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Simian Virus 40 (SV40)-Specific Proteins Associated with the Nuclear Matrix Isolated from Adenovirus Type 2-SV40 Hybrid Virus-Infected HeLa Cells Carry SV40 U-Antigen Determinants

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The distribution of simian virus 40 (SV40)-specific proteins in nuclear subfractions of pulse-chase-labeled HeLa cells infected with nondefective adenovirus type 2 (Ad2)-SV40 hybrid viruses was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The SV40-specific proteins of Ad2$^+$ND1, Ad2$^+$ND2, and Ad2$^+$ND5 specifically associate with the nuclear matrix and are virtually absent from the high-salt nuclear extract. In Ad2$^+$ND4-infected HeLa cells, the SV40-specific proteins with molecular weights of 64,000 (64K) and lower also specifically associate with the nuclear matrix. The SV40-specific 72K, 74K, and 95K proteins were found both in the nuclear matrix and in the high-salt nuclear extract. Analyses of the nuclear matrices isolated from hybrid virus-infected cells by immunofluorescence microscopy showed that SV40 U-antigen-positive sera from SV40 tumor-bearing hamsters react with SV40-specific proteins integrated into nuclear matrices of HeLa cells infected by Ad2$^+$ND1, Ad2$^+$ND2, and Ad2$^+$ND4, but not with nuclear matrices of HeLa cells infected by Ad2$^+$ND5. This suggests that SV40-specific proteins of Ad2$^+$ND1, Ad2$^+$ND2, and Ad2$^+$ND4 integrated into the nuclear matrix carry SV40 U-antigen determinants. The apparent discrepancy in the subcellular localization of SV40-specific proteins in hybrid virus-infected cells when analyzed by biochemical cell fractionation procedures and when analyzed by immunofluorescence staining is discussed.

Expression of the early part of the simian virus 40 (SV40) genome in SV40-infected and SV40-transformed cells leads to the appearance of three SV40-specific antigens: SV40 tumor antigen (T), SV40 tumor-specific transplantation antigen (TSTA), and SV40 U-antigen (6, 13, 19, 25, 34). T- and U-antigens can be detected in the nucleus (25, 31), whereas TSTA appears to be located in the plasma membranes (39). Recent data support the assumption that gene products of the early region of the SV40 genome, i.e., the early SV40 proteins, may themselves carry these antigenic determinants (3, 11, 12, 14, 32, 35, 36). The study of SV40 early proteins and their relation to SV40 early antigens in SV40-infected or SV40-transformed cells is hampered by the fact that these proteins are synthesized only in very small quantities and are not easily detected against the background of host-synthesized proteins.

The nondefective adenovirus type 2 (Ad2)-SV40 hybrid viruses isolated by Lewis and coworkers (23, 24) have proved to be useful for studying early SV40 proteins (2, 11, 12, 15, 16, 26, 40). The different hybrid viruses contain overlapping insertions of the SV40 genome recombined into the adenovirus DNA that encompass increasing portions of the early region of the SV40 genome (0.11 to 0.28 map unit for Ad2$^+$ND1, 0.11 to 0.39 map unit for Ad2$^+$ND5, 0.11 to 0.44 map unit for Ad2$^+$ND2, and 0.11 to 0.59 map unit for Ad2$^+$ND4 [22]). The expression of the SV40 information in the Ad2-SV40 hybrid viruses is controlled by the Ad2 genome and takes place at late times of infection. Since late in infection host protein synthesis is shut off by the adenovirus genome, the identification and characterization of SV40 early proteins encoded by the Ad2-SV40 hybrid viruses is greatly facilitated. The following SV40-specific proteins have been identified in HeLa cells infected with hybrid viruses: 28,000 molecular weight (28K) (Ad2$^+$ND1 [2, 12, 15, 16, 26, 40]); 42K and 56K (Ad2$^+$ND2 [11, 12, 40]); 28K, 42K, 56K, 60K, 64K, 72K, 74K, and 95K (Ad2$^+$ND2 [12]); and 42K (Ad2$^+$ND5 [12, 40]). These proteins are structurally related to each other, as shown by tryptic peptide analysis (11, 29); they are encoded, at least in part, by the early region of the SV40 genome, as shown by in vitro synthesis in

a cell-free system, using SV40-specific mRNA isolated from hybrid virus-infected cells (29; T. Hunter, G. Walter, K. Mann, and Ch. Lawrence, *in* P. May, R. Monier, and R. Weil, ed., *INSERM Symposium: Early Proteins of Oncogenic DNA Viruses*, in press); and they are immunologically related to SV40-specific proteins in SV40-induced tumors, as shown by immunoprecipitation with serum from hamsters bearing SV40 tumors (11, 12).

Upon infection, different hybrid viruses induce different SV40 early antigens: Ad2$^+$ND4 induces all three SV40 antigenic moieties (U-antigen, TSTA, and T-antigen); Ad2$^+$ND2 induces U-antigen and TSTA; and in Ad2$^+$ND1-infected cells only U-antigen can be detected (23, 24). This has permitted a mapping correlation to be established between the antigenic determinants of T, U, and TSTA and the region of the SV40 genome present in the different Ad2-SV40 hybrid viruses (22). The study of the relationship between the SV40-specific proteins encoded by the hybrid viruses and the SV40 antigens U, TSTA, and T induced in hybrid virus-infected cells may lead to an assignment of the different antigenic moieties to different protein domains of the early SV40 gene product(s).

In a first approach for studying this relationship, the intracellular localization of the SV40-specific proteins in HeLa cells infected by hybrid viruses has been determined by cell fractionation and pulse-chase experiments (11, 12). From these experiments the following conclusions were drawn. (i) The SV40-specific 95K protein in Ad2$^+$ND4-infected cells is a good candidate for an SV40-specific protein with T-antigen properties. Its molecular weight agrees reasonably with molecular weights described for T-antigen in SV40-transformed and SV40-infected cells (1, 9, 32, 38), and it is predominantly located in the nuclei of Ad2$^+$ND4-infected cells. (ii) The lower-molecular-weight SV40-specific proteins in Ad2$^+$ND4-, Ad2$^+$ND2-, and Ad2$^+$ND1-infected cells, on the other hand, may carry TSTA and U-antigen determinants. Such a structural relationship seems especially likely for the 56K and 42K proteins in Ad2$^+$ND2-infected cells with their predominant location in nuclei and plasma membranes (11, 12).

The objective of the current study was to determine the distribution of the hybrid virus SV40-specific proteins in nuclear subfractions and to investigate the possible relationship between these proteins and SV40 U-antigen. The accumulation of SV40-specific proteins in plasma membranes as well as in nuclei of hybrid virus-infected cells led to the assumption that the association of these proteins with the nuclei may be via membranous or supporting structures. Recent morphological and biochemical evidence has demonstrated that the framework of the nucleus is a proteinaceous nuclear matrix that spans the entire volume of the nucleus. Experimental evidence strongly suggests that this structure plays a fundamental role in the initiation and replication of DNA and in the synthesis, processing, and transport of RNA (for a review, see reference 5). A specific association of SV40 proteins to this structure, therefore, could be of functional importance.

MATERIALS AND METHODS

Viruses and cells. Seed stocks of the nondefective Ad2$^+$-SV40 hybrid viruses Ad2$^+$ND1, Ad2$^+$ND2, Ad2$^+$ND4, and Ad2$^+$ND5 were obtained from A. M. Lewis, Jr. Stocks of Ad2$^+$ND1, Ad2$^+$ND2, and Ad2$^+$ND5 were prepared in HeLa S_3 cells grown in minimal essential medium for suspension culture (Grand Island Biological Co., Grand Island, N.Y.; F-13) supplemented with 5% calf serum (growth medium). Stocks of Ad2$^+$ND4 were prepared in CV1 cells grown in Dulbecco-modified Eagle medium with 10% fetal bovine serum. The titer of the virus stocks used in these experiments was 10^9 PFU/ml for all stocks except Ad2$^+$ND4, which titrated 10^7 PFU/ml. Stocks were assayed on HeLa monolayers as described by Williams (42).

