfonyl fluoride was added to a final concentration of 300 µg/ml. Cells were extracted with 5 ml of IH buffer containing NP-40 per 150-mm dish for 5 min. The cells were scraped into a tube and vortexed four times for 1 min each at 30-s intervals on a Vortex Genie (Scientific Products, Inc.) set to high speed. Extracts were generally diluted with 1 volume of IH buffer without NP-40, and the resulting whole nuclei were pelleted at 800 × *g* for five min. These nuclei were washed once with IH buffer and pelleted at 200 × *g* for 10 min. All subsequent centrifugations were at this speed and time.

Whole nuclei (10<sup>7</sup>/ml) were suspended in TM sucrose buffer (4) plus 1% (vol/vol) Triton X-100 (Sigma Chemical Co.), vortexed to break up the pellet, and incubated for 5 min on ice to yield Triton nuclei. Triton nuclei were pelleted and washed once in TM buffer without Triton X-100.

Triton nuclei were then suspended in TM buffer plus 100 µg of DNase I (Sigma) per ml and allowed to digest for 4 h at room temperature with frequent shaking. These DNase-treated nuclei were pelleted and suspended in low Mg<sup>2+</sup> (LM) buffer (4). After a 5-min incubation on ice, the LM nuclei were pelleted and suspended in LM plus 2 M NaCl, incubated for 10 min on ice, pelleted, reextracted with 2 M NaCl, and repelleted. We have followed the terminology of others and defined these structures stable in 2 M NaCl as nuclear protein matrices.

**Extraction of matrices.** Matrices were further extracted by incubation overnight in 2% (wt/vol) sodium dodecyl sulfate (SDS) at room temperature and pelleted for 10 min at 200 × *g*.

**Indirect immunofluorescence.** Primary antibodies were used at titers (indicated in parentheses) for optimal staining of Formalin-fixed, NP-40-extracted whole cells. A concentration twofold higher than the one indicated for each antibody did not increase the intensity of staining, indicating antibody excess when used at the indicated titers. The reagents used to stain large T-ag were 905 (serum from a tumor-bearing hamster, 1:40), 116 (also serum from a tumor-bearing hamster [Division of Cancer Cause and Prevention, National Cancer Institute], 1:40), and 412 (culture fluid from a mouse monoclonal hybridoma [14], undiluted). The antibody against NVT antigen, designated 122, was the undiluted culture fluid from a mouse hybridoma (14).

Polyclonal antiserum 116 does not bind to the NVT antigen directly, but precipitates it indirectly through the complex NVT antigen forms with T-ag. Immunoprecipitation with all three T-ag-reactive antibodies (416, 905, or 116) demonstrated that none of these antibodies cross-reacts with the host 68K protein, which has been reported to bind to rare SV40 T-ag antibodies (23; unpublished data).

Conjugated second antibodies were obtained from Cappel Laboratories and used at titers of 1:10 for fluorescein isothiocyanate goat anti-mouse immunoglobulin G (IgG) or 1:40 for rhodamine-conjugated anti-hamster IgG as appropriate.

Whole nuclei or matrices were spun onto glass slides in a Shandon Elliott Cytospin at 1,200 rpm for 10 min. A 10-µl amount of primary antibody was placed on the

TABLE 1. Growth properties of cell lines<sup>a</sup>

Cell line	Relative doubling time <sup>b</sup>	Saturation density (cells/cm <sup>2</sup> [10 <sup>4</sup> ])	Plating efficiency <sup>c</sup> in Methocel (%)
3T3	0.23	5	0.001
SV101	0.53	>45	20
FISV101	0.61	9	0.01
LS <sub>1</sub>	<0.18	9	0.001
B8 <sup>d</sup>	0.52	>28	0.38 <sup>c</sup>

<sup>a</sup> Adapted from reference 35.

<sup>b</sup> Ratio of the doubling time in 1% calf serum to the doubling time in 10% calf serum. A value close to 1 indicates serum transformation.

<sup>c</sup> Number of colonies >0.2 mm in diameter per 100 cells plated.

<sup>d</sup> S. Chen, unpublished data.

<sup>e</sup> Plating efficiency in agarose.

