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Simian Virus 40 Tumor-Specific Proteins: Subcellular Distribution and Metabolic Stability in HeLa Cells Infected with Nondefective Adenovirus Type 2-Simian Virus 40 Hybrid Viruses

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HeLa cells infected with adenovirus type 2 (Ad2)-simian virus 40 (SV40) hybrid viruses produce several SV40-specific proteins. These include the previously reported 28,000-dalton protein of Ad2+ND1, the 42,000- and 56,000-dalton proteins of Ad2+ND2, the 56,000-dalton protein of Ad2+ND4, and the 42,000-dalton protein of Ad2+ND5. In this report, we extend the list of SV40-specific proteins induced by Ad2+ND4 to include proteins of apparent molecular weights of 28,000 42,000, 60,000, 64,000, 72,000, 74,000, and a doublet of 95,000. Cell fractionation studies demonstrate that the SV40-specific proteins are detectable in the nuclear, cytoplasmic, and plasma membrane fractions. By pulse-chase and cell fractionation experiments, three classes of SV40-specific proteins can be distinguished with regard to metabolic stability: (i) unstable in the cytoplasmic but stable in the nuclear and plasma membrane fractions; (ii) stable in the nuclear, cytoplasmic, and plasma membrane fractions; and (iii) unstable in all subcellular fractions. Immunoprecipitation of infected cell extracts demonstrates that most of the above proteins share antigenic determinants with proteins expressed in hamsters bearing SV40-induced tumors. Only the 42,000-dalton protein of Ad2+ND5 is not immunoprecipitable.

The nondefective adenovirus type 2 (Ad2)-simian virus 40 (SV40) hybrid viruses isolated by Lewis and coworkers (17, 18) are useful for studying nonstructural SV40-specific proteins. Each of the five nondefective hybrid viruses contains a segment of SV40 DNA covalently inserted in the Ad2 genome (4, 9, 11, 16, 25). Ad2+ND1 contains approximately 17 to 20% of the SV40 genome; Ad2+ND2 contains approximately 32 to 36.5%; Ad2+ND3 contains approximately 6.4 to 7%; Ad2+ND4 contains approximately 43 to 48%; and Ad2+ND5 contains approximately 28% (11, 15, 24, 25). During productive infection, the SV40 information in the hybrid viruses is expressed, leading to the induction of SV40-specific antigens. Ad2+ND4 induces T antigen, tumor-specific transplantation antigen (TSTA), and U antigen (18, 20); Ad2+ND2 induces TSTA and U; and Ad2+ND1 induces U antigen (18-20). Two other hybrid viruses, Ad2+ND3 and Ad2+ND5, do not induce any of the antigens in detectable amounts.

Previously, a protein with a molecular weight of 28,000 (28K protein) has been identified in Ad2+ND1-infected cells (7, 21). We recently reported a 42K protein in both Ad2+ND2 and Ad2+ND5-infected cells, and a 56K protein common to cells infected by Ad2+ND2 and Ad2+ND4 (30). The 56K and 42K proteins in Ad2+ND2-infected cells share common amino acid sequences, and are immunoprecipitable with serum from hamsters bearing SV40 tumors (5a). They have the interesting property of accumulating in a stable fashion in nuclei and plasma membranes of Ad2+ND2-infected HeLa cells while being metabolically labile in the cytoplasm (5a). In this report, we describe additional SV40-specific proteins in Ad2+ND4-infected cells. Furthermore, the intracellular distribution and the metabolic stability of the different SV40-specific proteins induced by the hybrid viruses have been studied. Immunoprecipitation with serum from hamsters bearing SV40 tumors identifies several of these proteins as SV40 tumor specific.

MATERIALS AND METHODS

Viruses and cells. Seed stocks of nondefective Ad2-SV40 hybrid viruses, Ad2+ND1, Ad2+ND2,

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$Ad2^+ND4$, and $Ad2^+ND5$, were obtained from A. M. Lewis, Jr. Stocks of Ad2, $Ad2^+ND1$, $Ad2^+ND2$, and $Ad2^+ND5$ were prepared in HeLa S_3 cells grown in minimum essential medium (MEM) for suspension culture (GIBCO no. F-13) supplemented with 5% calf serum (growth medium). Stocks of $Ad2^+ND4$ were prepared in CVI cells grown in Dulbecco modified Eagle medium with 10% fetal calf serum. The titer of the virus stocks used in these experiments was 10^9 PFU/ml for all stocks except $Ad2^+ND4$ which titered 10^7 PFU/ml. Stocks were assayed on HeLa monolayers as described by Williams (32).

Infection and labeling of cells. (i) Two-hour continuous labeling experiments. HeLa S_3 cells (2×10^7) in suspension were infected with 2 ml of undiluted virus stocks. After an adsorption period of 20 min, the cells were diluted with 100 ml of growth medium. At the times indicated in the figure legends, the cells were washed twice with 40 ml of Eagle medium minus amino acids and labeled in 10 ml of Eagle medium minus amino acids containing 10 μCi of a ^{14}C-labeled L-amino acid mixture (NEN, specific activity 10 mCi/mmol) per ml and 5% calf serum. After 2 h, the labeling medium was removed; the cells were washed with 40 ml of Eagle MEM and then subjected to cell fractionation (see below).

(ii) Pulse-chase experiments. HeLa S_3 cells (5×10^7) in suspension were infected with 5 ml of undiluted virus stocks. After the adsorption period, the cells were diluted with 300 ml of growth medium. At the times indicated in the figure legends, the cells were washed twice with 40 ml of Eagle medium minus amino acids and labeled in medium minus amino acids containing 40 μCi of ^{14}C-labeled L-amino acid mixture per ml and 5% calf serum. After 15 min, the cells were split into two parts and washed once with 20 ml of Eagle MEM. One part (pulse) was immediately subjected to cell fractionation (see below). The remaining cells (pulse-chase) were diluted with 100 ml of growth medium and kept in this medium for the time indicated in the figure legends. At the time indicated, the equivalent part was washed once with 20 ml of Eagle medium and subjected to cell fractionation. For immunoprecipitation, cells were infected as described and labeled at 37 h postinfection with 165 μCi of ^{35}S-labeled methionine (specific activity, 512 Ci/mmol) per ml for 2 h in methionine-free Eagle medium.

Cell fractionation. (i) Membrane isolation. Membranes of uninfected and infected HeLa cells were prepared according to the method of Brunette and Till (3). The cells were washed twice with 20 ml of 0.15 M NaCl and suspended in 2 ml of a 1 mM $ZnCl_2$ solution. After swelling for 10 min at room temperature, they were cooled in an ice bath for 5 min and then homogenized in a stainless steel Dounce homogenizer (clearance, 0.002 inch [ca. 0.005 cm]). The homogenization procedure was monitored by examining the cell lysate under a phase-contrast microscope. Homogenization was stopped after about 80% of the cells were broken (10 to 15 strokes). The cell lysate was spun at 2,000 rpm for 15 min in an IEC centrifuge, model PR-2, rotor head 269 ($800 \times g$). The supernatant was designated cytoplasm. The pellet of the low-speed centrifugation, containing membrane bags, unbroken cells, and nuclei, was subjected to centrifugation in the two-phase system containing polyethylene glycol 6000 and dextran 500 (3). Plasma membranes were found at the interphase; nuclei and unbroken cells were found in the pellet. Plasma membranes were further purified following the procedure of Brunette and Till (3). Phase-contrast microscopy of the purified plasma membranes suspended in water showed that the membrane fraction consisted of single empty bags and aggregates of bags (Fig. 1). No contamination by nuclei could be detected. We observed that the homogenization of cells in the presence of $ZnCl_2$ causes aggregation of cytoplasmic proteins which subsequently pellet during a 60-min centrifugation at $100,000 \times g$. Therefore, cell homogenization in the reconstitution experiments (see below) was performed in the absence of $ZnCl_2$. In these experiments, the cells were washed twice with 20 ml of 0.15 M NaCl and suspended in 2 ml of cold lysis buffer (5 mM $MgCl_2$–2 mM $CaCl_2$, buffered with 10 mM 2-[*N*-morpholino] ethanesulfonic acid [MES], pH 6.5). After swelling for 5 min in the cold, the cells were homogenized as described above, and the cell lysate was spun at 2,000 rpm for 15 min at $800 \times g$. The supernatant (cytoplasm) was further fractionated (see below). The pellet of the low-speed centrifugation was subjected to centrifugation in the two-phase system, and the plasma membranes were isolated and purified as described above.

(ii) Subfractionation of cytoplasm. The cytoplasm from the low-speed centrifugation of the cell lysate prepared in the absence of $ZnCl_2$ (see above) was separated into a $100,000 \times g$ cytoplasmic supernatant and a $100,000 \times g$ cytoplasmic pellet by centrifugation at $100,000 \times g$ for 60 min at 4°C.

(iii) Purification of nuclei. The pellet collected after the two-phase spin, consisting of unbroken cells and nuclei, was suspended in isotonic Tris buffer (0.14 M NaCl–5 mM KCl–0.5 mM $MgCl_2$–1 mM $CaCl_2$–25 mM Tris-hydrochloride, pH 7.4). Nonidet P-40 (NP-40) was added to a final concentration of 0.5%. After 10 min on ice, the nuclei were pelleted for 2 min at $800 \times g$ and washed three times with isotonic Tris buffer. This preparation was designated NP-40-nuclei. Nuclei, from which the outer nuclear membrane was removed, were prepared by treatment of NP-40 nuclei with a Tween-deoxycholate(DOC) mixture (26). NP-40 nuclei were resuspended in 1 ml of isotonic Tris buffer, and 150 μl of a mixture of 1 volume of 10% sodium deoxycholate and 2 volumes of Tween 40 in water were added. After 10 min on ice, the nuclei were pelleted for 2 min at $800 \times g$ and washed three times with isotonic Tris buffer. This nuclei preparation was designated Tween-DOC nuclei.