Infection and labeling of cells. HeLa S_3 cells grown in suspension (5×10^7 to 1×10^8 total) were infected with 1 ml of undiluted virus stocks per 10^7 cells. After an adsorption period of 20 min, the cells were diluted with growth medium (final concentration, 2×10^5 cells per ml). At the times indicated in the figure legends, the cells were washed twice with 40 ml of Earle salt solution, resuspended at 5×10^6 cells per ml, and labeled for 0.5 to 1 h in Eagle medium minus methionine containing 20 μCi of [^{35}S]methionine (Amersham/Searle, Arlington Heights, Ill.; specific activity, 500 to 600 Ci/mmol) per ml and 5% calf serum. After the labeling period, the labeling medium was removed, and the cells were split into two parts and washed once with 40 ml of Eagle minimal essential medium. One part (pulse) was immediately subjected to cell fractionation (see below). The remaining cells were diluted with growth medium (final concentration, 2×10^5 cells per ml) and kept in this medium for the time indicated in the figure legends. These cells (chase) were then washed once with 20 ml of Eagle minimal essential medium and subjected to cell fractionation (see below).

Cell fractionation, subfractionation of nuclei, and isolation of nuclear matrices. For cell fractionation, subfractionation of nuclei, and isolation of nuclear matrices (Hodge et al. [17], modified), 5×10^7 to 1×10^8 cells were washed once in Earle salt solution and 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 a 5-min incubation period on ice, Nonidet P-40 (NP-40; 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 × *g* for 5 min and washed several times with RSB. These nuclei were termed NP-40 nuclei. The combined supernatant fluids were kept as NP-40 cytoplasm. NP-40 nuclei were resuspended in the cold with RSB containing 1 mg of DNase I (Worthington Biochemicals Corp., Freehold, N.J.) per ml at a concentration of 5×10^8 nuclei per ml. A 2-ml amount 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 × *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 dithiothreitol–0.01 M NaCl–0.01 M EDTA) per 5×10^7 nuclei. After centrifugation at 500 × *g* for 10 min, nuclear matrices were washed once with 2 ml of TE (0.01 M Tris-hydrochloride [pH 7.4]–0.001 M EDTA) per 5×10^7 nuclei and resuspended in TE. Nuclear matrices were then processed either for gel electrophoresis or for 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 (Fig. 1a). A small percentage of the nuclear matrix fraction consisted of broken nuclear substructures. Figure 1b shows that the nuclear matrices isolated by this procedure resembled in shape intact nuclei.

Preparation of samples for gel electrophoresis. NP-40 nuclei and nuclear matrices were pelleted and then dissolved in electrophoresis sample buffer (0.0625 M Tris [pH 6.8]–3% sodium dodecyl sulfate [SDS]–5% 2-mercaptoethanol). Portions of the NP-40 cytoplasm 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 treated with a Branson Sonifier equipped with a microtip (position 4, three times for 10 s) 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. (27) and adjusted to approximately 10 to 15 μg/10 μl.

Polyacrylamide gel electrophoresis and fluorography. The polyacrylamide gel system of Laemmli (21) and Maizel (28) was employed. A total of 15 μg 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 (7).

Immunofluorescence staining. Ad2$^+$ND1- and

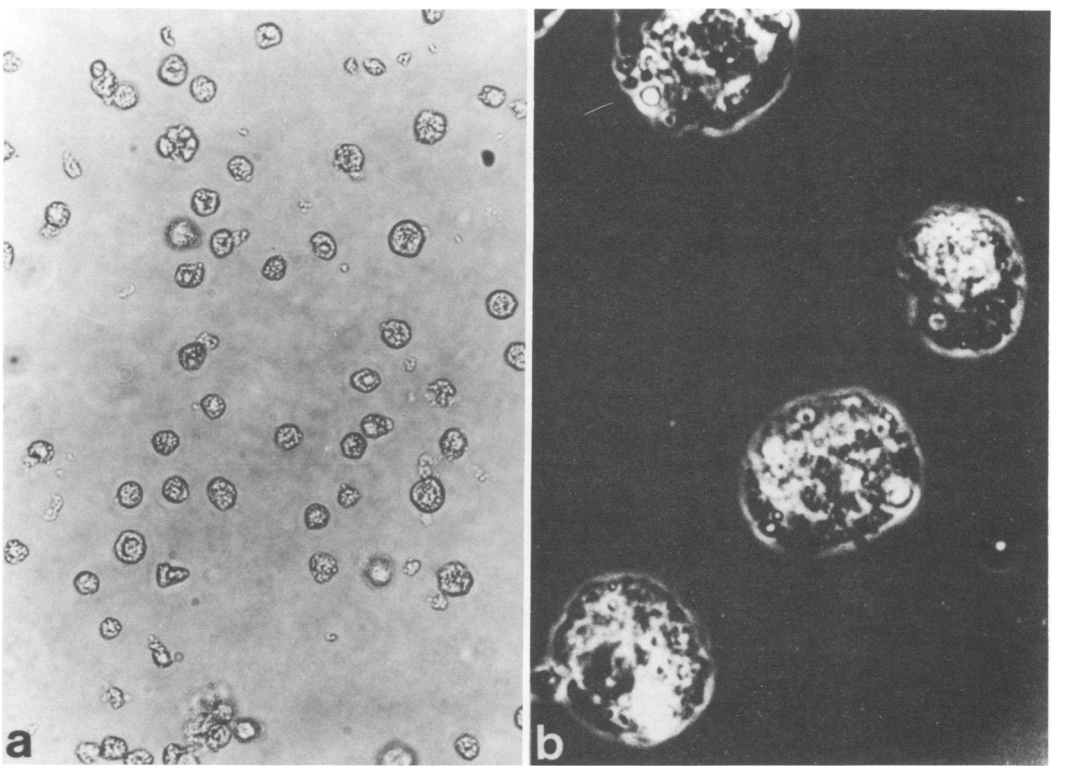

FIG. 1. *Nuclear matrix preparation from Ad2-infected HeLa cells. Nuclear matrices were isolated at 36 h postinfection as described in the text and suspended in TE buffer. Phase-contrast; magnifications are 250-fold (a) and 1,250-fold (b).*

Ad2+ND2-infected HeLa cells grown on glass cover slips (12-mm diameter) in Dulbecco-modified Eagle medium with 10% calf serum were washed once with phosphate-buffered saline, pH 7.4 (PBS [41]), 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. The first antibody (hamster SV40 tumor serum, diluted 1:10 with PBS [see below]) was then added, and the cover slips were incubated for 1 h at 37°C. After they were washed with PBS, fluorescein-labeled goat immunoglobulin prepared against hamster immunoglobulin (Cappel Laboratories, Inc., Cochranville, Pa.; 1:20 dilution with PBS of reconstituted material) was added, and the cover slips were held for another 1 h 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, Federal Republic of Germany) equipped with epifluorescent illumination. Pictures were taken with Planopo 40×, 63×, and 100× oil immersion objectives. Nuclear matrices suspended in TE were allowed to settle for 10 min in the cold onto glass cover slips (12-mm diameter). The supernatant fluid was removed carefully with a Pasteur pipette, and the cover slips were incubated either directly with antibody as described above or after fixation with 3.7% formaldehyde in PBS in the cold. The cover slips were then processed for immunofluorescence microscopy as described above. Before use, all sera were absorbed with Ad2+-infected HeLa cells.

SV40 tumor sera. SV40 tumor sera were produced in 4- to 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, was kindly provided by V. Defendi). Sera were collected at a tumor size of approximately 3 to 4 cm at 3 to 4 weeks after inoculation. Sera were tested by immunofluorescence microscopy for T-antibody titers on SV40-transformed 3T3 cells as described previously (31) and for U-antibody titers on HeLa monolayer cells infected by Ad2+ND1 as described previously (25). Sera having a titer of ≥1:40 (by immunofluorescence) on Ad2+ND1-infected HeLa cells were termed U-antigen positive and were used in these experiments.

