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Processing of Adenovirus 2-Induced Proteins

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Analysis of ^{35}S-methionine-labeled extracts of adenovirus 2-infected KB cells revealed 22 virus-induced polypeptide components. Most proteins of the virion were easily detected in extracts of whole cells labeled for short periods between 15 and 30 h after infection; however, several virion components were conspicuously absent. Radioactivity appeared in two of these virion components during a chase in nonradioactive medium, and this appearance was paralleled by a decrease in the radioactivity associated with two nonvirion adenovirus-induced proteins, results which imply precursor-product relationships for these components. Comparison of one of the chasable adenovirus-induced components (designated P-VII; mass of 20,000 daltons) and the major core protein (VII; mass of 18,500 daltons) of the virion showed that they have four common methionine-containing tryptic peptides; P-VII has an additional methionine residue which is not found in the major core protein. We propose that at least two of the adenovirus 2 virion components are derived by the cleavage of higher molecular weight precursor polypeptides.

Adenovirus 2, a nononcogenic member of the human adenoviruses, has been shown to contain at least 10 distinct virion polypeptides and a linear double-stranded DNA molecule with mass of 23×10^6 daltons (5, 14, 18). The virus particle consists of an icosahedral shell (capsid) with projecting spikes and an inner core of protein and DNA. The major protein components of the capsid (hexon, II; penton base, III; and fiber, IV) and the core (VII) have been isolated and characterized (see review, 20). Analysis of viral-specific macromolecular synthesis in productively infected KB and HeLa human tissue culture cells is aided by the fact that host cell DNA, RNA, and protein synthesis are progressively inhibited (3, 21, 23). Early viral message, representing 10 to 20% of the viral genome, can be detected in KB cells 2 to 6 h after infection (8). At least one virus-induced antigen is made before, and in the absence of, viral DNA replication (7). The late phase of virus multiplication begins approximately 6 to 8 h after infection with the onset of viral DNA synthesis (8). Capsid antigens can be detected shortly thereafter, and their appearance seems to be contingent upon viral DNA synthesis (6, 7). The virion proteins are transported to the nucleus, where virion assembly takes place. Newly synthesized virus can be detected by 24 h after infection; virus assembly occurs relatively slowly, and a large fraction of the capsid proteins and viral DNA apparently never assemble into virus particles.

The adenovirus 2 genome could theoretically code for 1.1×10^6 daltons of polypeptide or 20 to 50 average-sized proteins. Several species of adenovirus-specific RNA, ranging in size from 10 to 29 s, can be found in polysomes during the late stages of virus replication (13, 16). Because the largest of these species could code for a polypeptide nearly twice as large as the largest known capsid component (hexon, 120,000 daltons), it has been postulated that some of the adenovirus virion components might be derived from high-molecular-weight precursor polypeptides as are the poliovirus capsid components (16).

We have analyzed extracts of whole and partially fractionated cells which had been labeled with ^{35}S-methionine at various times after infection. After electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels, 22 virus-induced polypeptide bands were found by autoradiography. Whereas most of the virion proteins were observed in extracts from 1-h pulse-labeled cells, several, notably the major core protein (VII) and a hexon-associated protein (VI), were conspicuously absent. Furthermore, at least two of the nonvirion polypeptides from infected cell extracts were found to be unstable. One of these was shown to be related to the major core protein by tryptic fingerprint-

ing. Analysis of the synthesis of the major core protein and capsid component VI has led us to the conclusion that these virion components, and possibly others as well, are derived from adenovirus-induced precursor polypeptides. Unlike the poliovirus precursor, however, these precursors are only slightly larger than the final product. We suspect that this processing mechanism is related to assembly of the virus particle rather than being a way of separating different gene products.

MATERIALS AND METHODS

Cells and virus. KB cells (obtained from L. Prague, Rutgers University, New Brunwsick, N.J.) adapted to growth in plastic petri dishes (Nunc, 4-Shore, La Jolla, Calif.) were grown in Dulbecco modified Eagle (DME) medium (Grand Island Biological Co.) supplemented with 10% calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Mycostatin at 20 μg/ml was routinely added to the medium after virus infection. The adenovirus 2 stocks had titers of 10^{10} to 5×10^{10} PFU/ml and were generously provided by Ulf Pettersson. Immediately before infection, a sample of the stock was incubated for 30 min at 37 C with 100 μg of trypsin per ml.

Preparation of ^{35}S-methionine-labeled cell extracts. Confluent 60- or 90-mm plates of KB cells were infected by the addition of ca. 100 PFU of virus per cell (ca. 10^7 cells per 90-mm plate) in 0.5 ml of phosphate-buffered saline (PBS). Mock infections were with 0.5 ml of PBS only. After 30-min incubation at 37 C to allow adsorption, 5 ml (60-mm plates) or 10 ml (90-mm plates) of fresh DME supplemented with 10% calf serum was added to each plate. Immediately before labeling, the medium was removed and the plates were washed with 2 to 3 ml of methionine-free DME. A sample (1-5 ml) of methionine-free DME containing 10% calf serum was then added to each plate, followed immediately by one-tenth volume of normal DME supplemented with the desired amount of ^{35}S-methionine. ^{35}S-labeled methionine was prepared from $^{35}SO_4$-grown *Escherichia coli* as previously described (4). In general, 20 to 200 μCi at 2.5 to 25 Ci/mmol (3 μg of methionine per ml) was added per plate; under these conditions, the uptake of ^{35}S-methionine was linear for at least 6 h.

Cells were harvested from the plates by vigorous washing and were collected by centrifugation at 1,000 × *g* for 5 min at 4 C. The cell pellets were washed two to three times by suspension in cold PBS and, after the addition of phenylmethyl sulfonyl fluoride to 300 μg/ml, were frozen at −20 C or processed as described below. Nuclei were prepared from the washed cell pellets after resuspension in reticulocyte suspension buffer (10 mM Tris, pH 7.4; 10 mM KCl; 1.5 mM $MgCl_2$) by the addition of one-tenth volume of 10% Triton X-100 (in water), gentle vortexing, and incubation for 15 min at 0 C (17). Nuclei were separated from the cytoplasm by centrifugation at 2,000 × *g* for 5 min at 4 C. Observation by phase-contrast microscopy consistently indicated that greater than 90% of the cells lysed after the addition of the nonionic detergent.