TABLE 2. Cytochrome oxidase activity in nuclear protein matrices of various cell lines

Cell line <sup>a</sup>	Enzyme activity <sup>b</sup>
3T3	7.2
SV101	15
FISV101	11
BSA <sup>c</sup>	<0.18

<sup>a</sup> A 10- $\mu$ g amount of protein per sample.

<sup>b</sup>  $\Delta$ log [ferrocytochrome *c*]  $\text{min}^{-1}$   $\text{mg}$  of protein<sup>-1</sup>.

<sup>c</sup> Bovine serum albumin (10  $\mu$ g) was used as a control.

slide so as to cover the structures to be stained. The slides were incubated for 45 min at 37°C at 100% humidity. The slides were washed once in PBS, and 10  $\mu$ l of the appropriate second antibody was added to the slides, which were incubated an additional 45 min. The slides were washed in PBS, and cover slips were mounted with Aquamount. A Leitz Ortholux fluorescence microscope was used to visualize and photograph the fluorescence.

**Biochemical assays.** DNA was quantitated by the method of Burton (5) or with [<sup>3</sup>H]thymidine. For quantitating DNA by thymidine incorporation, cells were labeled for 24 h with 10  $\mu$ Ci of [<sup>3</sup>H]thymidine per ml, and samples were taken at each step of the extraction procedure. The samples were treated with 1 M NaOH for 2 h, precipitated with 30% trichloroacetic acid in the presence of 1% bovine serum albumin, collected on glass fiber filters (Whatman), and counted in Beta-Fluor (National Diagnostics). Protein was measured by the method of Lowry et al. (24). Cytochrome oxidase was assayed as described elsewhere (8).

**Radiolabeling and immunoprecipitation of tumor antigens.** For radiolabeling experiments, two 150-mm subconfluent plates were generally used. The cells were rinsed twice with warm PBS and incubated for 30 min with Dulbecco modified Eagle medium (DME) minus methionine plus 2% dialyzed fetal calf serum (GIBCO Laboratories), which was then changed to 5 ml of DME minus methionine plus 2% fetal calf serum containing 100  $\mu$ Ci of [<sup>35</sup>S]methionine per ml (Amersham; >700 Ci/mmol). The cells were labeled for 4 h. Matrices were prepared as described above, and the supernatants were collected at each step. The 2 M

NaCl supernatant was diluted 1:1 with LM buffer, the SDS supernatant was diluted 1:10 with LM buffer, and all supernatants were brought to 0.5% NP-40 for efficient antigen-antibody complexing. After the supernatants were spun at 48,000  $\times$  g to remove particulate matter, 10  $\mu$ l of 116 serum was added to a fraction of the NP-40 supernatant or to the entire supernatant from subsequent steps and incubated for 1 h on ice.

Immune complexes were collected with 40  $\mu$ l of 10% (vol/vol) washed Pansorbin (Calbiochem) for 20 min, pelleted at 2,000  $\times$  g for 5 min, and washed once in 140 mM NaCl-5 mM EDTA-10 mM Tris (pH 8.0) and once with 1 M NaCl-5 mM EDTA-10 mM Tris (pH 8.0). Proteins were eluted in sample buffer at 100°C as described elsewhere (28). Proteins were separated by polyacrylamide gel electrophoresis as described elsewhere (28), but on a gel in which the acrylamide-to-bisacrylamide ratio was 30:0.375. The gels were treated with En<sup>3</sup>Hance (New England Nuclear) and autoradiographed at -70°C on Kodak SB X-ray film. Densitometry scans were performed as described previously (6).

## RESULTS

Berezney and Coffey have reported that the nuclear matrix of rat liver cells retains the cytochrome oxidase activity found in intact rat liver nuclei (3). We assayed for the presence of cytochrome oxidase activity in our matrices. They were similar to those previously reported. Table 2 indicates that cytochrome *c* oxidase activity was retained by our nuclear protein matrices.

The amounts of protein and DNA remaining in the nucleus after each extraction step are indicated in Table 3. We defined NP-40-extracted nuclei as those containing 100% DNA and protein since these were the initial nucleus-derived structures that we isolated. The further extractions that we report here are similar to those previously reported by others (3).

