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Simian Virus 40-Specific Proteins in HeLa Cells Infected with Nondefective Adenovirus 2-Simian Virus 40 Hybrid Viruses

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The synthesis of simian virus 40 (SV40)-specific proteins in HeLa cells infected with the nondefective adenovirus 2 (Ad2)-SV40 hybrid viruses, Ad2$^+$ ND2, Ad2$^+$ ND3, Ad2$^+$ ND4, and Ad2$^+$ ND5, was investigated. Infected-cell proteins were labeled with radioactive amino acids late after infection, when host protein synthesis was shut off, and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. All polypeptides normally seen in Ad2-infected cells were found in cells infected by the hybrid viruses. In addition to the Ad2-specific proteins, cells infected with Ad2$^+$ ND2 contain two SV40-specific proteins with apparent molecular weights of 42,000 and 56,000, cells infected with Ad2$^+$ ND4 contain one protein with an apparent molecular weight of 56,000, and cells infected with Ad2$^+$ ND5 contain one protein with an apparent molecular weight of 42,000. Cells infected with Ad2$^+$ ND3 do not contain detectable amounts of proteins not seen during Ad2 infection. Pulse-chase experiments demonstrate that the SV40-specific proteins induced by Ad2$^+$ ND2, Ad2$^+$ ND4, and Ad2$^+$ ND5 are metabolically unstable. These proteins are not present in purified virions. Two nonstructural Ad2-specific proteins have been demonstrated in Ad2 and hybrid virus-infected cells which have a smaller apparent molecular weight after a short pulse than after a pulse followed by a chase. The molecular weight increase during the chase may be caused by the addition of carbohydrate to a polypeptide backbone.

The expression of the simian virus 40 (SV40) genome during productive infection occurs in two phases. The early phase is characterized by the induction of the SV40-specific antigens, T (13), tumor-specific transplantation antigen (TSTA) (8), and U (26), and by the induction of cellular DNA synthesis (10, 40). The late phase begins with the synthesis of viral DNA and structural proteins.

Little is known about the chemical and biological properties of the early antigens, T, TSTA, and U. They could be either proteins coded by the SV40 genome (early proteins) or, alternatively, cellular products induced or modified by the early viral proteins. The major obstacle to the detection and characterization of early SV40 proteins and early antigens is the continuation of host protein synthesis in SV40-infected cells. At present, no methods are available for selectively inhibiting host protein synthesis and making the clear detection of early proteins possible.

The nondefective adenovirus 2 (Ad2)-SV40 hybrid viruses, isolated by Lewis and co-workers, are a useful tool for the study of early SV40 proteins. They have the following properties: each of the five nondefective hybrid viruses, Ad2$^+$ ND1 (24), Ad2$^+$ ND2, Ad2$^+$ ND3, Ad2$^+$ ND4, and Ad2$^+$ ND5 (25), contains a single continuous segment of SV40 DNA covalently inserted at the same site in the Ad2 genome (3, 12, 16, 21, 32). The SV40 DNA segments differ in size: Ad2$^+$ ND1 contains 17 to 20%; Ad2$^+$ ND2, 32 to 36.5%; Ad2$^+$ ND3, 7%; Ad2$^+$ ND4, 43 to 48%; and Ad2$^+$ ND5, 28% of the SV40 genome (16, 20, 31, 32). The segments are completely overlapping and colinear with SV40 DNA (16, 20) (Fig. 1). They have a common end point which is located in the *Haemophilus influenzae* restriction endonuclease fragment G of SV40 DNA and is 0.11 map units of the SV40 genome away from the cleavage site of EcoR1 restriction endonuclease (16, 20, 31, 32). All SV40 DNA segments contain at their common end point a major fraction of the Hin G fragment (20) which is located in the late region of the SV40 genome. During productive infection with the nondefective hybrid viruses, the SV40 DNA segment contained in each hybrid virus is fully transcribed (18, 23). The transcripts are complementary to the minus (early) strand of SV40 DNA and consist of both early and

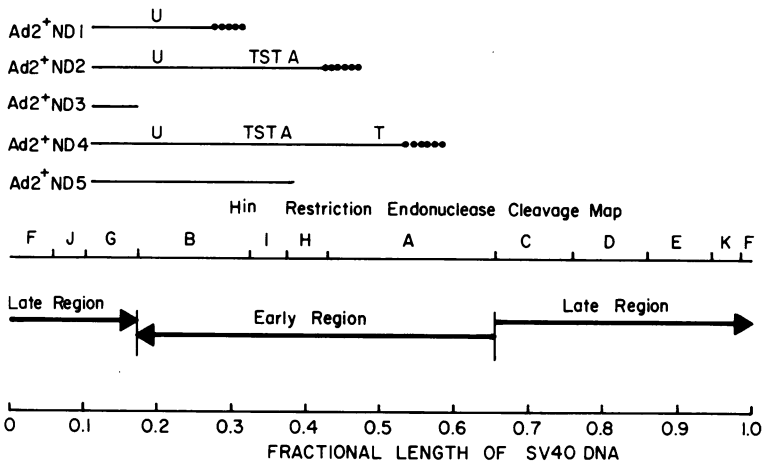

FIG. 1. *SV40 DNA segments in nondefective Ad2-SV40 hybrid viruses in relation to the Haemophilus influenza restriction endonuclease cleavage map of SV40. The position of the early and late gene regions in SV40 DNA is derived from the results of Khoury et al. (17) and Dhar et al. (5). Arrows indicate the direction of transcription. The dotted lines indicate the degree of variation in the length of the SV40 DNA segments as determined by different investigators (16, 20, 31, 32). The Haemophilus influenza cleavage map is derived from Danna et al. (4).*

antilate sequences (18). Ad2$^+$ ND4, containing the largest segment of the SV40 genome, induces the synthesis of three SV40-specific antigens: T, TSTA, and U (25, 27). Ad2$^+$ ND2 induces TSTA and U, and Ad2$^+$ ND1 induces only U antigen (25–27). The SV40 DNA regions in these hybrid viruses, which determine the expression of the early SV40 antigens, have been mapped on the SV40 genome (16, 20).