Reconstitution experiment. HeLa S_3 cells (5×10^7) in suspension were infected with 5 ml of undiluted $Ad2^+ND2$ virus stock and labeled with 40 μCi of a ^{14}C-labeled L-amino acid mixture per ml at 35 h postinfection as described. After 30 min, the cells were divided into two equal parts and washed once with 20 ml of Eagle MEM. The cells of one part (pulse) were immediately subjected to cell fractionation (see above). The cells of the other part (pulse-

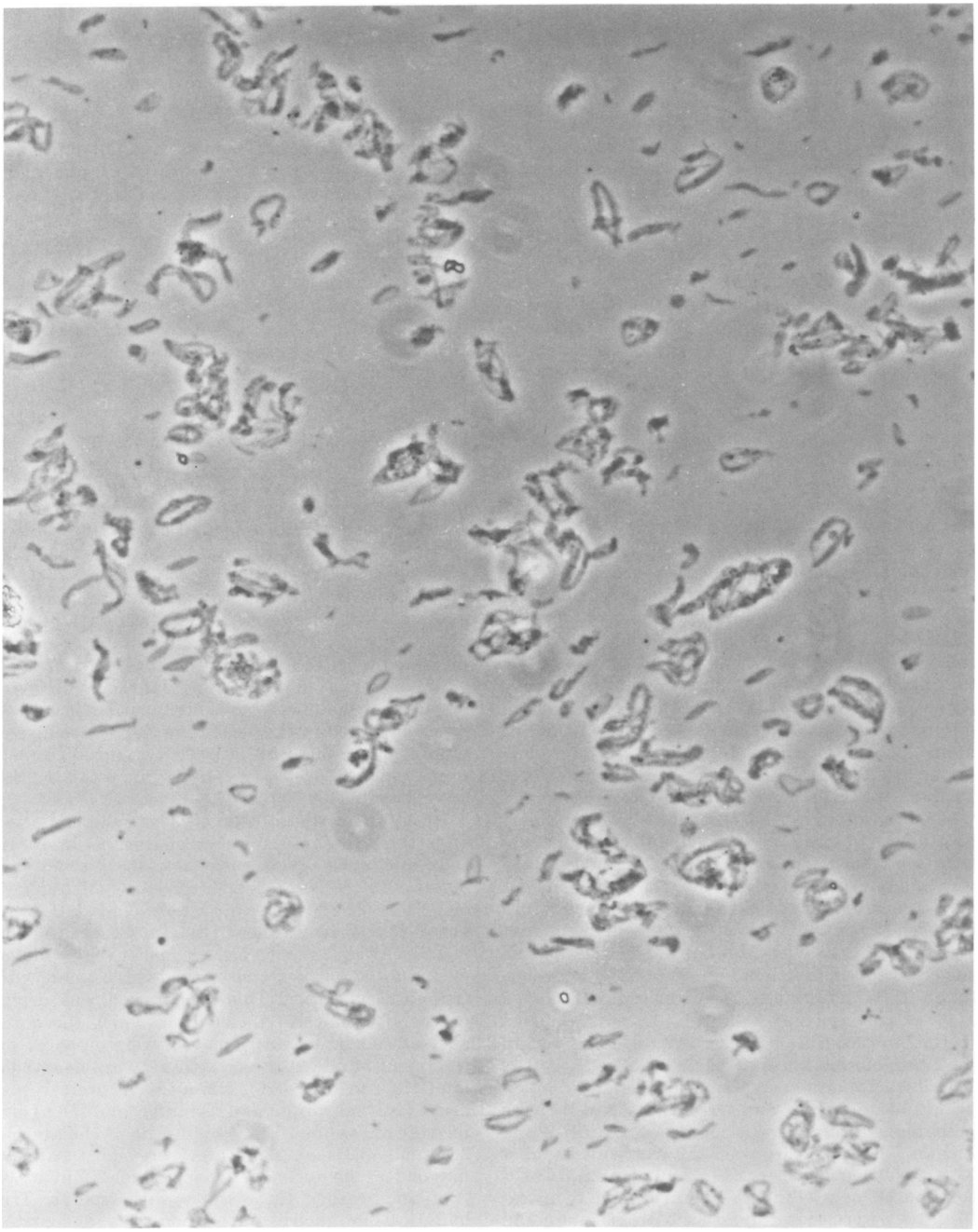


FIG. 1. *Plasma membrane preparation from Ad2-infected HeLa cells. Plasma membranes were isolated 36 h postinfection as described in Materials and Methods and suspended in water. The magnification is 400-fold.*

chase) were diluted with 150 ml of growth medium and kept in this medium for 5.5 h. They were then washed once with 20 ml of Eagle medium before being subjected to cell fractionation.

The cytoplasm and the 100,000 × *g* cytoplasmic supernatant of both the pulsed and the pulse-chased cells were incubated at 0°C with purified, unlabeled plasma membranes isolated from uninfected HeLa cells. After 20 min, the incubation mixture was spun for 15 min at 800 × *g*. The pelleted membranes were then repurified by centrifugation in the two-phase system as described above.

Preparation of samples for gel electrophoresis. Portions of the cell lysate (homogenate) and of the cytoplasm were diluted 1:1 with electrophoresis sample buffer (0.0625 M Tris, pH 6.8; 3% sodium dodecyl sulfate [SDS]; and 5% 2-mercaptoethanol). The 100,000 × *g* cytoplasmic supernatant was precipitated with ice-cold trichloroacetic acid (10% [vol/vol] final concentration), washed three times with cold acetone, air dried, and dissolved in electrophoresis sample buffer. Nuclei and plasma membranes were pelleted and then 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, 3× 10 s) and heated for 3 min in a boiling water bath. The protein content of the samples was determined by the method of Lowry et al. (22) and adjusted to approximately 15 μg/10 μl.

Polyacrylamide gel electrophoresis and fluorography. The polyacrylamide gel system of Laemmli (13) and Maizel (23) was employed. A 15-μg amount of protein in approximately 10-μl samples was applied per slot. Electrophoresis was performed at a constant current of 10 mA. After electrophoresis, the gels were prepared for fluorography as described by Bonner and Laskey (2). To obtain a linear response of the film to the radioactivity, the X-ray film was preexposed by a light flash (14).

Immunoprecipitation. Cytoplasmic extracts from Ad2 and Ad2$^+$SV40 hybrid virus-infected cells labeled with [^{35}S]methionine were prepared by lysing the cells in a buffer solution containing 0.14 M NaCl, 3 mM $MgCl_2$, 10 mM Tris-hydrochloride, pH 7.4, 0.5% NP-40, and 1% phenylmethylsulfonyl fluoride. Nuclei and debris were sedimented at 800 × *g* for 10 min. A 20-μl portion of the supernatant was incubated with either 5 μl of normal hamster serum (Flow Laboratories, Inc.) or 5 μl of serum from hamsters bearing SV40 tumors (received from Jack Gruber, Chief, Office of Program Resources and Logistics, National Cancer Institute, National Institutes of Health) for 60 min at 30°C. Then 0.4 ml of rabbit antiserum prepared against hamster gamma globulin (Cappel Laboratories) containing 0.1% NP-40 was added to equivalence. After 15 h at 4°C, the precipitate was collected by centrifugation and then washed and dissolved in 50 μl of sample buffer. A 10-μl portion of this sample was applied on a slab gel. Prior to use, all sera were absorbed with Ad2-infected HeLa cells and decomplemented by heating to 56°C for 30 min.

Safety precautions. Infections with nondefective Ad2-SV40 hybrids, labeling, and cell fractionation experiments were conducted in a containment laboratory, equipped according to the National Institutes of Health guidelines for handling nondefective Ad2-SV40 hybrids. All persons involved in this work had Ad2 antibody titers higher than 1/10 by plaque neutralization tests.

RESULTS

Subcellular distribution of SV40-specific proteins in cells infected by Ad2$^+$ND1, Ad2$^+$ND2, Ad2$^+$ND4, and Ad2$^+$ND5. HeLa cells in suspension were infected with different hybrid viruses and labeled with a ^{14}C-amino acid mixture for 2 h at a late time of infection when the synthesis of host cell protein was shut off. The labeled cells were fractionated into plasma membranes, cytoplasm, and a crude nuclear fraction as described in Materials and Methods. Purified nuclei were obtained by treatment of the crude nuclear fraction with 0.5% NP-40 (NP-40 nuclei) or by treatment of NP-40 nuclei with a mixture of Tween 40 and DOC (Tween-DOC nuclei; see Materials and Methods). The radioactive polypeptides in the initial homogenate, cytoplasm, NP-40 nuclei, Tween-DOC nuclei, and plasma membranes were analyzed by SDS-acrylamide gel electrophoresis and visualized by fluorography (Fig. 2 through 4). Approximately equal amounts of protein from each sample were applied to the gel. Therefore, the intensity of an individual band in the fluorograms is a measure of the specific activity and does not reflect the absolute amount of radioactive protein in a particular cell fraction.

Ad2$^+$ND1-infected cells. As described previously, the 28K protein induced by Ad2$^+$ND1 is present in infected cells in small quantities (30). The fluorogram shown in Fig. 2 demonstrates that this protein is present in all subcellular fractions. Most clearly it can be detected in nuclei and plasma membranes. For comparison, Fig. 2 also shows the distribution in different subcellular fractions of radioactive proteins from Ad2-infected cells. An interesting observation concerning the Ad2-specific protein Vc, should be pointed out. Vc (30) is a nuclear protein, not found in Ad2 virions, that is solubilized to a large extent by treatment of NP-40 nuclei with a mixture of Tween 40 and DOC. Since this mixture acts by removing the outer nuclear membrane (26), Vc may therefore be located in the outer nuclear membrane.

Ad2$^+$ND2-infected cells. The fluorogram shown in Fig. 3 demonstrates the subcellular distribution of SV40-specific proteins in Ad2$^+$ND2-infected cells. The previously described 56K and 42K proteins induced by Ad2$^+$ND2 are present in all cell fractions.