Preparation of ^{35}S-methionine-labeled adenovirus 2. Infected cultures of KB cells were labeled with ^{35}S-methionine essentially as described above from 18 to 30 h after infection. At 30 h, each plate was supplemented with additional DME to give a methionine concentration of 15 μg/ml (100 μM). At 48 h after infection, the cells were harvested and washed by centrifugation; virus was purified from the sonically disrupted, Freon 113 (Dupont Chemical Co.)-extracted cells by two bandings in CsCl as described by Pettersson and Sambrook (19).

SDS-polyacrylamide gel electrophoresis. The polyacrylamide gel system was basically that described by Laemmli (10), except that the gels were formed as slabs 1.3 mm thick and 110 mm long between glass plates. For analytical purposes, a stacking gel of 5% acrylamide and 0.13% bisacrylamide, containing up to 25 sample slots, was cast above the separating gel. The stacking gel for preparative separations was cast with one long sample well flanked on either side by analytical-sized wells into which suitable marker substances could be run. The acrylamide and bisacrylamide concentrations in the separating gel were chosen to give optimal separation of particular components and are listed in the appropriate figure legends.

Samples were prepared for electrophoresis by disruption in SDS sample buffer (0.0625 M Tris, pH 6.8; 2% SDS, Serva; 10% glycerol; 0.001% bromphenol blue; 0.1 M dithiothreitol), as previously described (1). Occasionally, cell pellets or fractions were incubated with RNase and DNase (1–5 μg/ml) for 10 min at 20 C to reduce their viscosity; this treatment did not affect the quality of the separation and was usually unnecessary. Analytical gels were loaded with 2 to 20 μliters of sample per slot (2 to 20 μg of protein; 50,000 to 200,000 counts/min). The center well of preparative gels was loaded with 200 to 1,000 μg of cell protein in 0.5 ml of sample buffer. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue (in 5% methanol, 7.5% acetic acid). Analytical gels were dried under vacuum and autoradiographed with Kodak single-sided medical X-ray film (SB54T). Side strips were cut from preparative gels and autoradiographed briefly to locate the radioactive bands. The desired bands were then cut from the remainder of the gel by using the stained bands as a visual guide. The protein was eluted from the gel by electrophoresis at 100 V for 6 to 12 h in Tris-glycine-SDS electrophoresis buffer (amounts per liter: 12.2 g of Tris, 57.7 g of glycine, and 4 g of SDS). Elution was conveniently accomplished by packing the gel slice between cellulose plugs in a disposable 10-ml plastic pipette which was capped at the anode end with a dialysis bag. By this technique, 80 to 100% of the radioactivity could be eluted, and most bands eluted in this manner had mobilities on SDS-polyacrylamide gels that were indistinguishable from the original bands.

Tryptic fingerprinting. ^{35}S-methionine-labeled samples were prepared for fingerprinting on MN300

cellulose thin-layer sheets (Brinkmann Instruments, Inc., Westbury, N.Y.) as previously described (1). The preparation included trichloroacetic acid precipitation in the presence of carrier bovine serum albumin, washing with acetone to remove SDS, oxidation with performic acid, and digestion with trypsin. The recovery of proteins eluted from SDS-polyacrylamide gels by trichloroacetic acid precipitation was variable; at low protein concentrations (e.g., less than 20 μg/ml), usually less than 50% of the radioactivity precipitated in 15% trichloroacetic acid. Two-dimensional separations were performed by spotting 5 to 20 μliters of the digested sample dissolved in 0.15 M NH_4OH in a 1-cm strip near one corner of the cellulose thin-layer sheet (20 by 20 cm). The sheets were developed first by chromatography in *n*-butanol, pyridine, acetic acid, water (300:60:200:240), and then at right angles to the first dimension by electrophoresis in pyridine, acetic acid, water (1:10:189, pH 3.5) at 50 V/cm for 35 min. The methionine-containing peptides were detected by autoradiography.

RESULTS

Proteins induced by adenovirus 2 infection of KB cells. Adenovirus 2, purified by the procedure of Pettersson and Sambrook (19) from infected KB cells which had been labeled during infection with ^{35}S-methionine, consistently yielded the band pattern shown in Fig. 1b when analyzed on SDS-polyacrylamide gels as described above. Thirteen labeled bands appeared to correspond to components which have previously been described by Maizel et al. (14) and Everitt et al. (5). Two additional bands can be seen which are very small (XI, 6,000 daltons; XII, 5,000 daltons) and difficult to resolve from polypeptide X (6,500 daltons). A comparison of a purified virus preparation by autoradiography and by Coomassie-blue staining of the polyacrylamide gel indicated that each of the stained bands was detectable by autoradiography. Therefore, each of the known capsid components of adenovirus 2 contains at least one methionine residue (20). That the incorporated label was in fact ^{35}S-methionine and not ^{35}S-cysteine was shown by acid hydrolysis of the acid-precipitable proteins from an adenovirus preparation and by identification of the ^{35}S-containing amino acids by thin-layer chromatography and electrophoresis (data not shown). More than 95% of the label was recovered as methionine or one of its oxidation products, and less than 1% of the label appeared in cysteine, cystine, or cysteic acid.