RESULTS

Localization of SV40-specific proteins in nuclear subfractions of hybrid virus-infected HeLa cells. SV40 T-antigen has so far been detected only in the nuclei of SV40-infected and SV40-transformed cells. The lower-molecular-weight SV40-specific hybrid virus proteins, on the other hand, specifically accumulate both in nuclei and in plasma membranes. This indicated that these proteins may be associated differently with the nuclei of hybrid virus-infected cells than is SV40 T-antigen with the nuclei of SV40-infected and SV40-transformed cells.

SV40 T-antigen can be solubilized by high-salt treatment of nuclei from SV40-infected or SV40-transformed cells (1, 10). When nuclei of Ad2+ND2-infected HeLa cells were extracted with high-salt buffers, these procedures failed to solubilize the SV40-specific 56K and 42K proteins. This was demonstrated by the following experiment. NP-40 nuclei (see above) of Ad2+ND2-infected and [^{35}S]methionine-labeled HeLa cells were extracted by the procedure of Ahmad-Zadeh et al. (1) with 0.5 M LiCl buffered at pH 7.4 with 2.5 mM Tris-hydrochloride–1 mM EDTA, and the nuclear lysate was spun at 30,000 × *g* for 30 min. Figure 2 shows that the soluble nuclear extract is devoid of SV40-specific 56K and 42K proteins and that these proteins are quantitatively recovered in the pelletable nuclear material. Similar results were obtained when the conditions for extracting T-antigen used by Tegtmeyer et al. (37) were applied (data not shown). The tight association of the SV40-

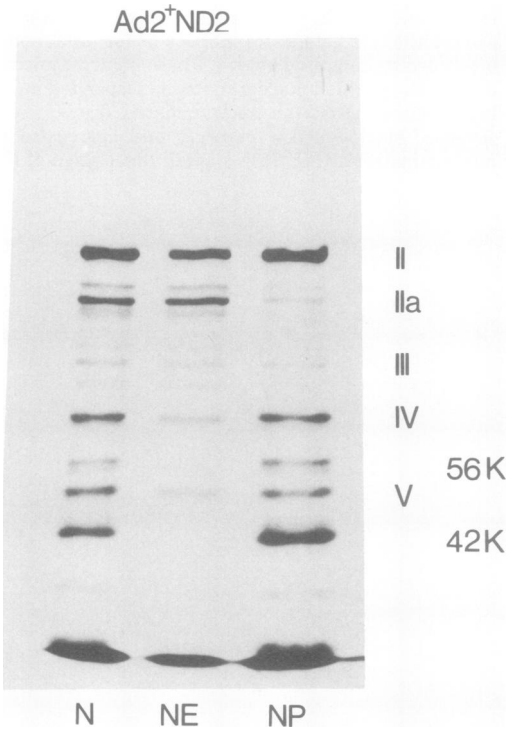

FIG. 2. *Distribution of SV40-specific 56K and 42K proteins in nuclei and nuclear subfractions of HeLa cells infected by Ad2+ND2; SDS-polyacrylamide gel fluorogram of ^{35}S-labeled polypeptides. HeLa S_3 cells were infected with Ad2+ND2 and labeled for 1 h (38 to 39 h after infection) with [^{35}S]methionine as described in the text. Extraction of the nuclei was by the method of Ahmad-Zadeh et al. (1) as described in the text. Portions of each fraction containing 15 μg of protein were applied to a 7.5% acrylamide gel and run for 4.5 h at 12 mA. N, NP-40 nuclei; NE, soluble nuclear extract; NP, pelletable nuclear material.*

specific 56K and 42K proteins with the nuclear particulate material in nuclei of Ad2$^+$ND2-infected HeLa cells further suggested that these proteins may be integrated into a structural component of the nucleus.

The distribution and the metabolic stability of the SV40-specific proteins in nuclear subfractions of HeLa cells infected by Ad2$^+$ND1, Ad2$^+$ND2, Ad2$^+$ND4, and Ad2$^+$ND5 were examined. HeLa cells in suspension infected with the different hybrid viruses were labeled with [^{35}S]methionine at late times of infection (pulse) and chased for several hours with excess unlabeled methionine. Both pulse-labeled and -chased cells were fractionated into cytoplasm, nuclei, high-salt nuclear extracts, and nuclear matrices as described above. The polypeptides in each fraction were analyzed by SDS-polyacrylamide gel electrophoresis, and the radioactive polypeptides were visualized by fluorography. Approximately equal amounts of protein from each sample were applied to the gel. Therefore, the intensity of an individual band in the fluorogram is a measure of the specific activity and does not reflect the absolute amount of radioactive protein in a particular subcellular fraction.

Ad2$^+$ND1-infected cells. In previous studies the SV40-specific 28K protein encoded by Ad2$^+$ND1 could only be detected in small quantities in Ad2$^+$ND1-infected HeLa cells (12, 40). Therefore, the subcellular localization of this protein and its metabolic stability in different subcellular fractions have not been analyzed in detail. A time course of the appearance of the SV40-specific 28K protein in Ad2$^+$ND1-infected HeLa cells, however, revealed that this protein is synthesized in appreciable quantities during the course of hybrid virus replication (Fig. 3). Analysis of the radioactive polypeptides of HeLa

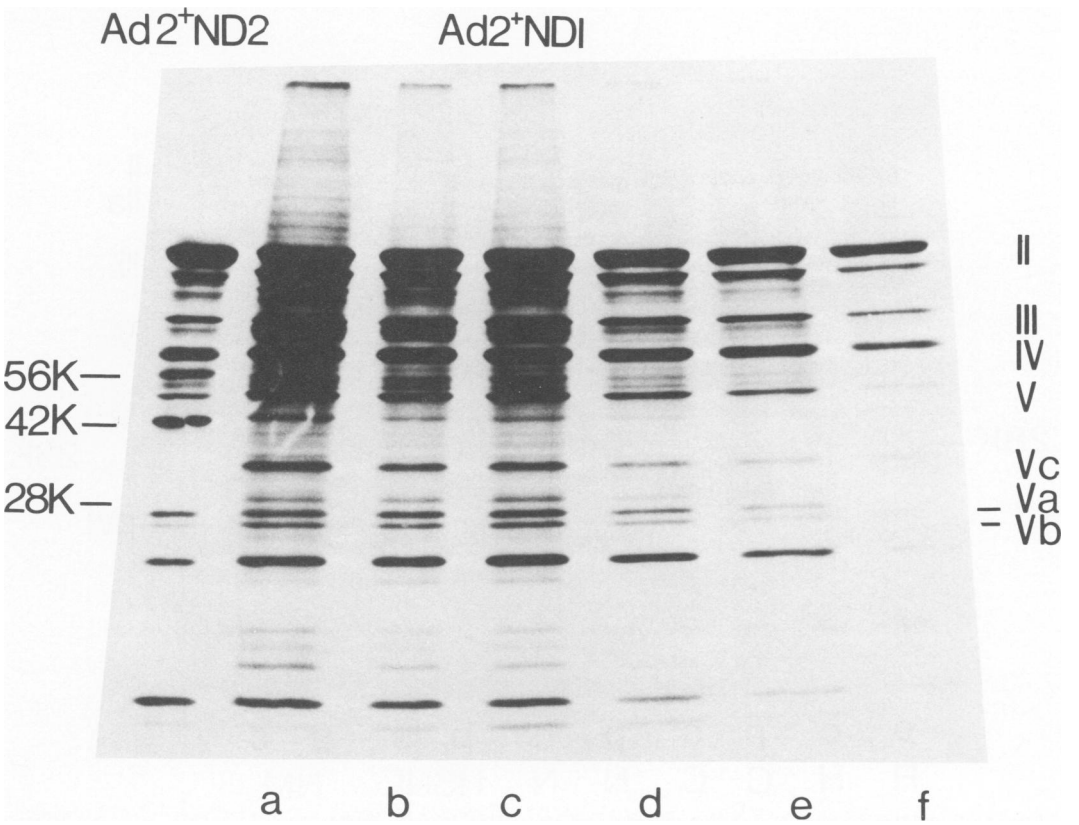

FIG. 3. *Time course of the synthesis of the SV40-specific 28K protein in HeLa cells infected by Ad2$^+$ND1; SDS-polyacrylamide gel fluorogram of ^{35}S-labeled polypeptides. Parallel cultures of HeLa cells in monolayers were infected with Ad2$^+$ND1 and labeled for 3 h with [^{35}S]methionine at different times of infection. The multiplicity of infection was approximately 100 PFU/cell. An 8.5 to 20% hyperbolic gradient gel was run for 5 h at a constant current of 12 mA. The sample order is: Ad2$^+$ND2-infected cells (marker), (a) Ad2$^+$ND1-infected cells labeled at 24 to 27 h, (b) 27 to 30 h, (c) 30 to 33 h, (d) 33 to 36 h, (e) 36 to 39 h, and (f) 39 to 42 h after infection.*