Ad2$^+$ND4-infected cells. Cells infected with Ad2$^+$ND4 synthesize a surprisingly large number of SV40-specific proteins (Fig. 3). (i) A 95K protein is most clearly detectable in homogenate and nuclei. This protein has a diffuse appearance indicating heterogeneity (see below). It is usually superimposed on a background either of host proteins that are not totally shut off by the infection or of Ad2-specific proteins. (ii) Two proteins, 74K and 72K, are present in all subcellular fractions. They are clearly separated from each other on gels from homogenate,

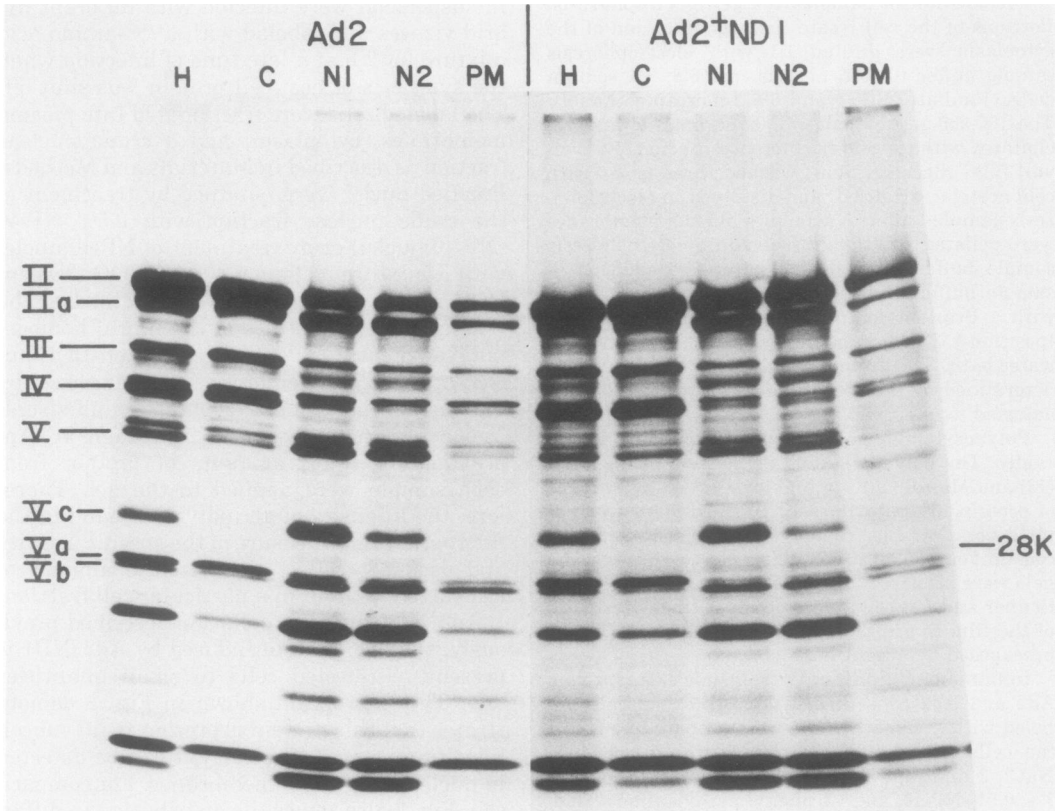


FIG. 2. *SDS-polyacrylamide gel fluorogram of ^{14}C-labeled polypeptides in different cell fractions of HeLa cells infected with Ad2+ND1. Parallel cultures of HeLa S_3 cells were infected with Ad2 or Ad2+ND1, respectively, and labeled for 2 h (46 to 48 h after infection) with ^{14}C-amino acids. The cells were fractionated as described in Materials and Methods. Portions of each fraction containing 15 μg of protein were applied to a hyperbolic 7.5 to 30% acrylamide gradient gel and run for 5.5 h at 10 mA. Subcellular fractions were designated as follows: (H) homogenate, (C) cytoplasm, (N1) NP-40 nuclei, (N2) Tween-DOC nuclei, and (PM) plasma membrane.*

cytoplasm, and plasma membranes. With samples from nuclei, the separation and detection of these two proteins is perturbed by the presence of the early Ad2-specific protein IIIb (approximate molecular weight of 74,000) which is located in the nucleus and absent in other cell fractions (30). (iii) A 64K protein was detected in pulse-chase experiments (see Fig. 7). (iv) A 60K protein, migrating slightly faster than the Ad2 protein IV (fiber) was detected in all subcellular fractions. (v) Two proteins, 56K and 42K, comigrating with the 56K and 42K proteins in Ad2+ND2-infected cells, are also found in all cell fractions. (vi) Occasionally we observed a 28K protein comigrating with the 28K protein in Ad2+ND1-infected cells. Figure 3 does not demonstrate this protein because the gel conditions are not optimal for its detection. However, the fluorogram of a gradient gel shown in Fig. 4 reveals a small amount of this protein in NP-40 nuclei of Ad2+ND4-infected cells. It is not detectable in NP-40 nuclei from Ad2+ND2 and Ad2+ND5-infected cells.

In previous experiments, only one SV40-specific 56K protein could be identified with certainty in Ad2+ND4-infected cells (30). This was somewhat surprising, since Ad2+ND4 has the capacity to code for an SV40-specific protein with a moleular weight of approximately 85,000, assuming a molecular weight of 3.25×10^6 for the SV40 genome (8) or for a 95K protein based on a molecular weight of 3.6×10^6 (28). The reasons for this result are now clear. First, the stock of Ad2+ND4 tested in our previous experiments (3) was contaminated with adeno-associated virus (AAV), whereas our present stock is AAV-free. Cells infected by Ad2+ND4 containing AAV synthesize three structural proteins of AAV (10, 27), in addition to Ad2- and SV40-specific proteins. One of these has an

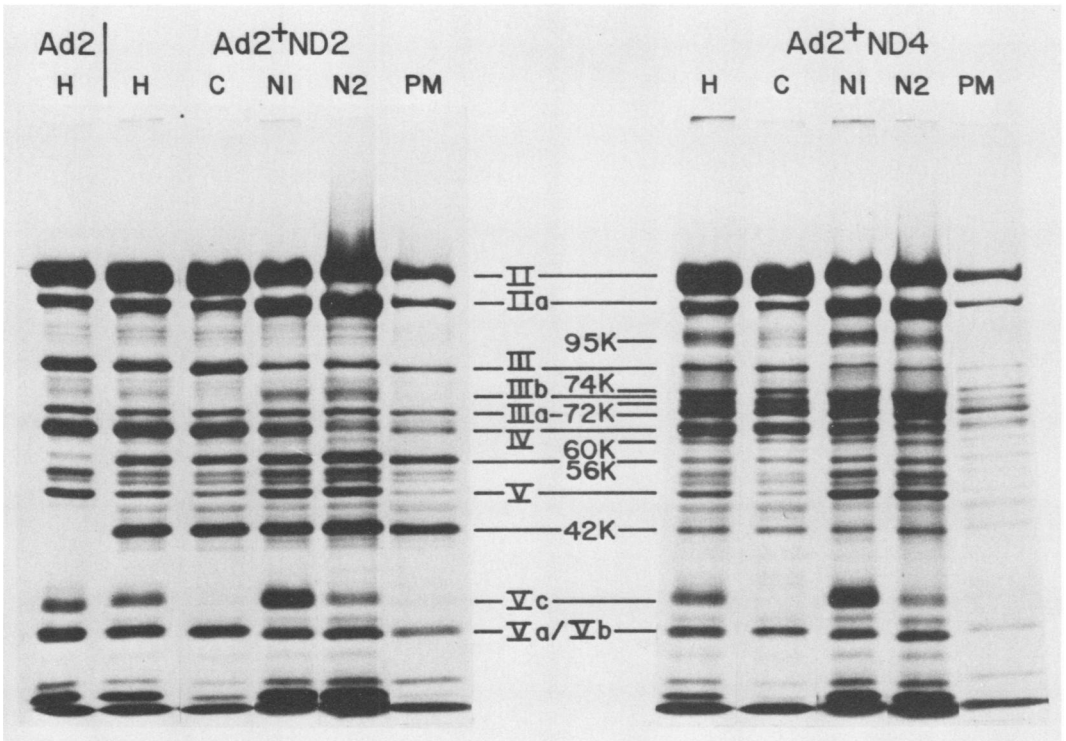


FIG. 3. *SDS-polyacrylamide gel fluorogram of ^{14}C-labeled polypeptides in different cell fractions of HeLa cells infected with Ad2$^+$ND2 and Ad2$^+$ND4. Parallel cultures of Hela S_3 cells were infected with Ad2$^+$ND2 or Ad2$^+$ND4, respectively, and labeled for 2 h (43 to 45 h after infection) with ^{14}C-amino acids. The cells were fractionated as described in Materials and Methods. Portions of each fraction containing 15 μg of protein were applied to a 7.5% acrylamide slab gel and run for 4.5 h at 10 mA. Designation of subcellular fraction as in the legend to Fig. 2. Homogenate of Ad2-infected cells is shown as control.*

approximate molecular weight of 96,000 and interferes with the detection of the SV40-specific 95K protein. The major structural protein of AAV (molecular weight, 65,000) interferes with the detection of the SV40-specific 74K, 72K, and 64K proteins. In addition, AAV at high multiplicity of infection suppresses the expression of Ad2-specific information in Ad2$^+$ND4-infected cells (H. Linke and G. Walter, unpublished data) and may also interfere with the expression of SV40-specific proteins. This may explain why the 42K protein, present in relatively small quantities, was not detected previously. A detailed report on the effect of AAV coinfection on the expression and detectability of SV40-specific proteins will be published elsewhere. Second, the analysis of subcellular fractions facilitates the identification of individual SV40-specific proteins, such as the 95K and 64K proteins in the nucleus and the 74K and 72K proteins in cytoplasm. Third, with regard to the 69K protein, it is evident now that the choice of the gel conditions is important for separating this protein from the Ad2 protein IV. For example, the 60K protein is clearly detectable on a linear 7.5% gel (Fig. 3) but not on a gradient gel where it migrates close to the Ad2 fiber protein IV (Fig. 4).

Ad2$^+$ND5-infected cells. As described previously (30), Ad2$^+$ND5 induces only one SV40-specific 42K protein. Figure 5 demonstrates that this protein is present mainly in the cytoplasm and in the nuclei, but only slightly present in the plasma membrane. The protein of slightly smaller apparent molecular weight than the 42K protein in the nuclear fractions could be a host cell protein, since it has been observed in several experiments in which the shutoff of host protein synthesis was incomplete. Table 1 summarizes the molecular weights of the SV40-specific proteins found in different hybrid virus-infected cells. Only proteins found reproducible under a variety of experimental conditions were considered as SV40-specific. Other novel bands that were observed only occasionally have not been studied.

