Isolation and Characterization of an Extremely Basic Protein from Adenovirus Type 5

KEIICHI HOSOKAWA* and MICHAEL T. SUNG

Worcester Foundation for Experimental Biology, Inc., Shrewsbury, Massachusetts 01545,* and Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois 62901

Received for publication 17 June 1975

By starch-gel electrophoresis and a staining method that is highly sensitive for arginyl residues, adenovirus type 5 was found to contain two minor basic polypeptides of extreme cathodic mobility in addition to the two known core proteins. The fastest-migrating polypeptide, named μ protein, and the second-fastest polypeptide are found in adenovirions and virus-infected KB cells but not in top components or in uninfected cells. The top components and infected cells contain an additional basic polypeptide, presumably P-VII, that migrates slightly slower than polypeptide VII. None of the basic polypeptides of adenovirions was electrophoretically identical to the host histone. The basic proteins of adenovirions were purified by urea phosphocellulose column chromatography and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The two minor basic core proteins, μ and another component, have similar mobilities in sodium dodecyl sulfate-polyacrylamide gels as a complex of polypeptides X-XII. After further purification on a Sephadex G-75 column, the μ protein was found to have a molecular weight of about 4,000. Amino acid analysis showed that the μ protein lacks tryptophan and 69% of the total amino acid residues are basic, that is, 54% arginine, 13% histidine, and 2% lysine. Only eight amino acids seem to contribute to make the μ polypeptide. There are 125 copies of the μ polypeptide per 1,000 copies of polypeptide VII in a virion.

Adenoviruses are nonenveloped, icosahedral DNA viruses (40). The viral particle consists of a DNA-protein core, which is encapsulated by a protein shell, the capsid (12). The molecular weight of virion particle is 175 \( \times 10^6 \), 13% of which is ascribed to DNA and the remainder to protein (11). One-third of virion protein exists in the core; the rest exists in the capsid.

The cores have been isolated successfully from virions by treatments such as exposure to 5 M urea (22), detergents (4, 29, 34), and organic solvents (17, 28) and freezing and thawing (27, 29) to break open the shell. It has been shown that the cores contain a linear duplex DNA molecule (molecular weight, 23 \( \times 10^6 \)) (11), 1,070 copies of polypeptide VII (molecular weight, 18,500) (1, 8), and 180 copies of polypeptide V (molecular weight, 48,500) (1, 8). Both core proteins are basic and resemble arginine-rich histones (16, 27, 30, 35). However, unlike the host histones, they contain tryptophan and are precipitated by virus-specific antisera (27). Moreover, the arginine-rich core proteins are coded for by the viral genome (1, 3). This is in contrast to papovaviruses, which incorporate cellular histones into the virion (10, 30).

Recently, evidence has been presented that polypeptide VII is derived by cleavage of a higher-molecular-weight (20,000) precursor, P-VII (1, 18), whereas polypeptide V is the direct translational product of its specific mRNA (1, 2, 18).

We plan to investigate the mechanism by which virion particles are assembled both in vivo and in vitro. To achieve this goal it is necessary to isolate all of the components from the virions and to study their properties. We report here the existence of a third arginine-rich protein in the adenovirions, in addition to the two core proteins previously reported.

MATERIALS AND METHODS

Reagents. CsCl, a product of American Potash and Chemical Corp., was recrystallized twice from hot water (13). Hydrolyzed starch was obtained from either Connaught Medical Laboratories, University of Toronto or Electrostarch Co. Phenol reagent, for the Lowry protein assay (30), and urea were obtained from Fisher Scientific Co. Methanesulfonic acid con-
taining 0.2% 3-(2-aminoethyl) indole was the product of Pierce Chemical Co.

Phosphocellulose (1.06 meg/g) was purchased from Schwartz/Mann. Sodium lauryl sulfate obtained from Sigma Chemical Co., was recrystallized from ethanol. Most of the other reagents for sodium dodecyl sulfate-polyacrylamide gel (SDS-gel) electrophoresis, including dyes, were the products of Eastman Organic Chemicals, Inc. Acrylamide and N,N'-methylenebisacrylamide were recrystallized from hot chloroform. Methylamine was purchased from J. T. Baker Chemical Co. RNase-free DNase was the product of Worthington Biochemicals Corp. Joklik modified minimal essential medium was obtained in a powder pack from Microbiological Associates. Fetal bovine serum was purchased from Flow Laboratories.