The expression of the SV40 information in the nondefective hybrid viruses is controlled by the Ad2 genome and takes place at a late time during productive infection, that is, after hybrid viral DNA synthesis has been initiated (23). It is this property of the hybrid viruses which facilitates the identification and characterization of early SV40 proteins, since late during infection host protein synthesis is shut off by the adenovirus genome. As a consequence, it may be possible to detect early SV40 proteins or SV40-specific antigens in hybrid virus-infected cells by labeling with radioactive amino acids late in the infection cycle and then analyzing the labeled proteins by gel electrophoresis and autoradiography. It has recently been shown that HeLa cells infected with Ad2$^+$ ND1 synthesize a polypeptide of molecular weight 28,000 which is not present in Ad2-infected cells (9, 28). In this report, we demonstrate the presence of specific proteins in cells infected by Ad2$^+$ ND2, Ad2$^+$ ND4, and Ad2$^+$ ND5. We have provisionally designated these proteins as "SV40 specific," since their expression is caused by the SV40 information contained in the hybrid virus genomes.

MATERIALS AND METHODS

Cells. HeLa S3 cells were grown in suspension in Eagle minimum essential medium supplemented with 5% calf serum or in monolayer in Dulbecco's modification of Eagle medium (DMEM) with 10% calf serum. HeLa S3 cells were only used for labeling experiments. For adenovirus plaque assays, a different type of HeLa cell with a flatter morphology was used. These cells were obtained from the Cold Spring Harbor Laboratory and grown in DMEM supplemented with 10% calf serum. CV_1 cells, a line of African green monkey kidney cells (AGMK), were obtained from the American Type Culture Collection and grown in DMEM supplemented with 10% fetal calf serum. Primary AGMK cells were purchased from Microbiological Associates, Inc., and grown in DMEM with 10% fetal calf serum.

Viruses. Seed stocks of nondefective Ad2-SV40 hybrid viruses Ad2$^+$ ND1, Ad2$^+$ ND2, Ad2$^+$ ND3, Ad2$^+$ ND4, and Ad2$^+$ ND5 were obtained from A. M. Lewis, Jr. Stocks of Ad2$^+$ ND1, Ad2$^+$ ND2, and Ad2$^+$ ND4 were prepared in CV_1 and HeLa S3 cells. Stocks of Ad2, Ad2$^+$ ND3, and Ad2$^+$ ND5 were prepared in HeLa S3 cells.

Hybrid stocks of Ad2$^+$ ND1, Ad2$^+$ ND2, Ad2$^+$ ND3, and Ad2$^+$ ND5 were shown to be free of adeno-associated viruses by cesium chloride density centrifugation, high multiplicity infections, and heat sensitivity assays which will be described elsewhere. All stocks of Ad2$^+$ ND4, however, were contaminated with adeno-associated virus.

Plaque titrations were routinely performed on HeLa and CV_1 cells. In one experiment, primary AGMK cells were used to assay Ad2, Ad2$^+$ ND1, Ad2$^+$ ND2, and Ad2$^+$ ND4. The assays were performed as described by Williams (41). The overlay medium for HeLa cells contained 0.6% agar (Difco) and 0.9% agar for CV_1 and AGMK cells. After incubation for 3 to 4

days, 2 ml of additional overlay medium was added. Overlay medium containing neutral red and 0.9% agar was added 6 to 7 days after infection of HeLa cells, and 8 to 10 days after infection of CV_1 and AGMK cells. $MgCl_2$ was omitted from the overlay medium when plaque assays were performed on CV_1 and AGMK cells. The plaque titers of virus stocks prepared in HeLa or CV_1 cells are shown in Table 1. Plaque formation in CV_1 cells was less efficient than in AGMK cells. Whereas Ad2$^+$ ND1 and Ad2$^+$ ND4 formed plaques with the same efficiency on HeLa and AGMK cells, Ad2$^+$ ND2 formed 10-fold less plaques on AGMK cells than on HeLa cells. Ad2 showed at least a 1,000-fold difference in plaque efficiency between HeLa and AGMK cells (Table 1).

The high titer stocks of Ad2$^+$ ND3 and Ad2$^+$ ND5 up to dilutions of 10^{-3} produced strong cytopathic effect (CPE) in the entire CV_1 monolayer, preventing the detection of plaques. Neither CPE nor plaques were observed at dilutions of 10^{-4}. Ad2 also produced total CPE on CV_1 cells up to a dilution of 10^{-3}, but a small number of plaques at a dilution of 10^{-4}. This observed CPE is presumably caused by a high concentration of free pentons in these high titer stocks and can be eliminated by trypsin treatment (9). Since in our experiments trypsin treatment of the high titer stocks was not performed, the titers of 10^3 PFU/ml of Ad2$^+$ ND3 and Ad2$^+$ ND5 on CV_1 cells only represent maximum estimates. A similar CPE was produced by the high titer Ad2 stock when assayed on AGMK cells. In this case, CPE was observed up to a dilution of 10^{-6}. It is likely, therefore, that the Ad2 titer of 10^6 PFU/ml on AGMK cells represents an overestimation. The titers of Ad2$^+$ ND3 and Ad2$^+$ ND5 stocks were not determined on AGMK.

Infection and labeling of cells. For labeling experiments, 2×10^6 HeLa S3 cells were seeded on 3-cm plastic petri dishes (NUNC, Denmark) and infected 24 h later with 0.2 ml of virus. After an adsorption period of 1 h, 2 ml of growth medium, DMEM containing 10% calf serum, was added per dish. For labeling cells, the culture medium was changed to labeling medium containing 2.5% of the normal level of amino acids, 5% calf serum, and 20 μCi of ^{14}C-labeled L-amino acid mixture per ml. A 0.6-ml amount of labeling medium was added per dish. After labeling, the cell layer was washed three times with DMEM. The cells were lysed on the dish with 0.2 ml of electrophoresis sample buffer (0.0625 M Tris, pH 6.8; 3% sodium dodecyl sulfate [SDS]; 5% 2-mercaptoethanol; 10% glycerol; 0.001% bromphenol blue; 2 mM phenylmethylsulfonyl fluoride). Glycerol and bromphenol blue were omitted until after boiling. The lysates were sonically treated for 10 s with a Branson sonifier at position 4 and heated for 2 min in a boiling water bath.

Polyacrylamide gel electrophoresis and scintillation autography (fluorography). The polyacrylamide gel system of Laemmli and Maizel as described by Laemmli (19) was employed, except that the gels were formed as slabs 1 mm thick and 9.5 cm long between glass plates. The general apparatus and methodology for gel electrophoresis have been described elsewhere (29). The ratio of acrylamide to bisacrylamide was 30 to 0.8. A 10-μl volume of cell lysate in electrophoresis sample buffer, containing 5 to 10 μg of protein and approximately 20,000 to 100,000 counts/min, were loaded per slot. Electrophoresis was performed at a constant current of 10 mA. After electrophoresis, the gels were prepared for fluorography, which is 10 times more sensitive than conventional autoradiography, as described by Bonner and Laskey (2). Briefly, the gels were dehydrated in dimethyl sulfoxide, soaked in a solution of 2,5-diphenyloxazole in dimethyl sulfoxide, immersed in water to remove dimethyl sulfoxide, dried and exposed to medical X-ray film (Kodak, RP Royal X-omat, RP/R54) at −70 C.

