JOURNAL OF VIROLOGY, Oct. 1977, p. 151-169

Vol. 24, No. 1
Printed in U.S.A.

Evidence for Simian Virus 40 (SV40) Coding of SV40 T-Antigen and the SV40-Specific Proteins in HeLa Cells Infected with Nondefective Adenovirus Type 2-SV40 Hybrid Viruses

KRISTINE MANN,† TONY HUNTER, GERNOT WALTER,* AND HAWLEY LINKE††
Tumor Virology Laboratory, The Salk Institute, San Diego, California 92112

Received for publication 12 April 1977

HeLa cells infected with the nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid viruses (Ad2+ND1, Ad2+ND2, Ad2+ND4, and Ad2+ND5) svnthesize SV40-specific proteins ranging in size from 28,000 to 100,000 daltons. By analysis of their methionine-containing tryptic peptides, we demonstrated that all these proteins shared common amino acid sequences. Most methionine-containing tryptic peptides derived from proteins of smaller size were contained within the proteins of larger size. Seventeen of the 21 methionine-containing tryptic peptides of the largest SV40-specific protein (100,000 daltons) from Ad2+ND4-infected cells were identical to methionine-containing peptides of SV40 T-antigen immunoprecipitated from extracts of SV40-infected cells. All of the methionine-containing tryptic peptides of the Ad2+ND4 100,000-dalton protein were found in SV40 T-antigen immunoprecipitated from SV40-transformed cells. All SV40-specific proteins observed in vivo could be synthesized in vitro using the wheat germ cell-free system and SV40-specific RNA from hybrid virus-infected cells that was purified by hybridization to SV40 DNA. As proof of identity, the in vitro products were shown to have methionine-containing tryptic peptides identical to those of their in vivo counterparts. Based on the extensive overlap in amino acid sequence between the SV40-specific proteins from hybrid virus-infected cells and SV40 T-antigen from SV40-infected and -transformed cells, we conclude that at least the major portion of the SV40-specific proteins cannot be Ad2 coded. From the in vitro synthesis experiments with SV40-selected RNA, we further conclude that the SV40-specific proteins must be SV40 coded and not host coded. Since SV40 T-antigen is related to the SV40-specific proteins, it must also be SV40 coded.

An early gene function of simian virus 40 (SV40) is involved in the control of SV40 DNA synthesis and in the process of cellular transformation by SV40. Complementation studies with temperature-sensitive mutants have identified a single early gene, known as gene *A*, in SV40 (9, 32). In infection of permissive cells with SV40, the gene *A* product is required for the initiation of both SV40 and host cell DNA synthesis (8, 10, 20, 57). It is also required for the initiation and subsequent maintenance of transformation in permissive cells infected by SV40 (6, 29, 30, 43, 45, 58).

SV40 tumor antigen (T-antigen), implicated as the gene *A* product, is found in the nuclei of permissive and nonpermissive cells infected by SV40 and in the nuclei of cells transformed by SV40 (5, 48, 59). It appears early after infection of permissive cells by SV40 (25, 52), and its presence is correlated with the initiation of SV40 and host DNA synthesis and with transformation of cells by SV40 (4, 20, 21). In addition to SV40 T-antigen, U-antigen and tumor-specific transplantation antigen (TSTA-antigen) are the two other SV40-specific antigens found consistently in SV40-infected and -transformed cells (18, 39).

Several lines of evidence suggest that SV40 T-antigen is SV40 coded. In cells infected with temperature-sensitive mutants in SV40 gene *A* (*tsA* mutants), there is overproduction at the restrictive temperature of a 100,000-dalton (100K) protein immunoprecipitable with antiserum from hamsters bearing SV40-induced tumors (SV40 anti-T serum) (59). T-antigen in cells infected with SV40 *tsA* mutants is more

† Present address: Department of Mathematics and Science, University of Alaska, Anchorage, AK 99504.
†† Present address: Molecular Biology Institute, University of California, Los Angeles, CA 90024.

heat labile than T-antigen from cells infected with wild-type virus (2, 60), and it is induced in mouse kidney cells in the presence of actinomycin D after microinjection of SV40 RNA complementary (cRNA) synthesized from SV40 DNA in vitro (19). Cell-free translation systems have provided evidence supporting the conclusion that SV40 T-antigen is SV40 coded (1, 3, 7, 49, 50, 54). Finally, infection with a deletion mutant of SV40 gene *A* results in a lower-molecular-weight protein with peptides related to the 100K protein from infections with wild-type SV40 (56).

We have used the nondefective adenovirus 2 (Ad2)-SV40 hybrid viruses to provide rigorous proof that SV40 T-antigen is indeed an SV40-coded protein. The hybrid viruses contain different lengths of SV40 DNA from the early region covalently inserted at the same site in the Ad2 genome (11, 22, 27, 35, 44). The spectrum of SV40-specific tumor antigens induced by each hybrid virus differs: Ad2$^+$ND1 induces only U-antigen (38, 39), Ad2$^+$ND2 induces U- and TSTA-antigens (38, 40), and Ad2$^+$ND4 induces U-, TSTA-, and T-antigens (38). Ad2$^+$ND3 and Ad2$^+$ND5 do not induce any detectable SV40-specific antigens (38). Previous studies have identified the SV40-specific proteins in HeLa cells infected with the Ad2-SV40 hybrid viruses (12, 13, 61). These proteins are the 28K protein of Ad2$^+$ND1-infected cells, the 42K and 56K proteins of Ad2$^+$ND2-infected cells, the 42K, 56K, 60K, 64K, 72K, 74K, and 95K doublet proteins of Ad2$^+$ND4-infected cells, and the 42K protein of Ad2$^+$ND5-infected cells. (The SV40-specific proteins are referred to on the basis of their apparent molecular weights determined from sodium dodecyl sulfate [SDS]-polyacrylamide gels. Thus the 28,000-dalton protein of Ad2$^+$ND1 is called the 28K protein, and so on.) There are no detectable SV40-specific proteins in Ad2$^+$ND3-infected HeLa cells. Most of the SV40-specific proteins are immunoprecipitable with SV40 anti-T serum from hamsters bearing SV40-induced tumors (12, 13), implying that structurally related proteins are expressed in SV40-induced tumors.

In this paper, we compare the tryptic peptides of the SV40-specific proteins in Ad2-SV40 hybrid virus-infected cells with the tryptic peptides of the SV40-specific proteins synthesized in vitro from SV40-specific RNA and finally with the tryptic peptides of SV40 T-antigen immunoprecipitated from SV40-infected monkey cells with SV40 anti-T serum. We show that all the SV40-specific proteins identified in the Ad2-SV40 hybrid virus-infected HeLa cells are SV40-coded and not host-coded proteins. In addition, we show that SV40 T-antigen is an SV40-coded protein.

MATERIALS AND METHODS

Viruses and cells. 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. High-titer stocks of Ad2, Ad2$^+$ND1, Ad2$^+$ND2, Ad2$^+$ND3, and Ad2$^+$ND5 were prepared in HeLa S3 cells grown in Eagle minimum essential medium (MEM) for suspension culture supplemented with 5% calf serum. Stocks of Ad2$^+$ND4 were prepared in CV1 cells grown in Dulbecco modified MEM (DMEM) supplemented with 10% fetal calf serum. Stocks were plaque titered on HeLa monolayers (62). The virus stocks used in these experiments had a titer of 10^9 PFU/ml except for Ad2$^+$ND1, which had a titer of 5×10^{10} PFU/ml, and Ad2$^+$ND4, which had a titer of 4×10^7 PFU/ml.

Large-plaque SV40 stock was prepared in TC7 cells grown in DMEM plus 10% fetal calf serum and an amino acid supplement of arginine, histidine, and glutamine (53). After being concentrated by pelleting, the titer of the SV40 stock was 5×10^9 PFU/ml.

Infection and labeling of cells. (i) Ad2-SV40 hybrid virus experiments. HeLa S3 cells in suspension were infected at a concentration of 10^7 cells/ml with Ad2 or Ad2-SV40 hybrid virus stock (multiplicity of 10^2 PFU/cell). In the case of Ad2$^+$ND4, HeLa S3 cells were infected at a concentration of 0.5×10^7 cells/ml with 4 ml of Ad2$^+$ND4 virus stock (multiplicity of 8 PFU/cell). After an absorption period of 20 min, the cells were diluted with MEM for suspension culture to a final concentration of 2×10^5 cells/ml. Between 30 and 40 h postinfection, when nucleoli had disappeared, the cells were washed once with MEM for suspension culture lacking methionine and labeled at a concentration of 10^6 cells/ml in medium lacking methionine and containing 165 μCi of [^{35}S]methionine (specific activity, 300 Ci/mmol; New England Nuclear NEG-009H) per ml and 5% dialyzed calf serum. Under these labeling conditions, the incorporation of [^{35}S]methionine into protein was linear for 4 h. After labeling, the cells were washed once with MEM for suspension culture and were resuspended in 1 ml of ice-cold lysis buffer (0.14 M NaCl-3 mM $MgCl_2$-10 mM Tris-hydrochloride, pH 7.4) with Nonidet P-40 (NP-40) added to a final concentration of 0.5%. After 10 min at 4°C, the cell lysate was spun in an IEC centrifuge (model PR-2, rotor head 269) at 2,000 rpm ($800 \times g$) for 10 min, the supernatant (cytoplasmic extract) was removed, and the nuclei were washed once with lysis buffer. The nuclear pellets were dissolved in 0.5 ml of electrophoresis sample buffer (0.0625 M Tris-hydrochloride [pH 6.8]-3% SDS-5% 2-mercaptoethanol – 10% glycerol-0.001% bromophenol blue-2 mM phenylmethylsulfonyl fluoride). The cytoplasmic extracts were diluted 1:1 with 2× electrophoresis sample buffer. The nuclear samples were sonically treated with a Branson Sonifier for 10 s at position 4. All samples were heated for 2 min in a boiling water bath.

(ii) SV40 virus experiments. CV1 cells (10^6) were

seeded on a 10-cm² plastic petri dish and infected 24 h later with 0.5 ml of SV40 virus stock at a multiplicity of 20 PFU/cell. After an adsorption period of 1 h, 10 ml of DMEM containing 10% fetal calf serum was added to the infected cells. At 77 h postinfection, the cells were washed with DMEM lacking methionine and labeled for 4 h with 2 ml of DMEM lacking methionine and containing 200 μCi of [^{35}S]methionine per ml and 5% dialyzed calf serum. At the same time, a mock-infected culture of CV1 cells was also labeled under the same conditions as the SV40-infected CV1 cells. After labeling, the cell layers were thoroughly washed and then lysed by the addition of 1 ml of cold pH 8 low-salt extraction buffer (0.14 M NaCl-3 mM $MgCl_2$-1 mM dithiothreitol-2 mM phenylmethylsulfonyl fluoride-10 mM Tris-hydrochloride, pH 8) with NP-40 added to a final concentration of 0.5%. After 20 min on ice, the lysate was collected by scraping the plate and fractionated by centrifugation at 800 × *g* for 10 min at 4°C into "pH 8 cytoplasm" and "nuclear pellet." The pH 8 cytoplasmic extracts were centrifuged in a Sorvall centrifuge, rotor SS-34, at 10,000 rpm (12,000 × *g*) for 20 min at 4°C to remove particulate matter before immunoprecipitation.

(iii) SV3T3 experiments. SV3T3 cells (0.75 × 10⁶) were seeded on a 10-cm² plastic petri dish in DMEM containing 10% calf serum. Thirty-six hours later, the cells were washed with DMEM lacking methionine and labeled for 3 h, using the same conditions as in the SV40 virus experiments. The cells were washed, lysed, and fractionated into pH 8 cytoplasm and nuclear pellet as described above. As a control, a plate of 3T3 cells was labeled and treated in the same manner.

