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Simian Virus 40 T- and U-Antigens: Immunological Characterization and Localization in Different Nuclear Subfractions of Simian Virus 40-Transformed Cells

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Simian virus 40 (SV40)-transformed cells and cells infected by the nondefective adenovirus 2(Ad2)-SV40 hybrid viruses Ad2$^+$ND1 and Ad2$^+$ND2 were analyzed for SV40 T- and U-antigens, respectively, using individual hamster SV40 tumor sera or serum for which U-antibodies were removed by absorption. These studies showed that (i) T- and U-antigens can be defined by separate classes of antigenic determinants and (ii) the U-antigenic determinants in SV40-transformed cells and in hybrid virus-infected cells are similar. The apparent discrepancy in the subcellular location of U-antigen in SV40-transformed cells (nuclear location) and in hybrid virus-infected cells (perinuclear location) as determined by immunofluorescence staining of methanol/acetone-fixed cells could be resolved by treating hybrid virus-infected cells with a hypotonic KCl solution before fixation. Upon this treatment hybrid virus-infected cells also showed nuclear U-antigen staining. The possibility of an association of T- and U-antigens with different nuclear subfractions in SV40-transformed cells was investigated. Detergent-cleaned nuclei of SV40-transformed cells were fractionated into nuclear matrices and a DNase-treated, high-salt nuclear extract. Analysis of the nuclear matrices by immunofluorescence microscopy with T$^+$U$^+$ and T$^+$U$^-$ hamster SV40 tumor serum revealed that U-antigen remained associated with the nuclear matrices, whereas T-antigen could not be detected in this nuclear subfraction. T-antigen, however, could be immunoprecipitated from nuclear extracts of the SV40-transformed cells.

In simian virus 40 (SV40)-infected and -transformed cells, three SV40-specific antigens are expressed: SV40 tumor antigen (T), SV40 tumor-specific transplantation antigen (TSTA), and SV40 U-antigen (3, 11, 17, 21, 30). SV40 T-antigen and SV40 U-antigen are both nuclear antigens (21, 26), whereas SV40 TSTA appears to be located in the plasma membranes (37). Recent data strongly suggest that gene products of the early region of the SV40 genome, i.e., the SV40 early proteins, may themselves carry these antigenic determinants (1, 6-10, 12, 24, 27, 28, 31, 33).

SV40 U-antigen was first detected in cells infected by the nondefective adenovirus 2 (Ad2)-SV40 hybrid virus Ad2$^+$ND1 (20, 21). This hybrid virus contains only a fraction of the SV40 genome (0.11 to 0.28 map unit) recombined into Ad2 DNA (19). The SV40 information contained within this hybrid virus is expressed late during the hybrid virus replication cycle and leads to the synthesis of an SV40-specific protein with a molecular weight of 28,000 (28K) (22). It has been shown that this SV40-specific 28K protein is coded for by the SV40 DNA fragment integrated into this hybrid virus (14, 25) and corresponds to the C-terminal part of the SV40 gene *A* product (25). With the aid of antibodies against Ad2$^+$ND1-infected cell extracts, SV40 U-antigen was subsequently also detected at early times in SV40-infected cells as well as in SV40-transformed cells (21). Furthermore, sera from SV40 tumor-bearing hamsters also may contain antibodies against SV40 U-antigen (21).

Staining of cells infected by the Ad2$^+$-SV40 hybrid viruses Ad2$^+$ND1 or Ad2$^+$ND2 for SV40 U-antigen results in a different staining pattern when compared with cells infected or transformed by SV40. In Ad2$^+$ND1- or Ad2$^+$ND2-infected cells, SV40 U-antigen is visualized only in the perinuclear or cytoplasmic region of the cells, with the nuclei being nearly devoid of stain, whereas in cells infected or transformed by SV40, U-antigen is found only in the nuclei (20, 21).

The different morphological appearance of SV40 U-antigen in Ad2$^+$ND1- or Ad2$^+$ND2-infected cells and in SV40-infected or -transformed cells is difficult to understand, because one function postulated for SV40 U-antigen, the helper

function for human adenoviruses to grow in monkey cells (29), is expressed in both hybrid virus-infected and SV40-infected monkey cells. If the SV40 helper function is directly mediated by SV40 U-antigen, one would expect SV40 proteins carrying U-antigenic determinants to be associated with the same subcellular structures in $Ad2^+$-SV40 hybrid virus-infected cells as in SV40-infected or -transformed cells.

Recently, biochemical and immunological evidence has been provided demonstrating that in HeLa cells infected by different $Ad2^+$-SV40 hybrid viruses, SV40-specific proteins carrying SV40 U-antigen determinants are stably associated with the nuclear matrices of the hybrid virus-infected cells. SV40-specific proteins with a close molecular relationship to SV40 T-antigen, on the other hand, are extracted from the nuclei during nuclear matrix isolation (7, 9). These data suggested that (i) also in hybrid virus-infected cells, the main location for SV40-specific proteins carrying U-antigen determinants is the nucleus and (ii) SV40 T- and U-antigens can be differentiated by their different structural organization within the nuclei.

In this report I demonstrate that SV40 U-antigen can be visualized also in the nuclei of $Ad2^+$-SV40 hybrid virus-infected cells, if the nuclei are made permeable for the antibody before fixation. Immunological analysis of SV40 U-antigen in $Ad2^+$-SV40 hybrid virus-infected and SV40-transformed cells shows that the antigenic determinants for SV40 U-antigen are similar in both systems. These antigenic determinants are different from the antigenic determinants of SV40 T-antigen. Furthermore, as in hybrid virus-infected cells, U- and T-antigens seem to be organized in a structurally different manner within the nuclei of SV40-transformed cells.

MATERIALS AND METHODS

Cells and viruses. HeLa cells, 3T3 cells, and SV40-transformed 3T3 cells (clone SV101) were grown in Dulbecco-modified Eagle medium supplemented with 10% calf serum. Seed stocks of the nondefective $Ad2^+$-SV40 hybrid viruses $Ad2^+ND1$ and $Ad2^+ND2$ were obtained from A.M. Lewis, Jr., and stocks were prepared in HeLa S_3 cells grown in suspension culture, using minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.; F13) supplemented with 5% calf serum.