The purity of the chemicals used for gel electrophoresis can influence the relative mobility of proteins and the quality of separations of complex mixtures of proteins (36). Therefore, we list the source of the reagents used in this study: acrylamide (Eastman 5521) was recrystallized from chloroform; bisacrylamide (Eastman 8383); *N,N,N',N'*-tetramethylethylenediamine (Eastman 8178); glycine (Eastman 445); SDS (BDH, specially pure, 30176); Tris (Trizma base, reagent grade, T-1503); ammonium persulfate (Baker 0762); and 2-mercaptoethanol (Eastman 4196).

The molecular weight of proteins was determined by comparing their mobility on SDS polyacrylamide gels with proteins of known molecular weight. The following marker proteins were used: Ad2 proteins as characterized by Maizel et al. (30), Ishibashi and Maizel (14), and Everitt et al. (7); β-galactosidase of *Escherichia coli* (135,000 daltons); bovine serum albumin (68,000 daltons); ovalbumin (43,000 daltons); chymotrypsinogen (25,700 daltons); and cytochrome *c* from horse heart (12,270 daltons).

Labeling and purification of virions. HeLa S3

TABLE 1. *Plaque-forming ability of Ad2-SV40 hybrid viruses*

Virus	Infectivity (PFU/ml)			Stock prepared in
	HeLa	CV_1	AGMK	
Ad2$^+$ ND1	9.2×10^6	7.0×10^4	1.1×10^7	CV_1
Ad2$^+$ ND2	2.4×10^6	1.2×10^2	2.5×10^5	CV_1
Ad2$^+$ ND3	4.5×10^9	$<10^3$	ND[a]	HeLa
Ad2$^+$ ND4	6.9×10^6	2.2×10^4	3.0×10^6	CV_1
Ad2$^+$ ND5	1.7×10^{10}	$<10^3$	ND	HeLa
Ad2	3.5×10^9	8.0×10^4	$<10^6$	HeLa

[a] ND, Not done.

cells, 2×10^6 per 10-cm dish, were seeded in DMEM containing 10% calf serum. After 24 h, parallel cultures were infected with Ad2, Ad2+ ND1, Ad2+ ND2, Ad2+ ND3, Ad2+ ND4, and Ad2+ ND5. The multiplicity of infection was approximately 1 to 2 PFU/cell. After 21 h of infection, the growth medium was replaced by labeling medium containing one-tenth of the normal level of amino acid, 5% calf serum, and 6.5 μCi of ^{14}C-labeled L-amino acid mixture per ml. Five milliliters of labeling medium was added per dish. After 52 h of infection, the labeling medium was removed, and the cells were washed once with DMEM and scraped from the culture dishes in 0.01 M Tris, pH 8.0. The virus purification was performed as described previously (30). After centrifugation in CsCl, the purified virions were dialyzed first against 0.01 M Tris, pH 7.4, at 4 C and then against 0.1% SDS at room temperature. They were concentrated by lyophilization and dissolved in electrophoresis sample buffer. This procedure for concentrating virus was preferred since precipitation of virus with trichloroacetic acid in some instances resulted in a selective loss of the virion polypeptide VII. The reason for this loss is unknown.

RESULTS

Induction of SV40-specific proteins by Ad2+ ND1, Ad2+ ND2, Ad2+ ND4, and Ad2+ ND5. Monolayers of HeLa cells were infected with Ad2 and the non-defective hybrid viruses, Ad2+ ND1, Ad2+ ND2, Ad2+ ND3, Ad2+ ND4, and Ad2+ ND5, and labeled from 38 to 40 h after the infection with ^{14}C-labeled amino acids. The infected cells were lysed in SDS containing buffer (Materials and Methods). The radioactive polypeptides of the lysates were analyzed by SDS acrylamide gel electrophoresis and visualized by fluorography (2) as described above. Figure 2 shows a fluorogram of a 7.5 to 30% acrylamide gradient gel. The patterns of Ad2-specific proteins from Ad2 and hybrid virus-infected cells were similar to those previously described (1, 14, 39). In addition to the adenovirus-specific proteins, Ad2+ ND1-infected cells contained a polypeptide with a molecular weight of 28,000 (28 K protein) which has been described previously (9, 28). It migrated very close to two adenovirus-specific proteins, Va and Vb, and in our hands was separable from these only in 7.5 to 30% gradient gels. Since it was present only in small quantity, a long exposure time of the fluorogram was required for its detection. Ad2+ ND2 induced two SV40-specific polypeptides with molecular weights of 42,000 (42 K protein) and 56,000 (56 K protein). Ad2+ ND4 induced one SV40-specific protein with a molecular weight of 56,000. In several Ad2+ ND4 infections this protein was consistently detected in smaller quantity than the 56 K protein in Ad2+ ND2-infected cells. A protein of 42,000 molecular weight was not detectable in Ad2+ ND4-infected cells, using a variety of different gel conditions and after long exposure of the gel to the X-ray film.

Three other proteins with apparent molecular weights of 96,000, 85,000, and 65,000 were synthesized only in Ad2+ ND4-infected cells. They were structural proteins of adeno-associated virus (15, 35) present as a contaminant in our Ad2+ ND4 stock. The 96,000 dalton AAV protein was clearly detectable in a 7.5 to 30% acrylamide gradient gel (Fig. 2), whereas the 85,000 and the 65,000 dalton AAV proteins comigrated with Ad2 protein III and IIIa/IV, respectively. Under alternate gel conditions, these AAV proteins have been separated from the Ad2 proteins, the 85,000 dalton AAV protein from Ad2 protein III by prolonged gel electrophoresis and the 65,000 dalton AAV protein from Ad2 proteins IIIa and IV by raising the pH of the polyacrylamide gel buffer from 8.8 to 9.3. Under these conditions, the 96,000, 85,000, and 65,000 molecular weight proteins from Ad2+ ND4-infected cells were shown to comigrate with the three structural proteins of purified AAV isolated from Ad2+ ND4-infected CV_1 and HeLa cells (manuscript in preparation).