Molecular stability of the SV40-specific proteins. Pulse-chase experiments demon-

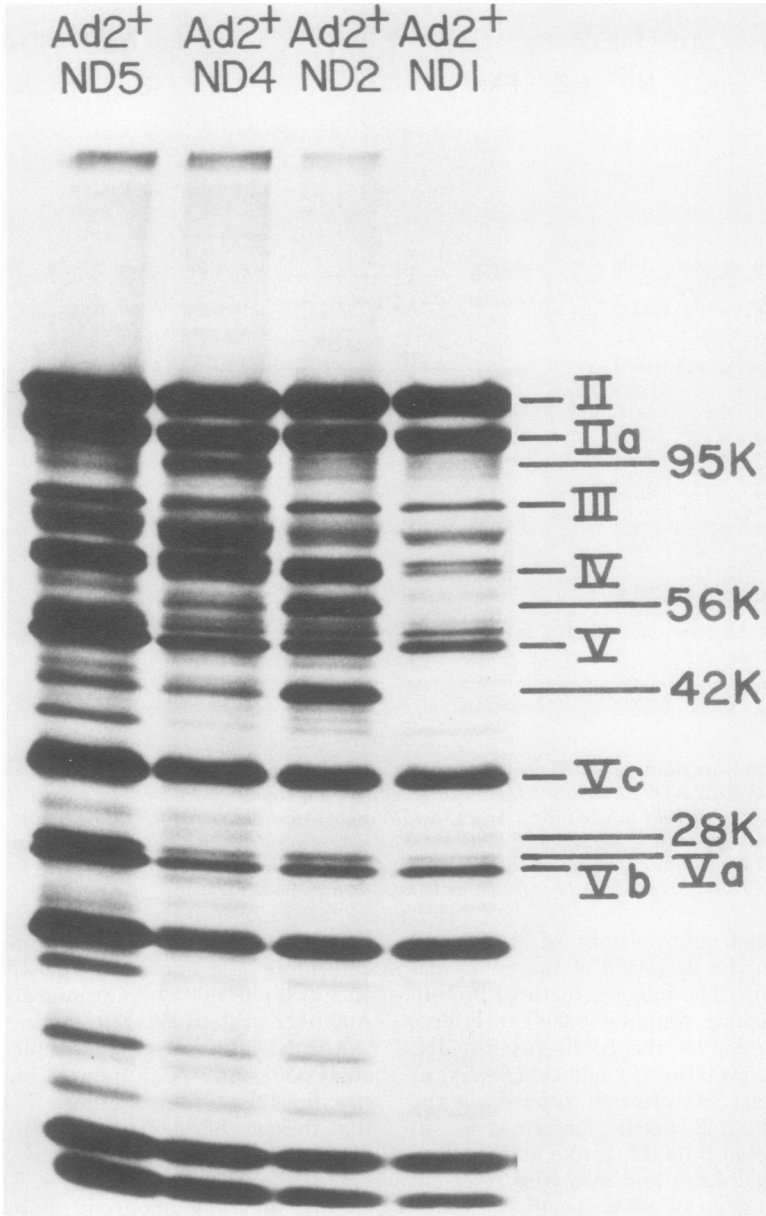


FIG. 4. *SDS-polyacrylamide gel fluorogram of ^{14}C-labeled polypeptides in NP-40 nuclei of HeLa cells infected with Ad2$^+$ND1, Ad2$^+$ND2, Ad2$^+$ND4, and Ad2$^+$ND5. Parallel cultures of HeLa S_3 cells were infected with Ad2$^+$ND1, Ad2$^+$ND2, Ad2$^+$ND4, or Ad2$^+$ND5, respectively, labeled for 2 h (46 to 48 h after infection with Ad2$^+$ND1; 43 to 45 h after infection with Ad2$^+$ND2 and Ad2$^+$ND4; and 44 to 46 h after infection with Ad2$^+$ND5) with ^{14}C-amino acids. The cells were fractionated as described in Materials and Methods. Portions of the NP-40 nuclei fraction containing 15 μg of protein were applied to a hyperbolic 8.5 to 20% acrylamide gradient gel and run for 5.5 h at 10 mA.*

strated previously that the 56K protein in Ad2$^+$ND2 and Ad2$^+$ND4-infected cells and the 42K protein in Ad2$^+$ND2 and Ad2$^+$ND5-infected cells are metabolically unstable (30). However, pulse-chase experiments and cell fractionation studies with Ad2$^+$ND2-infected cells showed that the 56K and 42K proteins are unstable only in the cytoplasm, which contains

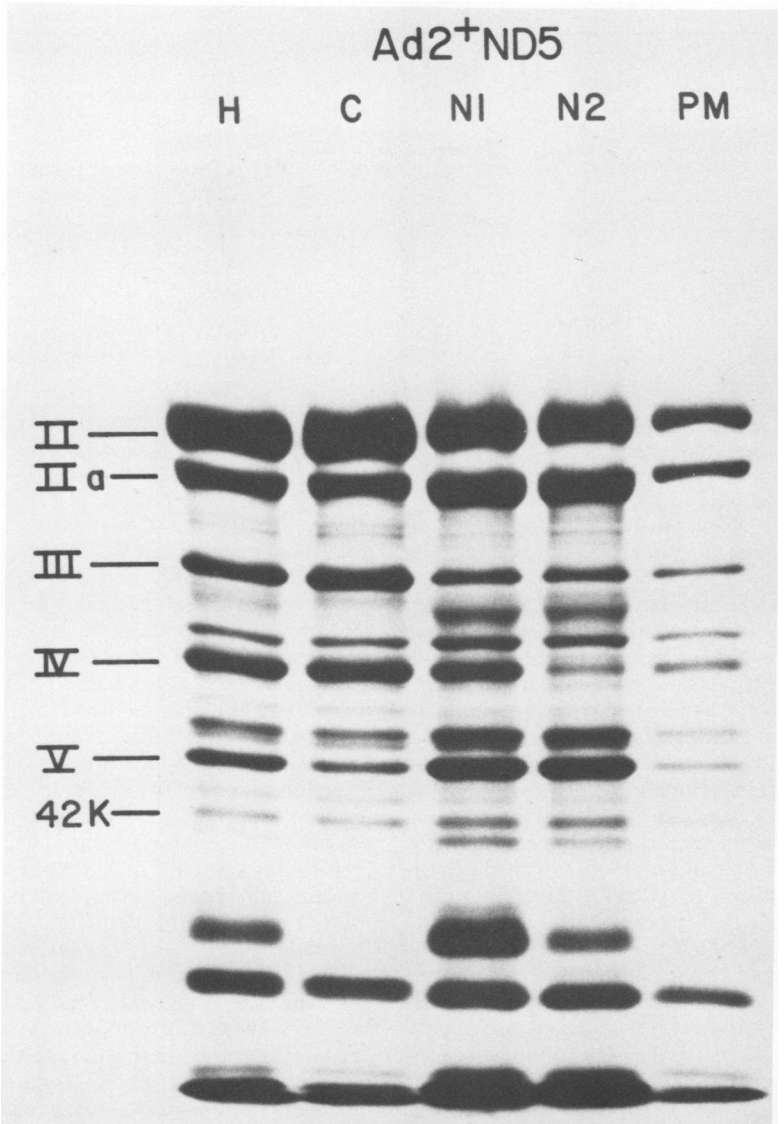


FIG. 5. *SDS-polyacrylamide gel fluorogram of ^{14}C-labeled polypeptides in different cell fractions of HeLa cells infected with Ad2+ND5. HeLa S_3 cells were infected with Ad2+ND5, labeled for 2 h (44 to 46 h after infection) with ^{14}C-amino acids and fractionated as described in Materials and Methods. Portions of each fraction containing 15 μg of protein were applied to a 7.5% acrylamide slab gel and run for 4.5 h at 10 mA. Designation of subcellular fractions were as in the legend to Fig. 2.*

the bulk of these proteins, but are stable in nuclei and plasma membranes (5a). It was of interest to investigate whether metabolic instability is a general property of all SV40-specific proteins induced by the different hybrid viruses and whether the stability of these proteins is influenced by their subcellular location. HeLa cells infected by the different hybrid viruses were labeled with ^{14}C-amino acids for 15 min (pulse) and chased for several hours with excess unlabeled amino acids. Both pulse-labeled and -chased cells were fractionated into nuclei, cytoplasm, and plasma membranes, and the radioactive polypeptides in each fraction were analyzed by SDS-acrylamide gel electrophoresis.

Ad2+ND2-infected cells. In previous experiments, AD2+ND2-infected cells were pulse-labeled for 30 min and chased for 5.5 h (5a). Figure 6 demonstrates the results of an experi-

TABLE 1. *Properties of the SV40-specific proteins found in different hybrid virus-infected cells*

Protein	Ad2+ ND1	Ad2+ ND2	Ad2+ ND4	Ad2+ ND5	Metabolic stability		Immunoprecipitation
					Nucleus and plasma membrane	Cytoplasm	
28K	+		+		Stable	Stable	−
42K		+			Stable	Unstable	+
			+		Unstable	Unstable	+
				+	Unstable	Unstable	−
56K		+	+		Stable	Unstable	+
60K			+		Stable	Stable	+
64K			+		Stable	Stable	+
72K			+		Stable	Stable[a]	+
74K			+		Stable	Stable[a]	+
95K (doublet)			+		Stable[b]	Unstable	+