14C-labeled amino acid mixture was purchased from Amersham/Searle. It is composed of 14 amino acids of more than 97% purity: alanine, arginine, aspartic acid, glutamic acid, leucine, isoleucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine. All carbon atoms are uniformly labeled, with a specific activity of 57 mCi/matom of carbon.

Cell culture and proliferation of virus. Human KB cells and adenovirus type 5 were kindly supplied by Harold S. Ginsberg, Department of Microbiology, Columbia University College of Physicians and Surgeons. KB cells were maintained as a suspension in Joklik modified minimal essential medium supplemented with 7% fetal bovine serum. Cell density was kept at 3 x 10^4 to 6 x 10^5/ml by daily addition of an equal volume of fresh medium. The cultures were examined periodically for mycoplasmas. Adenovirus was propagated by a modification of the procedure described by Green and Fiña (11). Two liters of KB cell culture, at a density of 6 x 10^4 cells/ml, was centrifuged at 1,500 x g for 2 min. The pelleted cells were suspended in 200 ml of Joklik modified minimal essential medium supplemented with 5% fetal bovine serum and inoculated with a 1-ml lysate of virus-propagated KB cells (3 x 10^7 cells, 2 x 10^11 PFU). The mixture was shaken for 1 h at 23 C and then transferred to 3.8 liters of the same medium used for infection (prewarmed to 37 C). The virus-infected culture was stirred at 37 C in a Spinner bottle. At 12 h after infection, the medium was fortified with 1 mM glutamine and 0.36 mM arginine. The cells were harvested 48 h after infection by centrifugation at 1,500 x g for 2 min, suspended in 40 ml of reticulocyte standard buffer (0.01 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], and 0.0015 M MgCl2 [25]), and stored at -70 C.

Purification of adenovirus. A suspension of virus-propagated cells was subjected three times to freezing and thawing and centrifuged at 15,000 x g for 10 min. The supernatant was saved and the pellet was extracted once more with 20 ml of reticulocyte standard buffer. The second extract was combined with the previous supernatant and layered gently on top of 5 ml of 50% (wt/vol) CsCl made with 0.03 M Tris-hydrochloride, pH 7.6, in a nitrocellulose tube. The tube was filled with reticulocyte standard buffer and centrifuged in a Beckman SW27 rotor at 28,000 rpm for 1 h at 20 C. A thick white band of adenoviruses formed in the middle of the CsCl cushion (5). The diffuse turbidity above the virion band was collected separately through a hole punched in the bottom of the tube, and was subjected to two successive centrifugations to density gradient equilibrium in 46% (wt/vol) CsCl -0.03 M Tris-hydrochloride (pH 7.6) using a Beckman SW50.1 rotor at 36,000 rpm for 16 h at 15 C. The final virus band and the bands of top components (11) were collected and dialyzed overnight against 1 liter of 0.03 M Tris-hydrochloride (pH 7.8; with two changes). The virions aggregated at the beginning and then redispersed to give an opalescent suspension. The purified virus suspension and top components were stored at 4 C.

Preparation of adenovirus labeled with 14C-amino acid mixture. The radioactive amino acid mixture (10 µCi) was added to 200 ml of virus-propagated cells at 12 h after infection; labeling was for 36 h. At 48 h after infection, the culture was mixed with 500 ml of carrier cell suspension, harvested by centrifugation, suspended in 35 ml of reticulocyte standard buffer and stored at -70 C.

Radioisotope-labeled adenoviruses and labeled virion proteins were prepared by the procedure described for unlabeled viruses.

Determination of adenoviruses. Since 13% of the adenovirion particles is DNA (11), the actual number of the virion particles can be determined from the amount of viral DNA.

A small amount of virus preparation was dissolved in 0.05 M sodium phosphate (pH 7.05)-0.5% SDS, and the absorbancy at 260 nm (A_{260}) was determined. One A_{260} unit of the virus is regarded as 0.28 mg of virus proteins or 1.1 x 10^{14} particles (22).