cells in monolayers infected by Ad2+ND1 showed that synthesis of the SV40-specific 28K protein could be detected at a time when host protein synthesis shutoff was not yet completed (24 h postinfection [Fig. 3a]). The rate of synthesis of this protein appeared to be constant during the next 9 h (time points a to c, Fig. 3) and then declined. Synthesis of the 28K protein in Ad2+ND1-infected HeLa cells was no longer detectable at about 39 h postinfection. For this experiment HeLa cells in monolayers with a flat morphology (obtained from the Cold Spring Harbor Laboratory) were used. In these HeLa cells, adenovirus replication is considerably slowed down as compared with adenovirus replication in HeLa S_3 cells, although a similar yield of adenovirus progeny is obtained with both cell types (W. Deppert, unpublished data). Figure 4 shows the distribution of the SV40-specific 28K protein in different subcellular fractions of Ad2+ND1-infected HeLa S_3 cells and its molecular stability in these fractions after pulse-chase labeling. After a 1-h pulse, the 28K protein could be detected in the cytoplasm as well as in the nuclei. Subfractionation of the nuclei into a high-salt nuclear extract and a nuclear matrix fraction showed that this protein is predominantly located in the nuclear matrix. After a 2-h chase the amount of radioactive 28K protein was markedly decreased in the homogenate and in the cytoplasm. In nuclei, on the other hand, the amount of radioactive 28K protein stayed constant, with the nuclear matrix being the subnuclear structure to which this protein is stably associated. If Ad2+ND1-infected and pulse-chase-labeled HeLa cells were subfractionated

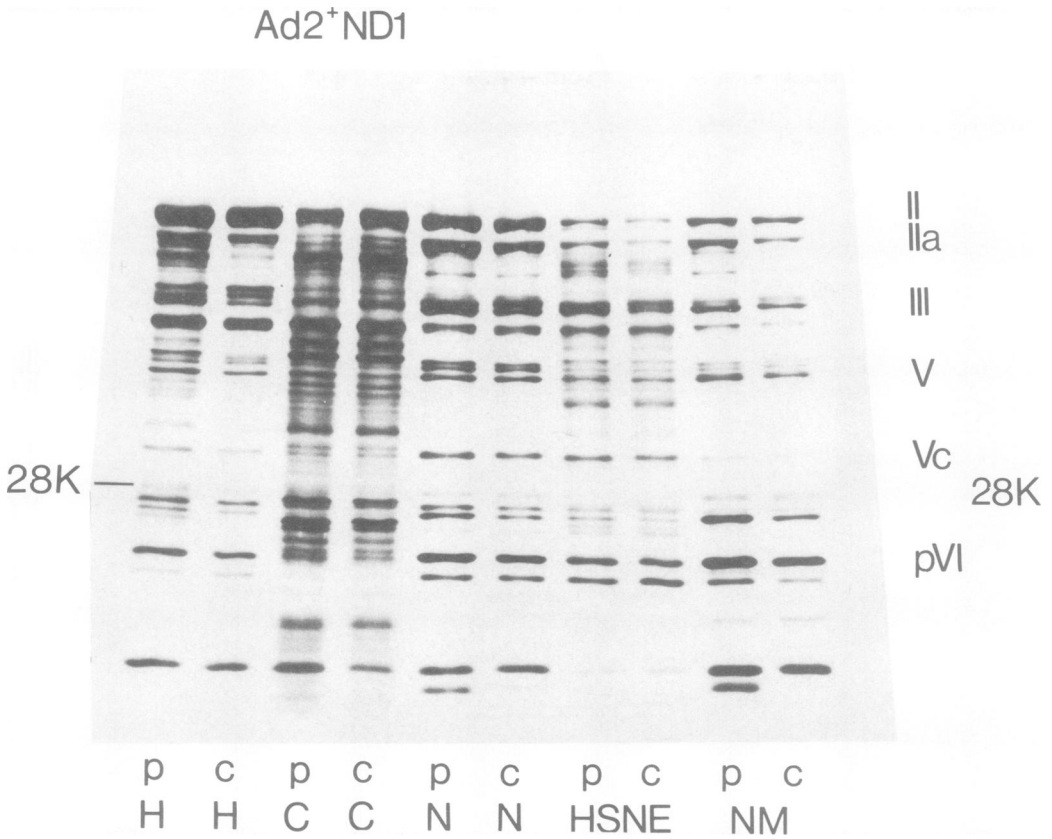

FIG. 4. *Distribution and metabolic stability of the SV40-specific 28K protein in different subcellular fractions of Ad2+ND1-infected HeLa S_3 cells; SDS-polyacrylamide gel fluorogram of ^{35}S pulse and pulse-chase-labeled polypeptides. HeLa S_3 cells were infected with Ad2+ND1 and pulse-labeled for 1 h with [^{35}S]-methionine at 24 h after infection as described in the text. Pulse-labeled cells (p) and cells chased for 2 h (c) were fractionated as described in the text. Samples of each fraction containing 15 μg of protein were applied to an 8.5 to 20% hyperbolic gradient acrylamide slab gel and run for 5 h at 12 mA. Subcellular fractions were designated as follows: H, homogenate; C, NP-40 cytoplasm; N, NP-40 nuclei; HSNE, high-salt nuclear extract; NM, nuclear matrices.*

to obtain plasma membranes (11, 12), it could be demonstrated that the SV40-specific 28K protein also stably associates with the plasma membranes (W. Deppert and Ch. Gambke, data not shown). In its metabolically stable association with nuclei and plasma membranes and its molecular instability in the cytoplasm, this protein, therefore, resembles the SV40-specific 42K and 56K proteins encoded by Ad2+ND2 and Ad2+ND4 (11, 12).

Ad2+ND2-infected cells. The stable association of the SV40-specific 42K and 56K proteins expressed in Ad2+ND2-infected HeLa cells with nuclei and plasma membranes has been described previously (11, 12). In a recent report, Jay et al. (18) have investigated the subcellular distribution of the SV40-specific proteins in KB cells infected by Ad2+ND2. They found that the SV40-specific 56K and 42K proteins were present in the cytoplasm and in the plasma membranes, but not in the nuclei. To ensure that the specific association of SV40-specific proteins with nuclei and plasma membranes of HeLa cells infected by Ad2+ND2 does not merely reflect a special characteristic of the cell line used in this and in previous studies, the subcellular distribution and metabolic stability of the SV40-specific 56K and 42K proteins were analyzed in CV1 cells infected by Ad2+ND2. Figure 5 shows that both the 56K and 42K proteins also associated with the nuclei of CV1 cells and that these proteins were metabolically more stable in the nuclear fraction than in the cytoplasmic fraction also in these cells. Figure 6 shows that in pulse-labeled and in pulse-chase-labeled HeLa cells the predominant location of these proteins within the nuclei is the nuclear matrix fraction. The small amount of radioactive 42K and 56K found in the high-salt nuclear extract after a 1-h pulse was not visible in the high-salt nuclear