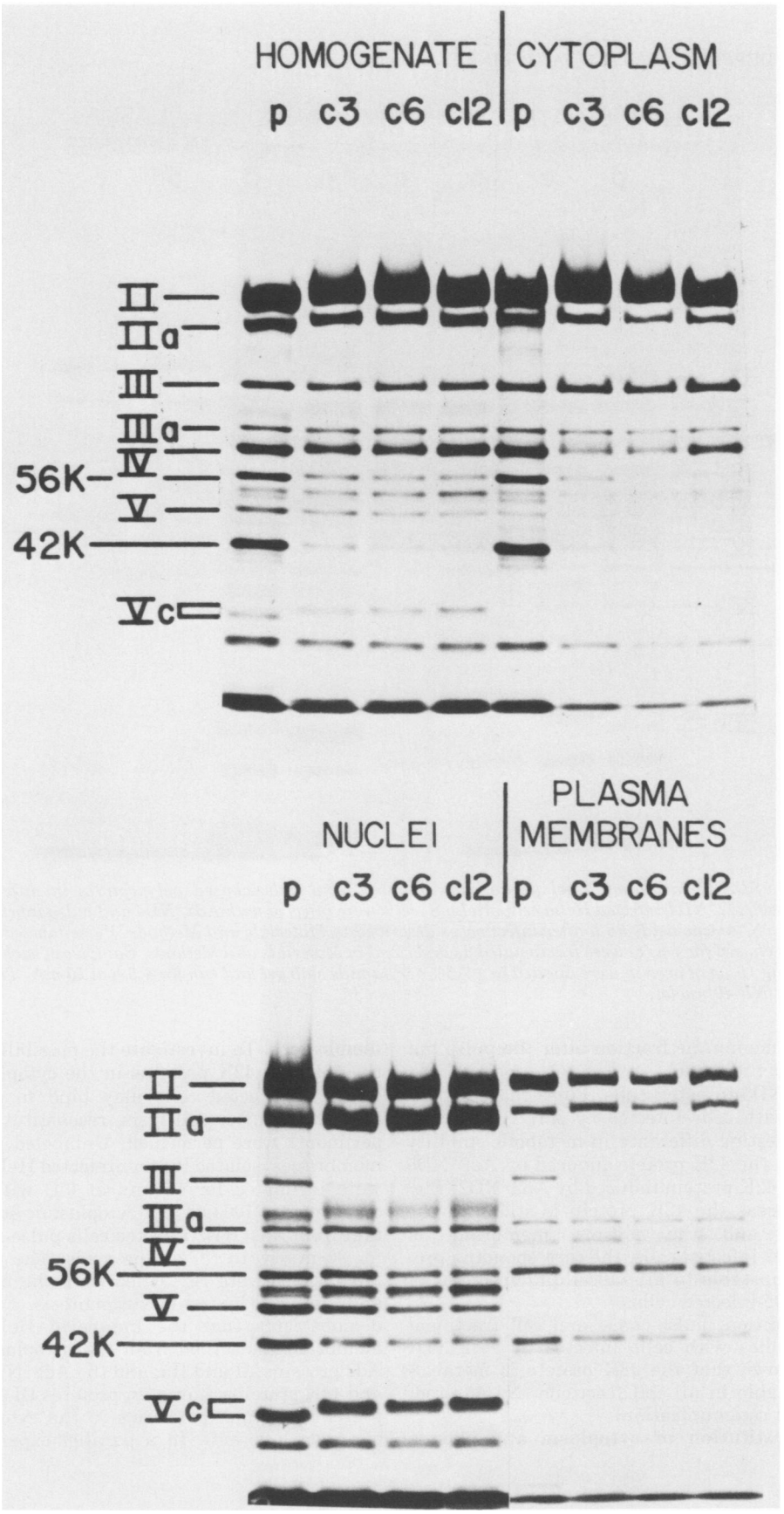
[a] Decreases slightly during chase.
[b] In the nucleus, lower band of the doublet is shifted to higher position during chase.

ment in which chase periods ranging from 3 to 12 h were chosen. In the cytoplasm, the amounts of radioactive 56K and 42K proteins were drastically reduced after 3 h of chasing. The 42K protein is undetectable after a chase of 6 h, whereas some 56K protein is still detectable even after a 12-h chase. In contrast, both proteins are considerably more stable in the nuclear and in the plasma membrane fraction. In both fractions, the amount of radioactive 56K protein is unchanged or slightly increased after a chase of 3 h. It then decreases during the 6-h chase and remains constant for another 6 h. The amount of 42K protein in nuclei and plasma membranes decreases during the first 3 h of chase and then remains constant.

By scanning the fluorograms and taking into account the total amounts of protein in each subcellular fraction, we calculated previously that after a 30-min pulse the cytoplasm contained the major protion of the radioactive 56K and 42K proteins, i.e., 67 and 79%, respectively, of the total amount present in homogenates. Nuclei and plasma membranes combined contain a minor fraction of 16 and 8%, respectively. In comparison, after a 5.5-h chase, the cytoplasm contains only 28% of the 56K protein and 17% of the 42K protein, whereas nuclei and plasma membranes together contain the bulk of the radioactive 56K and 42K proteins (5a).

Ad2+ND4-infected cells. The fluorogram shown in Fig. 7 demonstrates the result of a pulse-chase experiment with Ad2+ND4-infected cells. Here the 56K and 42K proteins in the cytoplasm are also unstable, but, whereas the 56K protein is stable in the nuclei and plasma membranes over a chase period of 4 h, the 42K protein appears to be unstable. The patterns of nuclei and plasma membranes, however, are difficult to evaluate with respect to the 42K protein since it is present in small quantity. The 60K protein is stable in all subcellular fractions. An SV40-specific protein with an approximate molecular weight of 64,000 is present in all cell fractions of both pulse-labeled and -chased cells. Under optimal gel conditions, this protein can be separated from the Ad2 protein IV (molecular weight, 62,000). It is most easily detectable in nuclei and plasma membranes that contain only small quantities of protein IV. The 74K and 72K proteins in the cytoplasm are slightly reduced in amount after the chase, but are stable in nuclei and plasma membranes. The appearance on the gel of the 74K protein in the nuclear fraction is perturbed by the Ad2-specific early protein IIIb. Both nuclei and cytoplasm of pulse-labeled cells contain a doublet of proteins migrating in the 95K region of the gel. After the chase, this doublet is detectable in reduced quantity in the cytoplasm, whereas in nuclei the smaller component is shifted to a slightly higher position. A trace of the 95K doublet protein is detectable in the

FIG. 6. *SDS-polyacrylamide gel fluorogram of pulsed and pulse-chased polypeptides in different cell fractions of Ad2+ND2-infected HeLa cells. HeLa S_3 cells were infected with Ad2+ND2 and pulse-labeled for 15 min with ^{14}C-amino acids 36 h after infection as described in Materials and Methods. Pulse-labeled cells (p) and cells chased for 3 h (c3), 6 h (c6), and 12 h (c12) were fractionated as described in Materials and Methods. Portions of each fraction containing 15 μg of protein were applied to a 7.5% acrylamide slab gel and run for 4.5 h at 10 mA. The nuclei represent NP-40 nuclei.*


HOMOGENATE
CYTOPLASM
p c3 c6 c12 p c3 c6 c12
II
IIa
III
IIIa
IV
56K
V
42K
Vc
NUCLEI
PLASMA MEMBRANES
p c3 c6 c12 p c3 c6 c12
II
IIa
III
IIIa
IV
56K
V
42K
Vc

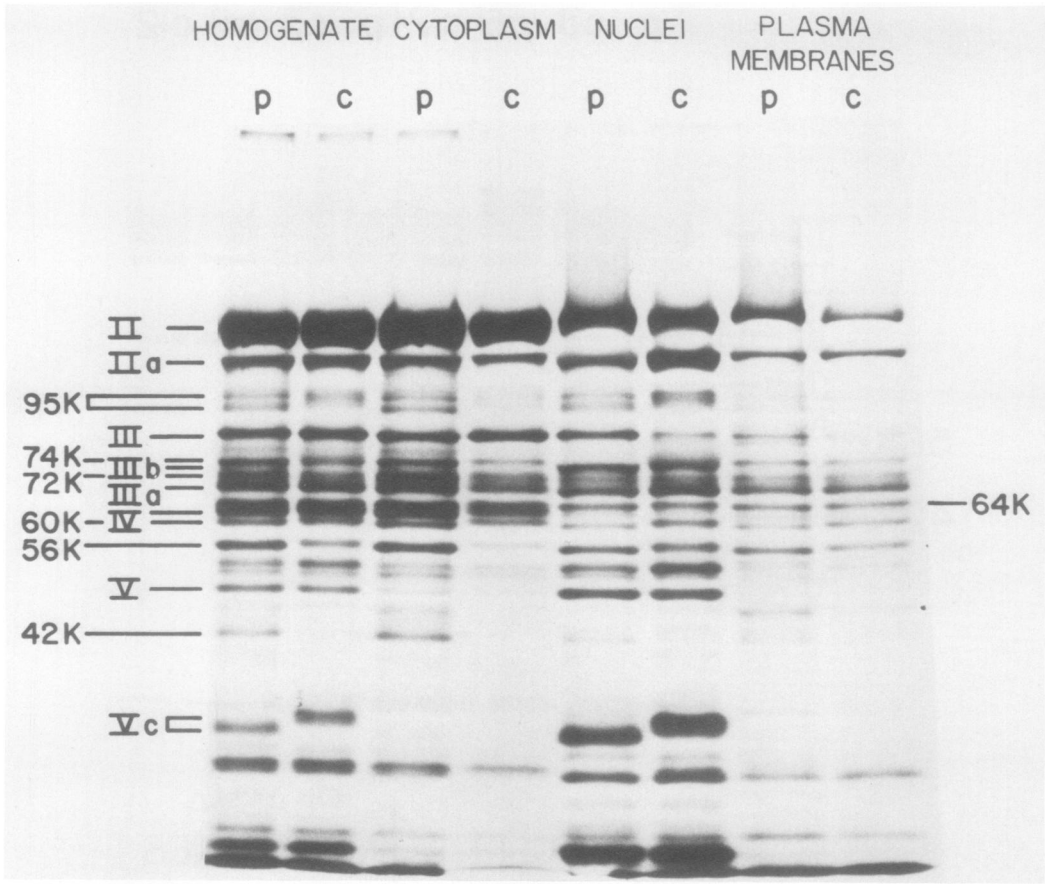


FIG. 7. *SDS-polyacrylamide gel fluorogram of pulsed and pulse-chased polypeptides in different cell fractions of Ad2⁺ND4-infected HeLa cells. HeLa S_3 cells were infected with Ad2⁺ND4 and pulse-labeled for 15 min with ¹⁴C-amino acids 38 h after infection as described in Materials and Methods. Pulse-labeled cells (p) and cells chased for 4 h (c) were fractionated as described in Materials and Methods. Samples of each fraction containing 15 μg of protein were applied to a 7.5% acrylamide slab gel and run for 4.5 h at 10 mA. The nuclei represent NP-40 nuclei.*

plasma membrane fraction after the pulse but not after the chase.

Ad2⁺ND5-infected cells. Pulse-chase experiments with cells infected by Ad2⁺ND5 reveal an interesting difference in metabolic stability between the 42K protein induced by Ad2⁺ND5 and the 42K protein induced by Ad2⁺ND2 (Fig. 8). Whereas the 42K protein is stable in the nucleus and the plasma membrane of Ad2⁺ND2-infected cells, the corresponding protein is unstable in all subcellular fractions of Ad2⁺ND5-infected cells.

In addition, pulse-chase and cell fractionation studies with cells infected by Ad2⁺ND1 have shown that the 28K protein is metabolically stable in all cell fractions (N. Axelrod, personal communication).