Labeling of cells, cell fractionation, subfractionation of nuclei, and isolation of nuclear matrices (13, modified). 3T3 and SV101 cells grown on 10-cm petri dishes (ca. 5×10^6 cells per plate) were washed twice with Earle salt solution and labeled for 2 h in Eagle medium minus methionine, containing 10 μCi of [^{35}S]methionine (Amersham Corp.; specific activity, 500 to 600 Ci/mmol) per ml and 5% calf serum (1 ml of labeling medium per 10-cm dish). After the labeling period the labeling medium was removed and the cells were washed twice with phosphate-buffered saline (PBS; pH 7.2; 38; cells were removed from the plates with trypsin-EDTA and washed twice with PBS. A total of 5×10^7 to 1×10^8 cells were suspended at a concentration of 10^7 cells per ml in RSB (0.01 M Tris-hydrochloride, pH 7.2; 0.01 M NaCl; 1.5 mM $MgCl_2$). After the mixture swelled for 5 min on ice, Nonidet P-40 (NP40; Shell Chemical Co., London) was added to a final concentration of 0.5% for 30 min at 0°C. In most preparations this treatment was sufficient to lyse the cells and to obtain "clean" nuclei as judged by phase-contrast microscopy. In some preparations further purification of the nuclei had to be achieved by Dounce homogenization. Nuclei were collected by centrifugation at $220 \times g$ for 5 min and washed several times with RSB. These nuclei were termed NP40 nuclei. The combined supernatants were kept as NP40 cytoplasmic extract. NP40 nuclei were resuspended in the cold with RSB containing 1 mg of DNase I (Worthington) per ml at a concentration of 5×10^8 nuclei per ml. Two milliliters of HSB (0.01 M Tris-hydrochloride, pH 7.4; 0.5 M NaCl; 0.05 M $MgCl_2$) was added per 5×10^7 nuclei, and the nucleohistone gel was digested for 30 min at 37°C. Nuclear matrices were separated from the high-salt nuclear extract by centrifugation at $500 \times g$ for 10 min and incubated at 37°C for 20 min in 2 ml of NEB (0.01 M Tris-hydrochloride, pH 7.4; 0.01 M dithithreitol; 0.01 M NaCl; 0.01 M EDTA) per 5×10^7 nuclei. After centrifugation at $500 \times g$ for 10 min, nuclear matrices were washed once with 2 ml of TE (0.01 Tris-hydrochloride, pH 7.4; 0.001 M EDTA) per 5×10^7 nuclei and resuspended in TE. Nuclear matrices were then processed for either gel electrophoresis or immunofluorescence staining (see below). Phase-contrast microscopy of the purified nuclear matrices suspended in TE buffer showed that the nuclear matrix fraction consisted of single nuclear matrices and aggregates of nuclear matrices. A small percentage of the nuclear matrix fraction consisted of broken nuclear substructures. Nuclear matrices isolated by this procedure resemble intact nuclei in shape but their diameters are only one-third to one-half those of intact nuclei (data not shown; cf. reference 7).

SV40 tumor sera. SV40 tumor sera were produced in 6-week-old male golden Syrian hamsters by subcutaneous inoculation of 10^6 SV40-transformed hamster cells. The SV40-transformed hamster cells (line H65/90B) as well as the procedure for preparing SV40 tumor sera were originally provided by V. Defendi, New York University. Sera were collected when tumors were 3 to 4 cm, approximately 4 weeks after inoculation.

Absorption of sera. Before the determination of T- and U-antibody titers on SV101 cells (see below), sera from tumor-bearing hamsters were absorbed on methanol-fixed 3T3 cells. Confluent monolayers of 3T3 cells grown on plastic petri dishes (9 cm in diameter) were washed twice with PBS and then fixed with methanol for 10 min at room temperature. After air drying, the cells were rinsed with PBS and 1-ml aliquots of the tumor sera, diluted 1:10 with PBS, were added per plate. The sera were absorbed at 4°C for 12 h per petri dish three times for a total of 36 h. For determination of U-antibody titers on hybrid virus-

infected cells (see below), the sera were absorbed as described above on Ad2-infected HeLa cells fixed with methanol approximately 24 h postinfection.

Immunofluorescence staining. 3T3 and SV101 cells grown on glass cover slips (12 mm in diameter) in Dulbecco-modified Eagle medium with 10% calf serum were washed once with PBS and fixed by treatment with methanol at −20°C for 5 min, followed by acetone treatment at −20°C for 30 s and air drying. Then the first antibody (serum from an SV40 tumor-bearing hamster, diluted with PBS as indicated) was added, and the cover slips were incubated for 45 min at 37°C. After washing with PBS, fluorescein-labeled goat gamma globulin prepared against hamster gamma globulin (Cappel Laboratories; 1:20 dilution with PBS of reconstituted material) was added, and the cover slips were held for a further 45 min at 37°C. After a second series of washes with PBS, the cover slips were mounted with Elvanol on microscope slides. The cells were viewed with a Zeiss microscope (Carl Zeiss, Oberkochen, F.R.G.) equipped with epifluorescent illumination. Pictures were taken with Planapo ×40 and ×63 oil immersion objectives. $Ad2^+ND1$- and $Ad2^+ND2$-infected HeLa cells grown on cover slips (12 mm in diameter) in Dulbecco-modified Eagle medium supplemented with 10% calf serum were processed for immunofluorescence microscopy approximately 32 h after infection as described above or as indicated in the figure legends. Nuclear matrices suspended in TE were allowed to settle for 10 min in the cold onto glass cover slips (12 mm in diameter). The supernatant fluid was removed carefully, using a Pasteur pipette, and the cover slips were incubated directly with antibody as described above. The cover slips were then processed for immunofluorescence microscopy.

Determination of T- and U-antibody titers. Antibody titers against SV40 T-antigen were determined on methanol/acetone-fixed SV101 cells as described (26). For determination of U-antibody titers in SV101 cells, T-antigen was heat inactivated (21). SV101 cells grown on cover slips were washed twice with PBS and incubated in a wet chamber at 50°C for 30 min. The cells were then washed twice with PBS and processed for immunofluorescence microscopy. Antibody titers against SV40 U-antigen in HeLa cells infected by $Ad2^+ND1$ were determined as described (21). Serum dilutions were done with filtered PBS.