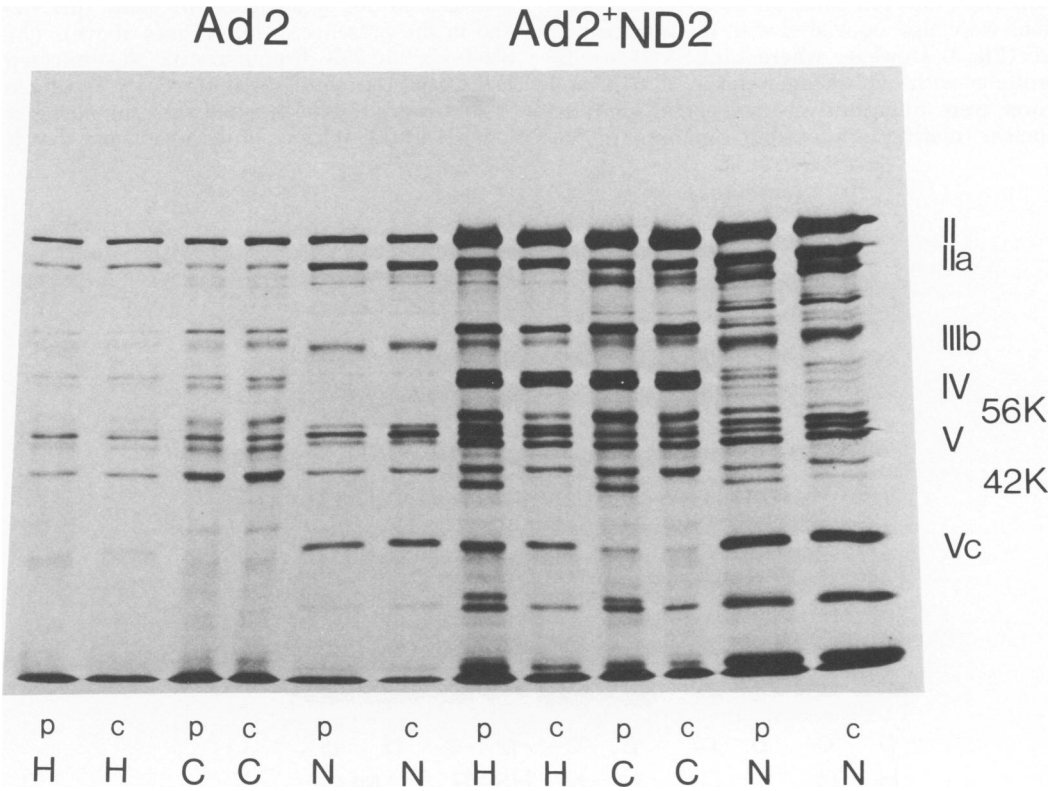

FIG. 5. *SDS-polyacrylamide gel fluorogram of ^{35}S pulse and pulse-chase-labeled polypeptides in different subcellular fractions of CV1 cells infected by Ad2+ND2. Parallel cultures of CV1 cells were infected at a multiplicity of 100 PFU/cell with Ad2 or Ad2+ND2 and pulse-labeled for 1 h with [^{35}S]methionine at 36 h after infection. Pulse-labeled cells (p) and cells chased for 3.5 h (c) were removed from plates with trypsin, washed once with normal growth medium, and then fractionated as described in the text to obtain detergent-cleaned nuclei (NP-40 nuclei). Samples of each fraction containing 15 μg of protein were applied to an 8.5% acrylamide gel and run for 5 h at 12 mA. The designations for subcellular fractions are as described in the legend to Fig. 4.*

extract after a 3-h chase, indicating a specific association of these proteins with the nuclear matrix.

Ad2$^+$ND5-infected cells. Analysis of the distribution of the SV40-specific 42K protein encoded by Ad2$^+$ND5 in nuclear subfractions of Ad2$^+$ND5-infected HeLa cells shows that this protein is also predominantly associated with the nuclear matrix fraction (Fig. 7). However, whereas both the SV40-specific 56K and 42K proteins of Ad2$^+$ND2 and the 28K protein of Ad2$^+$ND1 associated with the nuclear matrix in a metabolically stable fashion (Fig. 4, and 6), the 42K protein of Ad2$^+$ND5 was metabolically unstable in the nuclear matrix, and radioactive 42K protein could be detected in this fraction only in reduced quantities after a 2-h chase period (Fig. 7). This agrees with our previous finding that the 42K protein of Ad2$^+$ND5 is unable to stably associate with any subcellular fraction (12).

Ad2$^+$ND4-infected cells. In nuclei of Ad2$^+$ND4-infected cells, all SV40-specific proteins were also associated with the nuclear matrix (Fig. 8). However, whereas the SV40-specific proteins with molecular weights of 64K and lower were quantitatively associated with the nuclear matrix, appreciable amounts of the SV40-specific 72K and 74K proteins, and more pronounced, of the SV40-specific 95K protein, were solubilized by the high-salt extraction. These proteins, therefore, qualitatively behave like SV40 T-antigen in SV40-infected and SV40-transformed cells, which is also largely solubilized by high-salt treatment (1, 10). In the experiment shown in Fig. 8, the SV40-specific 95K protein could be detected in approximately equal amounts in the NP-40 cytoplasm of pulse-labeled and pulse-chase-labeled Ad2$^+$ND4-infected HeLa cells. Previous cell fractionation experiments with pulse-chase-labeled HeLa cells infected by Ad2$^+$ND4, on the other hand, have demonstrated that the SV40-specific 95K protein specifically accumulates in the nuclei and can no longer be detected in the cytoplasm after a 4-h chase (12). In these experiments, however, cell fractionation was performed at low pH (pH 6.5) in the absence of detergent, whereas cell fractionation in the experiment shown in Fig. 8 was carried out at a higher pH value (pH 7.2) and in the presence of NP-40 (see above). The SV40-specific 95K protein in Ad2$^+$ND4-infected HeLa cells, therefore, resembles SV40 T-antigen in that it is stably associated with the nuclei at low pH in the absence of detergent and that it

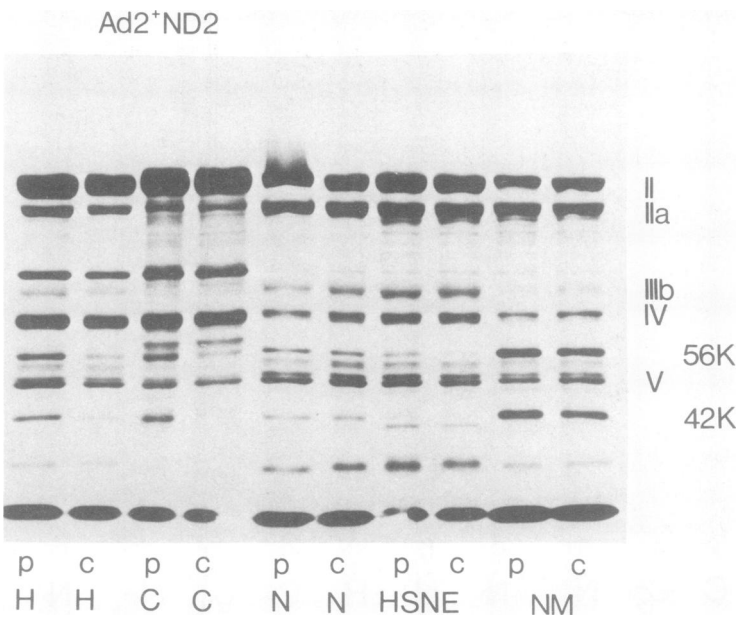

FIG. 6. *Distribution and metabolic stability of SV40-specific proteins in different subcellular fractions of HeLa S_3 cells infected by Ad2$^+$ND2; SDS-polyacrylamide gel fluorogram of ^{35}S pulse and pulse-chase-labeled polypeptides. HeLa S_3 cells were infected with Ad2$^+$ND2 and pulse-labeled for 1 h with [^{35}S]methionine at 35 h after infection as described in the text. Pulse-labeled cells (p) and cells chased for 3 h (c) were fractionated as described in the text. Samples of each fraction containing 15 μg of protein were applied to a 7.5% acrylamide gel and run for 4.5 h at 12 mA. The designations for subcellular fractions are as described in the legend to Fig. 4.*