Reconstitution of cytoplasm and plasma membranes. To investigate the possibility that the 56K and 42K proteins in the cytoplasm of Ad2⁺ND2-infected cells may bind to plasma membranes after cell lysis, reconstitution experiments were performed. Unlabeled plasma membranes isolated from uninfected HeLa cells were incubated for 15 min at 0°C with total cytoplasm or 100,000 × *g* cytoplasmic supernatant from Ad2⁺ND2-infected cells pulse-labeled for 30 min with ¹⁴C-amino acids. The plasma membranes were repurified from the mixture and analyzed by gel electrophoresis. Figure 9 demonstrates that the unlabeled HeLa cell membranes adsorbed from the cytoplasm the Ad2 proteins, II and IIa, and the Ad2⁺ND2 56K and 42K proteins; however, proteins III and IV, present in large quantities in the cytoplasm, adsorbed less well. In a parallel experiment,

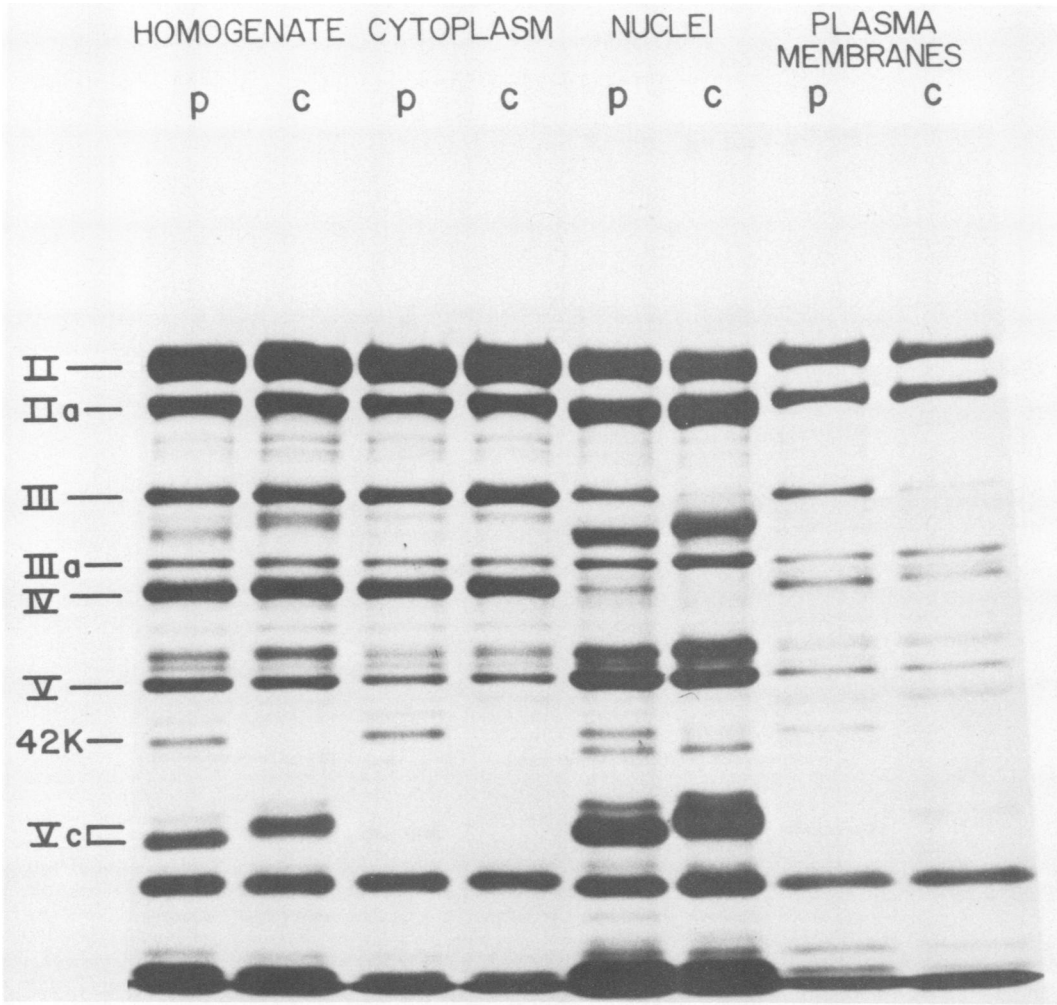


FIG. 8. *SDS-polyacrylamide gel fluorograms of pulsed and pulse-chased polypeptides in different cell fractions of Ad2⁺ND5-infected HeLa cells. HeLa S_3 cells were infected with Ad2⁺ND5 and pulse-labeled for 15 min with ^{14}C-amino acids 41 h after infection as described in Materials and Methods. Pulse-labeled cells (p) and cells chased for 3.5 h (c) were fractionated as described in Materials and Methods. Samples of each fraction containing 15 μg of protein were applied to a 7.5% acrylamide slab gel and run for 4.5 at 10 mA. The nuclei represent NP-40 nuclei.*

purified unlabeled HeLa cell membranes were mixed with cytoplasmic extracts from Ad2⁺ND2-infected cells that had been pulse-labeled with ^{14}C-amino acids for 30 min and chased with excess cold amino acids for 5.5 h. The polypeptide patterns of radioactive proteins adsorbed from the cytoplasm to the repurified plasma membranes are shown in Fig. 9. Only proteins II and IIa were bound in significant amounts to the reconstituted plasma membranes, whereas the 56K and 42K proteins were barely detectable. These experiments demonstrate that the 56K and 42K proteins bind to purified plasma membranes. The amounts of radioactive 56K and 42K proteins bound to purified plasma membranes in a reconstitution experiment are proportional to the amounts of radioactive 56K and 42K proteins present in the cytoplasm. In contrast, the amounts of radioactive 56K and 42K proteins found in plasma membranes isolated from Ad2⁺ND2-infected cells do not reflect the concentration of these proteins in the cytoplasm as shown by pulse-chase experiments.

Immunoprecipitation. It was recently shown that the 56K and 42K proteins of Ad2⁺ND2 are

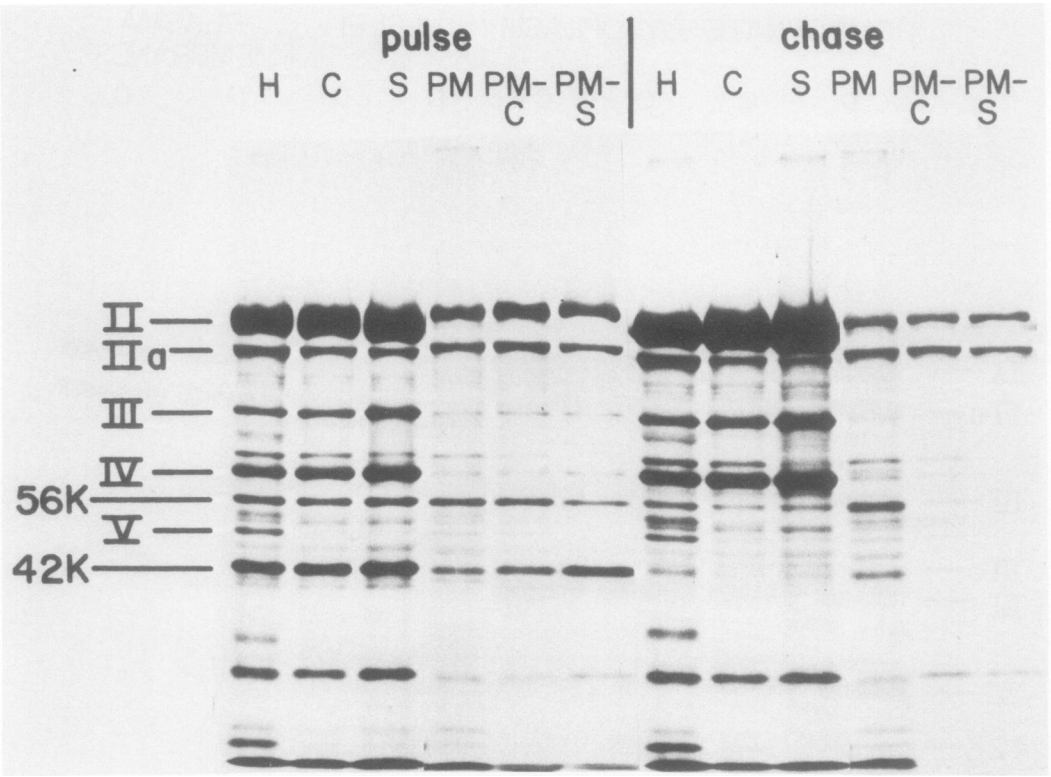


FIG. 9. *SDS-polyacrylamide gel fluorogram of pulsed and pulse-chased polypeptides in different cell fractions of Ad2⁺ND2-infected HeLa cells and of labeled polypeptides present in unlabeled plasma membranes of uninfected HeLa cells after reconstitution experiments. HeLa S_3 cells were infected with Ad2⁺ND2 and pulse-labeled for 30 min with ^{14}C-amino acids 35 h after infection as described in Materials and Methods. Pulse-labeled cells (pulse) and cells chased for 5.5 h (chase) were fractionated, and reconstitution experiments with unlabeled plasma membranes of uninfected HeLa cells were performed as described in Materials and Methods. Samples of each fraction containing 15 μg of protein were applied to a 7.5% acrylamide slab gel and run for 4.5 h at 10 mA. The following fractions were analyzed: (H) homogenate, (C) cytoplasm, (S) 100,000 × g cytoplasmic supernatant, (PM) plasma membrane, (PM-C) repurified plasma membranes of uninfected, unlabeled HeLa cells mixed with cytoplasm of labeled HeLa cells infected with Ad2⁺ND2, and (PM-S) repurified plasma membranes of uninfected, unlabeled HeLa cells mixed with 100,000 × g cytoplasmic supernatant of labeled HeLa cells infected with Ad2⁺ND2.*

immunoprecipitable with serum from hamsters bearing SV40 tumors (5a). Figure 10 demonstrates the results of immunoprecipitation experiments with extracts of Ad2⁺ND4- and Ad2⁺ND5-infected cells. The 60K, 64K, 72K, 74K, and 95K proteins of Ad2⁺ND4 were immunoprecipitable with SV40 antitumor serum (Fig. 10b and d). The 56K and 42K proteins of Ad2⁺ND4 were slightly detectable in the precipitates (Fig. 10d), presumably because they were present in small quantities in the infected cell extracts. The 42K protein of Ad2⁺ND5 was not immunoprecipitable (Fig. 10g). Though this protein occurred less abundantly than the Ad2⁺ND2 42K protein, attempts to immunoprecipitate equivalent absolute amounts of labeled 42K protein from both Ad2⁺ND2 and Ad2⁺ND5-infected cell extracts failed to precipitate any detectable 42K protein from Ad2⁺ND5. We were not able to immunoprecipitate the 28K proteins of Ad2⁺ND1 and Ad2⁺ND4. This may simply be a reflection of the absolute quantity of the protein in any given sample and, therefore, its apparent lack of antigenicity cannot be dismissed. Lewis and Rowe demonstrated that SV40 tumor bearing hamsters can develop T^+ U^- sera (19). When tested by immunofluorescence, our anti-T serum produced a very faint perinuclear staining, characteristic of U antigen (19), with Ad2⁺ND1 infected cells. Therefore, the inability of the SV40 anti-T serum to immunoprecipitate the