Selection of T^+U^+ and T^+U^- sera. T^+U^- serum was obtained by selective absorption of U-antibodies from T^+U^+ serum H10 (see Results; Table 1). Serum H10 was absorbed as described above on either 3T3 cells (control) or SV101 cells in which T-antigen was inactivated by heating the cells to 50°C for 30 min before fixation (21).

Immunoprecipitation. A 400-μl amount of either cytoplasmic extract or high-salt nuclear extract was incubated at 4°C with 10 μl of hamster SV40 tumor serum (serum H2 in Table 2; see Results) or normal hamster serum. After 1 h, 50 μl of settled staphylococcal protein A–Sepharose (Pharmacia) swollen in PBS was added. After gentle agitation overnight at 4°C the protein A–Sepharose was poured into a minicolumn. Washing and elution of the immunoprecipitates was performed as described by Schwyzer (35).

Preparation of samples for gel electrophoresis. NP40 nuclei and nuclear matrices were pelleted and then dissolved in electrophoresis sample buffer (0.0625 M Tris, pH 6.8; 3% sodium dodecyl sulfate; and 5% 2-mercaptoethanol). Portions of the NP40 cytoplasmic extract and the high-salt nuclear extract were precipitated with ice-cold trichloroacetic acid (10% [wt/vol] final concentration), washed three times with cold acetone, air dried, and dissolved in electrophoresis sample buffer. Immediately after the addition of sample buffer, the samples were sonically oscillated with a Branson Sonifier equipped with a microtip (position 4, 3× 10 s with 10-s intervals) and treated for 3 min in a boiling-water bath. The protein content of the samples was determined by the method of Lowry et al. (23) and adjusted to approximately 10 to 15 μg/10μl.

Polyacrylamide gel electrophoresis and fluorography. The polyacrylamide gel system of Laemmli (18) and Maizel (24) was used. A 15-μg amount of protein in approximately 10 μl was applied per slot. Electrophoresis was performed at a constant current of 12 mA with slab gels of 1-mm thickness. After electrophoresis the gels were prepared for fluorography as described by Bonner and Laskey (4).

RESULTS

Visualization of SV40 U-antigen in $Ad2^+$-SV40 hybrid virus-infected and SV40-transformed cells by immunofluorescence staining. Figure 1 shows the typical SV40 U-antigen staining pattern with $Ad2^+ND1$ (Fig. 1a)- and $Ad2^+ND2$ (Fig. 1b)infected HeLa cells and with SV40-transformed cells (SV101 cells) (Fig. 1c) as defined by Lewis and Rowe (21). In hybrid virus-infected cells (Fig. 1a and b), SV40 U-antigen appears as a broad perinuclear staining, with the nuclei being nearly devoid of stain. By contrast, in SV40-transformed cells SV40 U-antigen is only visible within the nuclei. Recent experiments, however, have shown that in hybrid virus-infected cells SV40-specific proteins carrying SV40 U-antigenic determinants are associated with the nuclei and can be visualized by immunofluorescence staining on isolated nuclear matrices (7, 9). These data further suggested that U-antigen could not be demonstrated within the nuclei of hybrid virus-infected cells either because the methanol/acetone fixation procedure used destroyed the antigenicity of the SV40-specific proteins (as is the case for SV40-specific proteins on isolated nuclear matrices) or because of their tight association with the nuclear matrices, the antibody is hindered from reacting with the SV40-specific proteins.

These probelms could be solved, however, when the nuclei of hybrid virus-infected cells were made permeable to antibody before fixation by treating the cells for 2 to 3 min with 0.4% KCl solution (34). Figure 2b demonstrates that after such a treatment, followed by Formalin

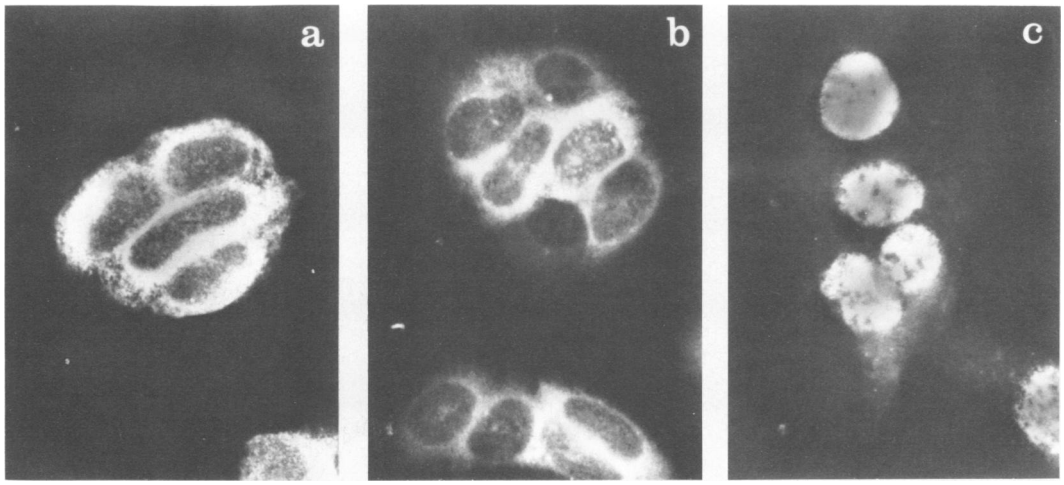

FIG. 1. *U-antigen staining of HeLa cells infected by the Ad2$^+$-SV40 hybrid viruses Ad2$^+$ND1 and Ad2$^+$ND2 and of SV40-transformed mouse cells (SV101 cells). HeLa cells on cover slips were infected with AD2$^+$ND1 (a) or Ad2$^+$ND2 (b) and processed for immunofluorescence microscopy 32 h after infection as described in the text. SV101 cells (c) were also grown on cover slips. T-antigen was heat inactivated at 50°C for 30 min, and the cells were processed for immunofluorescence microscopy as described. Hamster SV40 tumor serum H6 (see Table 1) was used at a dilution of 1:20. Control experiments with normal hamster sera or AD2-infected HeLa cells did not result in immunofluorescence staining of the cells. ×48.*