Although cells infected with Ad2+ ND3 and Ad2+ ND5 contain SV40-specific RNA sequences (23), they do not synthesize detectable amounts of SV40-specific antigens (25, 27). Interestingly, Ad2+ ND5-infected cells synthesized a polypeptide late in infection that was not detectable in Ad2-infected cells and that comigrated with the Ad2+ ND2-induced 42 K protein (Fig. 2). No additional proteins in Ad2+ ND3-infected cells could be detected by any of the analytical gel conditions that we tested.

Time of synthesis of SV40-specific proteins. To determine the time course of the appearance of the SV40-specific proteins, HeLa cells were infected with Ad2+ ND2, Ad2+ ND4, and Ad2+ ND5 and labeled with ^{14}C-labeled amino acids at different times after infection. Figure 3 shows that the 56 K and 42 K proteins induced by Ad2+ ND2 can first be detected at 23 h after infection simultaneously with the appearance of structural Ad2 proteins. Their synthesis continues late in infection when host protein synthesis is shut off almost completely. A similar time course was observed for the synthesis of the 42 K protein in cells infected by Ad2+ ND5 (data not shown). The beginning of synthesis of the 56,000 dalton protein in cells infected by Ad2+ ND4 could not be measured because this protein was present in very small quantity.

Metabolic stability of the SV40 proteins. Pulse-chase experiments were performed to examine the metabolic stability of the

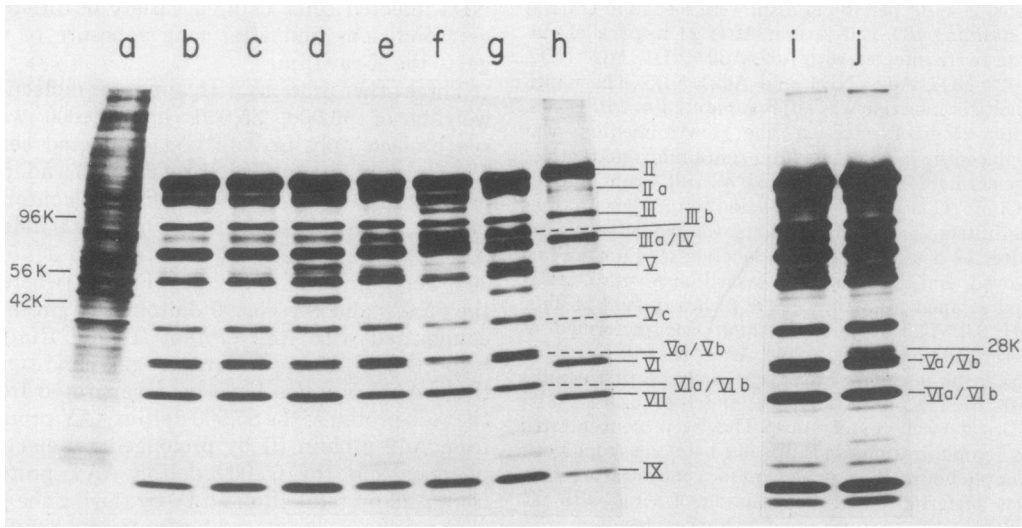

FIG. 2. *SDS polyacrylamide gel fluorogram of ^{14}C polypeptides from cells infected by nondefective Ad2-SV40 hybrid viruses. Parallel cultures of HeLa S3 cells were infected with the five nondefective Ad2-SV40 hybrid viruses and labeled with ^{14}C amino acids. The multiplicity of infection was approximately 10 to 50 PFU/cell. The time of labeling was from 38 through 40 h after infection. A mock-infected control was similarly treated. Samples of 10 μl were applied to a 7.5 to 30% acrylamide gradient gel. A constant current of 10 mA was applied for 5 h and 45 min. The sample order is: mock-infected cells (a), cells infected by Ad2 (b), Ad2$^+$ ND1 (c), Ad2$^+$ ND2 (d), Ad2$^+$ ND3 (e), Ad2$^+$ ND4 (f), Ad2$^+$ ND5 (g), purified Ad2 virions (h). The fluorographic exposure time for (a) to (h) was 40 h; (i) and (j) show exposure time of 6 days of samples (b) and (c). Procedures in detail are described in text. Ad2 polypeptides were designated with Roman numerals according to Maizel et al. (30) and Ishibashi and Maizel (14). Polypeptides VIa and VIb are not separated under the gel conditions used here. They are separable, however, on a 7.5 to 30% gradient slab gel of 20-cm length (data not shown). Polypeptides IIIb and Vc are described in "Metabolic stability of the SV40-specific proteins". No distinction was made between the designation of Ad2 virion proteins and Ad2-specific proteins in infected cells. The samples of Ad2$^+$ ND3- and Ad2$^+$ ND5-infected cells contained about twice as much protein as the other infected-cell samples. This resulted in incomplete separation of polypeptides II and IIa in samples (e) and (g).*

SV40-specific proteins. Ad2-, Ad2$^+$ ND2-, and Ad2$^+$ ND4-infected cells were labeled with radioactive amino acids from 39 to 41 h after infection and compared with cells labeled at the same time and chased with unlabeled amino acids for 12 h. Figure 4 demonstrates that within a period of 12 h, the 56 K and 42 K proteins induced by Ad2$^+$ ND2 and the 56 K protein induced by Ad2$^+$ ND4 disappeared almost completely. Further experiments with Ad2$^+$ ND2-infected cells labeled for 2 h and chased for either 2, 4, 6, or 10 h suggest that the half-life of the 56 K and 42 K proteins is less than 4 h (Fig. 5). Cells labeled for only 15 min show relatively more label incorporated in these two proteins than cells labeled for 2 h (Fig. 5), again indicating their metabolic instability. Pulse-chase experiments with Ad2$^+$ ND5 suggest that the half-life of the Ad2$^+$ ND5-induced 42 K protein is as short as that of the two Ad2$^+$ ND2-induced proteins (data not shown). It should be pointed out that the degradation of both Ad2$^+$ ND2-specific proteins during the chase did not result in the formation of discrete and stable smaller polypeptides of molecular weights between 42,000 and 12,000, since the polypeptide patterns of Ad2 and Ad2$^+$ ND2-infected cells after a 12 h chase looked indistinguishable in this molecular weight range.