When whole cell extracts of adenovirus 2-infected KB cells, labeled late in infection with ^{35}S-methionine, were analyzed on SDS-polyacrylamide gels, 22 bands were observed which were not obviously present in extracts from uninfected cells (Fig. 1c, d). Fourteen of these bands have apparent molecular weights which are indistinguishable from corresponding components found in purified virions. One virion-associated component (IV_{a1}) was not observed in extracts of infected cells. There is, however, a prominent cell component with the same mobility as IV_{a1}. Each virus particle would contain only one or two copies of component IV_{a1}, and thus it may not be a true virion component (5). For simplicity, we have not distinguished by nomenclature virion-like proteins found in whole cell extracts and those from purified virions. Nine additional adenovirus-induced bands have been consistently observed which do not correspond to any of the virion components. With the exception of band P-VII, which is described in detail below, each of these bands has been given a designation which corresponds to its approximate molecular weight (Table 1).

Four of the nonvirion adenovirus 2-induced components, designated 100K, 71K, 27K, and P-VII, are rather prominent bands and were readily seen in extracts from infected cells labeled late after infection (i.e., after 15 h). Band 27K is sometimes resolved into two components (Fig. 3), and the faster-migrating of these bands has been designated 26K. Components 27K and 26K can also be distinguished by the fact that 27K is apparently transported into nuclei less efficiently than is 26K; therefore 27K can be isolated from cytoplasmic extracts (unpublished data). A sixth prominent band in infected whole cell extracts (designated 11.5K) is only partially resolved from virion component IX. Acetic acid extraction of infected cell pellets preferentially removes virion component IX (unpublished data).

At least three additional adenovirus 2-induced bands (50K, 14K, and 13.5K) have been consistently detected in ^{35}S-methionine-labeled cell extracts. Band 50K migrates slightly more slowly than does the virion core component V; band 13.5K migrates just behind virion component VIII, but unlike VIII it appears in extracts from cells labeled for short periods. Band 14K, which migrates about halfway between components VII and VIII, is found in extracts from cells labeled for short periods, appears to be stable, and has about the same intensity by autoradiography as band 13.5K.

If each of the observed virus-induced bands represented a single, unique, virus-coded polypeptide, together they would account for about 80% of the coding capacity of the adenovirus 2 genome (1.1×10^6 daltons of protein). Evidence to be presented below will demonstrate that at

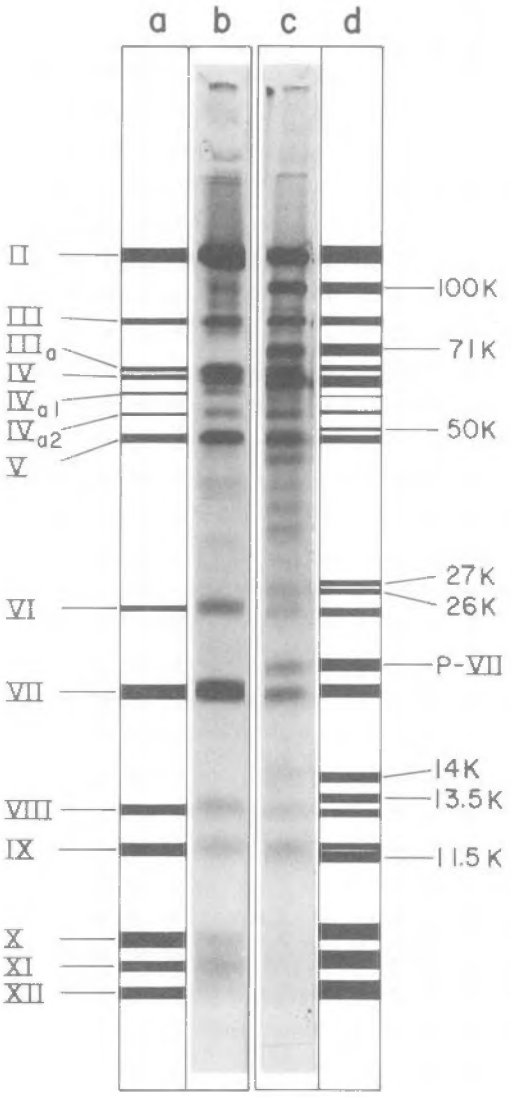

FIG. 1. *SDS-polyacrylamide gel autoradiogram of ^{35}S-methionine-labeled purified virus and extract of infected whole cells. The gel contained 15% acrylamide and 0.087% bisacrylamide. Adenovirus was purified as described in the text; the cell extract was labeled for 1 h with ^{35}S-methionine 18 h after infection, then chased for 12 h in the presence of 30 μg of nonradioactive methionine per ml. Parts a and d are artist's drawings representing an idealized gel in which all the virion components (a) and the 22 virus-induced components (d) that we have observed are illustrated. The nomenclature used to identify the bands is described in the text and in Table 1. Parts b and c are typical of the separations actually obtained; it is difficult to separate all 23 species on a single acrylamide gel. Band 50K is separated from component V in Fig. 2; bands 27K, 26K, 13.5K, and component VIII can be seen as resolved species in Fig. 3. The unidentified minor bands that are visible in the virus preparation (b) are present at less than one copy per virion (5) and probably result from minor host protein contamination or degradation during virus purification. The unidentified bands in c are host-cell proteins; a comparison of infected and mock-infected cell extracts can be made from the autoradiograms depicted in Fig. 2, 3, and 5.*

TABLE 1. *Adenovirus 2-induced proteins*

Band designation[a]	Molecular weight[b]	Relationship to virion
II	120,000[c]	Hexon
100K	100,000	
III	85,000	Penton base
71K	71,000	
IIIa	66,000	Virion component
IV	62,000[c]	Fiber
IV_{a1}	60,000	Virion component
IV_{a2}	56,000	Virion component
50K	50,000	
V	48,500[c]	Core
27K	27,500	Presumed precursor to component VI
26K	26,000	
VI	24,000	Hexon-associated
P-VII	20,000	Precursor to major core (VII)
VII	18,500[c]	Major core (AAP)
14K	14,500	
13.5K	13,500	
VIII	13,000[c]	Hexon-associated (appears during chase)
IX	12,000[c]	Hexon-associated
11.5K	11,500	
X	6,500[c]	Virion component (appears during chase)
XI	6,000	Virion component (appears during chase)
XII	5,000	Virion component (appears during chase)

[a] As assigned by Maizel et al. (14). Everitt et al. (5), or, for nonvirion adenovirus-induced components, expressed as a number based on the apparent molecular weight in SDS-polyacrylamide gels. All of the components have been found by autoradiography in purified virions or ^{35}S-methionine-labeled cell extracts.