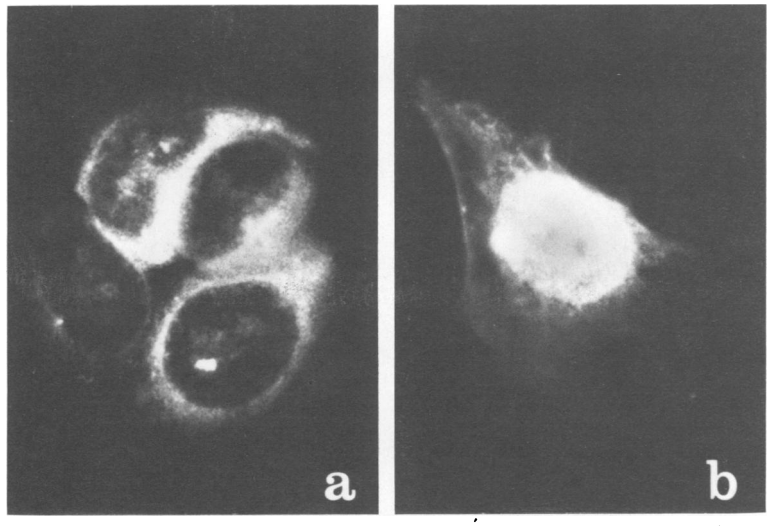

FIG. 2. *U-antigen staining of HeLa cells infected by the Ad2$^+$-SV40 hybrid virus Ad2$^+$ND2, using different fixation procedures. HeLa cells on cover slips were infected with Ad2$^+$ND2 and processed for immunofluorescence microscopy 32 h after infection. (a) Cells were fixed in methanol at −20°C for 5 min, followed by an acetone wash at −20°C for 30 s, and then incubated with antibodies as described in the text. (b) Cells were treated for 2 to 3 min with 0.4% KCl in distilled water and then fixed for 5 min in 3.7% Formalin in PBS. Incubation with antibodies was as described. Control experiments with normal hamster sera or Ad2-infected HeLa cells showed no fluorescence staining. ×74. A similar nuclear U-antigen fluorescence is obtained when Ad2$^+$ND1-infected cells are pretreated with KCl before fixation (data not shown).*

fixation, a nuclear U-antigen staining is obtained with hybrid virus-infected cells, too. As after methanol/acetone fixation without KCl pretreatment (Fig. 2a), some cytoplasmic (perinuclear) staining is visible also. Staining of hybrid virus-infected cells after KCl pretreatment, followed by methanol/acetone fixation, results in a similar nuclear U-antigen fluorescence as

shown in Fig. 2b (data not shown). This indicates that the failure to detect nuclear U-antigen fluorescence in hybrid virus-infected cells after methanol/acetone fixation without KCl pretreatment (Fig. 2a) is due to the antigen not being available for the antibody rather than to denaturation or extraction of the antigen by methanol/acetone treatment of the cells.

Immunological relationship of SV40 U-antigen in Ad2$^+$ND1-infected HeLa cells and in SV40-transformed cells. The immunological relationship between SV40 U-antigen in Ad2$^+$ND1-infected cells and in SV40-transformed cells was analyzed. Sera from SV40 tumor-bearing hamsters were screened for U-antibodies, and immunofluorescence titers against SV40 U-antigen were determined on Ad2$^+$ND1-infected HeLa cells and on heat-treated, SV40-transformed mouse cells (SV101 cells) as described in Materials and Methods. Immunofluorescence titers against SV40 T-antigen were determined in parallel. Table 1 shows that out of 12 sera tested, 10 were positive for U-antibodies on Ad2$^+$ND1-infected HeLa cells, with immunofluorescence titers ranging from 1:5 to 1:60. On SV101 cells anti-T-titers were from 1:200 to 1:400. On heat-treated SV101 cells, all sera were positive for U-antibodies, with immunofluorescence titers ranging from 1:40 to 1:320.

Comparison of the antibody titers against U- and T-antigens in individual hamster sera shows that it is possible to obtain sera with high antibody titers against T- and U-antigens (sera H6 and H11) as well as sera with high titers against T-antigen and low titers against U-antigen (sera H1, H4 and H5). This suggests that the antibody titers against U- and T-antigens developed independently of each other. Therefore, one may conclude that the T- and U-antigenic determinants are not related.

The antibody titers of these sera as determined on Ad2$^+$ND1-infected HeLa cells and on heat-treated SV101 cells correlate insofar as sera with a relatively high anti-U-titer on Ad2$^+$ND1-infected HeLa cells also have a high anti-U-titer on heat-treated SV101 cells and vice versa. This suggests that U-antigenic determinants in hybrid virus-infected and SV101 cells are similar.

These conclusions were tested by absorbing an individual hamster serum (serum H10 in Table 1; anti-T-titer, 1:300; anti-U-titer, 1:200) on heat-treated, i.e., only U-antigen-exposing, SV101 cells. Table 2 shows that the anti-T-titer was approximately the same after absorption as before absorption (1:250 after absorption as compared to 1:300 before absorption). The anti-U-titer, however, dropped drastically after absorption on heat-treated SV101 cells (1:20 after absorption as compared to 1:200 before absorption). This demonstrates that T and U are separate antigenic entities. No U-antibody titer was demonstrable after absorption on Ad2$^+$ND1- or Ad2$^+$ND2-infected HeLa cells, indicating that the U-antigenic determinants exposed in heat-treated SV101 cells are very similar to the U-antigenic determinants exposed in hybrid virus-infected cells.