Immunoprecipitation. All sera used for immunoprecipitation were absorbed three times with methanol-fixed uninfected CV1 or 3T3 cells, and complement was heat inactivated at 56°C for 30 min. Immediately before use, the sera were centrifuged at 10,000 rpm (12,000 × *g*) for 20 min at 4°C. For preparative purposes, 1 ml of pH 8 low-salt cytoplasmic extract from SV40-infected CV1 cells was used for immunoprecipitation as described previously (12), except that incubation with SV40 anti-T serum (received from Jack Gruber, Office of Program Resources and Logistics, National Cancer Institute, Bethesda, Md.) was performed at 4°C for 90 min. The immunoprecipitate was dissolved in 1 ml of sample buffer, heated for 2 min in a boiling water bath, and loaded onto preparative SDS-polyacrylamide gels. For analytical polyacrylamide gel electrophoresis, 50 μl of pH 8 cytoplasm from either mock-infected or SV40-infected CV1 cells and 50 μl from either 3T3 or SV3T3 cells were immunoprecipitated. The immunoprecipitates were dissolved in 50 μl of sample buffer, and 10-μl samples were applied per slot in an analytical SDS-polyacrylamide gel.

SV40 DNA-Sepharose. SV40 DNA form I was prepared from SV40-infected TC7 cells by the Hirt extraction procedure (24) followed by equilibrium centrifugation in CsCl-ethidium bromide (51). After removal of ethidium bromide with isopropanol, the DNA was ethanol precipitated and dissolved in 0.1 mM EDTA-5 mM phosphate buffer, pH 7.1. Sepharose 4B (Pharmacia) was activated by cyanogen bromide according to March et al. (42). The coupling of SV40 DNA to freshly activated Sepharose 4B was performed according to the procedures of Gilboa et al. (17). The amount of DNA covalently bound per milliliter of Sepharose 4B was approximately 50 μg.

To test the properties of the SV40 DNA-Sepharose, ^{3}H-labeled cytoplasmic RNA from Ad2-infected HeLa cells and ^{14}C-labeled SV40 cRNA synthesized by *Escherichia coli* RNA polymerase on SV40 form I DNA were hybridized together to the SV40 DNA-Sepharose and then eluted. Only 0.5% of the labeled Ad2 RNA bound, whereas 65% of the SV40 cRNA bound. The small percentage of Ad2 cytoplasmic RNA bound was not analyzed further. It appears unlikely that this binding is selective, since no predominant Ad2-specific mRNA was bound to SV40 DNA-Sepharose under these conditions as assayed by in vitro translation (see below).

Extraction of cytoplasmic RNA from Ad2- and Ad2-SV40 hybrid virus-infected HeLa cells. HeLa S3 cells in suspension were infected with Ad2 and Ad2-SV40 hybrid viruses as described above. Between 35 and 45 h postinfection, after the nucleoli had disappeared, the cells were pelleted and washed twice with MEM for suspension. The cells were suspended in cold reticulocyte standard buffer (0.14 M NaCl-3 mM $MgCl_2$-0.01 M Tris, pH 7.4) and lysed by 0.5% NP-40. After the nuclei were removed by centrifugation, SDS was added to the supernatant to a final concentration of 1%. The cytoplasmic fraction was then extracted two times at room temperature with phenol-chloroform (1:1), saturated, and equilibrated with 0.1 M NaCl-5 mM EDTA-50 mM Tris-hydrochloride, pH 7.5. The aqueous phase obtained was extracted with 1% isoamyl alcohol in chloroform and finally precipitated with ethanol at −20°C. The RNA was stored at −20°C in ethanol until used for translation, fractionation on oligodeoxythymidylate [oligo(dT)]-cellulose or hybridization to SV40 DNA-Sepharose. Where indicated, polyadenylate-containing RNA was selected from total cytoplasmic RNA by oligo-(dT)-cellulose chromatography.

Hybridization of RNA to SV40 DNA-Sepharose. The cytoplasmic RNA from Ad2- or Ad2-SV40 hybrid virus-infected HeLa cells was hybridized to SV40 DNA-Sepharose, essentially as described by Gilboa et al. (17). SV40 DNA-Sepharose was equilibrated in hybridization buffer (0.05 M Tris-hydrochloride-0.75 M NaCl-0.5% SDS-1 mM EDTA-50% deionized formamide, adjusted to pH 7.5). RNA (0.5 to 1.0 mg) extracted from Ad2-, Ad2⁺ND1-, Ad2⁺ND2-, Ad2⁺ND3-, Ad2⁺ND4-, or Ad2⁺ND5-infected HeLa cells was added to 1.5 ml of SV40 DNA-Sepharose and agitated in a water bath for 3 h at 37°C. Unhybridized RNA was removed after centrifugation, and the Sepharose was washed with three 5-ml washes of hybridization buffer. Hybridized RNA was then eluted stepwise at 37°C with: (i) three 2-ml washes of 98% deionized formamide in 10 mM Tris-hydrochloride-0.1 mM EDTA-0.01% SDS, adjusted to pH 8.5, and (ii) three 2-ml washes of 1 M NaCl. The washes within each elution were pooled. The two eluted RNA fractions were precipitated individually with ethanol at −20°C after addition of carrier yeast

tRNA to 10 μg/ml and addition of potassium acetate (pH 5.2) to 0.2 M in the case of eluate (i). The samples were reprecipitated to remove residual traces of formamide, dissolved in water, and lyophilized. They were then redissolved in water and stored in liquid nitrogen until translated.

In vitro translation of RNA in a wheat germ system. The cytoplasmic RNA fractions were translated in a wheat germ system, prepared essentially as described by Roberts and Paterson (55). The incubation mixture has been previously described (26), with inclusion of 0.8 mM spermidine to obtain the optimum rate of translation and synthesis of polypeptides over 100,000 daltons. The reaction mixture contained 250 μCi of [^{35}S]methionine per ml along with the other 19 unlabeled amino acids. The reaction mixtures containing approximately 100 μg of polyadenylate-containing RNA or 375 μg of total cytoplasmic RNA per ml were incubated for 2 to 3 h at 27°C.

Polyacrylamide gel electrophoresis and fluorography. The SDS-polyacrylamide gel system of Laemmli (31) and Maizel (41) was used. Electrophoresis was performed at a constant current of 10 mA. After electrophoresis, the gels were prepared for fluorography as described by Laskey and Mills (33).

Peptide mapping. Extracts from Ad2- and Ad2-SV40 hybrid virus-infected HeLa cells, immunoprecipitates of T-antigen from SV40-infected CV1 cells or from SV3T3 cells, and in vitro translation products from SV40-specific RNA, all labeled with [^{35}S]methionine, were prepared for electrophoresis as described above. Samples containing approximately 0.75 mg of protein in electrophoresis sample buffer were applied to 2-mm-thick preparative SDS-polyacrylamide slab gels. Electrophoresis was performed at a constant current of 20 mA per gel. After electrophoresis, the gels were dried under vacuum over a boiling-water bath, and labeled proteins were visualized by direct autoradiography. Those sections of the gel containing the Ad2- and SV40-specific proteins were cut out, and the proteins were eluted in 0.05 M ammonium bicarbonate containing 0.1% SDS and 10% 2-mercaptoethanol as described previously (12). The protein preparations were oxidized with performic acid as described by Hirs (23). The oxidized proteins were digested with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin and subjected to peptide analysis on thin-layer cellulose plates as described by Gibson (16).

RESULTS

Peptide analysis of in vivo SV40-specific proteins. The early region of the SV40 genome can code for approximately 100,000 daltons of protein. The different Ad2-SV40 hybrid viruses contain overlapping segments of different lengths of the SV40 early region inserted at the same site in the Ad2 genome (27, 34). If the SV40-specific proteins are SV40 coded, then the proteins induced by any one hybrid virus should have overlapping amino acid sequences with all the SV40-specific proteins induced by the other hybrid viruses. In addition, more than one SV40-specific protein is induced when cells are infected with either Ad2$^+$ND2 or Ad2$^+$ND4 (13, 61). Since the highest-molecular-weight protein induced in each case saturates the coding capacity of the SV40 DNA insert, it is likely that the different-molecular-weight SV40-specific proteins within each infection contain overlapping amino acid sequences with one another if they are SV40 coded. To investigate these possibilities, we carried out tryptic peptide analysis on purified SV40-specific proteins labeled in vivo.

[^{35}S]methionine-labeled proteins from Ad2$^+$-ND1-, Ad2$^+$ND2-, Ad2$^+$ND4-, and Ad2$^+$ND5 hybrid virus-infected HeLa cells were separated by preparative SDS-gel electrophoresis, extracted from the gel, and digested with trypsin. The tryptic peptides were analyzed by two-dimensional separation on thin-layer cellulose plates (see Materials and Methods). The peptide maps are presented in Fig. 1, 2, and 3. It can be seen that all the in vivo proteins identified as being SV40 specific are structurally related to each other. For the purposes of this discussion, the upper protein of the Ad2$^+$ND4 95K doublet will be referred to as the 100K protein. Only the Ad2$^+$ND4 100K protein of the 95K doublet was mapped, since during a 4-h label with [^{35}S]methionine the predominant protein labeled was the 100K. The peptides common to the series of proteins are numbered in order of appearance as one progresses in molecular weight from the 28K protein of Ad2$^+$ND1 to the Ad2$^+$ND4 100K protein. The molecular weights of the SV40-specific proteins increased in proportion to the size of the SV40 DNA inserts. Therefore, assuming that these proteins are SV40 coded, the lower numbers represent those peptides coded for by the 3′ end of the SV40 early region, and the higher numbers represent those peptides coded for by the 5′ end of the early region. The common methionine-containing peptides were not clustered but were distributed throughout the length of the SV40-specific proteins (Table 1).

An examination of the peptide maps shows that some peptides were more intensely labeled than others. This may be because they contained more than one methionine residue; alternatively, the variation in intensity could have been due to differential completeness of tryptic digestion. In addition, certain tryptic peptides, such as peptides 6 and 7, differed in their relative molar ratios in different proteins. The reason for this is not known, although again it may be due to differences in tryptic digestion. In general, the method we have used