[b] Determined from the component's mobility in SDS gels using myosin (200,000 daltons), glycerol-3-phosphate dehydrogenase (120,000 daltons), lactoperoxidase (92,600 daltons), bovine serum albumin (68,000 daltons), catalase (60,000 daltons), actin (47,000 daltons), chymotrypsinogen (25,700 daltons), myoglobin (17,200 daltons), cytochrome *c* (13,300 daltons), and the characterized adenovirus 2 virion components as moleular weight standards.

[c] As determined by reference 5 and by reference 14.

least two pairs of the 22 observed bands are related; consequently, the above estimate is probably somewhat high. Nevertheless, it is clear that this relatively simple fractionation procedure reveals a majority of the late species of viral-coded polypeptides.

Time course of virus-induced protein synthesis. KB cells, infected at high multiplicity with adenovirus 2, were pulse-labeled for 1 h with ^{35}S-methionine at various times after infection. The SDS-disrupted whole cell extracts from the resulting samples were analyzed by electrophoresis on polyacrylamide gels (Fig. 2). Most of the bands that are described above can be seen in the sample pulse-labeled 15 to 16 h after infection (Fig. 2), a result consistent with previous reports that most of the capsid antigens are detectable in infected cell extracts by 10 to 12 h after infection (6, 23). Synthesis of each of the adenovirus-induced bands, with the possible exception of 71K, continues until at least 30 h postinfection, at which point this experiment was terminated. Band 71K was detected earlier than were the other virus-induced polypeptides and seemed to reach a maximum rate of synthesis about 15 h postinfection. After that time its rate of synthesis declined until at 30 h its synthesis was hardly detectable. This band also undergoes a transformation from a rather sharp, well-defined band at 8 h postinfection to a somewhat diffuse band at 15 h postinfection, which occasionally can be resolved as a doublet band.

The most striking aspect of Fig. 2 is that only trace amounts of proteins VI and VII, which together constitute more than 15% of the virion protein (5), can be detected in the 1-h pulse-labeled samples at any time after infection. When the time-course experiment described above was repeated with a 3-h labeling period, substantially more label was observed in the positions of bands VI and VII at late times after infection; when extracts from cells pulse-labeled for 6 h or longer were analyzed, both VI

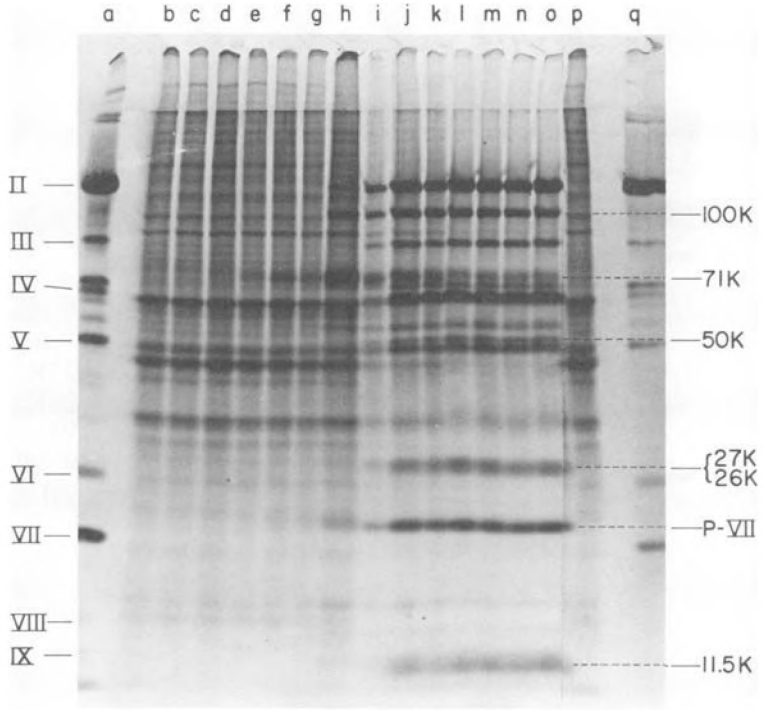

FIG. 2. *SDS-polyacrylamide gel autoradiogram of adenovirus 2-infected KB cells labeled at different times after infection. The gel contained 12.5% acrylamide and 0.1% bisacrylamide. A petri dish of infected KB cells was labeled for 1 h in medium containing ^{35}S-methionine (20 μCi/ml) and carrier methionine (3 μg/ml) as indicated below. Samples were processed as described in the text, and approximately 100,000 counts/min were applied to each sample well. The sample order is: a, purified ^{35}S-methionine-labeled virus; b, mock-infected cells. Samples c through o are adenovirus 2-infected cells labeled 3 to 4, 5 to 6, 7 to 8, 9 to 10, 12 to 13, 13 to 14, 15 to 16, 16 to 17, 18 to 19, 20 to 21, 24 to 25, 27 to 28, and 30 to 31 h after infection. p, Mock-infected cells; q, purified adenovirus 2.*

and VII appeared as prominent bands on the autoradiogram. These results suggested that capsid proteins VI and VII might be derived through the cleavage of precursor polypeptides of higher molecular weight.

Kinetics of appearance of capsid proteins VI and VII. To test the hypothesis that adenovirus 2 capsid proteins VI and VII are derived by cleavage of precursor polypeptides, and to try to detect the postulated precursors, a pulse-chase experiment was performed. A series of adenovirus-infected KB cell cultures were labeled with ^{35}S-methionine at 18 h post-infection for 1 h. The radioactive medium was then removed and replaced with nonradioactive medium containing 30 μg of methionine per ml. The incubation was continued, and at various times the cells were harvested for analysis on SDS-polyacrylamide gels (Fig. 3).