TABLE 1. *Distribution of SV40 T- and U-antibody titers in hamster SV40 tumor sera as determined by immunofluorescence staining*

Serum	Antibody titer[a] on:		
	SV101 cells	Heated SV101 cells[b]	Ad2$^+$ND1-infected HeLa cells
H1	300	60	0[c]
H2	400	200	20
H3	300	150	40
H4	300	80	5
H5	300	40	0
H6	400	320	60
H7	300	150	20
H8	200	100	10
H9	400	200	40
H10	300	200	20
H11	300	300	60
H12	300	100	10

[a] Antibody titers were determined as described in the text. Titers are given as the reciprocal of the serum dilution at which the staining intensity was 1+ on a scale of 4+ = brilliant and ± = very dull.

[b] SV101 cells were heat treated for 30 min at 50°C as described in the text and then processed for immunofluorescence staining.

[c] 0 = <5.

Localization of SV40 T- and U-antigens in different nuclear subfractions of SV40-transformed cells. Analysis of the distribution of SV40-specific proteins in nuclear subfractions of HeLa cells infected by different Ad2$^+$-SV40 hybrid viruses has shown that SV40-specific proteins carrying SV40 U-antigen determinants specifically associated with the nuclear matrix of these cells. SV40-specific proteins with a close molecular relationship to SV40 T-antigen (e.g., the SV40-specific 95K protein of Ad2$^+$ND4) (25), on the other hand, were partially extracted during the nuclear matrix isolation procedure (7, 9). Therefore, it was of interest to analyze whether a similar compartmentalization of U- and T-antigens could be detected in SV40-transformed cells.

3T3 and SV101 cells were labeled for 2 h with [^{35}S]methionine, and nuclei were prepared by lysing the cells with hypotonic buffer containing NP40. The nuclei were further fractionated into a high-salt nuclear extract and a nuclear matrix fraction (see Materials and Methods). Aliquots

of each subcellular fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and radioactive polypeptides were visualized by fluorography as described in Materials and Methods. Figure 3 shows a fluorogram of the radioactively labeled polypeptides of subcellular fractions of 3T3 cells compared with the radioactively labeled polypeptides of SV101 cells. The relative purity of the nuclear subfractions is documented by the accumulation of several proteins in either the high-salt nuclear extract fraction or the nuclear matrix fraction. The major protein component of the nuclear matrix fraction with an apparent polypeptide molecular weight of 65,000 (13) is virtually absent from the high-salt nuclear extract. On the other hand, several other proteins are exclusively found in the high-salt nuclear extract and are not detectable in the nuclear matrix fraction. Minor differences in the polypeptide pattern can be detected between the subcellular fractions of 3T3 and SV101 cells, but gel electrophoresis alone is not sufficient to determine whether particular polypeptides are SV40 specific. Analysis of the NP40 cytoplasmic extract and the high-salt nuclear extract of SV101 cells by indirect immunoprecipitation with serum from SV40 tumor-bearing hamsters (see Materials and Meth-

TABLE 2. *T- and U-antibody titers of serum H10 (Table 1) before and after absorption on heat-treated SV101 cells*

Serum	Antibody titer[a] on:		
	SV101 cells	Heated SV101 cells	Ad2⁺ND1-infected HeLa cells
H10, absorbed on 3T3 cells (control)	300	200	20
H10, absorbed on heated SV101 cells	250	20	0[b]

[a] Antibody titers were determined as described in Table 1.
[b] 0 = <5.

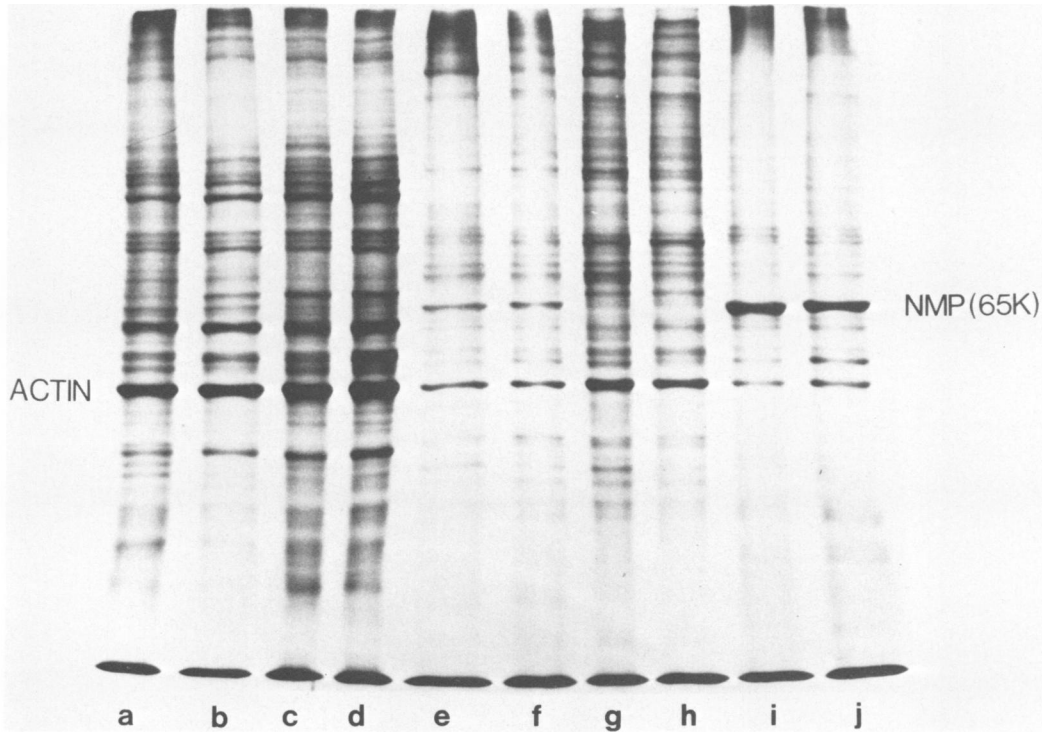

FIG. 3. *Polypeptide pattern of subcellular fractions of 3T3 and SV101 cells; fluorogram of ^{35}S-labeled polypeptides. 3T3 cells and SV101 cells were labeled for 2 h with [^{35}S]methionine and fractionated into different subcellular fractions as described in the text. Portions of each fraction containing 15 μg of protein were applied to a 10% sodium dodecyl sulfate-polyacrylamide slab gel and run for 5 h at 12 mA. The sample order is: (a) 3T3 homogenate; (b) SV101 homogenate; (c) 3T3 NP40 cytoplasmic extract; (d) SV101 NP40 cytoplasmic extract; (e) 3T3 nuclei; (f) SV101 nuclei; (g) 3T3 high-salt nuclear extract; (h) SV101 high-salt nuclear extract; (i) 3T3 nuclear matrices; (j) SV101 nuclear matrices. NMP is the major nuclear matrix protein.*