Preparation of protein components from adenovirus. To a suspension of adenovirions (25 A_{260} units/ml), 1/10 volume of 20% sodium acetate and 2 volumes of ethanol were added. After standing for 5 h at 0 C, the precipitate virions were collected by centrifugation at 28,700 x g for 10 min. The sediments were dissolved in the original volume of water and subjected to freezing and thawing 10 times. Magnesium acetate and Tris-hydrochloride (pH 7.5) were added to give final concentrations of 0.02 and 0.01 M, respectively. The suspension was warmed to 37 C, DNase was added at 10 µg/ml, and incubation continued for 1 h with occasional stirring. The suspension was cleared by the addition of solid urea to a final concentration of 6 M.

Phosphocellulose column chromatography. Phosphocellulose was soaked in 0.1 N NaOH and washed by decantation at least 10 times with water until the yellowish color was removed. The slurry of phosphocellulose was poured onto a Büchner funnel and washed with 0.1 N HCl until the effluent became fully acidic. The phosphocellulose was washed repeatedly with water, then with 0.02 M sodium acetate (pH 4.7) to equilibrate the pH, and finally with acetone. The creamy white phosphocellulose powder was dried in air and stored for later use.

For column preparation, phosphocellulose powder was equilibrated in 6 M urea-0.02 M methylamine phosphate (pH 5.8) (buffer A) and packed in a
jacketed column (1 by 60 cm) maintained at 20°C with circulating water. The viral protein was dissolved in buffer A and applied to the column. The proteins that failed to be adsorbed were eluted with 100 ml of buffer A. Chromatographic elution was carried out with KCl in linearly increasing concentrations from 0.01 to 1.0 M in buffer A. Fractions of 5 ml were collected, and aliquots were removed for determination of protein by the Lowry method (20). Any precipitated KCl was removed by centrifugation at 4°C before the absorbance was measured.

In phosphocellulose column chromatography of 14C-amino acid-labeled virion protein, elution was done with an NaCl gradient (pH 4.7) in place of KCl (pH 5.6). The elution pattern was similar to that with a KCl gradient, except that all capsid proteins were first adsorbed onto the column and then eluted at the beginning of the salt gradient. Each chromatographic fraction was mixed with 1 drop of 0.1% bovine serum albumin as a carrier and then with 1/10 volume of 50% trichloroacetic acid-2.5% sodium tungstate, pH 2.5 (36). The mixture was allowed to stand at 0°C for 30 min, and the precipitated protein was collected on a Whatman GF/C glass filter disk (pore size, 0.45 μm) by suction. The filter disk was dried under a heating lamp and put into a vial containing 10 ml of toluene-based scintillation mixture [2.5-diphenyloxazole, 4 g; 1,4-bis-(5-phenyloxazolyl)benzene, 100 mg; toluene, to a final volume of 1 liter]. The radioactivity was measured in a scintillation spectrometer.

**Amino acid analysis of virion protein.** Samples were hydrolyzed in 0.5 ml of constant-boiling HCl in evacuated, sealed tubes at 110°C for 20 h. After removal of HCl by rapid evaporation over NaOH, the amino acid analysis was performed on a Durrum model 500 amino acid analyzer.

For tryptophan analysis, the methanesulfonic acid method was used (24). Samples were first dried in new, disposable culture tubes in vacuo over NaOH to completely remove HCl. To each tube containing samples, 20 μl of 43 HCl and methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole was added. The samples were placed in a desicator, to the bottom of which 9 ml of 8 N H2SO4 was added. The desiccator was sealed, evacuated, and clamped, and the protein samples were hydrolyzed in an autoclave for 18 h at 125°C. Amino acid analysis was again performed on a Durrum amino acid analyzer.

**Starch-gel electrophoresis.** Urea-aluminum lactate-starch gel was made by the method of Sung and Smithies (38). Vertical gel electrophoresis was performed at a potential gradient of 4 V/cm at 4°C until the methyl green dye marker migrated close to the cathode end of the slab. This was usually 1 h. The slab was then sliced horizontally to yield three pieces of equal thickness. The middle piece was used for the guanidinium-specific staining method of Sung and Smithies (38); staining was for 40 min in 0.5% amido black-1% acetic acid solution and differentiation was in 1 N H2SO4. Upon standing, bands of black to blue color gradually appeared. After photography, the gel slice may be destained by the conventional method, with frequent changes of 1% acetic acid.