We prepared matrices from normal (3T3), SV40-transformed (SV101), and SV40-revertant (FISV101) cell lines and examined the proteins in them by polyacrylamide gel electrophoresis and autoradiography in the hope of identifying SV40-specific proteins associated with the nu-

TABLE 3. DNA and protein retained after each extraction step in a typical preparation of matrices

Treatment	% of total remaining		
	DNA (Burton)	DNA ([ <sup>3</sup> H]-thymidine)	Protein (Lowry)
NP-40 <sup>a</sup>	(100)	(100)	(100)
Triton X-100	96	94	56
DNase <sup>b</sup>	67	74	48
LM buffer	13	13	24
2 M NaCl <sup>c</sup>	<1	0.1	10

<sup>a</sup> These are the first nuclear structures isolated and are defined as 100%.

<sup>b</sup> DNase removes roughly 30% of the nuclear DNA directly and nicks the remaining DNA. Of the total DNA, 50% is then subsequently removed by the low  $\text{Mg}^{2+}$  wash, which abolishes electrostatic interactions between the DNAs.

<sup>c</sup> The structures remaining after 2 M NaCl treatment are nuclear matrices.

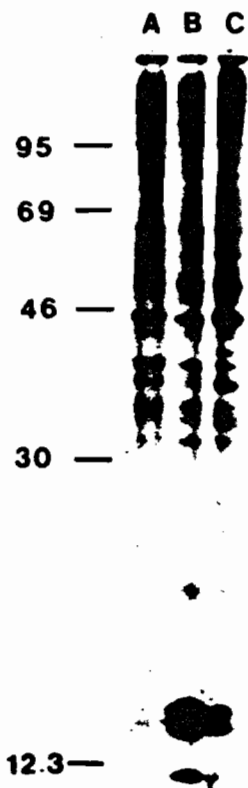


FIG. 1. Autofluorograph of [ $^{35}\text{S}$ ]methionine-labeled proteins from matrices of (lane A) normal 3T3 cells, (lane B) SV40-transformed SV101 cells, and (lane C) revertant FISV101 cells. Markers indicate the molecular weight ( $\times 10^3$ ). The doublet just above the 12.3K marker was variable from experiment to experiment and corresponds to histones. No difference could be detected either by direct observation or densitometry scan of the autoradiograph.

clear matrix (Fig. 1). These profiles are similar to those reported by Pardoll et al. (25), who reported 5 major bands and 10 to 20 minor bands in matrix preparations.

Densitometry scans of the autofluorograms of [ $^{35}\text{S}$ ]methionine-labeled samples indicated few differences among the gross protein profiles of matrices derived from 3T3, SV101, and FISV101. Neither by direct observation nor by densitometry scans could any virus-specific proteins be detected above the background of matrix proteins. This is in marked contrast to the results obtained in the lytic cycle with polyoma (a related papovavirus) (4).

These results could indicate either that no SV40-specific proteins are associated with the nuclear matrix or that their level cannot be detected above the background of matrix-specific proteins. To address this question, we examined the matrices by indirect immunofluorescence (Fig. 2).

The pattern of fluorescence for T-ag in the standard nuclear matrix after 2 M NaCl extraction (Table 3, Fig. 2B) resembled that produced by whole cells extracted with 1% NP-40 in IH buffer (pH 7.4) (Fig. 2A). When the matrix extractions were performed at pH 7.4, the internal matrix framework fluoresced distinctly, whereas residual nucleoli remained characteristically unstained. No difference could be detected between the intensities of fluorescence of the large T-ag in the matrices of SV40-transformed mouse fibroblasts and of the revertants derived from them. This qualitative identity of fluorescence was seen with two polyclonal antisera and one monoclonal antibody, all directed against T-ag.

Normal 3T3 nuclei do not fluoresce when prepared either from whole cells extracted in 1% NP-40 or as nuclear protein matrices and stained with antibodies either against T-ag or NVT antigen (data not shown).

The staining pattern of the NVT antigen revealed by indirect immunofluorescence in the nuclear matrix of cells containing SV40 T-ag (Fig. 2D) was very similar to that seen in NP-40-extracted cells (Fig. 2A). This could represent T-ag binding to NVT antigen, which is itself a part of the matrix, or NVT antigen binding to T-ag, which is a part of the matrix.