ods) revealed (Fig. 4) that the NP40 cytoplasmic and the high-salt nuclear extracts of SV40-transformed cells contained three SV40-specific proteins with apparent polypeptide molecular weights of about 94K, 90K, and 84K, which were specifically immunoprecipitated with serum from SV40 tumor-bearing hamsters (Fig. 4b and f) but not with normal hamster serum (Fig. 4d and h). These proteins were absent from the NP40 cytoplasmic extract and the high-salt nuclear extract of untransformed 3T3 cells (data not shown). Therefore, the immunoprecipitated proteins most likely represent SV40 T-antigen. The presence of three SV40-specific proteins in the immunoprecipitates most probably is due to proteolysis of the 94K protein during either cell fractionation or incubation of the subcellular fractions with serum (5). Quantitative evaluation of the experiment shown in Fig. 4 revealed that about 80 to 90% of the immunoprecipitated SV40 T-antigen was already extracted by the detergent treatment used to obtain detergent-cleaned nuclei, and the residual 10 to 20% were extracted by the DNase/high-salt treatment applied to obtain nuclear matrices.

The nuclear matrices obtained by the nuclear fractionation procedure were analyzed by immunofluorescence microscopy with T^+U^+ and T^+U^- hamster SV40 tumor serum (see Materials and Methods). Figure 5 shows that the intensity of the T-staining of SV101 cells with the T^+U^+ and T^+U^- sera was similar at a dilution of 1:250 (Fig. 5a and d). Immunofluorescence staining of heat-treated SV101 cells for U-antigen with the T^+U^+ serum was positive up to a serum dilution of 1:200 (Fig. 5b). At a 1:20 dilution of the T^+U^- serum, however, no U-antigen fluorescence could be detected (Fig. 5e). Analysis of nuclear

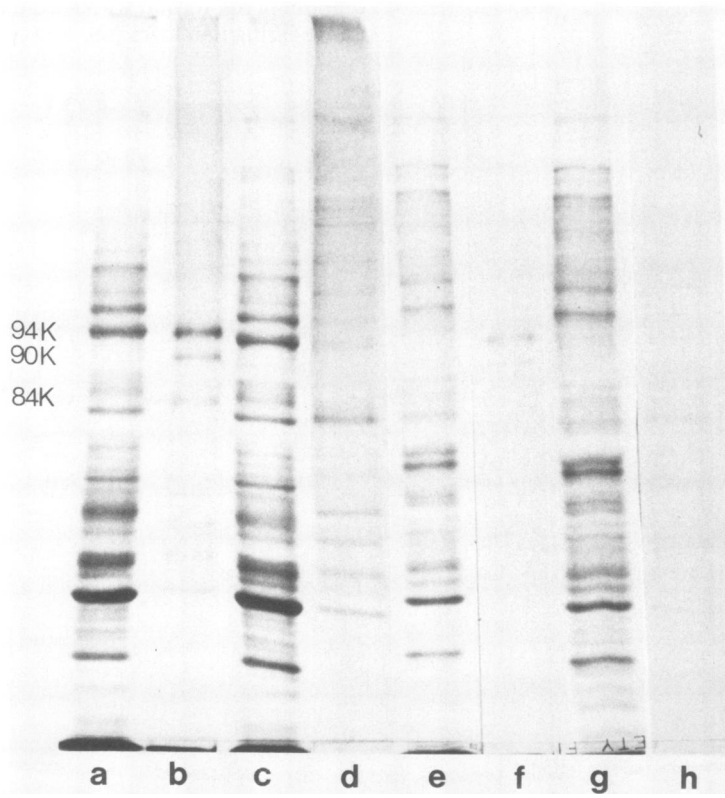

FIG. 4. *Immunoprecipitation of SV40 T-antigen from NP40 cytoplasmic extract and high-salt nuclear extract of SV101 cells; fluorogram of ^{35}S-labeled polypeptides. NP40 cytoplasmic extract and high-salt nuclear extract of SV101 cells were prepared and immunoprecipitated as described in the text. Aliquots of the immunoprecipitates were analyzed on a 7.5% acrylamide slab gel and run for 5 h at 12 mA. The sample order is: (a) SV101 NP40 cytoplasmic extract; (b) same, immunoprecipitated with hamster SV40 tumor serum; (c) SV101 NP40 cytoplasmic extract; (d) same, immunoprecipitated with control serum; (e) SV101 high-salt nuclear extract; (f) same, immunoprecipitated with hamster SV40 tumor serum; (g) SV101 high-salt nuclear extract; (h) same, immunoprecipitated with control serum. Tracks (a), (c), (e), and (g) were exposed for 20 h; tracks (b), (d), (f), and (h) were exposed for 3 days.*