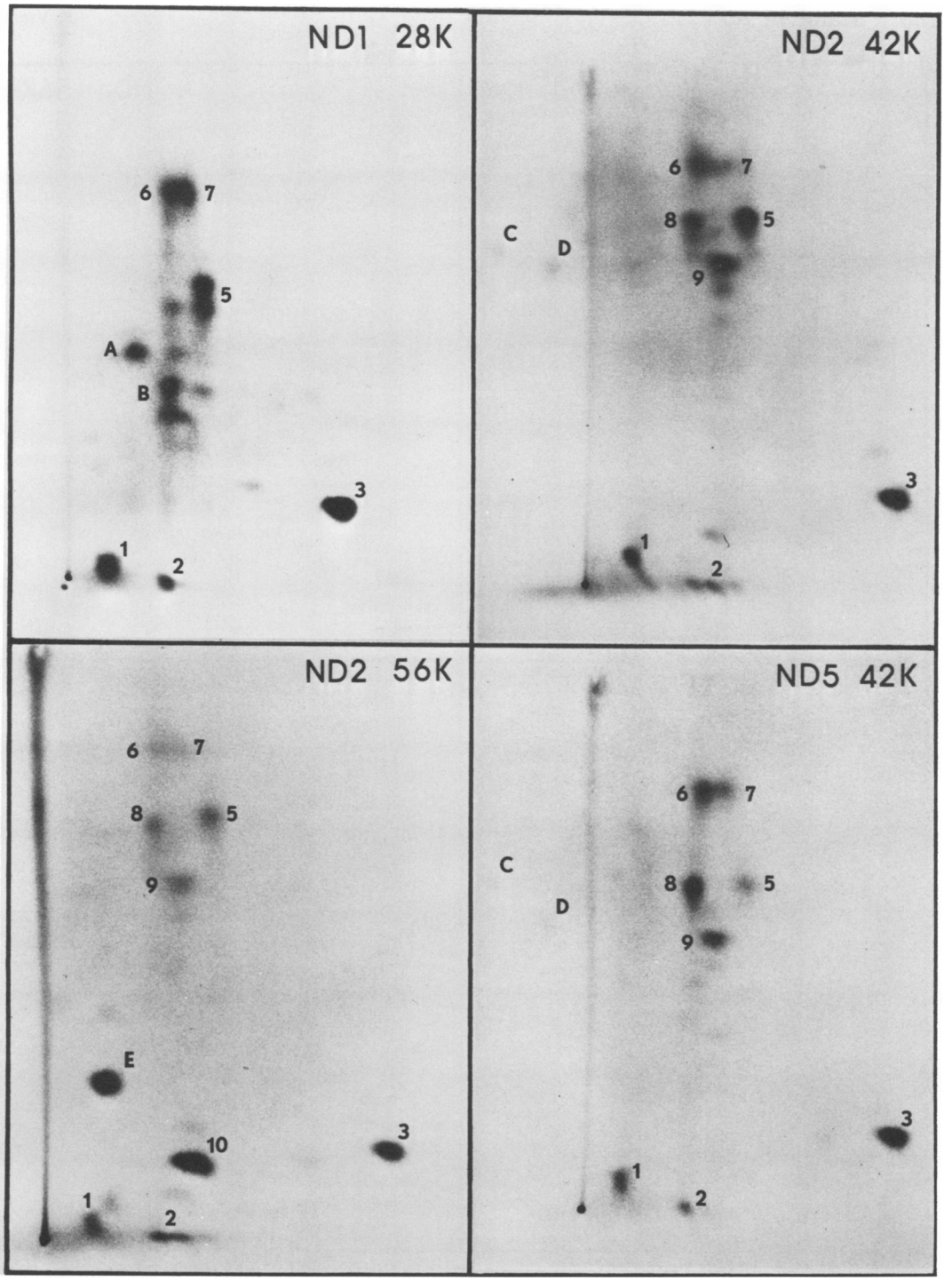

FIG. 1. *Tryptic peptide maps of [^{35}S]methionine-labeled in vivo SV40-specific proteins from Ad2-SV40 hybrid virus-infected HeLa cells. In this and the following peptide maps, tryptic digests were prepared and analyzed by two-dimensional separation on thin-layer cellulose plates as described in Materials and Methods. Electrophoresis at pH 4.7 was in the horizontal dimension, with the origin on the left and the cathode on the right. Ascending chromatography was in the vertical dimension. [^{35}S]methionine-labeled tryptic peptides were visualized by autoradiography. Peptide maps of Ad2^{+}ND1 28K, Ad2^{+}ND2 42K and 56K, and Ad2^{+}ND5 42K proteins can be seen. The Ad2^{+}ND1 28K protein is referred to as ND1 28K in the figure, and so on.*

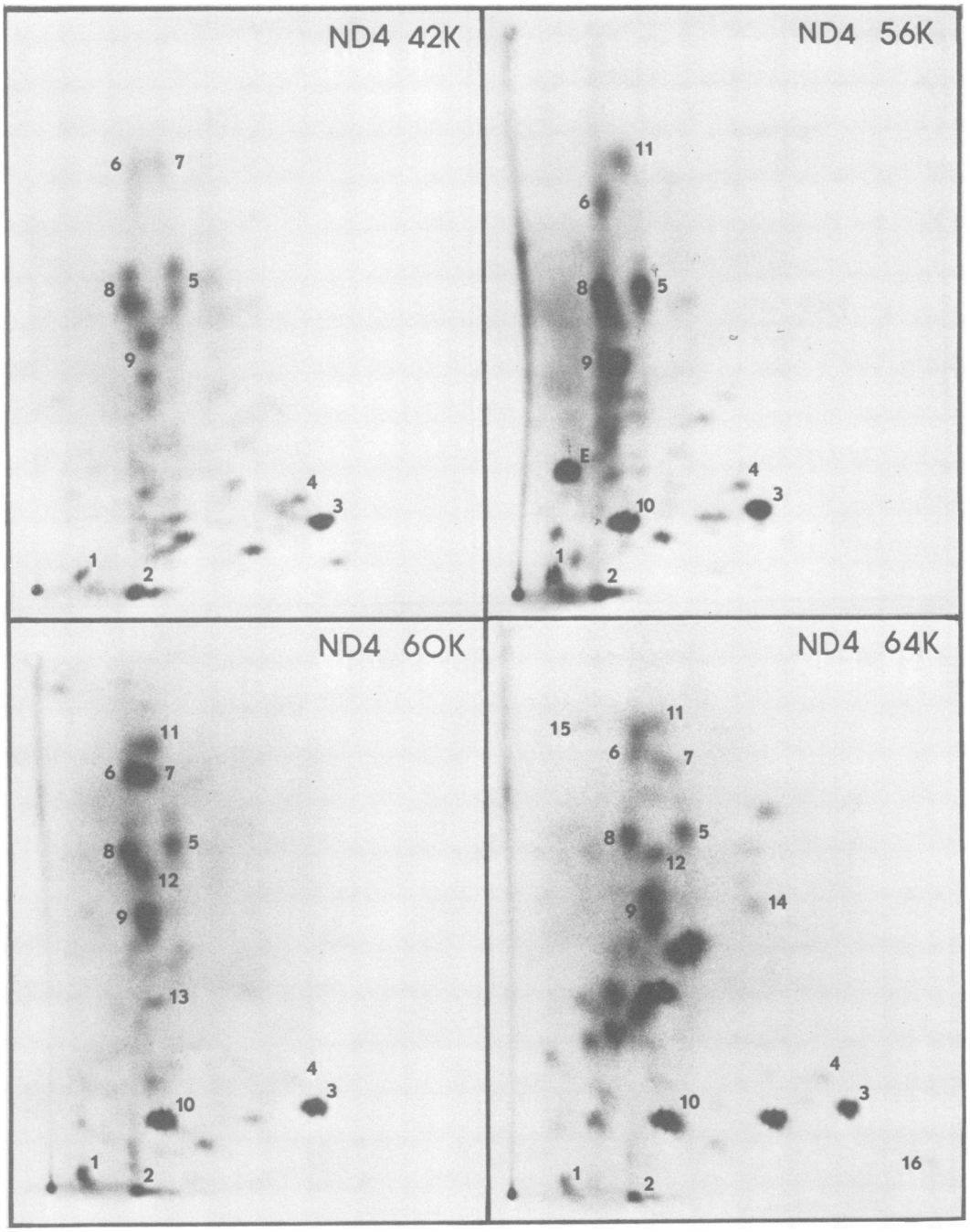

FIG. 2. *Tryptic peptide maps of in vivo Ad2⁺ND4 42K, 56K, 60K, and 64K proteins labeled with [³⁵S]methionine.*

for peptide mapping gives extremely reproducible results, although we have noted the following discrepancies. Peptide 4 is found in all the Ad2⁺ND4 proteins; it cannot be detected in the Ad2⁺ND1 28K protein, in the Ad2⁺ND2 42K and 56K proteins, or in the Ad2⁺ND5 42K protein. There is a weakly labeled peptide just above peptide 2 in the map of the Ad2⁺ND2 42K protein which is not seen in that of the Ad2⁺ND5 42K protein; the peptide map of the

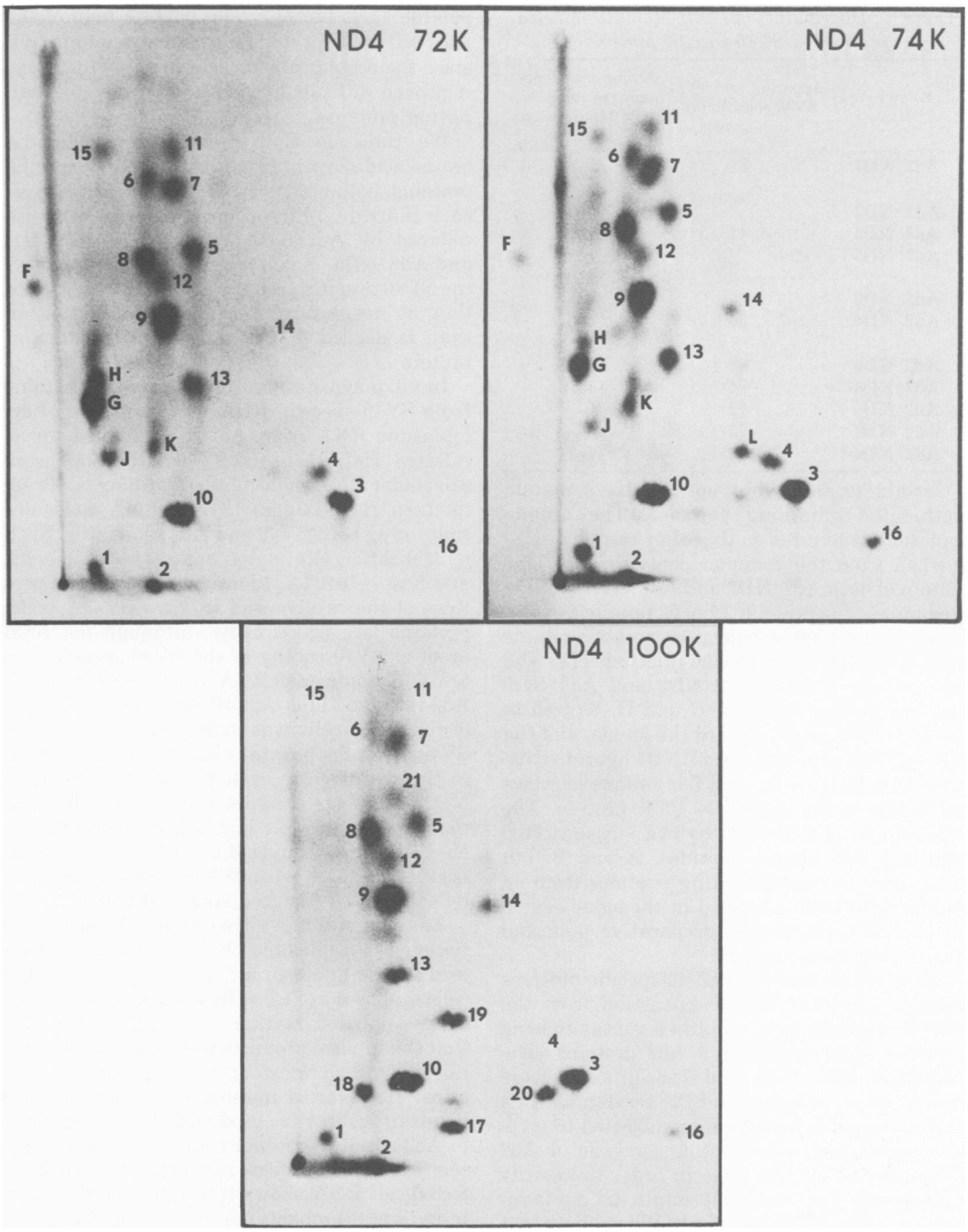

FIG. 3. *Tryptic peptide maps of in vivo Ad2⁺ND4 72K, 74K, and 100K proteins labeled with [³⁵S]methionine.*

Ad2⁺ND4 42K protein has too many impurities in this area of the map to determine whether or not it is there. In some of the tryptic peptide maps, peptides 5, 6, 8, and 9 appear as doublets, as in the Ad2⁺ND1 28K, Ad2⁺ND2 42K, and Ad2⁺ND4 42K and 64K proteins (Fig. 1 and 2). This variation is not found consistently with every map of the same protein; it is possibly due to incomplete oxidation of sulfur-containing peptides.