We wished to determine whether T-ag associated with the nuclear matrix could be removed without destroying the matrix. We took standard nuclear matrices after 2 M NaCl extraction and further extracted them with 2% SDS in LM buffer. When the nuclei were examined by immunofluorescence after this treatment with SDS, they were still positive for T-ag, but the intensity of staining was diminished (Fig. 2C) when compared with the intensity of whole cells extracted with NP-40 (Fig. 2A) or that of standard matrices stained in parallel (Fig. 2B).

These results suggested that it was possible to separate T-ag from the matrix. SV101 contains both the 94 and 100K forms of SV40 T-ag. Immunoprecipitation with anti-T-ag serum (one of the polyclonal sera used in the immunofluorescence study) was carried out to determine which species of T-ag was responsible for the fluorescence. This analysis also determined how much T-ag was released at each step in the preparation of the matrices.

Figure 3 shows an autofluorogram of an immunoprecipitation of the supernatant proteins extracted from SV101 cells by each step in the preparation of nuclear protein matrices. The relative areas enclosed by the curves of the densitometry scans of an autofluorogram of the soluble T-ag released by successive treatment with NP-40, Triton X-100, 2 M NaCl, DNase, and SDS were quantitated by densitometry scan

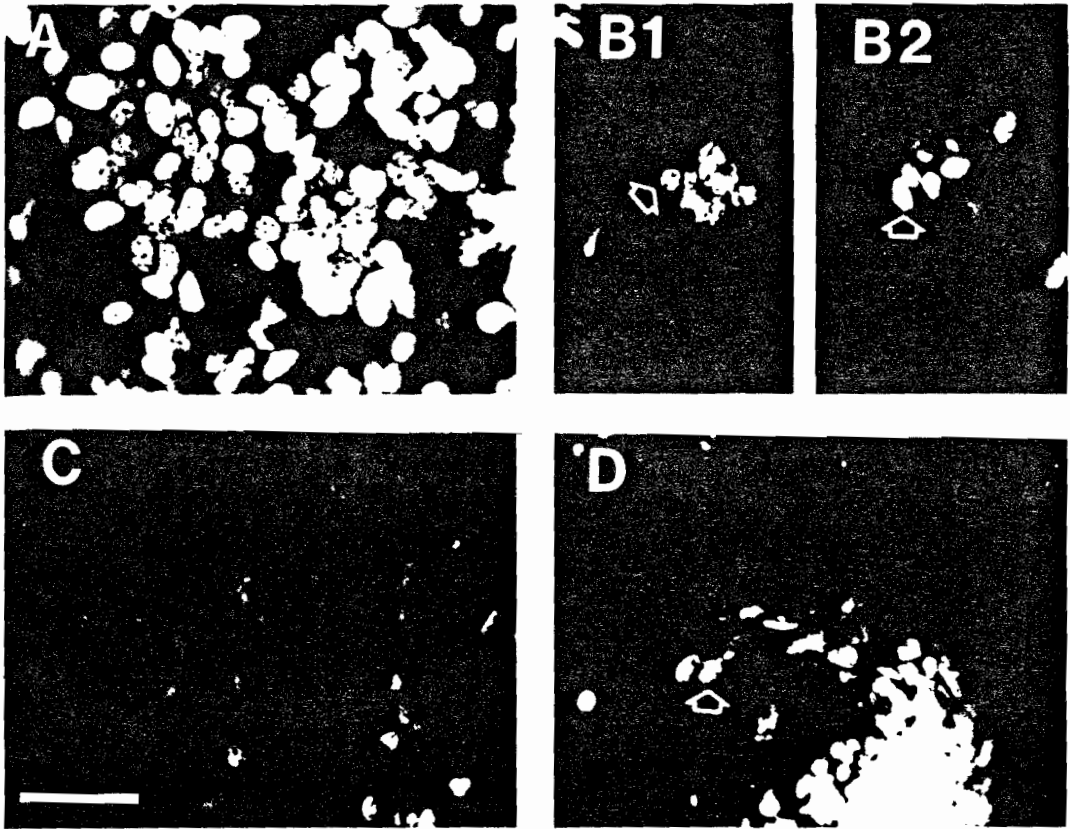


FIG. 2. Photographs of indirect immunofluorescence of structures derived from SV101 cells and stained for SV40 T-ag. (A) Cells were washed with IH buffer, extracted with IH buffer plus 1% NP-40 as described in the text, washed once with IH buffer, and spun onto slides in the cytofuge. (B) Standard matrices stained after 2 M NaCl extraction. (C) Matrices after further extraction with 2% SDS. (A), (B), and (C) were stained with hamster anti-T-ag serum 905, followed by rhodamine-conjugated goat anti-hamster IgG antibody. Note the diminished intensity in (C) compared with (B). (D) Matrices prepared as in (B), but stained with mouse monoclonal Pab 122 (which is directed against NVT antigen) followed by rhodamine-conjugated goat anti-mouse IgG antibody. In all cases, notice that the nucleoli are dark, characteristic of the distribution of T-ag normally seen. The arrows in (B) and (D) indicate particularly clear examples of T-ag staining in individual matrices, which generally clump together. The matrices are smaller than intact nuclei, as previously reported (3). Bar = 50  $\mu$ m.

and are summarized in Table 4. More than 95% of the total large T-ag and the NVT antigen were extracted during lysis in 0.5% NP-40 (Fig. 3, lane a, and Table 4). The T-ag immunoprecipitated from the NP-40 fraction probably represents the fraction of these proteins not bound to