**SDS-gel electrophoresis.** Electrophoresis was carried out with an SDS-gel of 10% polyacrylamide by the method of Maizel (21). The separation gel was 10 cm high with a 1-cm stacking gel of 3% polyacrylamide on top in a glass tube of 6-mm diameter. A sample solution in a total volume of 75 μl was mixed with 5 μl of 1 M Tris-hydrochloride (pH 7.0), 10 μl of 20% SDS, 5 μl of β-mercaptoethanol, and 5 μl of 0.3% bromothymol blue and boiled for 2 min to dissociate protein components (15, 22). The protein sample was mixed with 250 μl of the same 3% polyacrylamide solution and polymerized on the top of the stacking gel. Both electrodes were soaked in 0.05 M Tris-glycine buffer (pH 8.5), and the electrophoresis was performed at 100 V at 23°C for 4 h. The gels were stained with 0.25% Coomassie brilliant blue in 7.5% acetic acid-15% methanol for 1 h and then destained with frequent changes of 7.5% acetic acid-15% methanol.

**Sephadex column chromatography.** Sephadex G-75 was soaked in 0.5 M NaCl-0.01 M HCl and packed into a column (2.2 by 200 cm). A preparation of μ protein purified by phosphocellulose column chromatography, in a total volume of 3 ml, was applied on the top of the column, and molecular sieving gel filtration was performed with the same solution. The molecular weight of polypeptide μ was determined by using highly purified histones as calibration standards.

**RESULTS**

**Basic proteins of adenovirion.** The utility of the aluminum-lactate-starch gel for basic nuclear proteins was demonstrated previously (37, 38). The major advantage is that the staining method is highly sensitive and specific for the guanidinium groups of arginyl residues in basic proteins. We have attempted to investigate the basic core proteins of adenovirus as to whether there are minor arginine-rich DNA-binding proteins. To this end, a method was devised to permit the complete recovery of all protein components of adenoviruses. The purified viruses were first subjected to repeated freezing and thawing to produce cracks on the shell (22, 29) and then treated with DNase to release DNA-combining proteins from the core. Solid urea was finally added to facilitate the solubilization of total viral proteins. For comparison, protein samples from the empty shells were also prepared by the same procedure. Furthermore, basic protein fractions were extracted, with dilute HCl, from whole cells and nuclei of both adenovirus-infected KB cells and the control uninfected cells. All of the protein samples were examined by starch-gel electrophoresis.

Figure 1 is a photograph of gels stained by the guanidinium-specific stain method and the conventional amido black stain method. The guanidinium-specific stain for arginine-rich proteins is approximately 100 times more sensitive.
than the conventional amido black stain (38). The heterogeneity of the adenovirus protein is illustrated in sample 6 of Fig. 1A and B. There are three major components that stain intensely black (zones a, b, and c of Fig. 1A) and are presumably arginine rich. Zone a is unique; it migrates slightly faster than the tracking dye methyl green. It is also found in the acid extracts of virus-infected cells (sample 2) and nuclei from infected cells (samples 3 and 8). In contrast, it is absent in the acid extract of uninfected cells (sample 1), nuclei from uninfected cells (sample 7), and top components (sample 5). Clearly, zone a is a virion component possibly of the core or a core-associated protein. Due to its low molecular weight and/or low concentration, it is not detectable by the usual amido black staining procedure (sample 6, Fig. 1B).

Component b is the most histone-like in electrophoretic mobility; it migrates slightly ahead of histone I and behind histones II and III (37). The fact that it is present only in trace amount in the top components (sample 5, Fig. 1B), is acid soluble (sample 4, Fig. 1B), and stains black (Fig. 1A) suggests that it is one of the arginine-rich core proteins (16, 27, 34). Similarly, component c is thought to be another arginine-rich core protein. 