TABLE 1. *Distribution of [^{35}S]methionine-labeled peptides in SV40-specific proteins*

Ad2-SV40 hybrid virus	Apparent mol wt of SV40-specific protein ($\times 10^3$)	Order of appearance of common [^{35}S]Met-labeled peptides
Ad2$^+$ND1	28	1-7
Ad2$^+$ND2 Ad2$^+$ND4 Ad2$^+$ND5	42	8, 9
Ad2$^+$ND2	56	10
Ad2$^+$ND4	56	11
Ad2$^+$ND4	60	12, 13
Ad2$^+$ND4	64	14-16
Ad2$^+$ND4	72	-
Ad2$^+$ND4	74	-
Ad2$^+$ND4	100	17-21

Certain proteins, but not all, have unique methionine-containing peptides. The unique peptides are lettered so that they can be distinguished from the common peptides. The 56K protein of both Ad2$^+$ND2 and Ad2$^+$ND4 has one predominant peptide, E, that is unique and not found in the higher-molecular-weight proteins from Ad2$^+$ND4 hybrid virus-infected cells. The 42K protein of both Ad2$^+$ND2 and Ad2$^+$ND5 has two unique peptides, C and D, located to the left of the origin toward the anode, and the 72K and 74K proteins of Ad2$^+$ND4 hybrid virus-infected cells have at least five unique peptides not found in the Ad2$^+$ND4 100K protein. The 28K protein of Ad2$^+$ND1 hybrid virus-infected cells has two unique peptides, A and B, but these may be contaminating peptides from an Ad2 protein that is located in the same region on the SDS-acrylamide preparative gels and has not yet been mapped.

Some of the Ad2$^+$ND4 SV40-specific proteins mapped after the initial extraction from the SDS-acrylamide gels contained contaminating peptides from neighboring Ad2 proteins. The Ad2$^+$ND4 60K, 72K, and 74K proteins were repurified on preparative SDS-acrylamide gels and reextracted before being subjected to tryptic peptide analysis. Peptide analysis of Ad2 IIIa and Ad2 IV was done in order to identify Ad2-specific peptides that might be contaminating these SV40-specific proteins (data not shown). Repurification of the extracted Ad2$^+$-ND4 60K protein on preparative SDS-acrylamide gel electrophoresis resulted in the elimination of several peptides specific to Ad2 IV. These contaminating peptides could be found in the Ad2$^+$ND4 64K protein (Fig. 2, undesignated peptides). The 64K protein has not yet been repurified, so we are unable to determine whether peptide 13 is present. The peptide map of the Ad2$^+$ND4 42K protein shows many impurities; since the protein was present in small amounts, it proved difficult to purify it free of contaminating proteins.

We conclude that there are overlapping amino acid sequences in all of the SV40-specific proteins, both among those proteins induced by each individual hybrid virus and among those induced by Ad2$^+$ND1, AD2$^+$ND2, Ad2$^+$ND4, and Ad2$^+$ND5. It is, therefore, possible that all the SV40-specific proteins are SV40 coded since they do not require more genetic information than is needed to code for 100,000 daltons of protein.

In vitro synthesis of SV40-specific proteins from SV40-specific RNA. Hybridization of cytoplasmic RNA from Ad2-SV40 hybrid virus-infected HeLa cells to SV40 DNA-Sepharose selects for RNA molecules containing SV40 sequences, including any hybrid RNA molecules containing both SV40 and Ad2 sequences. Such hybridization eliminates unlinked Ad2-specific and host cell RNA. Identity of the tryptic peptides of the in vivo and in vitro SV40-specific proteins is a prerequisite, although not final proof of SV40 coding of the SV40-specific proteins. Cytoplasmic RNA from mock-infected, Ad2-infected, and Ad2-SV40 hybrid virus-infected HeLa cells was extracted, hybridized to SV40 DNA-Sepharose, eluted, and translated in the wheat germ system (see Materials and Methods). Four samples from each hybridization experiment were translated: (i) unfractionated RNA, (ii) unhybridized RNA, (iii) hybridized RNA eluted with 98% formamide, and (iv) hybridized RNA eluted with 1 M NaCl.

Analysis of the in vitro products from unfractionated cytoplasmic RNAs from mock-infected, Ad2-infected, and hybrid virus-infected cells is shown in Fig. 4. By comparison with the in vivo labeled marker track, it can be seen that the in vitro products from the Ad2-infected cell RNA had most of the Ad2-specific proteins. However, a number of the less intense bands in the in vitro product did not correspond to Ad2-specific proteins seen in vivo. Comparison with the in vitro product from mock-infected-cell RNA shows that some of these proteins were probably derived from host cell mRNA, although many host cell proteins had completely disappeared, possibly reflecting shutoff of host protein synthesis by Ad2 late in infection. The other background proteins may represent premature termination products from translation of the Ad2 RNA in the wheat germ system (26).

In addition to the Ad2-specific proteins and

host cell proteins, the in vitro product from the different Ad2-SV40 hybrid virus-infected HeLa unfractionated cytoplasmic RNAs had proteins that co-migrated with the in vivo SV40-specific proteins (Fig. 4). In general, the relative levels of the SV40-specific proteins synthesized in vitro were similar to those seen in vivo (13). However, proteins that were metabolically unstable in vivo (e.g., the 42K proteins of Ad2+ND4 and Ad2+ND5) appeared more predominantly in the in vitro product, presumably because there is less turnover in vitro. Translation of oligo(dT)-cellulose-selected RNA fractionated from the total cytoplasmic RNAs gave patterns of proteins very similar to those obtained with unfractionated RNA, including all the SV40-specific proteins (data not shown). We conclude that the mRNA's for the SV40-specific proteins are at least in part polyadenylated.

The hybridization reaction did not appear to affect the quality of the mRNA, since the pattern of the in vitro translation product from unhybridized RNA was very similar to that from unfractionated RNA (Fig. 5 and 6). However, there were two new proteins with molecular weights of 32K and 80K in the product from unhybridized RNA. The mRNA's for these proteins may have been generated during hybridization by denaturation. For instance, we found that mRNA activities coding for proteins of 31K, 32K, and 66K were unmasked by exposing Ad2-infected HeLa cytoplasmic RNA to denaturing conditions (C. Lawrence and T. Hunter, unpublished data).

Translation of RNA selected by hybridization to SV40 DNA results in proteins with molecular weights corresponding to those of the in vivo SV40-specific proteins: the Ad2+ND1 28K protein, the Ad2+ND2 42K and 56K proteins, the Ad2+ND4 42K, 56K, 60K, 64K, 72K, and 74K

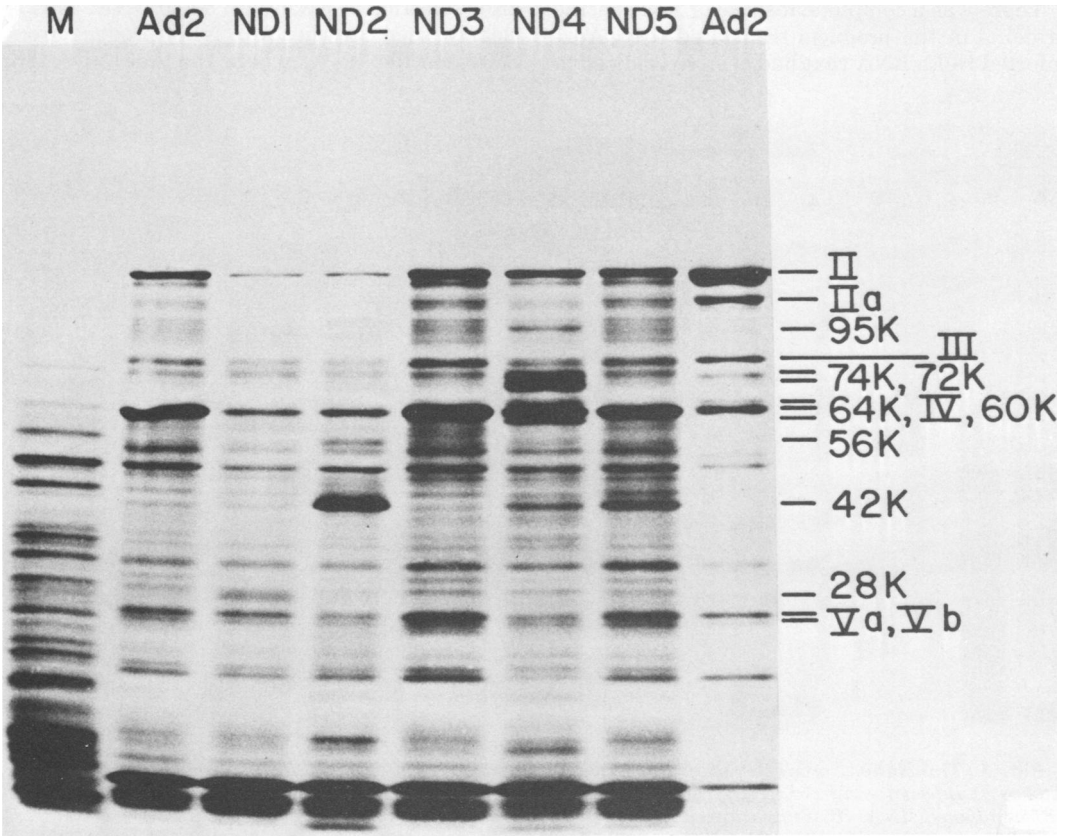

FIG. 4. *Translation products of unfractionated cytoplasmic RNA from mock-infected (M), Ad2-infected, and Ad2-SV40 hybrid virus (Ad2+ND1, Ad2+ND2, Ad2+ND3, Ad2+ND4, and Ad2+ND5)-infected HeLa cells. The Ad2 in vivo proteins can be seen in the right-hand track. Total cytoplasmic RNA was translated in the presence of [35S]methionine in the wheat germ system as described in Materials and Methods. Two microliters of the reaction in 10 μl of sample buffer was applied to a hyperbolic 7.5 to 20% SDS-polyacrylamide gel run at a constant current of 10 mA. The fluorogram of the gel is shown.*

proteins and a single 95K protein, and the Ad2+ND5 42K protein (Fig. 5 and 6). Anderson et al. (3) have obtained in vitro synthesis of an Ad2+ND1 28K protein and Ad2+ND4 60K and 95K proteins from SV40-specific RNA.

The relative levels of proteins in the translation products of the unhybridized RNA and SV40-specific RNAs are not directly comparable because we used 1/100 of the total unhybridized RNA and 1/6 of the total SV40-specific RNA in the translation reactions shown. Even so, the in vitro products synthesized from the SV40-specific RNA showed a marked decrease in the Ad2 and host cell proteins, with a strong enhancement of those proteins identified as SV40 specific. In the case of Ad2+ND1, for example, the 28K protein was barely detectable in the product translated from unfractionated RNA (Fig. 5), and yet in the product translated from the RNA hybridized to SV40 DNA it was the predominant protein synthesized in vitro.