cellular substructures since 0.5% NP-40 disrupts the nuclear envelope sufficiently to allow any unbound large T-ag, NVT antigens, or T-ag/NVT antigen complex to escape. Most (>95%) of the detectable small t-ag was released in this step as well (Table 4).

Detergent extraction with Triton X-100 displaced less than 1% of the total large T-ag and NVT antigen (Fig. 3, lane b, and Table 4). This small fraction could represent the T-ag associated with the nuclear envelope as Triton X-100 is particularly effective at dissolving that structure (9).

Treatment with DNase, which nicks DNA extensively and which released approximately 30% of the DNA in isolated nuclei (Table 3), released no further detectable immunoprecipitable tumor antigens (Fig. 3, lane c, and Table 4).

TABLE 4. SV40-specific and SV40-induced antigens removed at each extraction

Treatment	% of antigen solubilized		
	T-ag	NVT antigen	t-ag
NP-40	95	96	96
Triton X-100	0.1	<0.2	<0.3
DNase I	<0.1	<0.2	<0.3
2 M NaCl	1	<0.4	4
2% SDS	4	3	<0.3



FIG. 3. Autofluorograph of immunoprecipitations of soluble antigens from each step in the preparations of matrices from SV101 cells. Normal serum (N) and tumor serum (T) are indicated. The tumor serum was 905, the same one used in Fig. 2. Lane a, 1-day exposure of the immunoprecipitation of 1/10 of the total NP-40 fraction. Lanes b through f, 3-day exposure of the same experiment. Lane b, Immunoprecipitation of the antigens released from the nucleus by extraction with Triton X-100. Lane c, Immunoprecipitation of the antigens released by DNase I treatment. Lane d, Immunoprecipitation of the antigens released by treatment with LM buffer. Lane e, Immunoprecipitation of the antigens released by 2 M NaCl extraction. Note the strong band corresponding to histones released at this step. Lane f, Immunoprecipitation of the antigens from 1/2 of the SDS fraction. The high background is apparently due to the unusual composition of the buffer (containing SDS) in which this immunoprecipitation was carried out. Although 95% of the T-ag was removed by NP-40 (Table 4), clearly, there is a small fraction of T-ag specifically interacting with the nuclear matrix and only released from the matrix in 2% SDS.

In view of the ability of the large T-ag to bind DNA (21, 29, 30, 32), it is important to extract as much of the DNA as possible and to be aware of the DNA released at each step to insure that the detected tumor antigens are only those specifically associated with the protein of the nuclear protein matrix and not with the DNA of the matrix. Washing with LM buffer released no detectable T- or t-ag (Fig. 3, lane d, and Table 4) but did release roughly 50% of the nicked DNA (Table 3). The results shown here strongly suggest that the large T-ag was directly associated with the matrix and was not binding to the matrix through DNA.