Two characteristic changes which occurred during the chase of both Ad2 and hybrid virus-infected cells (Fig. 4 and 5) have been described recently. Anderson et al. (1) demonstrated that the polypeptide probably corresponding to VIa/VIb in this paper is the precursor of virion polypeptide VII. Ishibashi and Maizel (14) have shown that the polypeptides Va, Vb, VIa, and VIb are present in "young" virions and absent in "aged" virions. They have suggested that Va, Vb, and VIa could be the precursors to VI, VIII, and VII, respectively.

Two other changes were observed to be common to both adenovirus and hybrid virus-infected cells. A polypeptide designated Vc in Fig. 4 has a slightly smaller apparent molecular weight after a 2 h pulse than after a 2 h pulse

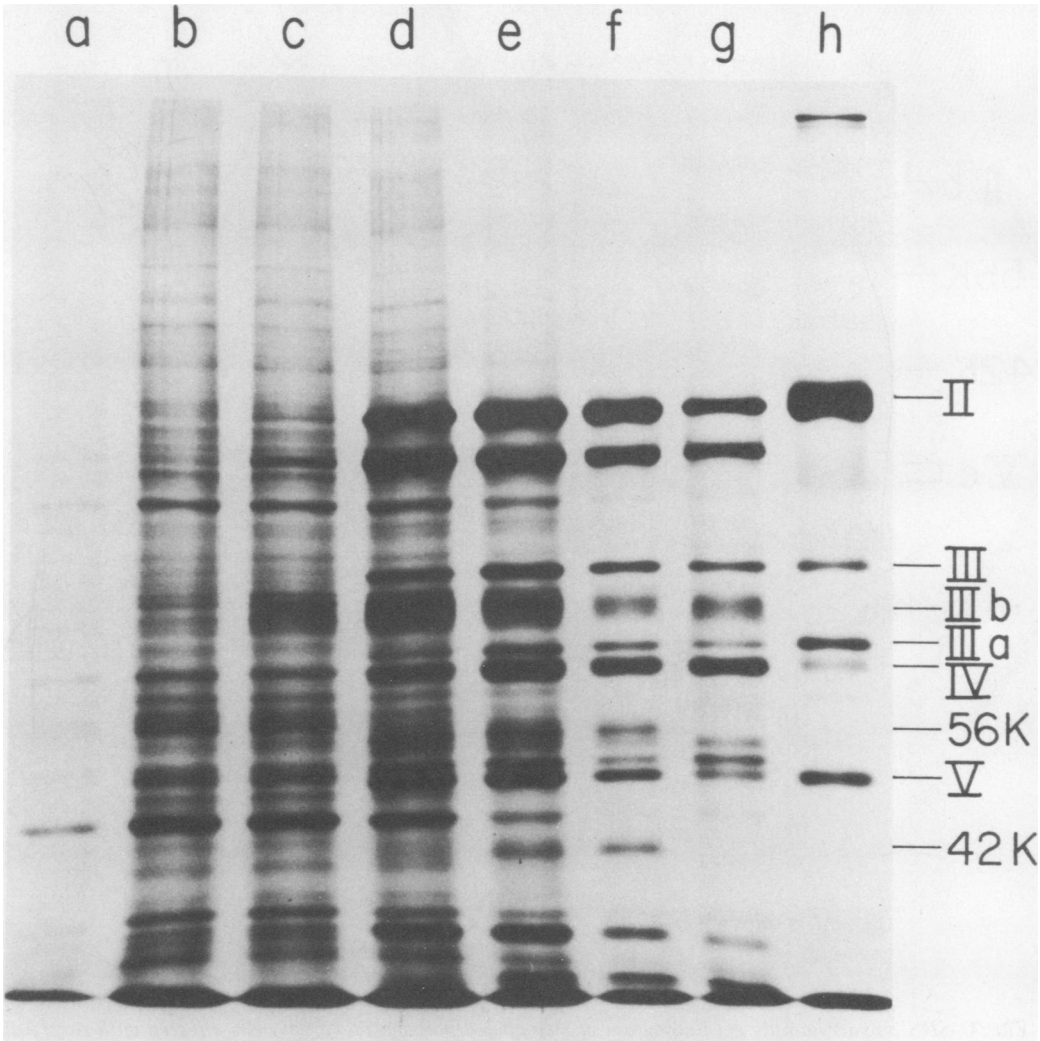

FIG. 3. *SDS polyacrylamide gel fluorogram of ^{14}C-labeled polypeptides from cells infected by Ad2$^+$ ND2; time course of the synthesis of the 56 K and 42 K proteins. Parallel cultures of HeLa S3 cells were infected with Ad2$^+$ ND2 and labeled for 2 h with ^{14}C-labeled amino acids (see text) at different times after infection. The multiplicity of infection was approximately 100 PFU/cell. A 7.5% acrylamide gel was run for 6 h at a constant current of 10 mA. The sample order is: (a) mock-infected cells, (b) Ad2$^+$ ND2-infected cells labeled 9 through 11 h, (c) 15 through 17 h, (d) 21 through 23 h, (e) 27 through 29 h, (f) 34 through 36 h after infection, (g) Ad2-infected cells, (h) purified Ad2 virions.*