There was a complete absence of Ad2-specific proteins in the products translated from Ad2-infected HeLa RNA that had been hybridized to SV40 DNA (Fig. 5). Although the majority of the Ad2 proteins, such as hexon, were absent from the in vitro products from hybrid virus-infected HeLa SV40-specific RNA, there were some proteins present, in addition to the SV40-specific proteins, that are thought to be Ad2 specific. These Ad2-specific proteins, labeled A through E (Fig. 5 and 6), had molecular weights of 100K, 62K, 31K, 26K, and 20K. The 31K, 26K, and 20K proteins were the only proteins seen in the Ad2+ND3 product from SV40-specific RNA. The 100K and 62K proteins are thought to correspond to Ad2 IIa and IV. By comparative peptide mapping, we have shown that the 26K protein corresponds to Ad2 Vb, but have not found Ad2 in vivo counterparts for the 31K and 20K proteins. Proteins A through E were not present in the same relative abundance as in the product from unfractionated RNA, and they contrasted with the SV40-specific proteins, which showed strong enrichment after selection of RNA with SV40 DNA. This is clearly seen in the Ad2+ND5 product from SV40-specific RNA, where the Ad2+ND5 42K

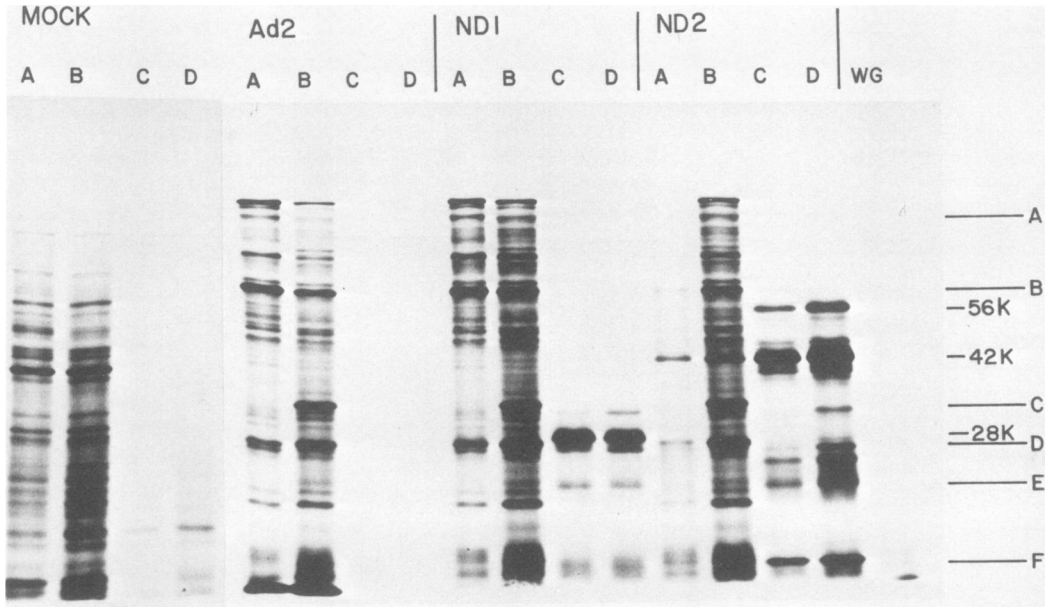

FIG. 5. *Translation products of fractionated cytoplasmic RNA from mock-infected, Ad2-infected, Ad2+ND1-infected, and Ad2+ND2-infected HeLa cells. In each case four fractions were translated: (A) unfractionated RNA; (B) unhybridized RNA; (C) hybridized RNA eluted with 98% formamide; (D) hybridized RNA eluted with 1 M NaCl. SV40-specific RNA was isolated from 500 μg of total cytoplasmic RNA as described in Materials and Methods. Total unfractionated cytoplasmic RNA was translated at a final concentration of 375 μg/ml. One-hundredth of the unhybridized RNA recovered and one-sixth of both of the eluted RNA fractions were translated in the presence of [35S]methionine in the wheat germ system in 10-μl reactions as described in Materials and Methods. Two microliters of each reaction in 10 μl of sample buffer was run on a 10% SDS-polyacrylamide gel. The fluorogram of the gel is shown. The translation products from the wheat germ system without any RNA added can be seen in the track labeled WG.*

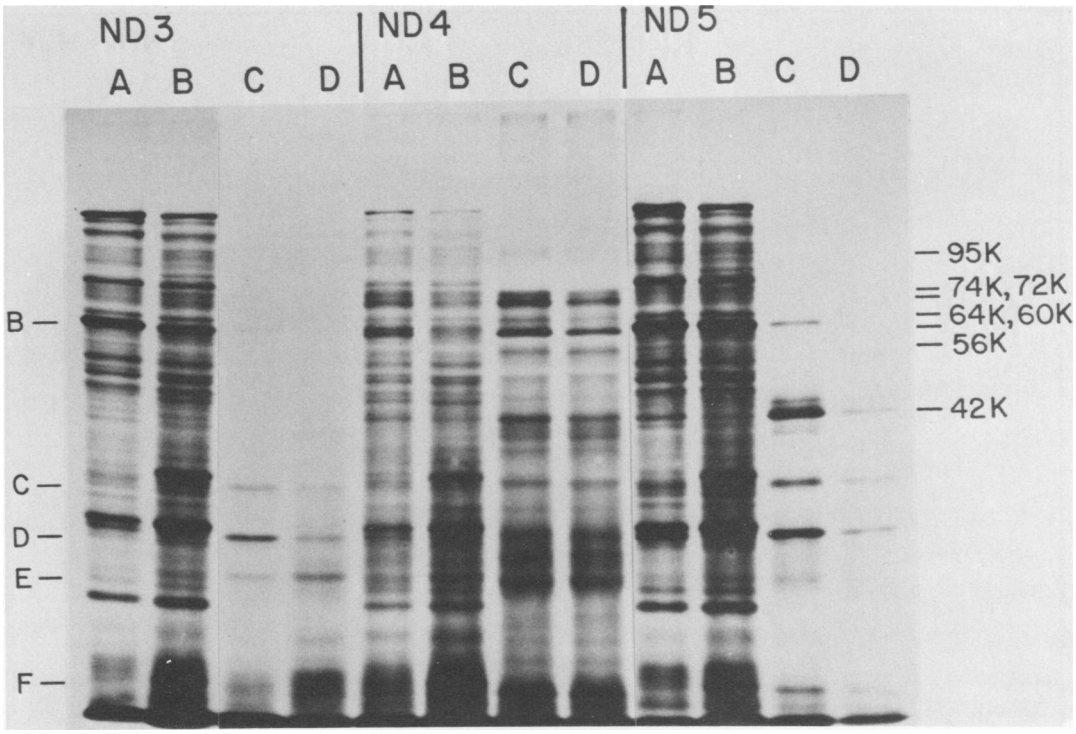

FIG. 6. *Translation products of fractionated cytoplasmic RNA from Ad2⁺ND3-, Ad2⁺ND4-, and Ad2⁺ND5-infected HeLa cells. The same legend applies as for Fig. 5.*

protein was enriched and the 62K protein, presumably Ad2 fiber, was weakly labeled relative to the product from the unfractionated or unhybridized RNA (Fig. 6). The 15K protein, labeled F, was only seen in the translation products from SV40-specific RNA from Ad2⁺ND2-, Ad2⁺ND4-, and Ad2⁺ND5-infected cells; it is an SV40-specific protein containing peptides 5, 6, 7, 8, and 9 (data not shown). The other weakly labeled proteins were presumably translation products from endogenous wheat germ message since they were also seen in the product from SV40-selected, mock-infected HeLa RNA (Fig. 5) and in the product obtained when no RNA was added to the wheat germ system (Fig. 5).

Peptide analysis of in vitro-synthesized SV40-specific proteins. The tryptic peptides of the in vitro SV40-specific proteins synthesized from SV40-specific RNA were compared with those of the corresponding in vivo SV40-specific proteins. SV40-specific RNA from Ad2⁺ND1-, Ad2⁺ND2-, Ad2⁺ND3-, Ad2⁺ND4-, and Ad2⁺ND5-infected HeLa cells was translated in the wheat germ system in the presence of [^{35}S]-methionine. The translation products were purified and digested with trypsin in the same manner as the in vivo proteins. There was almost exact correspondence between the tryptic peptide pattern of any one in vitro protein (Fig. 7–9) and its in vivo counterpart (Fig. 1–3). The peptide map of a mixture of the in vivo and in vitro Ad2⁺ND4 60K proteins showed complete correspondence between the peptides of the two proteins (data not shown). The peptides of the Ad2⁺ND2 56K and the Ad2⁺ND4 42K and 56K in vitro proteins, however, did not completely correspond with those of the in vivo proteins. The in vitro Ad2⁺ND4 42K protein, but not the in vitro Ad2⁺ND2 42K protein, contained a strong additional peptide that appeared to correspond to peptide 10 found in all proteins of molecular weight greater than 42,000. The explanation for an additional peptide is not known. In the in vitro Ad2⁺ND2 56K and Ad2⁺ND4 56K proteins, the characteristic unique peptide E was more weakly labeled relative to peptides 3 and 10 than its in vivo counterpart. If peptide E arises as a result of modification, then it might be present in decreased amount in the in vitro protein.

Peptide analysis of SV40 T-antigen. When extracts of SV40-infected monkey cells (CV1 cells) were immunoprecipitated with serum from hamsters with SV40-induced tumors (SV40 anti-T serum), two proteins with apparent molecular weights of 100K and 90K were