No radioactive material above background was detected in the positions of bands VI and VII from samples harvested immediately after the 1-h labeling period; however, after 2 h of chasing in the nonradioactive medium, a small amount of both VI and VII could be detected, and the amount of radioactive material in these bands increased with increasing time of chase. Concomitantly with the appearance of capsid proteins VI and VII, the amount of radioactive material in the bands designated 27K and P-VII appeared to decrease by comparison with most other virus-induced bands.

The autoradiogram depicted in Fig. 3 was scanned with a Joyce-Loebl densitometer to determine the areas under bands 27K, PVII, VI, and VII. Several other bands which did not appear to change in relative amount were used to provide loading controls, and the amount of

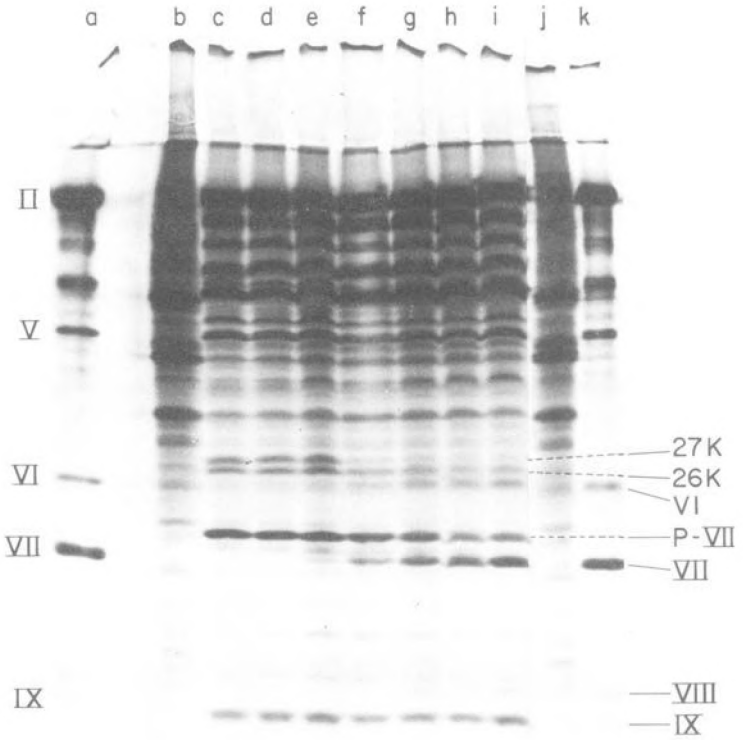

FIG. 3. *SDS-polyacrylamide gel autoradiogram of adenovirus 2-infected KB cells pulse-labeled for 1 h and then chased in nonradiactive medium. The gel contained 20% acrylamide and 0.067% bisacrylamide. Petri dishes of infected cells were labeled as described in Fig. 2 between 18 and 19 h after infection at high multiplicity. After labeling, the radioactive medium was removed and replaced with prewarmed medium containing 30 μg methionine per ml. At the times indicated below, the samples were harvested and processed as described in the text; 100,000 counts/min were applied to each sample well. The sample order is: a purified adenovirus 2; b, mock-infected cells (not chased). Samples c through i are infected cells harvested 0, 1, 2, 4, 6, 9, and 12 h after removal of the radioactive medium. j, Mock-infected cells; k, purified adenovirus 2.*