In urea-aluminum-lactate-starch gel, the histones with molecular weights ranging from 11,000 to 20,000 have relatively similar mobilities. The much reduced

---

**Fig. 1.** Starch-gel electrophoresis of proteins from adenovirus type 5 compared with acid-soluble proteins of normal and adenovirus 5-infected KB cells. The proteins of the complete adenovirion and empty shell were prepared as described in the text, except that urea was added to bring the final concentration to 4 M. Acid-soluble proteins of adenovirus were prepared by treating the adenovirion with 0.3 N HCl for 1 h, and then the suspension was made up to 4 M with solid urea. A 1-ml solution of virion or top component protein was derived from 4 x 10¹² particles. Nuclei were prepared by subjecting KB cells to repeated freezing and thawing, followed by pelleting by centrifugation. Approximately 10⁶ cells or nuclei from each normal or adenovirus-infected culture of KB cells were extracted with 0.5 ml of 0.35 N HCl, and the centrifuged supernatant with urea added to 4 M was used for starch-gel electrophoresis. An 80-µl volume of each protein solution was applied to sample slots of the starch gel. Electrophoresis was carried out as described in the text. Guanidinium-specific stain: 1, uninfected KB cells; 2, infected KB cells; 3, infected nuclei; 4, adenovirus 5 plus HCl; 5, top components; 6, adenovirus 5; 7, uninfected nuclei; 8, the second acid extract of infected nuclei. Following the first HCl extraction, the pellet obtained after centrifugation was extracted again with HCl. (B) Conventional amido black stain of the same gel slab.
cathodic mobility of component c in comparison to component b suggests that c is much larger in size than in charge differences. The quantitative differences between b and c in protein-bound amido black dye indicate that b is a major component. Based on the above information, we tentatively classify b as polypeptide VII and c as polypeptide V (22).

The capsid proteins of the adenovirus can be identified on the basis of their acid insolubility and low electrophoretic mobility in urea-aluminum-lactate starch-gel and by the fact that they stain blue rather than black by the differential method. The group of zones near the origin of sample 6 in Fig. 1B stained blue when viewed with transmitted light. However, in Fig. 1A, due to the contrasting black zones and greyish background, they appear as negative-stained zones. These zones are absent in the acid extract of the virus (sample 4, Fig. 1B) and are present, although in different proportions, in the top components (sample 5). We classify these as capsid proteins.

There are several other arginine-rich components deserving brief mention. These include a minor component, d, which is present in the virion (samples 6 and 4, Fig. 1A) and in acid extracts of virus-infected cell (sample 2, Fig. 1A) and their nuclei (samples 3 and 8, Fig. 1A), and a component, e, which is in the acid extracts of virus-infected cells (sample 2, Fig. 1B) and their nuclei (samples 3 and 8, Fig. 1B).

Protein e is of interest. It is found in the top components but not in the purified virion particles and yet it is present in large quantity in virus-infected cells. Judging from its abundance in arginine and from its electrophoretic mobility, with respect to the major core protein VII, it may be P-VII, the precursor of VII (1, 3, 14, 40). Also indicative of precursor polypeptide is an intensely stained component migrating within a cluster of bands behind zone c (sample 5 in Fig. 1A). It is present only in the top components but absent in the adenovirion.

Isolation of component a. The extreme cathodic mobility of component a is truly exceptional; to our knowledge no basic proteins known to date have a greater cathodic mobility than methyl green. Even the most basic protein, protamine, migrates behind the tracking dye in the starch-gel system used here (see Fig. 4). We sought to isolate and identify this specific, arginine-rich component. Preliminary tests revealed that it could be adsorbed onto a phosphocellulose column and eluted between 1.0 and 1.5 M KCl in 6 M urea and 0.006 M β-mercaptoethanol buffered at pH 5.6 with 0.01 M methylamine phosphate. Figure 2 shows the chromatographic separation of viral proteins on a phosphocellulose column, eluted with a linear salt gradient. The virion protein was adsorbed as described above. Elution was carried out with a 0.0 to 1.0 M KCl linear gradient in buffer A. The column was finally stripped with 1.5 M KCl in buffer A. Aliquots were removed from the column chromatographic peak fractions (arrows in Fig. 2) and analyzed by SDS-gel electrophoresis. The SDS-gel pattern of whole virion proteins as prepared by the DNase treatment is shown in Fig. 3C. The overall pattern is similar to that found previously (1, 7, 8, 22), and the major polypeptide assignment is according to Maizel et al. (22). Several minor components are also clearly visible in the gel. The stained gels in Fig. 3 (gels 2 and 3) reveal that the initial protein peaks (Fig. 2, no. 1 through 4) are capsid proteins which were not adsorbed onto the column under the conditions employed. Peak 5, although Lowry positive, did not show any detectable zones on the SDS-gel. Peaks 7 and 11, which were eluted at 0.4 and 0.65 M KCl, respectively, correspond to the viral core proteins V and VII, respectively. They were further verified as the bands c and b from the starch-gel electrophoresis (Fig. 1). Peak 8 may be a complex of polypeptides X-XII (1), according to mobility in SDS-gel, and was identified as the second-fastest band on starch-gel electrophoresis (Fig. 1). The last peak (no. 12) that appeared in the 1.5 M KCl eluant showed a faint staining zone near the anodic end of the SDS-gel. The band position corresponds to a group of protein bands X-XII on the SDS-gel. This was verified to be component a by starch-gel electrophoresis and the guanidinium-specific stain method as shown in Fig. 4. That component a was the sole protein species present in the “1.5 M KCl strip” was confirmed in a separate experiment in which the gradient elution of the column was extended from 0 to 1.5 M KCl and component a emerged from the column at 1.05 M KCl after polypeptide VII was eluted. The above data clearly indicate that component a is an authentic basic protein that is rich in arginine and possesses extreme electrophoretic mobility. Since this protein is present in only a miniscule amount and is of small molecular size, we designated it the μ protein.