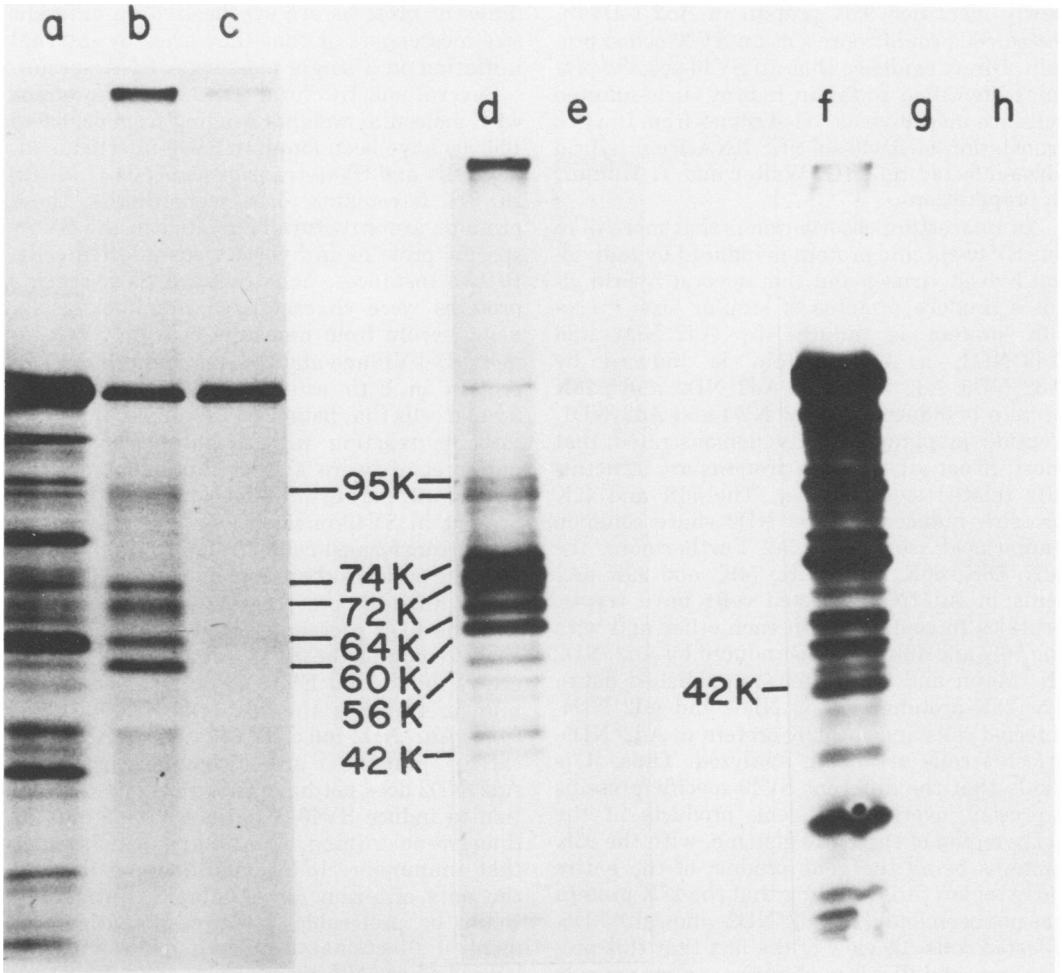


FIG. 10. *SDS-polyacrylamide gel fluorogram of [^{35}S]methionine polypeptides immunoprecipitated from hybrid-infected HeLa cell preparations with serum from SV40 tumor-bearing hamsters. HeLa S_3 cells were infected with Ad2$^+$ND4 or Ad2$^+$ND5 and labeled with [^{35}S]methionine. Extracts were prepared and immunoprecipitated as described in Materials and Methods. The sample order is (a) cell extract of Ad2$^+$Nd4-infected cells; (b) and (d) same, immunoprecipitated with SV40 antitumor serum; (c) and (e), same, immunoprecipitated with control serum; (f) cell extract of Ad2$^+$ND5-infected cells; (g) same, immunoprecipitated with SV40 antitumor serum; (h) same, immunoprecipitated with control serum. Immunoprecipitations shown in (b) and (c), and (d) and (e) were performed with cell extracts from separate infections. The presence of large quantities of gamma globulin in the immunoprecipitates causes a distortion of the gel pattern in the region of 50,000 daltons. To compare the migration of the precipitated polypeptides with the corresponding polypeptides in the extracts, an equivalent amount of gamma globulin was mixed with the extracts a and f prior to electrophoresis. Samples a, b, and c; d and e; and f, g, and h were analyzed on three separate 7.5% acrylamide slab gels and run for approximately 4.5 h at 10 mA.*

28K protein could also be due to a low titer of SV40 U antibody. Table 1 summarizes the properties of SV40-specific proteins induced by the different hybrid viruses.

DISCUSSION

As pointed out previously (30), the 28K, 56K, and 42K proteins induced by Ad2$^+$ND1, Ad2$^+$ND2, and Ad2$^+$ND5, respectively, correspond in size to the maximum coding capacity of the SV40 DNA segments contained in these hybrid viruses. Ad2$^+$ND4 could code for an SV40 95K protein, assuming that the SV40 DNA in Ad2$^+$ND4 represented 48% of the total SV40 genome and based on a molecular weight of 3.6×10^6 for the SV40 genome. Thus, the

newly identified 95K protein in Ad2+ND4-infected cells could represent an SV40-coded protein. Direct evidence that all SV40-specific proteins identified so far in hybrid virus-infected cells are indeed virus coded comes from in vitro translation of SV40-specific RNA from hybrid virus-infected cells (G. Walter and T. Hunter, in preparation).

An interesting observation is that more than one SV40-specific protein is induced by individual hybrid viruses and that several hybrid viruses produce proteins of similar size: i.e., a 56K protein is induced by Ad2+ND4 and Ad2+ND2; a 42K protein is induced by Ad2+ND5, Ad2+ND4, and Ad2+ND2; and a 28K protein is induced by Ad2+ND4 and Ad2+ND1. Peptide mapping directly demonstrated that most, if not all, of these proteins are structurally related to each other. The 56K and 42K proteins induced by Ad2+ND2 share common amino acid sequences (5a). Furthermore, the 42K, 56K, 60K, 64K, 72K, 74K, and 95K proteins in Ad2+ND4-infected cells have tryptic peptides in common with each other and with the 56K and 42K proteins induced by Ad2+ND2 (K. Mann and G. Walter, unpublished data). The 28K proteins in Ad2+ND1- and Ad2+ND4-infected cells and the 42K protein in Ad2+ND5-infected cells are being analyzed. Thus, it is likely that the different SV40-specific proteins represent overlapping gene products of the early region of the SV40 genome, with the 95K proteins being the gene product of the entire early region. It is puzzling that the 28K protein has not been found in Ad2+ND2- and Ad2+ND5-infected cells. In view of the fact that this protein is most difficult to identify, one cannot rule out that it is present in very small amounts.

The mechanism by which two or more SV40-specific proteins could be generated by one hybrid virus is presently unknown. We have excluded the possibility that the large number of SV40-specific proteins in Ad2+ND4-infected cells is due to a mixture of several different hybrid viruses present in the stock of Ad2+ND4. Ten different plaques of Ad2+ND4 were isolated and used to infect HeLa cells. The infected cells were labeled with ^{14}C-amino acids and analyzed by acrylamide gel electrophoresis. The number and quantity of SV40-specific proteins was identical in all 10 samples (unpublished data). No evidence that the smaller proteins are derived from the larger ones by post-translational cleavage was obtained from the pulse-chase experiments. Attempts to demonstrate a precursor-product relationship by treating infected cells with protease inhibitors have failed. It is possible, therefore, that the different proteins are synthesized on different size messengers or that they arise by internal initiation on a single messenger RNA species.

Several nonstructural SV40-specific proteins with molecular weights ranging from 60,000 to 100,000 have been found in SV40-infected monkey cells and SV40-transformed cells (1, 5, 12, 29, 31). It remains to be seen whether these proteins are structurally related to the SV40-specific proteins in hybrid virus-infected cells. In two instances, nonstructural SV40-specific proteins were characterized immunologically using serum from hamsters bearing SV40 tumors. Del Villano and Defendi identified a 70K protein in both acutely infected and transformed cells that bound specifically to immunosorbents reacting with T antigen (5). Tegtmeyer et al. were able to immunoprecipitate with antitumor serum a 100K protein, that was present in SV40-infected monkey cells and in SV40-transformed cells (29). Both groups infer that the proteins they identified are SV40 T antigen. Furthermore, Tegtmeyer et al. suggest that the 100K protein is coded for by the entire early region of the SV40 genome (29). We recently found that SV40 antitumor serum immunoprecipitates the 56K and 42K proteins from Ad2+ND2-infected cells (5a). Although these proteins are immunoprecipitable, Ad2+ND2 does not have enough SV40 information to induce SV40 T antigen, as defined by fluorescent staining. This finding demonstrates that immunoprecipitation cannot be used as the only criterion for defining T antigen. It would be preferable to define T antigen by using a functional rather than an immunological assay. We have shown that the SV40-specific proteins induced by Ad2+ND4 of molecular weights 42,000, 56,000, 60,000, 64,000, 72,000, 74,000, and 95,000 are also immunoprecipitable with antitumor serum. These data imply that structurally related proteins are expressed in SV40 tumors. It remains to be seen which of these proteins represents T antigen in a functional sense.