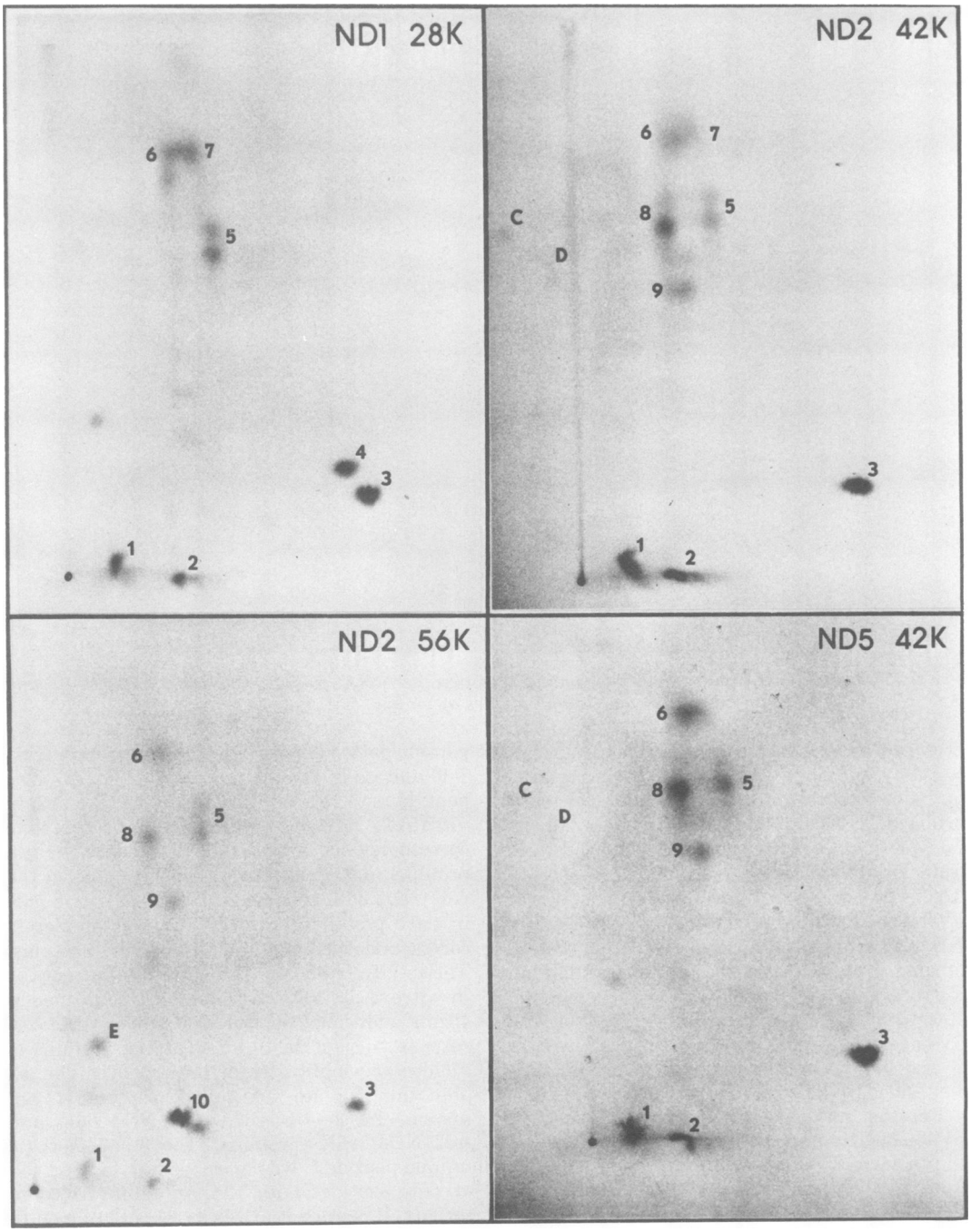

FIG. 7. *Tryptic peptide maps of [^{35}S]methionine-labeled SV40-specific proteins synthesized in vitro. Tryptic digests were prepared from proteins translated from SV40-specific RNA isolated by hybridization to SV40 DNA-Sepharose as described in Materials and Methods. Peptide maps of in vitro Ad2^{+}ND1 28K, Ad2^{+}ND2 42K and 56K, and Ad2^{+}ND5 42K proteins can be seen.*

precipitated. These proteins were not precipitated with normal hamster serum and were not precipitated from extracts of uninfected cells. The peptide map of the SV40 90K protein (Fig. 10) labeled with [^{35}S]methionine and precipitated with SV40 anti-T serum showed numerous peptides in common with the Ad2^{+}ND4 100K protein (Fig. 3). It is obvious that the 90K

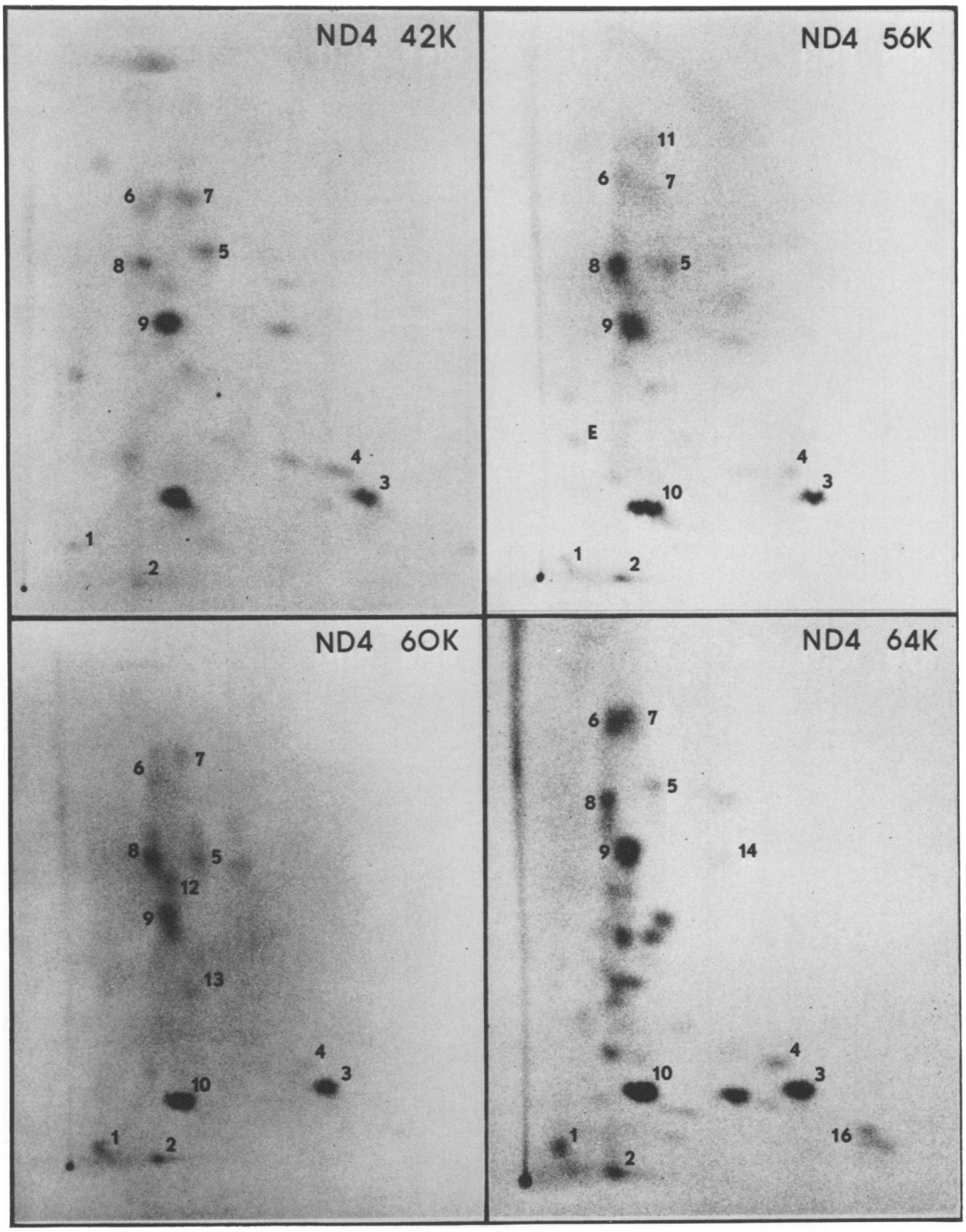

FIG. 8. *Tryptic peptide maps of in vitro Ad2⁺ND4 42K, 56K, 60K, and 64K proteins labeled with [³⁵S]methionine.*

protein was strongly related to the whole series of SV40-specific proteins in Ad2-SV40 hybrid virus-infected cells. Seventeen of the 21 [^{35}S]-methionine-labeled peptides seen in the Ad2⁺-ND4 100K protein were also found in the SV40 90K protein. Peptides 11, 12, 13, and 21 were not detectable in the 90K protein, but we do not know the meaning of their absence. Three of these peptides first appeared in the Ad2⁺ND4 56K (peptide 11) and 60K (peptides 12 and 13) proteins and must, therefore, be coded by nucleotide sequences somewhere in the middle of

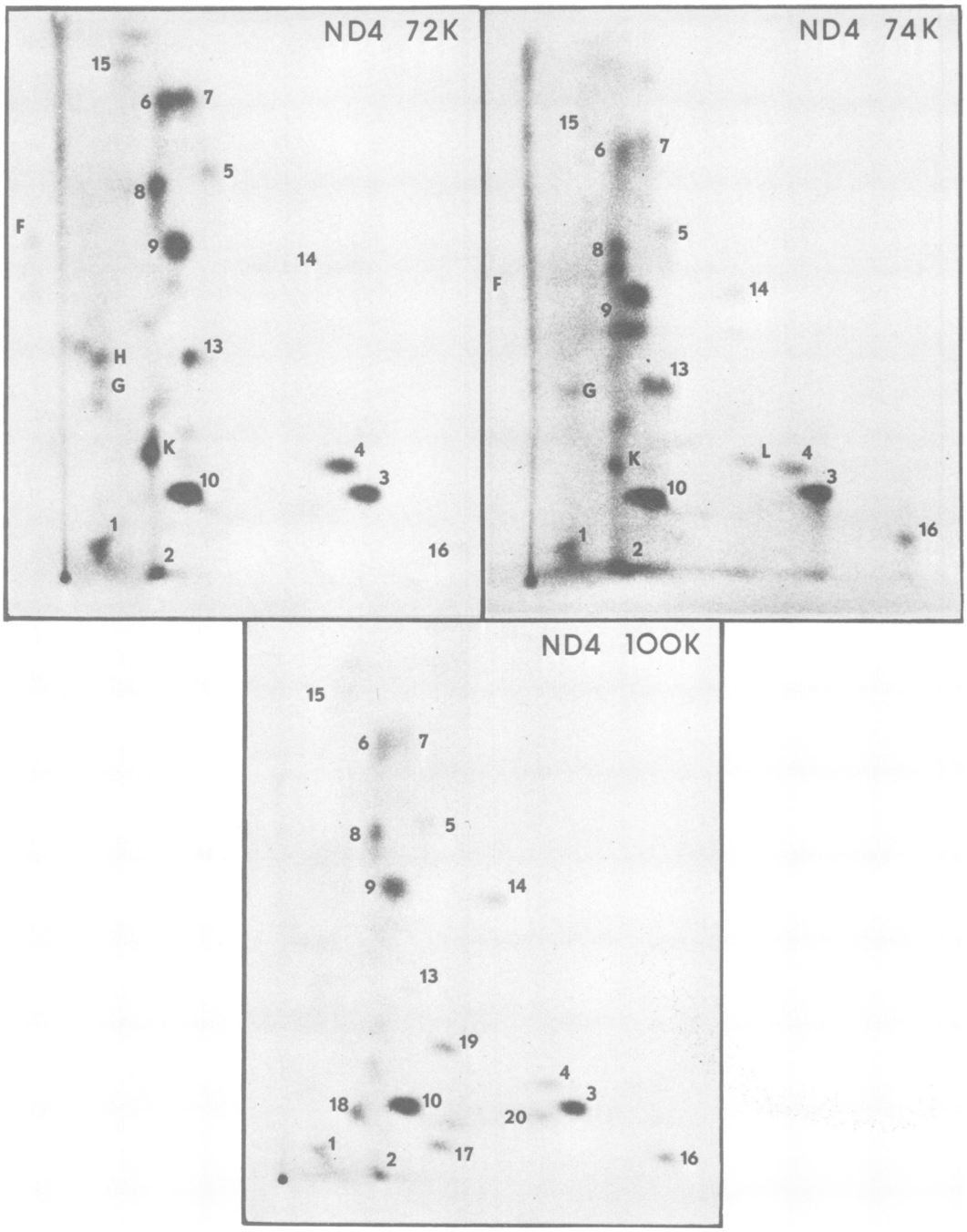

FIG. 9. *Tryptic peptide maps of in vitro Ad2⁺ND4 72K, 74K, and 100K proteins labeled with [³⁵S]methionine.*

the early region of the SV40 DNA. Only two peptides, M and N, in the SV40 90K protein were not found in the Ad2⁺ND4 100K protein. The peptide map of a mixture of the SV40 90K protein with the Ad2⁺ND2 56K protein (Fig. 10) indicates that peptide M differed from unique peptide E found in the Ad2⁺ND2 56K protein. It is probable that there are even more peptides in common between the SV40 100K protein precipitated with SV40 anti-T serum and the