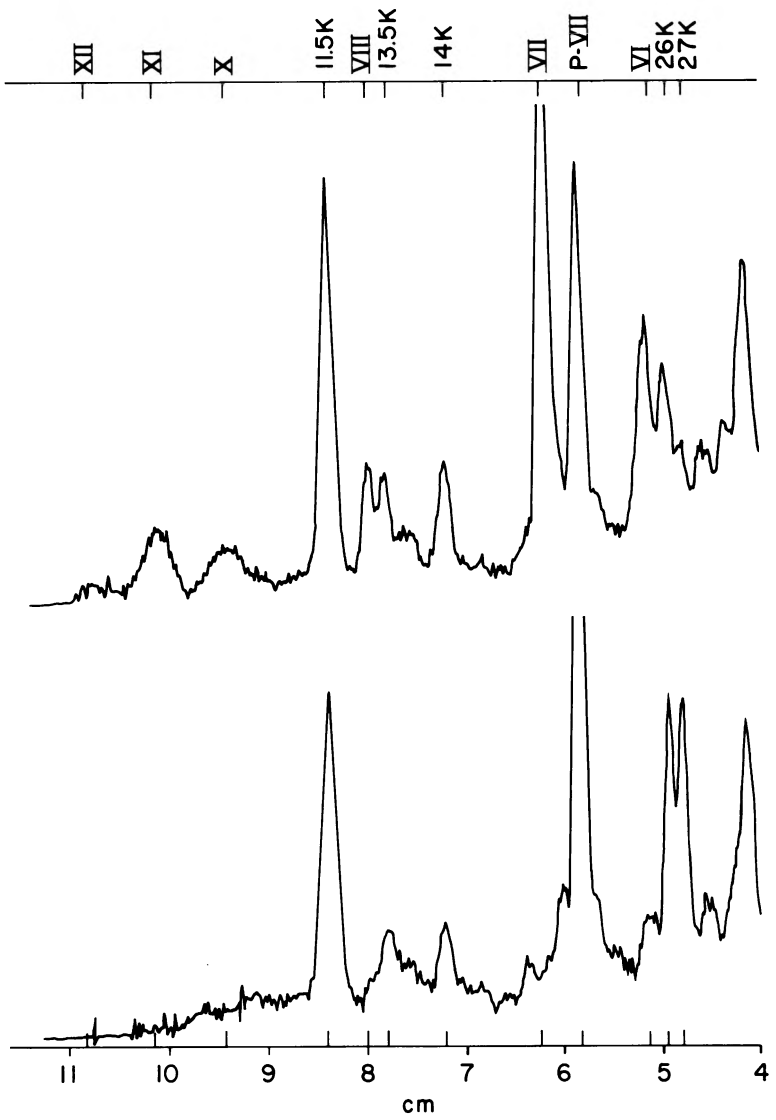

FIG. 4. *Densitometer tracing of an SDS-polyacrylamide gel autoradiogram. The lower curve is a tracing of a portion of the autoradiogram shown in Fig. 3c depicting an infected culture labeled for 1 h and harvested without a chase. The upper curve is a tracing of a portion of the autoradiogram shown in Fig. 3i depicting an infected culture labeled for 1 h and harvested after a 12-h chase in nonradioactive medium. The scale at the bottom indicates the distance (in centimeters) of migration from the stacking gel/separating gel interface. The position of various adenovirus-induced components is indicated at the top of the figure.*

radioactive material in bands 27K, PVII, VI, and VII was computed in relation to that of the control bands. Portions of the scans from the no-chase sample (Fig. 3c) and the 12-h chase sample (Fig. 3i) are given in Fig. 4.

Although quantitation of the radioactivity in a complex pattern such as that obtained from whole cell extracts is subject to numerous uncertainties, it does appear that about one-half of the radioactivity incorporated into P-VII in 1 h is lost from the P-VII position in 6 h of chase. There was still a substantial amount of P-VII (25 to 40% of the initial) after 12 h of chase, but by 48 h postinfection virutally no P-VII was found in cell extracts. After 6 h of chase the amount of radioactivity found in component VII was approximately equal to that lost from band P-VII (the accuracy of measurement was not sufficient to detect the expected loss of 10% of the counts; see following section). The disap-

pearance of band 27K and the appearance of component VI roughly paralleled that of P-VII and VII, respectively. Band 27K, however, contained less than one-third the radioactivity of band P-VII after 1 h of labeling, and the background due to residual host synthesis and band 26K severely limits the accuracy with which 27K and VI can be measured. In spite of these limitations, the experiment supports the proposal that 27K is the precursor to capsid component VI and that P-VII is the precursor to core component VII.

Fingerprint analysis of bands P-VII and the major core protein VII. Polypeptides P-VII and VII were obtained from nuclei of ^{35}S-methionine-labeled cells by preparative SDS-polyacrylamide gel electrophoresis as described above. A sample from each extracted band was rerun on an analytical SDS gel and shown to be essentially free of the other component (Fig. 5A). Band VII was also obtained in a similar manner from purified ^{35}S-methionine-labeled virus. After the removal of SDS and other salts by trichloroacetic acid and acetone precipitations, samples of the extracted material were oxidized by performic acid and digested with trypsin. Two-dimensional fingerprints were then prepared on cellulose sheets (20 by 20 cm) as described above (Fig. 5B, C).

Autoradiograms of the two-dimensional tryptic fingerprint of the major core protein obtained from purified ^{35}S-methionine-labeled virus (not shown) indicated that this protein has at least four methionine-containing tryptic peptides. The same fingerprint was obtained from the band VII material extracted from nuclei of adenovirus-infected cells (Fig. 5B). The material extracted from band P-VII yielded five methionine-containing tryptic peptides, four of which had mobilities in each of the two dimensions equal to those of the four corresponding methionine-containing peptides obtained from band VII, the major core protein. Furthermore, densitometer tracings of the one-dimensional separations (not shown) indicated that the five methionine-containing peptides from band P-VII, like the four peptides from band VII, were present in approximately equal amounts.

The simplest interpretation of these results is that the major core protein contains a minimum of four methionine residues and is closely related in sequence to another slightly larger protein which contains one additional methionine residue. Results of the pulse-chase experiment indicate that the major core protein is derived from this precursor by the removal of approximately 15 amino acids, including one methionine. Virion component VI probably has only one methionine residue (5; unpublished

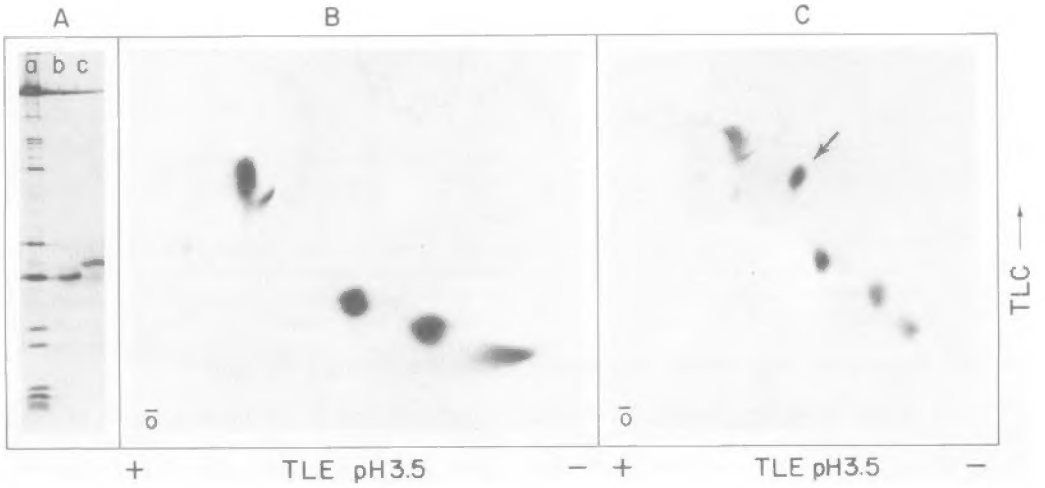

FIG. 5. *Two-dimensional tryptic fingerprints of P-VII and the major core protein. The SDS-polyacrylamide gel autoradiogram in part A compares purified VII and P-VII with purified adenovirus; B and C are autoradiograms of the thin-layer tryptic fingerprints of VII and P-VII, respectively; the arrow (C) points to the extra methionine-containing peptide of P-VII. One-dimensional separations by electrophoresis and chromatography (not shown) of the same digests showed that the four methionine-containing peptides visible in B had identical mobilities with the four corresponding peptides from the precursor P-VII (C). Approximately 50,000 counts/min of the appropriate trypsin-digested sample was applied in a 1-cm strip near the lower left corner of each fingerprint. Development was first by chromatography, then by electrophoresis as described in the text.*

data), and the single methionine-labeled tryptic peptide seems to have the same mobilities as does the single peptide from component 27K. This similarity is not sufficient, however, to unambiguously prove that these proteins have similar amino acid sequences.