Of major interest is the finding that all SV40-specific proteins in cells infected by the different hybrid viruses can be found in purified plasma membranes. Proteins identified in purified plasma membranes could be either genuine components of plasma membranes in intact cells, or they could be adsorbed from the cytoplasm to plasma membranes in the course of cell fractionation. In the case of the 56K and 42K proteins, this problem does not arise because these proteins are metabolically unstable in the cytoplasm but are stable in the plasma membranes. Hence, it was possible to isolate

plasma membranes after a 12-h chase period containing a large quantity of radioactive 56K and 42K proteins, although the cytoplasmic pool of radioactive 56K and 42K proteins was almost depleted by this time (Fig. 6). We, therefore, conclude that the 56K and 42K proteins in Ad2+ND2-infected and the 56K proteins in Ad2+ND4-infected cells are plasma membrane proteins in intact cells.

Further support for the conclusion that the 56K and 42K proteins induced by Ad2+ND2 are real plasma membrane proteins comes from reconstitution experiments. The amounts of radioactive 56K and 42K proteins bound to isolated plasma membranes were proportional to the amounts of radioactive 56K and 42K proteins present in the cytoplasm; i.e., unlabeled plasma membranes bind more radioactive 56K and 42K proteins from cytoplasm containing a pool of these proteins with high specific radioactivity than from cytoplasm of cells containing a pool of these proteins with low specific radioactivity (Fig. 9). Therefore, the radioactive 56K and 42K proteins present in high amounts in plasma membranes after a long chase period cannot be bound to membranes during their isolation, but must represent an integral part of plasma membranes in intact cells. On the other hand, it seems likely that the 56K and 42K proteins found in plasma membranes after a short pulse are, at least in part, adsorbed from the cytoplasm after cell lysis. Reconstitution experiments may not be suitable for determining whether the metabolically stable proteins such as the 60K, 64K, 72K, and 74K proteins induced by Ad2+ND4 are plasma membrane proteins in vivo. Only those proteins that are metabolically unstable in the cytoplasm and stable in the plasma membrane can be identified as genuine plasma membrane proteins by pulse-chase and by reconstitution experiments.

By pulse-chase experiments, three classes of SV40-specific proteins can be distinguished with regard to their metabolic stability. (i) The 56K and 42K proteins in Ad2+ND2-infected cells are unstable in the cytoplasm and stable in nuclei and plasma membranes. Although the reasons for this behavior are unknown, it is possible that these proteins escape from proteolytic digestion in the cytoplasm by insertion into the lipid bilayer of nuclear or plasma membranes (6). Preliminary evidence suggests that the location of the 56K and 42K proteins in the nucleus is the nuclear membrane. Another possibility is that these proteins, when present in plasma membranes (or in the nuclear membrane), are modified and therefore become resistant to degradation. (ii) The higher molecular weight SV40-specific proteins in Ad2+ND4-infected cells are stable in all subcellular fractions, as is the 28K protein induced by Ad2+ND1. It is possible that a specific amino acid sequence in the 56K and 42K proteins is recognized by cellular proteolytic enzymes. The same region may not be exposed on the higher molecular weight SV40-specific proteins in Ad2+ND4-infected cells and may be absent in the 28K protein. (iii) The 42K protein induced by Ad2+ND5 is neither stable in nuclei and plasma membranes, nor stable in the cytoplasm. This finding is of particular interest since Ad2+ND5 does not induce any detectable SV40 antigens (20). Ad2+ND2, on the other hand, does induce U antigen and TSTA, and the 42K and the 56K proteins accumulate in the plasma membrane and in the nucleus in a stable form. This correlation between the ability of Ad2+ND2 to induce SV40 U antigen and TSTA and the stable incorporation of the 42K protein in nuclei and plasma membranes on one hand, and the absence of both events in Ad2+ND5-infected cells on the other, is compatible with the idea that the 42K protein in Ad2+ND2-infected cells is structurally related to SV40 U antigen and TSTA. Due to a small difference in structure, the 42K protein in Ad2+ND5-infected cells may not be able to associate in a stable form with the nuclear membrane or the plasma membrane. Peptide mapping experiments demonstrated that the 42K proteins of Ad2+ND2 and Ad2+ND5 share common peptides (K. Mann, T. Hunter, and G. Walter, unpublished data). On the other hand, the finding that the 42K protein of Ad2+ND5 is not immunoprecipitable, in contrast to the 42K protein of Ad2+ND2, indicates a structural difference between these proteins. The elucidation of this difference may shed light on the nature and function of the SV40-specific antigens U and TSTA.

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LITERATURE CITED

1. **Anderson, C. W., and R. F. Gesteland.** 1972. Pattern of protein synthesis in monkey cells infected by simian virus 40. J. Virol. 9:758–765.

2. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83–88.
3. **Brunette, D. M., and J. E. Till.** 1971. A rapid method for the isolation of L-cell surface membranes using an aqueous two-phase polymer system. J. Membr. Biol. 5:215–224.
4. **Crumpacker, C. S., P. H. Henry, T. Kakefuda, W. P. Rowe, M. J. Levin, and A. M. Lewis, Jr.** 1971. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. III. Base composition, molecular weight, and conformation of the $Ad2^+ND_1$ genome. J. Virol. 7:352–358.
5. **Del Villano, B. C., and V. Defendi.** 1973. Characterization of SV40 T antigen. Virology 51:34–46.
5a. **Deppert, W., and G. Walter.** 1976. Simian virus 40 (SV40) tumor-specific proteins in nucleus and plasma membrane of HeLa cells infected by adenovirus 2-SV40 hybrid virus $Ad2^+ND2$. Proc. Natl. Acad. Sci. U.S.A. 73:2505–2509.
6. **Goldberg, A. L., and J. F. Dice.** 1974. Intracellular protein degradation in mammalian and bacterial cells. Annu. Rev. Biochem. 43:835–869.
7. **Grodzicker, T., C. Anderson, P. A. Sharp, and J. Sambrook.** 1974. Conditional lethal mutants of adenovirus 2-simian virus 40 hybrids. I. Host range mutants of $Ad2^+ND1$. J. Virol. 13:1237–1244.
8. **Helling, R. B., H. M. Goodman, and H. W. Boyer.** 1974. Analysis of endonuclease R·*Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. J. Virol. 14:1235–1244.
9. **Henry, P. H., L. E. Schnipper, R. J. Samaha, C. S. Crumpacker, A. M. Lewis, Jr., and A. S. Levine.** 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VI. Characterization of the DNA from five nondefective hybrid viruses. J. Virol. 11:665–671.
10. **Johnson, F. B., H. L. Ozer, and M. D. Hoggan.** 1971. Structural proteins of adenovirus-associated virus type 3. J. Virol. 8:860–863.
11. **Kelly, T. J., Jr., and A. M. Lewis, Jr.** 1973. Use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. J. Virol. 12:643–652.
12. **Kiehn, E. D.** 1973. Protein metabolism in SV40-infected cells. Virology 56:313–333.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
14. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gel by fluorography. Eur. J. Biochem. 56:335–361.
15. **Lebowitz, P., T. J. Kelly, Jr., D. Nathans, T. N. H. Lee, and A. M. Lewis, Jr.** 1974. A colinear map relating the simian virus 40 (SV40) DNA segments of six adenovirus-SV40 hybrids to the DNA fragments produced by restriction endonuclease cleavage of SV40 DNA. Proc. Natl. Acad. Sci. U.S.A. 71:441–445.
16. **Levin, M. J., C. S. Crumpacker, A. M. Lewis, Jr., M. N. Oxman, P. H. Henry, and W. P. Rowe.** 1971. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. II. Relationship of adenovirus 2 deoxyribonucleic acid and simian virus 40 deoxyribonucleic acid in the $Ad2^+ND_1$ genome. J. Virol. 7:343–351.
17. **Lewis, A. M., Jr., M. J. Levin, W. H. Wiese, C. S. Crumpacker, and P. H. Henry.** 1969. A non-defective (competent) adenovirus-SV40 hybrid isolated from the Ad2-SV40 hybrid population. Proc. Natl. Acad. Sci. U.S.A. 63:1128–1135.
18. **Lewis, A. M., Jr., A. S. Levin, C. S. Crumpacker, M. J. Levin, R. J. Samaha, and P. H. Henry.** 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. V. Isolation of additional hybrids which differ in their simian virus 40-specific biological properties. J. Virol. 11:655–664.
19. **Lewis, A. M., Jr., and W. P. Rowe.** 1971. Studies on nondefective adenovirus-simian virus 40 hybrid viruses. I. A newly characterized simian virus 40 antigen induced by the $Ad2^+ND_1$ virus. J. Virol. 7:189–197.
20. **Lewis, A. M., Jr., and W. P. Rowe.** 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VIII. Association of simian virus 40 transplantation antigen with a specific region of the early viral genome. J. Virol. 12:836–840.
21. **Lopez-Revilla, R., and G. Walter.** 1973. Polypeptide specific for cells with adenovirus 2-SV40 hybrid $Ad2^+ND1$. Nature (London) New Biol. 244:165–167.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
23. **Maizel, J. V. Jr.,** 1971. Polyacrylamide gel electrophoresis of viral proteins, p. 179–246. *In* K. Maramorosh and H. Koprowski (ed.), Methods in virology, vol. 5. Academic Press Inc., New York.
24. **Morrow, J. F., and P. Berg.** 1972. Cleavage of simian virus 40 DNA at a unique site by a bacterial restriction enzyme. Proc. Natl. Acad. Sci. U.S.A. 69:3365–3369.
25. **Morrow, J. F., P. Berg, T. J. Kelly, Jr., and A. M. Lewis, Jr.** 1973. Mapping of simian virus 40 early functions on the viral chromosome. J. Virol. 12:653–658.
26. **Penman, S.** 1969. Preparation of purified nuclei and nucleoli from mammalian cells, p. 35–48. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
27. **Rose, J. A., J. V. Maizel, Jr., J. K. Inman, and A. J. Shatkin.** 1971. Structural proteins of adenovirus-associated viruses. J. Virol. 8:766–770.
28. **Tai, H. T., C. A. Smith, P. A. Sharp, and J. Vinograd.** 1972. Sequence heterogeneity in closed simian virus 40 deoxyribonucleic acid. J. Virol. 9:317–325.
29. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Randell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene *A*. J. Virol. 16:168–178.
30. **Walter, G., and H. Martin.** 1975. Simian virus 40-specific proteins in HeLa cells infected with nondefective adenovirus 2-simian virus 40 hybrid viruses. J. Virol. 16:1236–1247.
31. **Walter, G., R. Roblin, and R. Dulbecco.** 1972. Protein synthesis in simian virus 40-infected monkey cells. Proc. Natl. Acad. Sci. U.S.A. 69:921–924.
32. **Williams, J. F.** 1970. Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. J. Gen. Virol. 9:251–255.