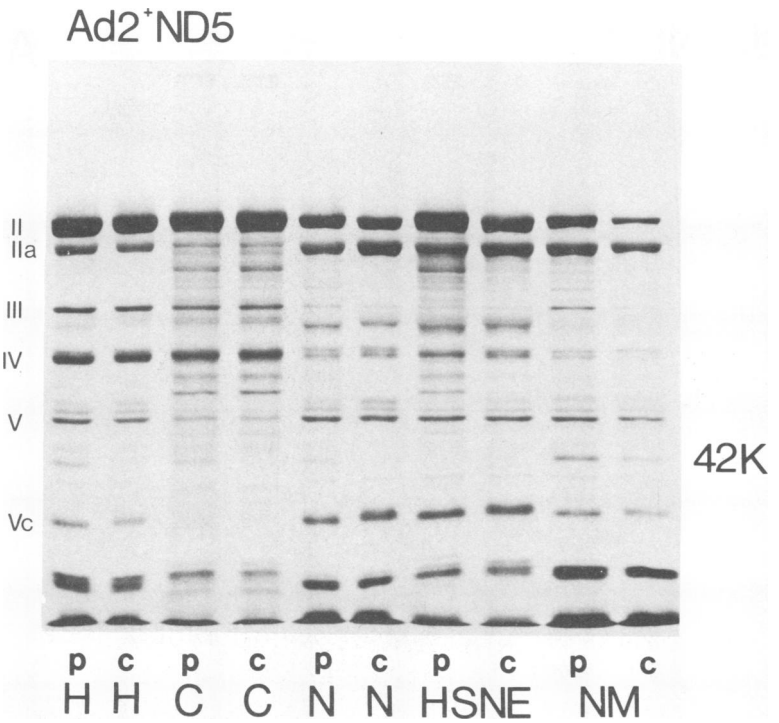

FIG. 7. *Distribution and metabolic stability of the SV40-specific 42K protein in different subcellular fractions of HeLa S_3 cells infected by Ad2+ND5; SDS-polyacrylamide gel fluorogram of ^{35}S pulse and pulse-chase-labeled polypeptides. HeLa S_3 cells were infected with Ad2+ND5 and pulse-labeled for 1 h with [^{35}S] methionine at 40 h after infection as described in the text. Pulse-labeled cells (p) and cells chased for 2 h (c) were fractionated as described in the text. Samples of each fraction containing 15 μg of protein were applied to an 8.5% acrylamide gel and run for 5 h at 12 mA. The designations for subcellular fractions are as described in the legend to Fig. 4.*

can be partially extracted at higher pH in the presence of detergent (38).

Analysis of nuclear matrices for SV40-specific proteins by immunofluorescence microscopy. In SV40-infected and SV40-transformed cells, SV40 U-antigen appears by immunofluorescence staining as a diffuse nuclear staining, which is indistinguishable from the morphology of SV40 T-antigen staining (23, 25). In these cells, SV40 U-antigen differs immunologically from SV40 T-antigen only in its stability to heating at 50°C for 30 min (25). SV40 U-antigen in cells infected by Ad2+ND1 and Ad2+ND2, on the other hand, can be detected by immunofluorescence microscopy only in the perinuclear region of the cell and seems to be absent from the nucleus (23, 25).

The perinuclear location of SV40 U-antigen in hybrid virus-infected cells as opposed to the nuclear location of SV40 U-antigen in SV40-infected and SV40-transformed cells was attributed to the absence of the "virus-mediated transport mechanism" (8), which transfers early SV40 antigens from the cytoplasm to the nucleus in hybrid virus-infected cells (25). Biochemical analyses, however, have demonstrated that SV40-specific proteins are transported into the nucleus of hybrid virus-infected cells (11, 12) and are stably associated with the nuclear matrix structure (this paper). To resolve the apparent discrepancy in the subcellular localization of SV40-specific proteins when analyzed by biochemical cell fractionation procedures and when analyzed by immunofluorescence staining, nuclear matrices isolated from hybrid virus-infected HeLa cells were analyzed by immunofluorescence staining with SV40 U-antigen-positive sera from SV40 tumor-bearing hamsters (see above). Immunofluorescence staining of Ad2+ND1 (Fig. 9a)- and Ad2+ND2 (Fig. 9b)-infected HeLa cells in monolayers with these sera resulted in a perinuclear staining characteristic for SV40 U-antigen in hybrid virus-infected cells (25). Immunofluorescence staining of SV40-transformed cells heated to 50°C for 30 min (U-antigen staining [25]), on the other hand, resulted in a staining pattern indistinguishable from SV40 T-antigen staining (data not shown).

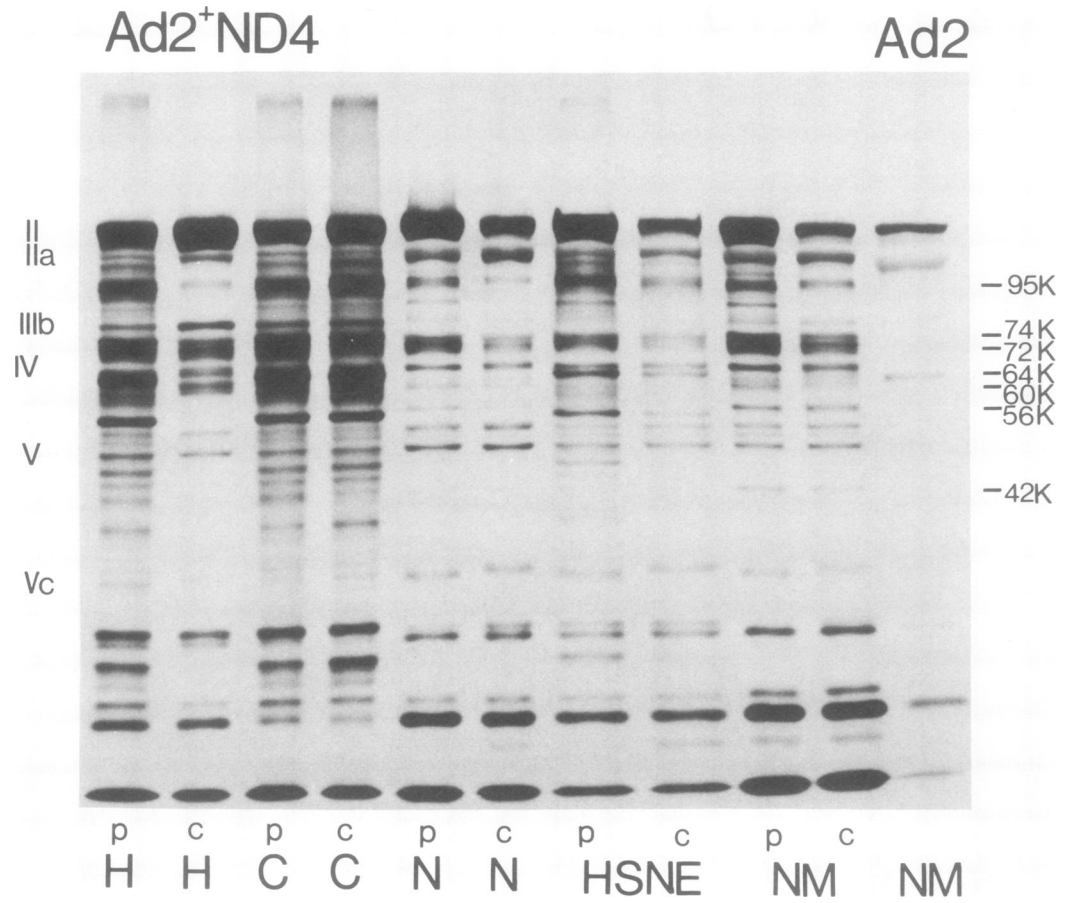

FIG. 8. *Distribution and metabolic stability of SV40-specific proteins in different subcellular fractions of HeLa cells infected by Ad2+ND4; SDS-polyacrylamide gel fluorogram of ^{35}S pulse and pulse-chase-labeled polypeptides. HeLa S_3 cells were infected with Ad2+ND4 and pulse-labeled for 0.5 h with [^{35}S]methionine at 36 h after infection as described in the text. Pulse-labeled cells (p) and cells chased for 4 h (c) were fractionated as described in the text. Samples of each fraction containing 15 μg of protein were applied to an 8.5% acrylamide gel and run for 5 h at 12 mA. The designations for subcellular fractions are as described in the legend to Fig. 4.*