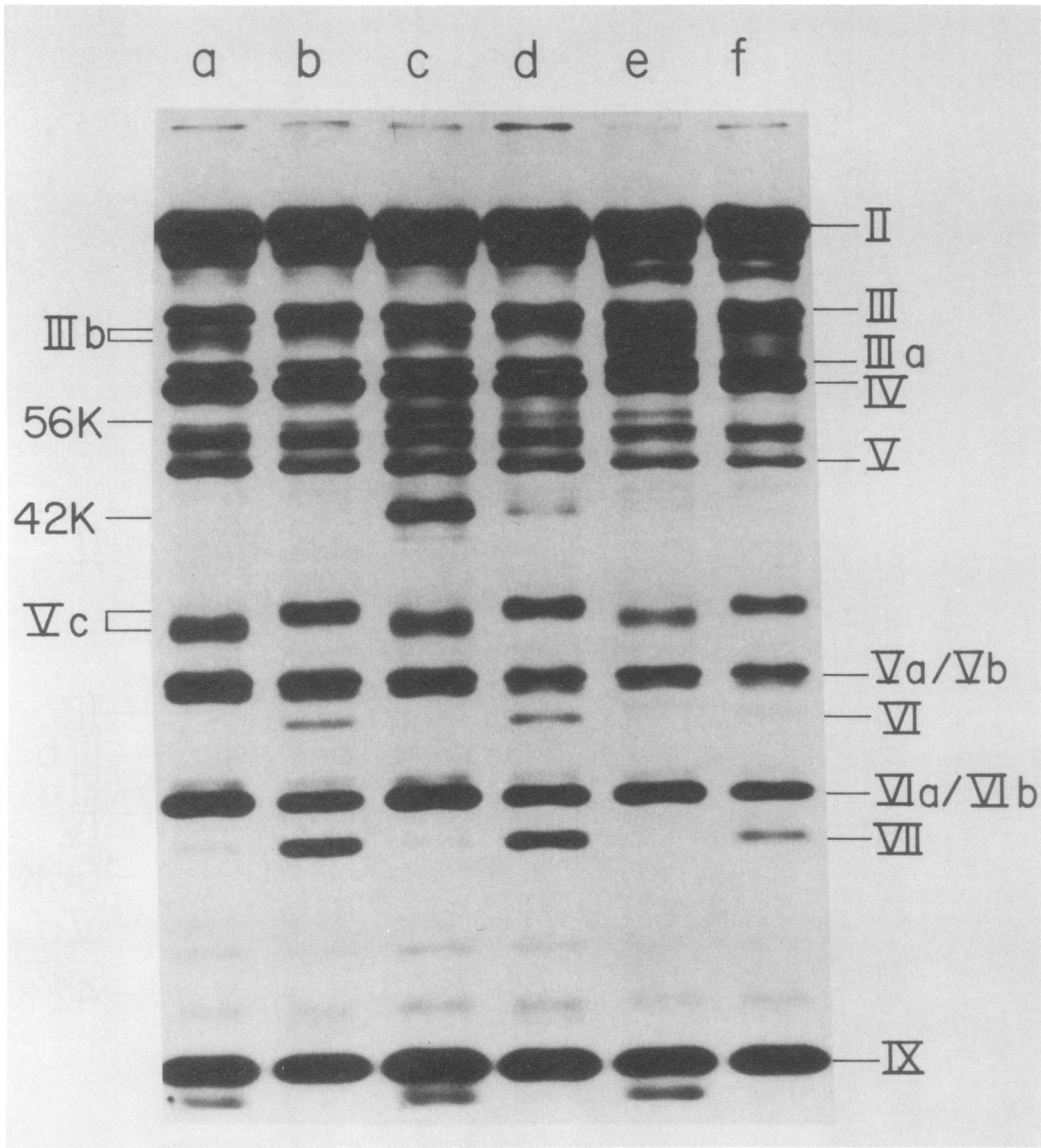

FIG. 4. *SDS polyacrylamide gel fluorogram of ^{14}C-labeled polypeptides from cells infected with Ad2, Ad2$^+$ ND2, and Ad2$^+$ ND4. Comparison of cells pulse-labeled for 2 h (39 through 41 h postinfection) with cells pulse-labeled for 2 h and chased for 12 h (41 through 53 h postinfection). The infection is described in legend to Fig. 2. The cells were labeled as described in text. Before chasing with DMEM plus 10% calf serum, the cell layer was rinsed three times with DMEM. Samples (a) through (f) were applied to an 11.5% gel. The running time was 5 h at 10 mA. (a) Ad2 pulse, (b) Ad2 chase, (c) Ad2$^+$ ND2 pulse, (d) Ad2$^+$ ND2 chase, (e) Ad2$^+$ ND4 pulse, (f) Ad2$^+$ ND4 chase.*

followed by a 12 h chase. A similar phenomenon occurred with a protein in the region between III and IIIa, designated IIIb. In cells labeled for 15 min, proteins Vc and IIIb appeared slightly smaller in size than after a 2 h label (Fig. 5). During the chase of 2, 4, and 6 h, they were replaced in a stepwise fashion by proteins of larger apparent molecular weight. No further increase in size occurred after 6 h. The stepwise increase in size of protein IIIb is perturbed by the presence of a large amount of serum albumin in the 2 h pulse sample. Neither Vc nor IIIb seem to be present in purified virions (Fig. 5). The apparent molecular weights of Vc and

IIIb after a 15 min pulse are 30,500 and 74,000, respectively, and 34,000 and 79,000, respectively, after a 2 h pulse followed by a 10 h chase. These results, combined with the observation that these proteins have a more diffuse appearance on gels after a chase than after a short pulse of 15 min (Fig. 5), suggest that the increase in molecular weight during the chase might be the result of a sequential addition of carbohydrate to a polypeptide backbone. Figure 3 demonstrates that IIIb is synthesized in infected cells earlier than the major structural Ad2 proteins. Therefore, it is likely that IIIb is identical to an Ad2-induced early protein of similar molecular weight: E_1 described by Walter and Maizel (39), the 71 K protein described by Anderson et al. (1), and the DNA-binding protein described by Levine et al. (22).

Analysis of virion proteins. It was important to determine whether the new SV40-specific proteins present in hybrid virus-infected cells were structural components of hybrid virions. To investigate this possibility, Ad2 and the five hybrid viruses were labeled with ^{14}C-labeled amino acids, purified, and