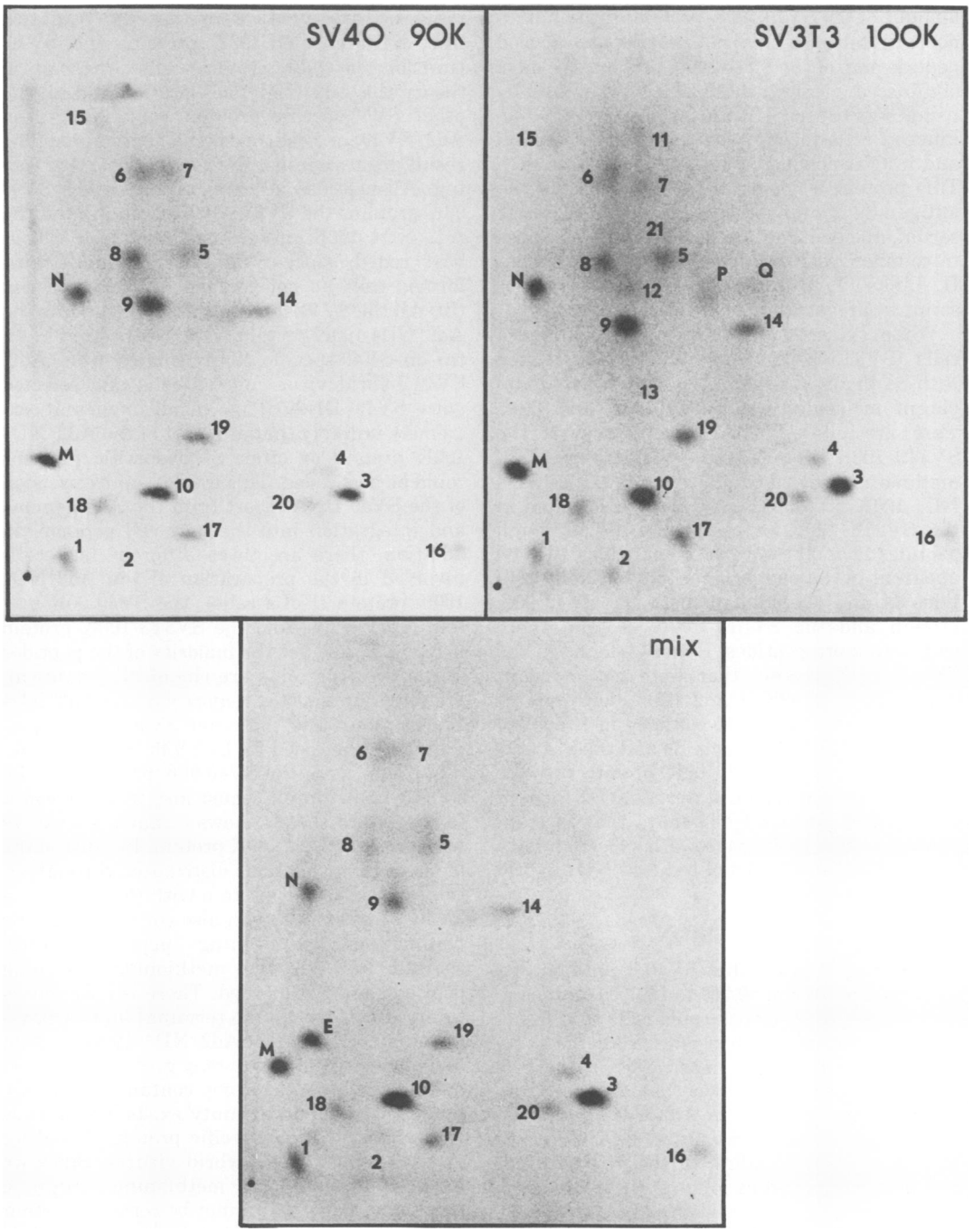

FIG. 10. *Tryptic peptide maps of [^{35}S]methionine-labeled in vivo SV40 T-antigen. The SV40 90K protein was immunoprecipitated from SV40-infected CV1 cells, and the SV3T3 100K protein was immunoprecipitated from SV3T3 cells, both with SV40 anti-T serum, as described in Materials and Methods. A peptide map of an equal mixture of SV40 90K protein and Ad2$^+$ND2 56K protein can also be seen.*

Ad2$^+$ND4 100K protein, although these proteins migrated somewhat differently on SDS-polyacrylamide gels. The SV40 100K protein migrated slightly more slowly than the Ad2$^+$ND4 100K protein. Due to a predominance of the SV40 90K protein and a decreased

amount of the SV40 100K protein in the immunoprecipitate, we have not yet prepared a good peptide map of the SV40 100K protein. We have obtained in vitro synthesis of a 100K protein by using SV40-specific RNA isolated from SV40-infected cells in both the wheat germ system and mRNA-dependent reticulocyte lysate (47). This protein is identical in size to in vivo T-antigen, is immunoprecipitable by SV40 anti-T serum, and has methionine-containing peptides in common with in vivo T-antigen (K. Mann, H. Linke, T. Hunter, and G. Walter, manuscript in preparation).

When extracts of SV40-transformed mouse cells (SV3T3 cells) were immunoprecipitated with SV40 anti-T serum, two proteins with apparent molecular weights of 100K and 110K were precipitated. The peptide map of the SV3T3 100K protein shows that all the [^{35}S]-methionine-labeled peptides found in the Ad2$^+$-ND4 100K protein (Fig. 3) were also found in the SV3T3 100K protein (Fig. 10), although peptides 13 and 21 were too weakly labeled to be apparent in the photograph. In addition to peptides M and N, found in both the SV40 90K protein and the SV3T3 100K protein, there were two more peptides, P and Q, found in the SV3T3 100K protein that were not found in either the SV40 90K or Ad2$^+$ND4 100K proteins (Fig. 10). The M and N peptides in the SV40 90K and SV3T3 100K proteins and the P and Q peptides in the SV3T3 100K protein may be coded for by that region of the SV40 DNA (from 0.59 to 0.67 on the SV40 map) that is transcribed in the early phase of SV40 productive infection but is not part of the Ad2$^+$ND4 hybrid virus genome (28, 34).

DISCUSSION

Our studies prove that SV40 T-antigen, defined as a protein of 90,000 to 100,000 daltons in SV40-infected or -transformed cells that is immunoprecipitable with serum from SV40 tumor-bearing hamsters, is an SV40-coded protein. We have also shown that all the SV40-specific proteins found in Ad2-SV40 hybrid virus-infected cells are SV40 coded. The experimental evidence leading to these conclusions can be summarized as follows. (i) Tryptic peptide mapping has demonstrated that the SV40-specific proteins in hybrid virus-infected cells ranging in size from 28K to 100K represent an overlapping set of gene products such that the smaller proteins are completely overlapped by the larger. (ii) The majority of methionine-containing tryptic peptides of the largest SV40-specific protein, the Ad2$^+$ND4 100K protein, which is representative of the whole overlapping set, are identical to those of T-antigen from SV40-infected (90K protein) and SV40-transformed (100K protein) cells. Although in theory the Ad2$^+$ND4 100K protein and all the other SV40-specific proteins could have been Ad2, SV40, or host coded, the peptide mapping result argues against the possibility of Ad2 coding. All peptides in common among the SV40 90K protein, the SV3T3 100K protein, and the Ad2$^+$ND4 100K protein can only be SV40 or host coded, since SV40-infected and -transformed cells do not contain Ad2 information. (iii) All the SV40-specific proteins including the Ad2$^+$ND4 100K protein were synthesized in vitro on SV40-specific RNA isolated from Ad2-SV40 hybrid virus-infected cells and selected with SV40 DNA. This result makes it extremely unlikely that any part of the Ad2$^+$ND4 100K protein or other SV40-specific proteins could be host coded. This would require excision of the SV40 DNA insert from the Ad2 genome and integration into the host cell genome. In addition, there are three different host cells involved in the preparation of the Ad2$^+$ND4 100K protein (HeLa cells), the SV40 90K protein (CV1 cells), and the SV3T3 100K protein (3T3 cells), and yet the majority of the peptides of the three proteins are chemically identical. We conclude that the majority of the Ad2$^+$ND4 100K protein and the other SV40-specific proteins must be coded for by SV40. Furthermore, SV40 T-antigen (the SV40 90K protein and the SV3T3 100K protein) must also be SV40 coded to the extent that it shows common sequences with the Ad2$^+$ND4 100K protein. Because of the identity of the peptides distributed throughout the Ad2$^+$ND4 100K protein with the peptides of SV40 T-antigen, we can also conclude that the non-methionine-containing peptides, interspersed between the methionine-containing peptides, are SV40 coded. There is only uncertainty about the amino-terminal and carboxy-terminal ends of the Ad2$^+$ND4 100K protein and of T-antigen, which may give rise to one or more tryptic peptides not containing methionine. The same uncertainty exists for the ends of the largest SV40-specific proteins coded for by each of the other hybrid viruses. Since we have studied only the methionine-containing tryptic peptides, we cannot be certain whether both ends of the T-antigen molecule are SV40 coded. This question might be approached by studying the tryptic peptides of the proteins labeled with a ^{14}C-amino acid mixture.

Since the [^{35}S]methionine-labeled peptides of the Ad2$^+$ND1 28K protein are coded for by the 3′ end of the SV40 early mRNA (15, 61), their reiteration in all the higher-molecular-weight

products from the SV40 early region suggests that all the SV40-specific proteins have a common C-terminal region. This implies that the different-molecular-weight products must be generated through independent initiations, either on a single mRNA or on mRNA's of different sizes, rather than being generated by independent terminations. Preliminary results indicate that each of the SV40-specific proteins, including the smaller proteins of Ad2+ND2 and Ad2+ND4, is made on a different-size mRNA (C. Lawrence and T. Hunter, unpublished data). On the basis of this result and the peptide maps, it seems unlikely that the shorter proteins from Ad2+ND2 and Ad2+ND4 contain any Ad2 sequences, since these proteins are probably initiated within the SV40 sequence. N-terminal peptides can be specifically labeled with *N*-formyl-[^{35}S]methionyl-tRNA$_f^{Met}$ in an in vitro protein-synthesizing system. We have found that all of the SV40-specific proteins are labeled with [^{35}S]formyl-methionine in vitro and that for those proteins examined only a single tryptic peptide is labeled. Initial results from peptide analysis of the [^{35}S]formyl-methionine-labeled proteins support the idea of independent initiation, since the N-terminal peptides of the Ad2+ND1 28K protein and the Ad2+ND2 56K protein appear to be different (C. Lawrence, T. Hunter, G. Walter, and K. Mann, unpublished data). The fact that all the SV40-specific proteins share a common C-terminal region and are initiated independently indicates that the smaller proteins in Ad2+ND2 and Ad2+ND4 are not generated by proteolytic processing, a finding that is borne out by in vivo pulse-chase experiments (13).

Independent initiation of each SV40-specific protein could result in a unique methionine-containing peptide not found in the other SV40-specific proteins if (i) the initiation site of the protein is internally located within a peptide normally generated by tryptic digestion of the higher-molecular-weight SV40-specific proteins, and (ii) the newly generated peptide still contains methionine. In many of the proteins, however, there is more than one unique peptide, a situation that could not result from internal initiation alone. Unique peptides could also result from modification, such as phosphorylation or glycosylation. If the unique peptides have arisen through modification, then the process must also be occurring in vitro, since the unique peptides are found in both the in vivo protein and its in vitro equivalent.

The results of others suggest that there are covalently linked Ad2-SV40 RNA molecules in cells infected by Ad2+ND1 (3, 15), Ad2+ND3 (15), and Ad2+ND4 (3, 46). Hybridization of cytoplasmic RNA to SV40 DNA-Sepharose will select for hybrid Ad2-SV40 RNA molecules as well as for RNA containing only SV40 sequences. Some of these hybrid Ad2-SV40 RNA molecules seem to be mRNA's for recognized Ad2 proteins, since we have found that SV40-specific RNA from hybrid virus-infected cells codes for the 100K, 62K, 31K, and 26K proteins (Fig. 5 and 6). Anderson et al. (3) have also observed the synthesis of 100K, 38K (which may correspond to our 31K protein), and 26K proteins in the translation products of SV40-specific RNA isolated from Ad2+ND1-infected cells. Therefore, the inserted SV40 sequences in the hybrid viruses do not appear to affect the products of the known late Ad2 genes mapping nearest to the insert (100K, 62K, 38K, and 26K) (36). The early Ad2 gene coding for the 15.5K protein also maps near the position of the SV40 inserts, but may be deleted in the hybrid viruses (27, 37). We see no evidence of the Ad2 15.5K early protein in our translation products (Fig. 5 and 6). Even though there are covalently linked Ad2-SV40 hybrid mRNA molecules, there is to date no evidence that the SV40 sequence is inserted into a functional Ad2 gene. If this is true, then the SV40-specific proteins may not contain any Ad2 amino acid sequences covalently linked to SV40 sequences.