Figure 10 shows that nuclear matrices isolated from Ad2+ND1 (Fig. 10a), Ad2+ND2 (Fig. 10b), and Ad2+ND4-infected cells (Fig. 10c) could be stained with these sera when unfixed or Formalin-fixed preparations were analyzed. Methanol-acetone-fixed nuclear matrices isolated from Ad2+ND2-infected HeLa cells, on the other hand, had virtually lost their ability to react with these sera (Fig. 10e). The loss of antigenicity after treatment of the nuclear matrices with methanol-acetone seems to be due to denaturation rather than to extraction of the SV40-specific proteins, since methanol-acetone treatment did not extract these proteins from nuclear matrices (data not shown). It cannot, however, be excluded that methanol-acetone fixation in combination with the PBS washing steps involved in the preparation of the nuclear matrices for immunofluorescence microscopy (see above) may result in extraction of these proteins. Differences in the morphology of the U-antigen staining could be observed with nuclear matrices isolated from HeLa cells infected by different hybrid viruses. Whereas nuclear matrices isolated from Ad2+ND1- and Ad2+ND4-infected cells were uniformly stained (Fig. 10a and c), staining of nuclear matrices isolated from Ad2+ND2-infected cells occurred in patches (Fig. 10b). No immunofluorescence staining of nuclear matrices isolated from Ad2+ND5-infected HeLa cells could be obtained with SV40 tumor sera (Fig. 10d). This is in accordance with earlier reports that Ad2+ND5 does not induce any detectable SV40 antigens (24).

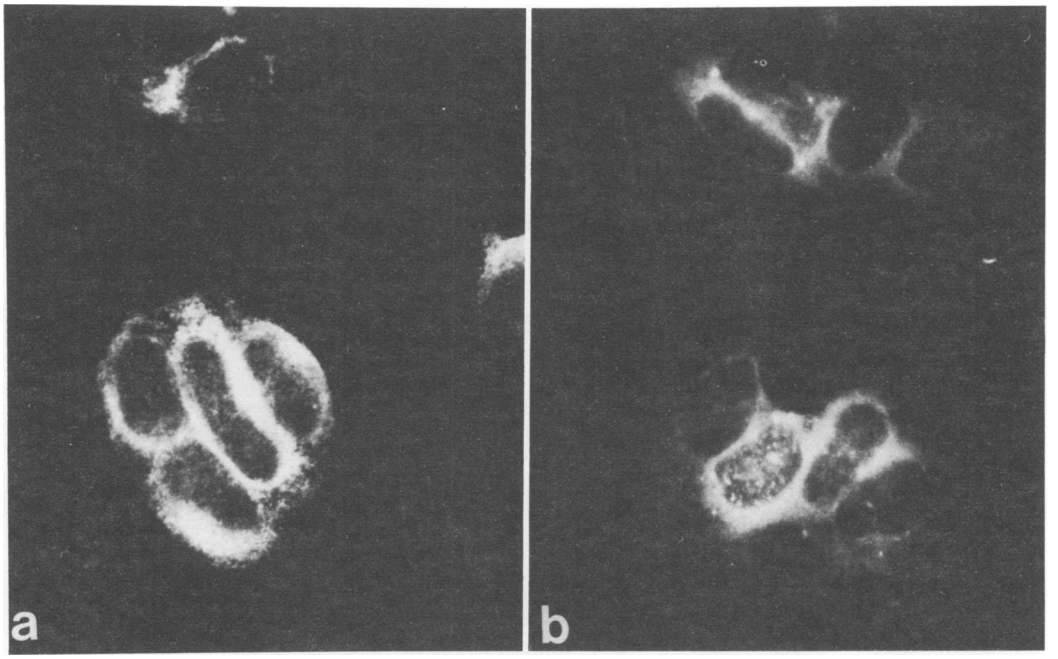

FIG. 9. *Immunofluorescence staining of HeLa cells infected with Ad2+ND1 (a) and Ad2+ND2 (b) with sera from SV40 tumor-bearing hamsters and fluorescein-labeled anti-hamster immunoglobulin. HeLa cells on cover slips were infected with Ad2+ND1 (a) or Ad2+ND2 (b) and processed for immunofluorescence microscopy at 32 h after infection (Ad2+ND1) or at 24 h after infection (Ad2+ND2) as described in the text. Magnifications are 650-fold (a) and 440-fold (b). Control experiments with normal hamster serum instead of the SV40 tumor serum showed no immunofluorescence staining.*

DISCUSSION

In previous reports it was documented by biochemical cell fractionation procedures that SV40-specific proteins encoded by different hybrid viruses stably associate with the nuclei of infected HeLa cells (11, 12). The fact that many of these proteins also were found to accumulate in nuclei and plasma membranes prompted us to suggest that the association of these proteins with the nuclei may be via membranous or supporting structures (11). This study shows that the SV40-specific proteins encoded by the different hybrid viruses all associate with the nuclear matrix structure. Whereas the SV40-specific 28K protein of Ad2+ND1, the SV40-specific 42K and 56K proteins of Ad2+ND2, and the SV40-specific 42K, 56K, 60K, and 64K proteins of Ad2+ND4 are located exclusively in the nuclear matrix, the SV40-specific 72K, 74K, and 95K proteins of Ad2+ND4 were also found in the high-salt nuclear extract. These proteins, therefore, contain molecular features in common with SV40 T-antigen, since they are at least partially extractable from the nuclei by high-salt treatment. The SV40-specific 95K protein shows further analogy to SV40 T-antigen, because it stably associates with the nuclei at low pH in the absence of detergent, but is partially extracted at higher pH in the presence of detergent.

It was found previously that SV40 tumor sera are capable of specifically immunoprecipitating the SV40-specific proteins synthesized in Ad2+ND2- and Ad2+ND4-infected HeLa cells (11, 12). In these experiments the 28K protein of Ad2+ND1 and the 42K protein of Ad2+ND5 were not immunoprecipitable by the SV40 tumor sera used. These sera, however, had a low titer of SV40 U-antibody. The sera used in this study, on the other hand, had a reasonably high titer of SV40 U-antibody (see above) and were able to immunoprecipitate the SV40-specific 28K protein of Ad2+ND1 (data not shown). The same sera, however, still failed to react with the SV40-specific 42K protein in Ad2+ND5-infected cells (data not shown). Therefore, the following correlation can be established: in Ad2+ND5-infected HeLa cells, SV40 U-antigen is not expressed. The SV40-specific 42K protein of Ad2+ND5 is not immunoprecipitable with SV40 tumor sera; this protein is not stably associated with the nuclear matrix; and nuclear matrices isolated from Ad2+ND5-infected HeLa cells do not react with SV40 U-antigen-positive sera. In HeLa cells infected by Ad2+ND1, Ad2+ND2, and Ad2+ND4, on the other hand, SV40 U-an-