Tumor antigens released by treatment with 2 M sodium chloride also were immunoprecipitated. This extraction released a small fraction (less than 2%) of the total T-ag (Fig. 3, lane e). T-ag extractable with 2 M NaCl has been observed before (9). In addition, the remainder of the small t-ag, approximately 4% of the total, was released at this step (Table 4).

By several criteria, the nuclear protein matrices that we isolated from SV40-transformed mouse cell lines were identical to the structures isolated by Coffey and others. The matrices

were still positive for T-ag by immunofluorescence, and the intensity of staining was roughly equal to that given by the residue after the extraction of whole cells with NP-40.

The immunoprecipitation of the supernatant from nuclear matrices derived from further extraction with 2% SDS confirmed the prediction that T-ag was released by SDS treatment. The SDS supernatant contained another fraction of T-ag and NVT antigen which represented approximately 4% of the total (Fig. 3, lane f, and Table 4). The T-ag released by SDS can be immunoprecipitated only if NP-40 is added to the supernatant before the antibody (data not shown).

The association between the 100K form of SV40 T-ag and anchorage-independent growth led us to examine a subclone which contains the 100K form but no detectable 94K T-ag to see whether the 100K form interacts differently from the 94K form with the nuclear protein matrix. Figure 4 shows the results of an immunoprecipitation of the supernatants of the various extractions of cell line B8 (which contains only the 100K form of T-ag). Clearly, the 100K form of large T-ag was associated with the matrix (Fig.

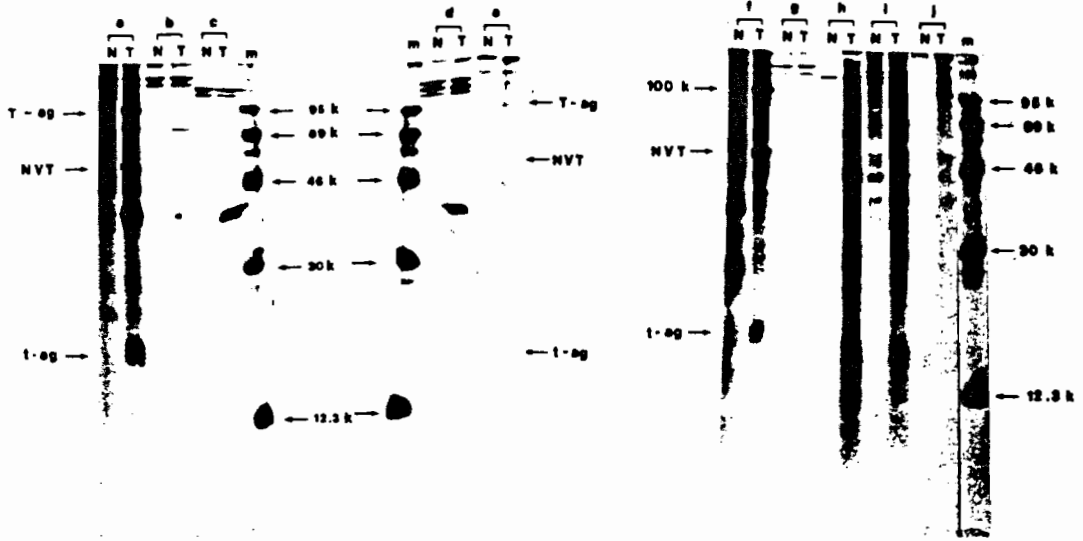


FIG. 4. Comparison of the extraction profiles of  $LS_1$  cells, which contain only 94K (wild-type) T-ag (lanes a through e), with B8 cells, which contain only the 100K variant of T-ag (lanes f through j). Lanes a and f, Immunoprecipitation of 1/10 of the NP-40 fraction. Lanes b and g, Immunoprecipitation of the entire Triton X-100 fraction. Lanes c and h, Immunoprecipitation of the entire DNase I fraction. Lanes d and i, Immunoprecipitation of the entire 2 M NaCl fraction. Lanes e and j, Immunoprecipitation of 1/2 of the SDS fraction. Revertant  $LS_1$  contains 94K T-ag associated with its matrix. The 100K variant is also capable of interacting with the nuclear protein matrix as shown in lane J. Both cell lines released approximately the same amount of T-ag after SDS treatment.