The structure of the Ad2-SV40 hybrid RNA molecules deduced from nucleic acid hybridization experiments contains about 1,000 nucleotides of Ad2 RNA at the 5′ end of the RNA (15). Clearly the Ad2-SV40 hybrid RNA molecules that are mRNA's for Ad2 proteins must contain much longer sequences of Ad2 RNA. Since the transcription of the *r* strand of the Ad2 genome late in infection occurs from very few promoters (14), most of the late genes are expressed as large precursors from which individual mRNA's are subsequently processed. In the case of the hybrid viruses, such precursor transcripts will contain SV40 sequences. We propose that some small fraction of the mRNA's for the 100K, 31K (probably corresponding to the 38K protein of Lewis et al. [36]), and 26K proteins reach the cytoplasm before they are completely processed. Because of the restriction on internal initiation in eukaryotic cells, it is likely that such mRNA's will have the expressed Ad2 cistron at their 5′ ends, with sequences including the SV40 region at their 3′ ends. In keeping with this idea, we note that 100K, 38K, and 26K proteins map just to the 5′ side of the SV40 insert on the *r* strand (36). Some of the mRNA's for the 62K Ad2 IV protein appear to contain SV40 sequences. Since Ad2

IV maps just to the 3′ side of the SV40 insert on the *r* strand (36), incomplete processing products could contain SV40 sequences. However, in this case, it is harder to see how the Ad2 IV gene can be expressed on such molecules, since it would not be at the 5′ end of the RNA.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants CA 15365 and CA 17096 from the National Cancer Institute. K.M. was the recipient of a postdoctoral fellowship from the Medical Research Council of Canada.

LITERATURE CITED

1. **Ahmad-Zadeh, C., B. Allet, J. Greenblatt, and R. Weil.** 1976. Two forms of simian virus 40-specific T-antigen in abortive and lytic infection. Proc. Natl. Acad. Sci. U.S.A. 73:1097-1101.
2. **Alwine, J. C., S. I. Reed, J. Ferguson, and G. R. Stark.** 1975. Properties of T antigens induced by wild-type SV40 and tsA mutants in lytic infection. Cell 6:529-533.
3. **Anderson, C. W., J. B. Lewis, D. R. Baum, and R. F. Gesteland.** 1976. Simian virus 40-specific polypeptides in Ad2$^+$ND1- and Ad2$^+$ND4-infected cells. J. Virol. 18:685-692.
4. **Black, P. H.** 1966. Transformation of mouse cell line 3T3 by SV40: dose response relationship and correlation with SV40 tumor antigen production. Virology 28:760-763.
5. **Black, P. H., W. P. Rowe, H. C. Turner, and R. J. Huebner.** 1963. A specific complement-fixing antigen present in SV40 tumor and transformed cells. Proc. Natl. Acad. Sci. U.S.A. 50:1148-1156.
6. **Brugge, J. S., and J. S. Butel.** 1975. Involvement of the simian virus 40 gene *A* function in the maintenance of transformation. J. Virol. 15:619-635.
7. **Carroll, R. B., and A. E. Smith.** 1976. Monomer molecular weight of T antigen from simian virus 40-infected and transformed cells. Proc. Natl. Acad. Sci. U.S.A. 73:2254-2258.
8. **Chou, J. Y., J. Avila, and R. G. Martin,** 1974. Viral DNA synthesis in cells infected by temperature-sensitive mutants of simian virus 40. J. Virol. 14:116-124.
9. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. 13:1101-1109.
10. **Chou, J. Y., and R. G. Martin.** 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. J. Virol. 15:145-150.
11. **Crumpacker, C. S., P. H. Henry, T. Kadefuda, 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$^+$ND1 genome. J. Virol. 7:352-358.
12. **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.
13. **Deppert, W., G. Walter, and H. Linke.** 1977. SV40 tumor-specific proteins: subcellular distribution and metabolic stability in HeLa cells infected with nondefective adenovirus 2-SV40 hybrid viruses. J. Virol. 21:1170-1186.
14. **Flint, J.** 1977. The topography and transcription of the adenovirus genome. Cell 10:153-166.
15. **Flint, S. J., Y. Wenerka-Lutz, A. S. Levine, J. Sambrook, and R. A. Sharp.** 1975. Adenovirus transcription. II. RNA sequences complementary to simian virus 40 and adenovirus 2 DNA in Ad2$^+$ND$_1$- and Ad2$^+$ND$_3$-infected cells. J. Virol. 16:662-673.
16. **Gibson, W.** 1974. Polyoma virus proteins: a description of the structural proteins of the virion based on polyacrylamide gel electrophoresis and peptide analysis. Virology 62:319-336.
17. **Gilboa, E., C. L. Prives, and H. Aviv.** 1975. Purification of SV40 messenger RNA by hybridization to SV40 DNA covalently bound to Sepharose. Biochem. 14:4215-4220.
18. **Girardi, A. J., and V. Defendi.** 1970. Induction of SV40 transplantation antigen (TrAg) during the lytic cycle. Virology 42:688-698.
19. **Graessmann, M., and A. Graessmann.** 1976. "Early" simian-virus-40-specific RNA contains information for tumor antigen formation and chromatin replication. Proc. Natl. Acad. Sci. U.S.A. 73:366-370.
20. **Hatanaka, M., and R. Dulbecco.** 1966. Induction of DNA synthesis by SV40. Proc. Natl. Acad. Sci. U.S.A. 56:736-740.
21. **Henry, P., P. H. Black, M. N. Oxman, and S. M. Weissman.** 1966. Stimulation of DNA synthesis in mouse cell line 3T3 by simian virus 40. Proc. Natl. Acad. Sci. U.S.A. 56:1170-1176.
22. **Henry, P. H., L. E. Schnipper, R. J. Samaha, C. S. Crumpacker, A. M. Lewis, Jr., and A. S. Levin.** 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.
23. **Hirs, C. H. W.** 1967. Performic acid oxidation. Methods Enzymol. 11:197-199.
24. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.
25. **Hoggan, M. D., W. P. Rowe, P. H. Black, and R. J. Huebner.** 1965. Production of tumor specific antigens by oncogenic viruses during acute cytolytic infections. Proc. Natl. Acad. Sci. U.S.A. 53:12-19.
26. **Hunter, A. R., P. Farrell, R. J. Jackson, and T. Hunt.** 1977. The role of polyamines in cell-free protein synthesis in the wheat germ system. Eur. J. Biochem. 75:149-157.
27. **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.
28. **Khoury, G., P. Howley, D. Nathans, and M. Martin.** 1975. Post-transcriptional selection of simian virus 40-specific RNA. J. Virol. 15:433-437.
29. **Kimura, G., and R. Dulbecco.** 1973. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. Virology 52:529-534.
30. **Kimura, G., and A. Itagaki.** 1975. Initiation and maintenance of cell transformation by simian virus 40: a viral genetic property. Proc. Natl. Acad. Sci. U.S.A. 72:673-677.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
32. **Lai, C.-J., and D. Nathans.** 1975. Mapping the genes of the simian virus 40. Cold Spring Harbor Symp. Quant. Biol. 39:53-60.
33. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335-341.
34. **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.
35. **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+ND1 genome. J. Virol. 7:343-351.

36. **Lewis, J. B., J. F. Atkins, C. W. Anderson, P. R. Baum, and R. F. Gesteland.** 1975. Mapping of late adenovirus genes by cell-free translation of RNA selected by hybridization to specific DNA fragments. Proc. Natl. Acad. Sci. U.S.A. 72:1344-1348.
37. **Lewis, J. B., J. F. Atkins, P. R. Baum, R. Solem, R. F. Gesteland, and C. W. Anderson.** 1976. Location and identification of the genes for adenovirus type 2 early polypeptides. Cell 7:141-151.
38. **Lewis, Jr., A. M., A. S., Levine, 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.
39. **Lewis, Jr., A. M., 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+ND1 virus. J. Virol. 7:189-197.
40. **Lewis, Jr., A. M., 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.
41. **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.
42. **March, S. C., I. Parikh, and P. Cuatrecasas.** 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. 60:149-152.
43. **Martin, R. G., and J. Y. Chou.** 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. 15:599-612.
44. **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.
45. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene *A* function and maintenance of transformation. J. Virol. 15:636-644.
46. **Oxman, M. N., M. J. Levin, and A. M. Lewis, Jr.** 1974. Control of simian virus 40 gene expression in adenovirus-simian virus 40 hybrid viruses. Synthesis of hybrid adenovirus 2-simian virus 40 RNA molecules in cells infected with a nondefective adenovirus 2-simian virus 40 hybrid virus. J. Virol. 13:322-330.
47. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. 67:247-256.
48. **Pope, J. H., and W. P. Rowe.** 1964. Detection of specific antigen in SV40-transformed cells by immunofluorescence. J. Exp. Med. 120:121-127.
49. **Prives, C., H. Aviv, E. Gilboa, E. Winocour, and M. Revel.** 1975. The cell-free translation of early and late classes of SV40 messenger RNA. Colloq. Inst. Nat. Sante R. Med. 47:305-312.
50. **Prives, C., E. Gilboa, M. Revel, and E. Winocour.** 1977. Cell-free translation of simian virus 40 early messenger RNA coding for viral T-antigen. Proc. Natl. Acad. Sci. U.S.A. 74:457-461.
51. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. 57:1514-1521.
52. **Rapp, F., T. Kitahara, J. S. Butel, and J. L. Melnick.** 1964. Synthesis of SV40 tumor antigen during replication of simian papovavirus (SV40). Proc. Natl. Acad. Sci. U.S.A. 52:1138-1142.
53. **Robb, J. A., and R. Lopez-Revilla.** 1976. Temperature dependent synthesis of early and late SV40 cytoplasmic RNA during tsD*101 mutant virion infection. Intervirology 6:122-128.
54. **Roberts, B. E., M. Gorecki, R. C. Mulligan, K. J. Danna, S. Rozenblatt, and A. Rich.** 1975. Simian virus 40 DNA directs synthesis of authentic viral polypeptides in a linked transcription-translation cell-free system. Proc. Natl. Acad. Sci. U.S.A. 72:1922-1926.
55. **Roberts, B. E., and B. M. Paterson.** 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. Proc. Natl. Acad. Sci. U.S.A. 70:2330-2334.
56. **Rundell, K., J. K. Collins, P. Tegtmeyer, H. L. Ozer, C.-J. Lai, and D. Nathans.** 1977. Identification of simian virus 40 protein A. J. Virol. 21:636-646.
57. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. 10:591-598.
58. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene *A* in transforming infection. J. Virol. 15:613-618.
59. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene *A*. J. Virol. 16:168-178.
60. **Tenen, D. G., P. Baygell, and D. M. Livingston.** 1975. Thermolabile T (tumor) antigen from cells transformed by a temperature-sensitive mutant of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. 72:4351-4355.
61. **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.
62. **Williams, J. F.** 1970. Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. J. Gen. Virol. 9:251-258.