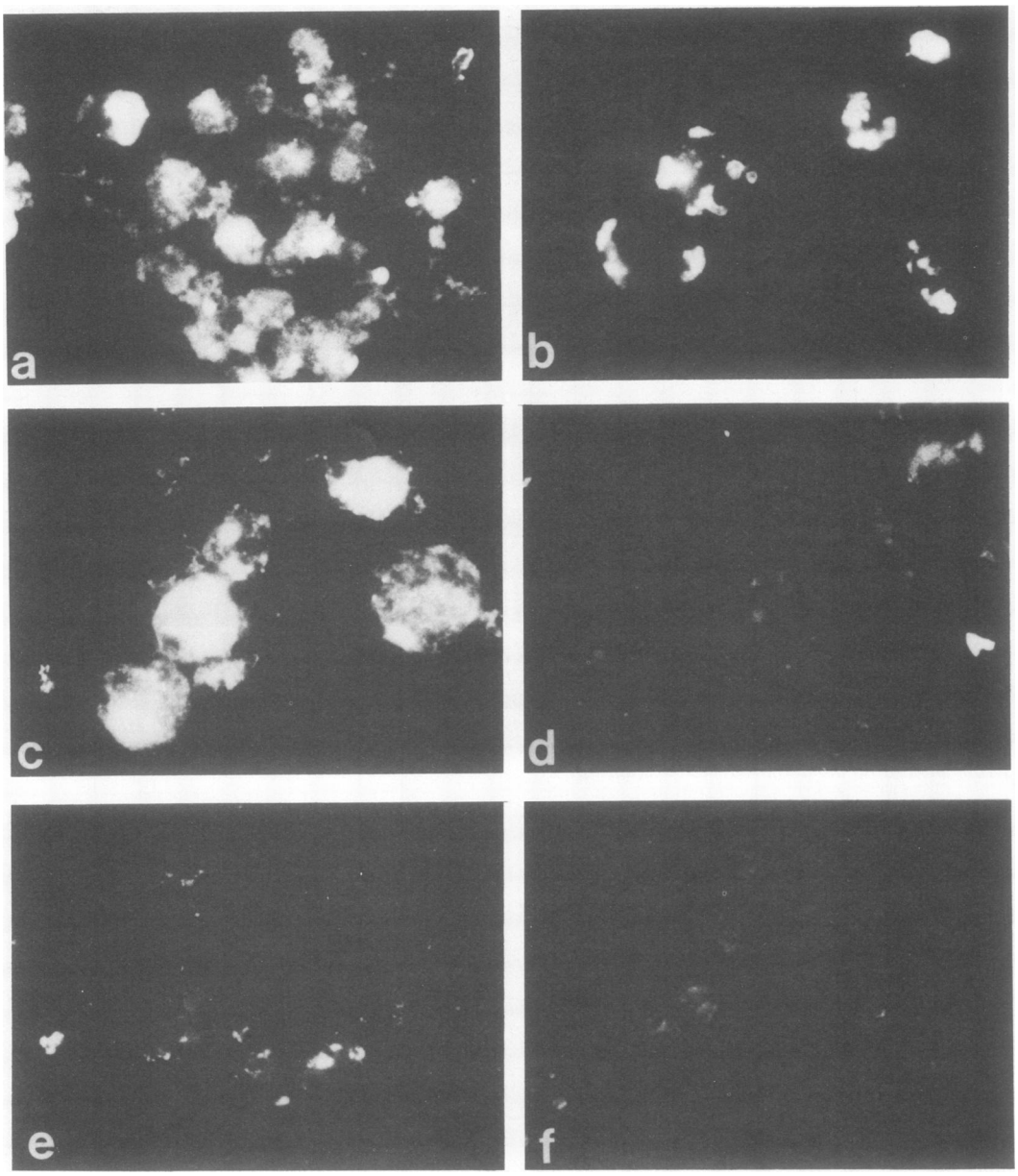

FIG. 10. *Immunofluorescence staining of nuclear matrices isolated from hybrid virus-infected HeLa cells with sera from SV40 tumor-bearing hamsters and fluorescein-labeled anti-hamster immunoglobulin. Nuclear matrices of hybrid virus-infected HeLa cells were prepared and processed for immunofluorescence microscopy as described in the text. (a) Nuclear matrices of Ad2$^+$ND1-infected cells (unfixed; magnification is 750-fold); (b) nuclear matrices of Ad2$^+$ND2-infected cells (Formalin fixed; magnification is 1,200-fold); (c) nuclear matrices of Ad2$^+$ND4-infected cells (unfixed; magnification is 1,200-fold); (d) nuclear matrices of Ad2$^+$ND5-infected cells (unfixed; magnification is 750-fold); (e) nuclear matrices of Ad2$^+$ND2-infected cells (methanol-acetone fixed; magnification is 750-fold); (f) control experiment (nuclear matrices of Ad2$^+$ND2-infected cells stained with normal hamster serum instead of with the SV40 tumor serum; magnification is 750-fold). Nuclear matrices of Ad2$^+$ND1-, Ad2$^+$ND4-, and Ad2$^+$ND5-infected cells also did not show immunofluorescence staining when treated with normal hamster serum instead of with the SV40 tumor serum. Exposure time for (a) to (c) was approximately 30 s; exposure time for (d) to (f) was approximately 2.5 min.*

tigen is expressed. The SV40 proteins encoded by these hybrid viruses are immunoprecipitable and are stably associated with the nuclear matrices. Furthermore, nuclear matrices isolated from HeLa cells infected by these hybrid viruses give positive immunofluorescence staining with SV40 U-antigen-positive tumor sera. This correlation leads to the conclusion that the SV40-specific proteins associated with the nuclear matrices of Ad2$^+$ND1, Ad2$^+$ND2, and Ad2$^+$ND4 themselves carry SV40 U-antigen determinants. In the case of Ad2$^+$ND4, immunofluorescence staining of the nuclear matrices with SV40 tumor sera may also partially reflect SV40 T-antigen staining, since the SV40 tumor sera used contained antibodies against SV40 T-antigen as well as SV40 U-antigen, and SV40 specific proteins with molecular features in common with SV40 T-antigen (SV40-specific 72K, 74K, and 95K proteins) also are associated with the nuclear matrix of Ad2$^+$ND4-infected cells. The question arises of why SV40 U-antigen staining in Ad2$^+$ND1- and Ad2$^+$ND2-infected cells is perinuclear (25) (Fig. 9) and no nuclear fluorescence is obtained, although the SV40 proteins of these hybrid viruses are stably associated with the nuclear matrix structure and isolated nuclear matrices can be stained with SV40 U-antigen-positive tumor sera (Fig. 10). Two possible explanations can be envisioned. (i) Due to their integration into the nuclear matrix, SV40-specific proteins in the nuclei of hybrid virus-infected cells may be sterically hindered in reacting with antibody; or (ii) alternatively, denaturation of these proteins by methanol-acetone fixation, as in the case of isolated nuclear matrices, is responsible for the lack of nuclear fluorescence in Ad2$^+$ND1- and Ad2$^+$ND2-infected cells.

The accumulation of SV40-specific proteins in the nuclei of hybrid virus-infected cells is in variance to a recent report by Jay et al. (18), who did not find the 56K and 42K proteins of Ad2$^+$ND2 associated with the nuclei of KB cells, although these authors used a cell fractionation procedure very similar to the one described by us (11, 12). The fact that SV40-specific proteins of all hybrid viruses investigated in this and previous studies (11, 12) associate with the nuclei of infected cells excludes that this variance is due to differences in the virus stocks used in these experiments. The subcellular location of SV40-specific proteins may be important for the analysis of SV40-induced functions, e.g., the enhancement of the growth of human adenovirus in monkey cells ("helper" function [33]). The finding that the SV40-specific proteins of Ad2$^+$ND2 also associate with the nuclei of monkey cells (Fig. 5) shows that the accumulation of these proteins in the nuclei of hybrid virus-infected cells is not restricted to the HeLa cells used in this and in previous investigations (11, 12), but also occurs in cells in which these proteins exert a biological function. The discrepancy between the results of Jay et al. (18) and ours (11, 12; this study) cannot be explained at the moment.

The association of SV40-specific proteins with the nuclear matrix in hybrid virus-infected cells may be of functional importance. Grodzicker et al. (15, 16) have demonstrated that mutations introduced into the SV40 segment of the Ad2$^+$ND1 genome abolished the ability of the mutant viruses to grow in monkey cells. Mutant Ad2$^+$ND1 hybrid virus unable to grow in monkey cells, e.g., hybrid virus, which had lost the helper function, also had defects in the SV40-specific 28K protein. One, therefore, can assume that the helper function is mediated by SV40-specific proteins in hybrid virus-infected cells. The nature of the restriction of Ad2 growth in monkey cells is not yet understood, and the decreased synthesis of late adenovirus proteins observed in Ad2-infected monkey cells (4) could be due either to a block in the translational machinery of the cells (30) or to a defect in the late adenovirus mRNA (20). If the latter were the case, one may speculate that SV40-specific proteins exert their helper function in association with the nuclear matrix, since the nuclear matrix seems to play an important role in the synthesis, processing, and transport of RNA (5).

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