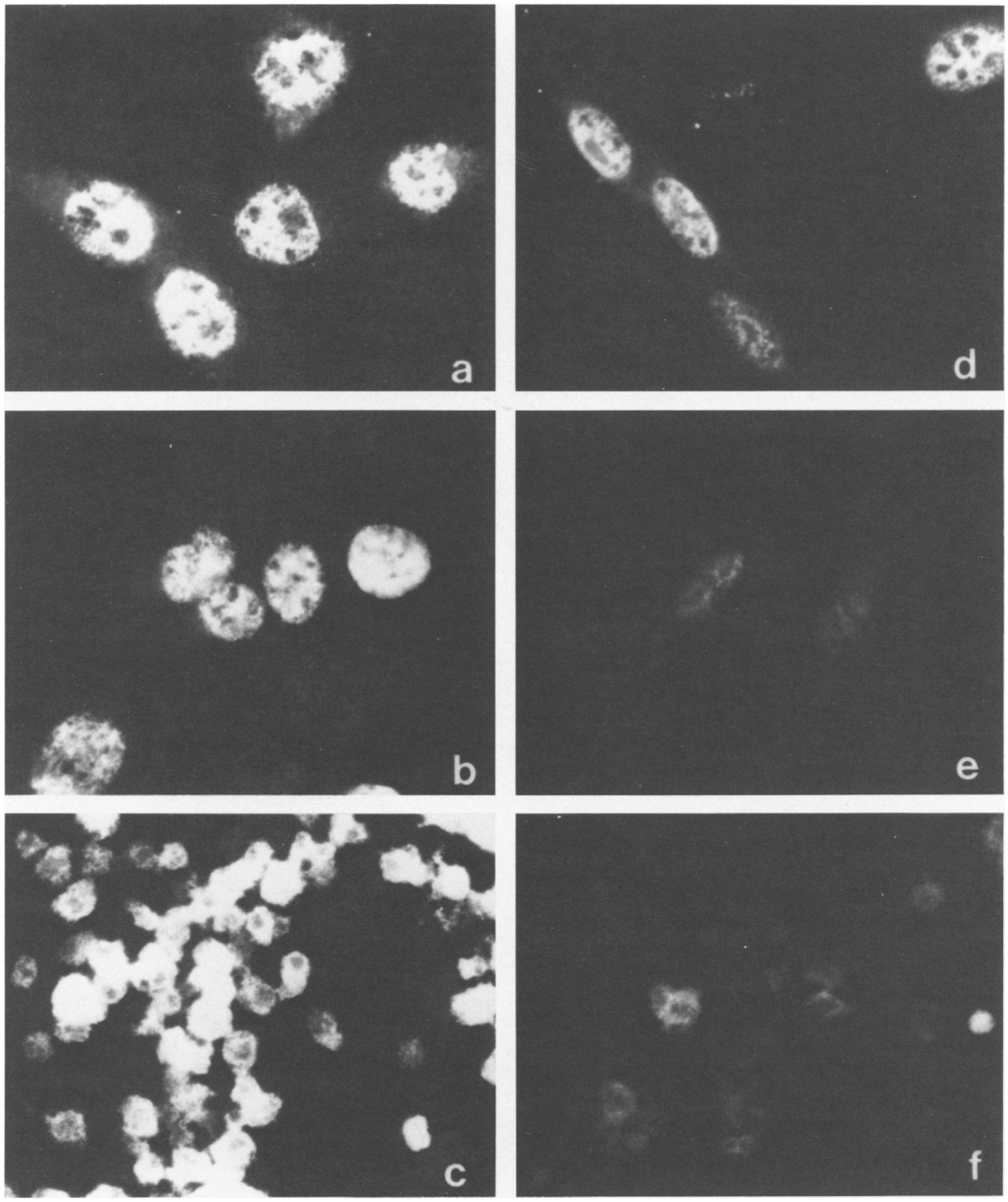

FIG. 5. *Immunofluorescence staining of SV101 cells, heat-treated SV101 cells, and SV101 nuclear matrices with T^+U^+ and T^+U^- hamster SV40 tumor serum and fluorescein-labeled anti-hamster gamma globulin. SV101 cells, heat-treated SV101 cells, and SV101 nuclear matrices were prepared and processed for immunofluorescence microscopy as described in the text. T^+U^+ serum was serum H10 absorbed on 3T3 cells; T^+U^- serum was serum H10 absorbed on heat-treated SV101 cells (see Table 2). (a) SV101 cells, treated with T^+U^+ serum at a serum dilution of 1:250; (d) SV101 cells, treated with T^+U^- serum at a serum dilution of 1:250; (b) heated SV101 cells, treated with T^+U^+ serum at a serum dilution of 1:200; (e) heated SV101 cells, treated with T^+U^- serum at a serum dilution of 1:20; (c) SV101 nuclear matrices, treated with T^+U^+ serum at a serum dilution of 1:20; and (f) SV101 nuclear matrices, treated with T^+U^- serum at a serum dilution of 1:10. ×68. Exposure times: (a to d) 30 s; (e and f) 2.5 min.*

matrices with T^+U^+ serum at a dilution of 1:20 resulted in a bright immunofluorescence staining (Fig. 5c), whereas at a 1:10 dilution of the T^+U^- serum no fluorescence staining of the nuclear matrices could be obtained. The immunofluorescence staining of nuclear matrices with T^+U^+ serum, therefore, most probably is not due to T-antigen molecules which were not extracted by the nuclear matrix isolation, but rather to molecules exposing U-antigen determinants. This interpretation was further substantiated by reconstitution experiments, in which nuclear matrices isolated from 3T3 cells were incubated for 30 min with the NP40 cytoplasmic extract or the high-salt nuclear extract of SV101 cells and then repurified. Analysis of these nuclear matrices with either T^+U^+ or T^+U^- serum did not result in any immunofluorescence staining (data not shown).

On isolated SV101 nuclear matrices the U-antigen titer as determined by serum dilution was lower than on heat-treated SV101 cells (ca. 1:50 on nuclear matrices as compared to approximately 1:200 on heat-treated SV101 cells, using T^+U^+ serum H10; Table 2). This may reflect that either SV40-specific proteins carrying U-antigenic determinants were partially extracted or, alternatively, that the isolation procedure decreased the antigenicity of U-antigen-expressing molecules.

DISCUSSION

SV40 U- and T-antigens are both nuclear antigens in SV40-infected and -transformed cells. Serum from SV40 tumor-bearing hamsters may contain antibodies against both antigens (T^+U^+ sera; 21). This paper shows that antibodies against U-antigen can be absorbed from these sera without affecting their titer against T-antigen (T^+U^- sera). SV40 T- and U-antigens in SV40-infected and -transformed cells, therefore, can be defined by separate classes of antigenic determinants. The T^+U^- serum obtained by absorption on heat-treated, i.e., only U-antigen-exposing, SV101 cells failed to react with cells infected by the nondefective $Ad2^+$-SV40 hybrid virus $Ad2^+ND1$, e.g., a hybrid virus inducing only U-antigen (19). Therefore, one can conclude that the antigenic determinants for U-antigen in hybrid virus-infected cells and SV40-transformed cells are very similar.