4, lane j). The 100K form of T-ag released from the matrix by SDS treatment was approximately 3% of the total 100K T-ag in B8 cells. This amount is roughly the same as that released from SV101 matrices.

Revertants derived from SV40-transformed cells still contain at least the wild-type-sized T-ag (94K), whereas the 100K form is lost specifically in those revertants now requiring anchorage for growth. We examined  $LS_1$  cells, which have both an anchorage and a high serum concentration requirement for growth and produce only the 94K form of T-ag, for the association of T-ag with the nuclear protein matrix to determine whether reversion in these two phenotypes is accompanied by a loss of the interaction of T-ag with the matrix. Figure 4, lane e, clearly shows that the 94K T-ag was still associated with the matrix, even in revertant cells. The 94K T-ag released by SDS from matrices derived from revertant cells also represents about 3% of the total T-ag and is similar to the amount released from the matrices of fully transformed SV101 cells.

## DISCUSSION

SV40 T-ag is responsible for the induction of cellular DNA synthesis in the lytic cycle (12, 16, 17). Since cells which are stably transformed to

anchorage-independent and serum-independent growth by SV40 are able to continue to synthesize their DNA and divide, even under restrictive conditions that would stop normal cells, a possible target for the SV40 T-ag in transformation is the set of cellular molecules responsible for the regulation of DNA replication.

We have demonstrated here that small amounts of both the T-ag of SV40 and its tightly bound host NVT protein are associated with the nuclear protein matrix in SV40-transformed mouse cells. The T-ag represents approximately 5% of the total T-ag. Matrix-bound T-ag does not bind to the matrix through DNA, since releasing 80% of the DNA by sequential extractions with DNase and LM buffer did not release any detectable T-ag. After treatment with 2 M NaCl, matrices have less than 0.1% of the DNA remaining, yet still contain 5% of the total T-ag.

Although 5% of the T-ag is a small portion of the total, the possibility of its having a significant function is not ruled out. For example, a minority subpopulation of T-ag has been reported by Scheller and co-workers (30) to be bound preferentially by a particular monoclonal antibody to T-ag. In that case, the 5% of T-ag molecules which bind to the monoclonal antibody represents over 60% of the origin-binding fraction of T-ag.

Subclones of SV101 which exclusively con-

tain the 100K form of T-ag have been isolated and examined. The 100K protein is retained in the matrix to about the same levels as is the 94K antigen.

Revertants of SV40-transformed cells can exhibit a reacquisition of growth control for anchorage and serum. The T-ag-containing revertant LS<sub>1</sub>, an example of this class of revertant, continues to synthesize DNA under low-serum restrictive conditions (39) and still contains T-ag in its matrix. This result is consistent with the idea that T-ag-dependent DNA synthesis is a direct result of T-ag interaction with the matrix of this revertant. We are examining revertants containing T-ag which continue to synthesize DNA under restrictive conditions to examine whether this relationship is causal or coincidental.

Nuclear protein matrix proteins increase their phosphorylation just before the cells enter the S phase (1). If this phosphorylation is a prerequisite for the S phase to begin, one might expect to find a kinase activity associated with the nuclear matrix. SV40 T-ag does indeed have a tightly associated, but not intrinsic, kinase activity (13, 33). NVT antigen, which is tightly bound to T-ag (22), has an associated kinase activity (20). We have shown here that approximately 5% of the total NVT antigen is associated with the nuclear matrix. The incorporation of T-ag into the matrix and, therefore, the subsequent localization of kinase activity in the matrix might be a necessary event in the T-ag-dependent initiation of cellular DNA synthesis in the transformed state.

NVT antigen, which is present in the nucleus of non-SV40-transformed cells (10, 19), might be found in the matrix in the absence of T-ag and may represent a critical controlling element in the replication of cellular DNA. We are currently examining this possibility.

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