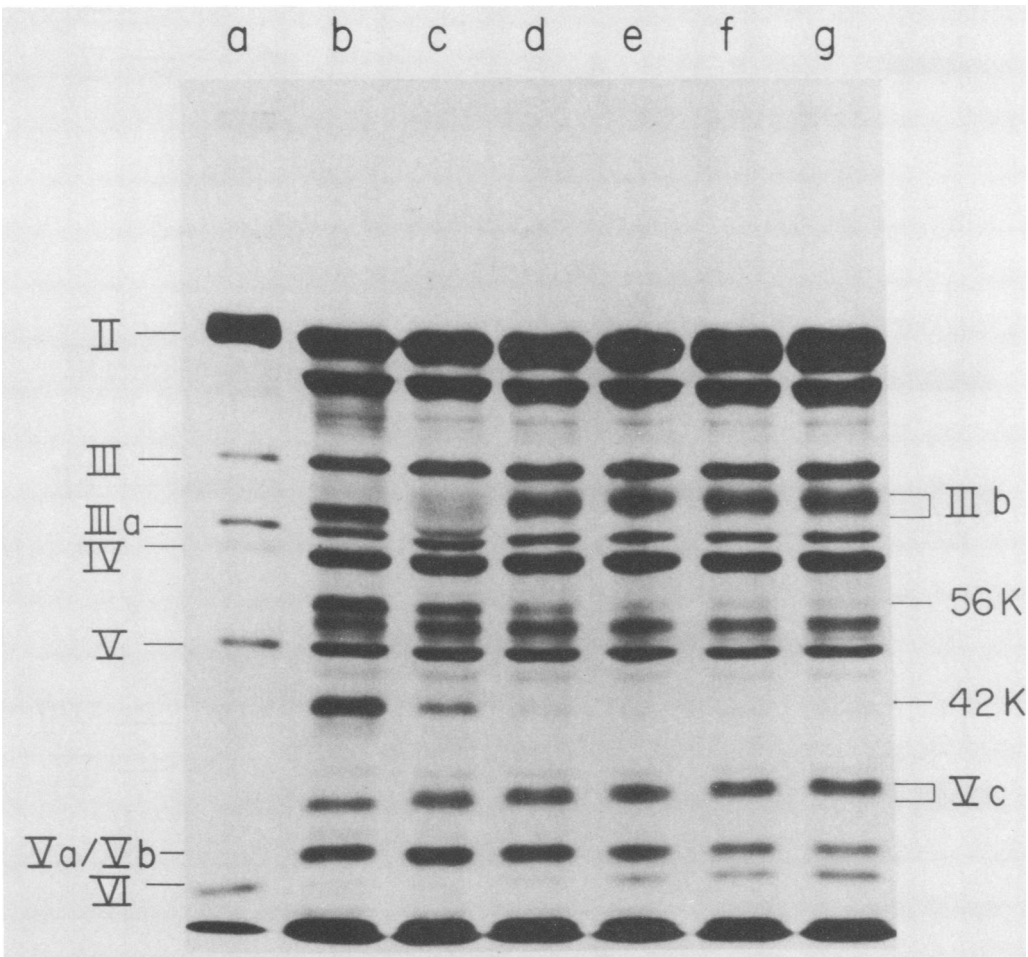

FIG. 5. *SDS polyacrylamide gel fluorogram of ^{14}C-labeled polypeptides from Ad2$^+$ ND2-infected cells; metabolic stability of the Ad2$^+$ ND2-induced 56 K and 42 K proteins. Parallel cultures of HeLa S3 cells were infected with Ad2$^+$ ND2 at a multiplicity of 100 PFU/cell. One culture was labeled for 15 min 30 h after infection with labeling medium containing neither unlabeled amino acids nor serum and 30 μCi of ^{14}C-labeled amino acids per ml. The other cultures were labeled 30 h after infection for 2 h as described in text. The chase was performed as described in legend to Fig. 4. The samples were applied to a 7.5% gel and run for 5 h at 10 mA. The sample order is: (a) purified Ad2 virions. Ad2$^+$ ND2-infected cells: (b) 15-min label, (c) 2-h label, (d) 2-h label followed by a 2-h chase, (e) 2-h label followed by a 4-h chase, (f) 2-h label followed by a 6-h chase, (g) 2-h label followed by a 10-h chase.*

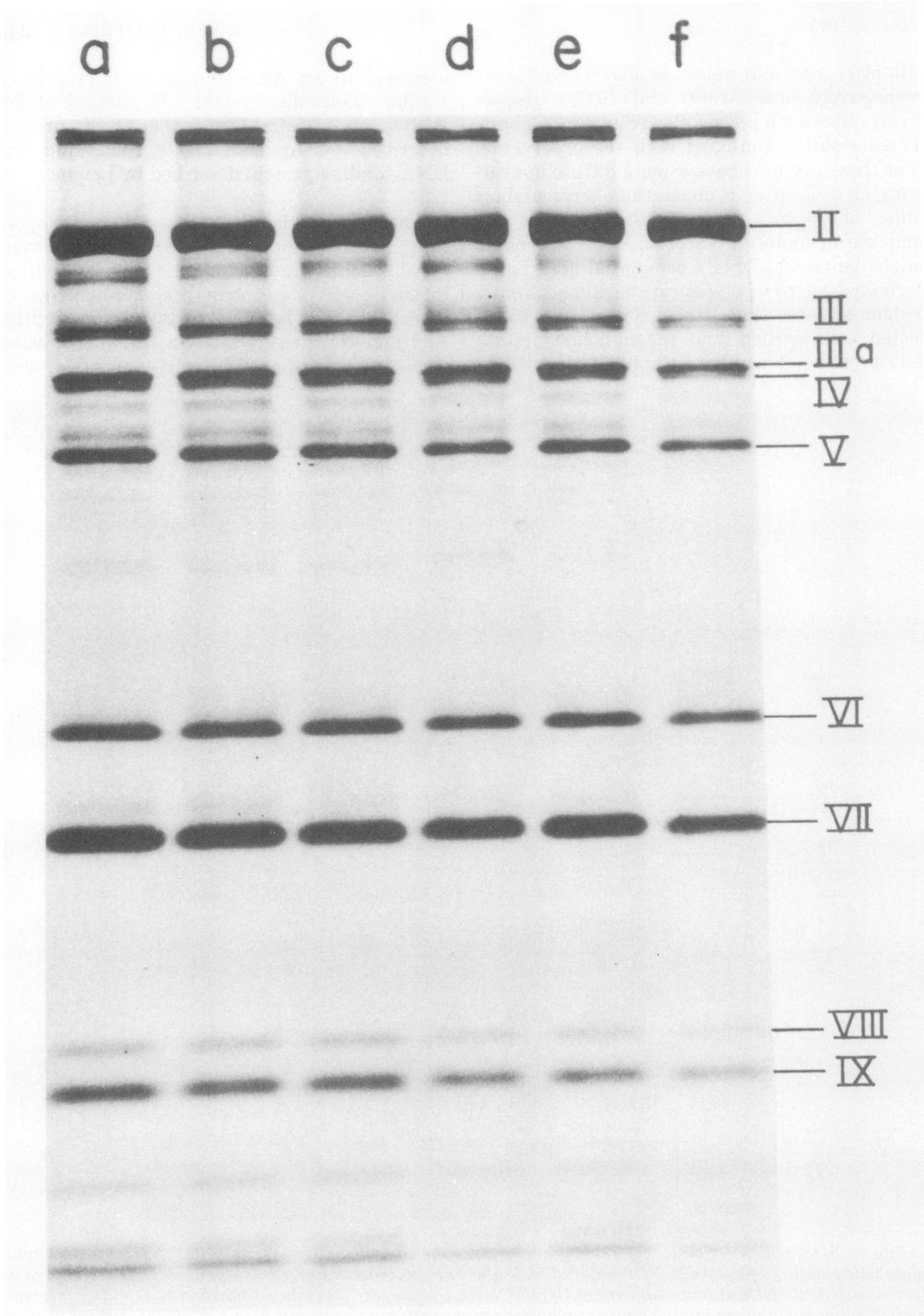

FIG. 6. *SDS polyacrylamide gel autoradiogram of ^{14}C-labeled polypeptides of purified virions. The procedures for labeling and purification of virions were described in text. Samples were applied to a 12.5% gel. The running time was 6 h and 15 min at 10 mA. (a) Ad2$^+$ ND1, (b) Ad2$^+$ ND2, (c) Ad2$^+$ ND3, (d) Ad2$^+$ ND4, (e) Ad2$^+$ ND5, (f) Ad2.*