However, staining for U-antigen of hybrid virus-infected cells and SV40-infected or -transformed cells after methanol/acetone fixation leads to an apparently different subcellular location of SV40 U-antigen in these cells; in hybrid virus-infected cells U-antigen appears to be located only in the perinuclear region of the cells, whereas in SV40-infected or -transformed cells SV40 U-antigen is strictly nuclear. The experiments shown here demonstrate that using a different fixation procedure which allows the permeation of antibody into the nuclei of Formalin-fixed or methanol/acetone-fixed cells results in a nuclear staining for SV40 U-antigen in hybrid virus-infected cells also. As in SV40-infected or -transformed cells, SV40 specific proteins carrying U-antigenic determinants are also associated with the nuclei of hybrid virus-infected cells. These results are further confirmation for our earlier reports providing biochemical and immunological evidence for the association of SV40-specific proteins carrying U-antigenic determinants with the nuclei of hybrid virus-infected cells (7–10). These data, however, are in variance to reports by Jay et al. (15, 16), who do not find any association of SV40-specific proteins with the nuclei of $Ad2^+ND2$-infected cells. For $Ad2^+ND1$-infected cells, these authors report an association of the SV40-specific 28K protein with the outer nuclear membrane, but not with the nucleus itself. The reason for these discrepancies is not yet understood (see reference 7 for further discussion).

In addition to the nuclear location of SV40 U-antigen in hybrid virus-infected cells, a perinuclear U-antigen staining can be observed in these cells which cannot be found in SV40-infected and -transformed cells. This perinuclear U-antigen fluorescence seems to be due to a particular property of the SV40-specific hybrid virus proteins themselves and is not due to an adenovirus-specific function, because superinfection of SV40-transformed monkey cells with adenovirus does not change the morphology of the SV40-specific T- and U-antigen staining (Deppert, unpublished data). The reason for the different properties of the SV40-specific hybrid virus proteins during fixation and staining of hybrid virus-infected cells is not yet known. A possible explanation is that these proteins differ in some properties from the SV40 gene *A* product because they constitute only fragments of the SV40 gene *A* product (25) and, therefore, may exhibit characteristics of only certain protein domains of the full-length A protein.

The presence of two different nuclear antigens in SV40-infected and -transformed cells raised the question of whether these antigens may be associated with different nuclear substructures. Experiments with $Ad2^+$-SV40 hybrid virus-infected cells had shown that SV40-specific proteins carrying U-antigenic determinants were associated with the nuclear matrices of hybrid virus-infected cells, whereas the SV40-specific 95K protein of $Ad2^+ND4$, the SV40-specific hy-

brid virus protein most closely resembling SV40 T-antigen (25), was partially extracted from the nuclei during nuclear matrix isolation (7, 9).

In contrast to the hybrid virus system, where identification of SV40-specific proteins is greatly facilitated by the fact that these proteins are expressed at late times of infection when host protein synthesis is shut off, SV40-specific proteins in SV40-transformed cells are synthesized only in very small quantities and are not easily detected against the background of host-synthesized proteins. SV40-specific proteins in SV40-transformed cells, therefore, can only be identified by indirect methods, e.g., immunoprecipitation or immunofluorescence microscopy using sera from SV40 tumor-bearing animals. When isolated nuclear matrices of SV101 cells were analyzed by immunofluorescence microscopy with either T^+U^+ or T^+U^- serum, it was found that SV40-specific proteins exposing T-antigenic determinants had been completely extracted during the isolation procedure. SV40-specific proteins exposing U-antigenic determinants, on the other hand, could be visualized by immunofluorescence staining with the T^+U^+ serum, indicating a specific association of SV40 U-antigen with the nuclear matrix in SV40-transformed cells also. Indirect immunoprecipitation of the NP40 cytoplasmic extract and the high-salt nuclear extract of SV101 cells revealed the presence of three SV40-specific proteins with apparent molecular weights of 94K, 90K, and 84K in these subcellular fractions. These proteins, by definition, represent SV40 T-antigen (14, 33).

Robb (31) and Prives and Beck (27) have provided evidence that these proteins are quantitatively immunoprecipitable with either T^+U^- or T^-U^+ serum, suggesting that extracted T-antigen exposes both U- and T-antigenic determinants. Together with recent data showing that extracted T-antigen also has TSTA activity (1, 6, 32), it can be assumed that the SV40 antigenic moieties U, TSTA, and T are all contained within the same polypeptide chain. These data also imply that on the level of the extracted SV40 A protein a molecular differentiation between U, TSTA, and T is no longer possible. Immunofluorescence analysis of SV101 cells, heat-treated SV101 cells, and isolated SV101 nuclear matrices with T^+U^+ and T^+U^- sera, however, has demonstrated that a clear differentiation can be made between molecules exposing T-antigenic sites and those exposing U-antigenic sites. In addition, it was found that T- and U-antigens are associated with different nuclear subfractions.

Assuming that the U- and T-antigenic sites are located within the same molecules, the interesting question of how this compartmentalization of the SV40 A protein is achieved arises. Preliminary evidence indicates that in hybrid virus-infected cells SV40-specific proteins carrying SV40 U-antigenic determinants are not phosphorylated (W. Deppert and C. Gambke, unpublished data), whereas SV40 T-antigen is a phosphoprotein (36). A tempting hypothesis is, therefore, that the differentiation of the SV40 A protein into U- and T-antigens and its specific association with different nuclear subfractions could be achieved by its phosphorylation state. Alternatively, U-antigen in association with the nuclear matrix of SV40-transformed cells could be a fragment of the A protein derived from it by proteolytic cleavage. Clearly, these questions can only be answered by identification of the molecule(s) reacting with the T^+U^+ serum in isolated nuclear matrices.

A specific association of U-antigen exposing SV40 A protein with the nuclear matrix of SV40-transformed cells may be of functional importance, since the nuclear matrix seems to play a fundamental role in the initiation and replication of DNA and in the synthesis, processing, and transport of RNA (for a review see reference 2). Further experiments, however, are necessary to elucidate in which of these functions the SV40 A protein cooperates with the nuclear matrix.

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