Intracellular site of the processing of P-VII. In order to determine where the conversion of P-VII to VII occurs, we fractionated labeled, infected cells into nuclear and cytoplasmic components.

Adenovirus 2-infected KB cells were pulse-labeled with ^{35}S-methionine 24 to 27 h postinfection and were immediately harvested by washing in ice-cold medium. The washed cells from two petri dishes were resuspended in cold reticulocyte suspension buffer and lysed by the addition of Triton X-100 as described above. The nuclear pellet and cytoplasmic fraction obtained after centrifugation of the lysate were then analyzed on SDS-polyacrylamide gels without further purification (Fig. 6).

The results of this experiment indicate that a substantial fraction of the precursor polypeptides 27K and P-VII synthesized within the 3-h labeling period are found in the cell nucleus; in addition, nearly all of the processed core protein is also found in the nucleus. To obtain the results depicted in Fig. 6, equal amounts of radioactivity from the nuclear fraction and the cytoplasmic fraction were applied to the gel. Quantitation of the autoradiogram indicates that the ratio of radioactive nuclear P-VII to cytoplasmic P-VII is in excess of 10 to 1. Even though the cytoplasmic fraction contained four times as much total radioactivity as did the nuclear fraction, we can say that at least two-thirds of the radioactive P-VII is found in the nuclear fraction. Recent attempts to selectively extract bands P-VII and VII from isolated nuclei indicate that these polypeptides, unlike much of the hexon and fiber protein, are firmly bound within the nucleus. Consequently, it is unlikely that the distribution of P-VII and VII found in the above experiment resulted from precipitation or extensive aggregation of these proteins under the conditions in which the nuclei were prepared. From these results we tentatively conclude that processing of the adenovirus major core precursor polypeptide occurs after its entry into the nucleus rather than in the cytoplasm or as a consequence of transport through the nuclear membrane.

DISCUSSION

We have analyzed the pattern of protein synthesis during the late stages of adenovirus 2 replication in productively infected KB cells. By 24 h after a high multiplicity infection, host cell protein synthesis is reduced to less than 10% of the normal rate, and at least 22 adenovirus-induced proteins can be detected in extracts of whole cells that were labeled with ^{35}S-methionine. It is unlikely that extensive degradation of the newly synthesized virus-induced peptides has occurred, because the infected cells were labeled at high specific activity for relatively short periods and the samples were prepared for analysis in the presence of inhibitors of proteolysis within minutes of harvesting. The sum of the masses of the adenovirus-induced proteins that we have observed amounts to approximately 920,000 daltons. About 80% of the adenovirus 2 coding capacity is accounted for if each of these is a unique virus-coded species; however, as yet there is no direct evidence that any of the observed virus-induced proteins is, in fact, virus coded. Additional adenovirus-induced proteins will probably be discovered as techniques are further refined.

Adenovirus 2 and adenovirus 5 belong to the same subgroup and are closely related (20). Russell and Skehel (21) have used techniques similar to ours to identify virus-induced proteins in adenovirus 5-infected cells; their results are compatible with our results using adenovirus 2. Although it is not possible to make direct comparisons, several of the adenovirus 2 nonvirion proteins (e.g., 100K, 71K, 50K, and 11.5K) may correspond to the nonvirion components found in adenovirus 5-infected cells. We find only one band (71K, 71,000 daltons) that can be detected earlier than the adenovirus 2 virion components, and this component may correspond to the adenovirus 5 "ICSP-3" (64,000 daltons) that also appears early and is made in the absence of DNA synthesis. Any comparison of our time-course results with those of Russell and Skehel is difficult, however, because different host cells were used.

It is well known that the capsid components of several RNA viruses, including poliovirus, are derived by cleavage of a precursor polypeptide of high molecular weight so that only one ribosome initiation site is required for all of the gene products (2). Thus far, however, there has been no indication that the capsid components of any DNA virus, including adenovirus 2 (20), are derived in a similar fashion. It has been shown, however, that some of the capsid components of several DNA bacteriophages are derived from precursors only slightly larger than the processed capsid component (10, 15). The processing of bacteriophage capsid components is probably related to the packaging of their DNA and assembly of the bacteriophage particle, rather