analyzed on a slab gel. Figure 6 shows that all six virus preparations have indistinguishable polypeptide patterns characteristic of Ad2 virions (1, 7, 14, 30). No proteins of 56,000, 42,000, and 28,000 molecular weight were detectable in the hybrid virions, suggesting that these proteins are not part of the virion structure. Several minor polypeptides migrating between polypeptides II and III, IV and V, V and VI, were found in all virus preparations. They may correspond to components of similar mobility described previously (1, 7, 14). A protein of slightly higher mobility than protein III was found only in virus preparations which had been dialyzed, but not in trichloroacetic acid-precipitated virus (see above). It is likely that this protein is derived from protein II or III by proteolytic degradation which may have occurred during dialysis (34).

DISCUSSION

We have demonstrated in cells infected by nondefective Ad2-SV40 hybrid viruses the synthesis of SV40-specific proteins not present in Ad2-infected cells. The expression of these proteins takes place late in the infection cycle. A common property of the SV40-specific proteins induced by Ad2+ ND2, Ad2+ ND4, and Ad2+ ND5 is their metabolic instability. It seems likely that the 56 K protein induced by Ad2+ ND4 and the 42 K protein induced by Ad2+ ND5 are identical with the proteins of corresponding molecular weights in Ad2+ ND2-infected cells.

TABLE 2. *Relationship between the size of the SV40 DNA segments in nondefective AD2-SV40 hybrid viruses, the estimated coding capacity of these segments, and the size of the SV40-specific proteins in hybrid virus-infected cells*[a]

Hybrid virus	Fraction of SV40 genome (%)	Mol wt of protein ($\times 10^{-3}$)		Reference
		Expected	Found	
Ad2+ ND1	17.2	31	28	31
	18	32.5		16
	20	36		20
AD2+ ND2	32	57.5	56; 42	16
	36.5	64		20
Ad2+ ND3	6.4	11.5	None	20
	7	12.5		16
Ad2+ ND4	43	77	56	16
	46.3	83		20
	48	86		32
Ad2+ ND5	27.3	49	42	20
	28	50		16

[a] The coding capacity estimate was based on a value of 3.25×10^6 molecular weight for the size of the SV40 genome (11). The values for the expected molecular weights would be smaller if the antilate RNA transcribed from the *Haemophilus influenzae* G fragment is not translated.

In Table 2, the apparent molecular weights of these proteins are compared with the coding capacity of the SV40 DNA segments contained in the hybrid viruses. It is conceivable that the proteins of molecular weights 28,000, 56,000, and 42,000 induced by Ad2+ ND1, Ad2+ ND2, and Ad2+ ND5, respectively, may represent the gene products of the complete SV40 DNA fragments contained in these viruses. Although this possibility is appealing, it should be pointed out that several complicating factors permit only an approximate estimation of the coding capacity of the SV40 segments contained in the hybrid viruses. First, the values for the fractional lengths of these segments, as reported from several laboratories, vary (16, 20, 31, 32). Second, the published values for the molecular weight of SV40 DNA show considerable variations ranging from 2.5×10^6 to 3.6×10^6 (38). Two recent estimates of 3.25×10^6 (11) and 3.6×10^6 daltons (37) are presumably more accurate than some of the earlier values. Third, all nondefective hybrid viruses contain the same amount, approximately 6% of the SV40 genome, of late SV40 DNA which is transcribed in infected cells into antilate RNA (18, 23) (Fig. 1). It is not known whether or not this antilate RNA is translated. Fourth, at present we cannot rule out that the SV40-specific proteins induced by nondefective hybrid viruses are hybrid molecules coded for by both Ad2 and SV40 DNA. Recent experiments by Oxman et al. (33) suggest that a fraction of the SV40-specific RNA in cells infected by Ad2+ ND4 is covalently linked to Ad2 RNA. However, it is not known whether or not the Ad2-specific segments in the hybrid RNA molecules are translated. If the SV40-specific proteins in hybrid virus-infected cells do represent SV40 gene products, they must contain overlapping amino acid sequences since the early region of the SV40 genome can code only for approximately 100,000 daltons of protein. We expect that the isolation of these proteins and the comparison of their tryptic peptides will clarify this point.

Considering the coding capacity of Ad2+ ND4, one might have expected to find a protein of approximately 80,000 molecular weight in Ad2+ ND4-infected cells (Table 2). It seems likely that such a protein is present in Ad2+ ND4-infected cells but was not detected either

because it comigrated with an Ad2- or AAV-specific protein or because it is very unstable. On the other hand, it cannot be excluded at the moment that AAV interferes with the expression of SV40-specific proteins in Ad2$^+$ ND4-infected cells. Future experiments with Ad2$^+$ ND4 free of AAV should clarify this point.

An interesting point to be considered is the possible relationship between the SV40-specific antigens induced by Ad2$^+$ ND1, Ad2$^+$ ND2, and Ad2$^+$ ND4 (Fig. 1) and the SV40-specific proteins in cells infected by these viruses. The molecular weight of SV40 T antigen has been reported to be 70,000 (6). Therefore, it is unlikely that the 56 K protein in Ad2$^+$ ND4-infected cells is T antigen. However, the 56 K proteins induced by Ad2$^+$ ND4 and Ad2$^+$ ND2 may represent TSTA or contain the antigenic determinants of both TSTA and U in one polypeptide chain, whereas the 42 K and 28 K proteins induced by Ad2$^+$ ND2 and Ad2$^+$ ND1, respectively, may only have U antigen specificity. Ad2$^+$ ND5 does not induce SV40-specific antigens, although cells infected by this virus synthesize a protein of 42,000 daltons. The lack of detectable antigens in Ad2$^+$ ND5-infected cells could be explained by the finding that the 42 K protein, a possible candidate for U antigen, is metabolically unstable and therefore never accumulates in quantities sufficient to be detectable by immunological techniques.

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