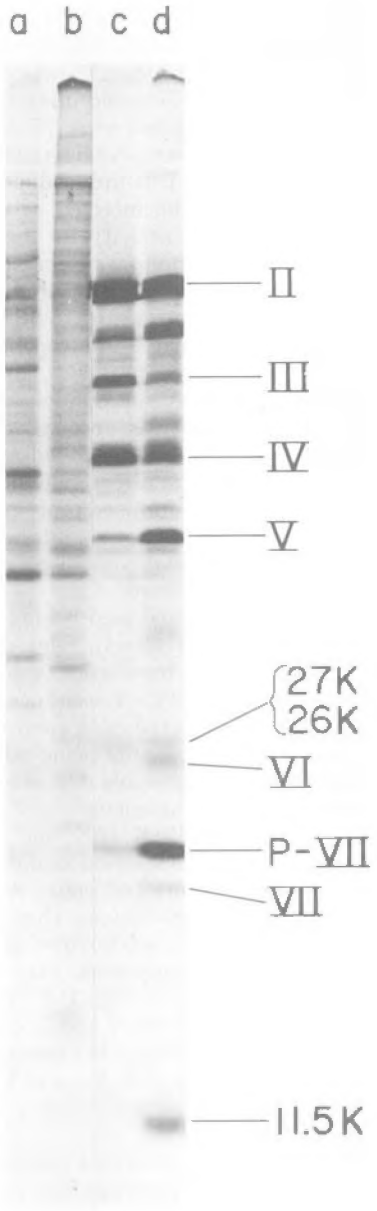

FIG. 6. *SDS-polyacrylamide gel autoradiogram of nuclear and cytoplasmic extracts of pulse-labeled adenovirus-infected and mock-infected KB cells. The gel contained 15% acrylamide and 0.08% bisacrylamide. The infected culture was pulse-labeled with ^{35}S-methionine for 3 h, beginning 24 h after infection; the mock-infected culture was labeled in a similar fashion, and nuclei and cytoplasm were prepared as described in the text. The adenovirus-infected nuclei contained approximately 25% of the incorporated counts; the nuclei from mock-infected cells contained 40% of the incorporated counts. Each sample well was loaded with 100,000 counts/min of extract. The sample order is: a, cytoplasm from mock-infected cells; b, nuclei from mock-infected cells; c, cytoplasm from adenovirus-infected cells; d, nuclei from adenovirus-infected cells.*

than being a way of separating the products of several genes.

While analyzing the pattern of protein synthesis after infection by adenovirus 2, we were surprised to find that two major capsid components, the hexon-associated component VI and the major core protein VII, were synthesized at a much lower rate than that expected. It was found, however, that after a brief labeling period radioactive material could be chased into components resembling VI and VII by allowing protein synthesis to continue in the presence of nonradioactive medium. Furthermore, it was found that two of the noncapsid virus-induced polypeptide bands that could be observed on SDS-polyacrylamide gels decreased in amount in relation to most of the virus-induced proteins during the chase period. These two proteins, which we have tentatively designated 27K and P-VII, each have molecular weights slightly larger than their presumptive products, VI and VII. At present we have only circumstantial evidence that 27K is in fact the precursor to VI, whereas P-VII has been shown to be closely related to VII by tryptic fingerprinting of their methionine residues. We have also noticed that several additional minor capsid components, notably VIII, X, XI, and XII, appear more prominently in chased samples than in samples harvested immediately after a short pulse. These components are present in sufficiently small quantities so that it will be difficult to distinguish whether they arise from the natural breakdown of larger virus components or are necessary virion constituents derived from a precursor or precursors in a manner similar to that of the major core protein.

The major core protein and its precursor polypeptide differ in mass by approximately 1,500 daltons, a difference which corresponds to the loss of about 15 amino acid residues. It is interesting to note that the major core protein is one of the few adenovirus capsid components to have a free amino-terminal residue (11, 12), suggesting that a fragment may have been cleaved from the amino-terminal portion of this component. It has been shown that during the maturation of bacteriophage T4, component P23* is derived from P23 by the loss of about 10,000 daltons of protein from the amino-terminal end of the precursor (10). If a similar situation exists for the production of the adeno-

virus major core, one would expect a fragment of about 15 amino acids, one of which is a methionine residue, to be released from the amino terminus of the precursor. We have looked for such a fragment in acetic acid extracts of frozen and thawed cell preparations by passage of the extract through Sephadex G-50 and Bio-Gel P-2. No fragment was found, but it is possible that the fragment was insoluble under the extraction conditions employed or was rapidly degraded after its release. We are presently purifying sufficient material to obtain both carboxyl-terminal and amino-terminal amino acids of both P-VII and VII, as well as fingerprints of 27K and VI.

The SDS-polyacrylamide gel system we have used is capable of resolving components that differ in their apparent molecular weight by about 4%; this accuracy can be maintained when comparing different samples only because the samples can be analyzed side by side on the same slab gel. We would not expect to resolve components that differed by 15 amino acid residues but that had masses in excess of about 40,000 daltons. Consequently, it is possible that the larger adenovirus 2-induced proteins have undetected precursors similar to that of the major core protein. We do not expect this to be the case for the remaining major capsid components, because they are believed to have blocked amino-terminal residues (10). The results of Russell and Skehel (21) do not rule out the possibility that the adenovirus 5 major core protein (core 2) is derived in a manner similar to that of adenovirus 2.

At present there is no evidence for the existence of high-molecular-weight precursors to the adenovirus virion components (21, 23). We have not detected any adenovirus 2-induced polypeptides of mass greater than 120,000 daltons, although we have not yet examined the pattern of protein synthesis after extremely short labeling periods. The processing mechanism of adenovirus 2 described above more closely resembles that of bacteriophage T4 than that of poliovirus. A possible rationale for the existence of this processing system is that the removal of certain amino acid sequences is necessary to induce a conformational change in the proteins which, in turn, control the virus assembly process. In support of this hypothesis, we find that a substantial fraction of the precursor to the major core is rapidly transported to the nucleus, where we presume processing takes place. In addition, it is known that the assembly of adenovirus 2 occurs relatively slowly, even though there is a large excess of many of the virion components and free viral DNA in the cell at late times after infection (23). In our pulse-chase study, it required nearly 12 h to convert half of the major core precursor synthesized in 1 h to the product form. This process, therefore, could be a rate-limiting step in virus assembly.

A recent report by Sundquist et al. (22) has demonstrated that empty capsids are precursors to assembled virus. Empty capsids from adenovirus 2 contain several polypeptides which are not present in complete virions. Two of these peptides (D and E) probably correspond to our 27K (the presumed precursor to VI) and 26K. In addition, empty capsids are deficient in virion component VI. We have recently found that significant amounts of 27K, 26K, and P-VII are found in purified virus if the virus is harvested immediately after a short labeling period; these three bands progressively disappear from virus purified from pulse-labeled infected cells during a subsequent chase (manuscript in preparation). Taken together, these results indicate that the cleavage of polypeptides 27K and P-VII is a relatively late step in virus maturation.

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