Properties of μ protein. The phosphocellulose column eluant fractions containing the μ protein, peak no. 12 in Fig. 3, were pooled, dialyzed against 0.01 M HCl to remove salt and urea, and then lyophilized. The protein was acid hydrolyzed and its amino acid composition...
Fig. 2. Phosphocellulose column chromatography of whole virion proteins of adenovirus type 5. The virion protein was prepared from 230 A_{260} units of adenovirus type 5 as described in the text. The sample was dialyzed overnight against buffer A and applied on the top of a phosphocellulose column (1 by 50 cm). The column was then washed with 90 ml of buffer A, and linear gradient elution was carried out with 0 to 1 M KCl made with buffer A in a total volume of 600 ml (fractions 38 through 169). Fractions of 4.5 ml were collected. The column was finally washed with 2 M KCl-buffer A. Protein was determined by the Lowry method (20) on an aliquot from each fraction.

was determined. As shown in Table I, the $\mu$ protein has a very unique composition. (i) It contains five amino acids contributing to more than two-thirds of its total composition—arginine, glycine, histidine, alanine, and serine. (ii) More than one-third of the total amino acids are basic. (iii) It is very low in hydrophobic residues. (iv) No tryptophan is present. Since the $\mu$ protein preparation sometimes showed a diffusely stained faint zone around the position of polypeptides V and VII (Fig. 4), it was purified further by passage through a Sephadex G-75 column to remove possible trace contaminants. From the elution profile, the molecular weight of polypeptide $\mu$ was estimated to be around 4,000. This is in reasonable agreement with the molecular weight of 5,000 to 6,500 estimated by SDS-gel electrophoresis (1, 8). The amino acid composition (Table 1) of the finally purified $\mu$ protein is very similar to that from the first analysis. However, some trace amino acid residues are no longer present (for example, valine and leucine), and isoleucine is present only in trace amounts. In contrast, the arginine content increased to 53.9% of total amino acid residues, indicating that the $\mu$ polypeptide is almost like polarginine. As much as 69% of the total amino acid residues are basic: arginine, 53.9%; histidine, 13.5%, and lysine, 1.7%. Apparently only eight amino acids contribute to polypeptide $\mu$: arginine, histidine, alanine, glycine, serine, methionine, lysine, and proline.

To determine the number of molecules of $\mu$ proteins in a virion particle, adenovirus type 5 was propagated in KB cells in the presence of a mixture of $^4$C-amino acids. The labeled proteins of the adenovirions were chromatographed on a phosphocellulose column to determine the relative distribution of radioactivity in each of the protein peaks. The yield of the column was at least 80%. The specific activity of $^4$C-amino acids in the proteins may be calculated from their known molecular weights provided that the 14 amino acids are equally distributed in all polypeptides. The number of copies of $\mu$ proteins per virion can be evaluated by comparing the ratio of the specific radioactivities between polypeptide $\mu$ and polypeptide VII. Assuming
that there are 1,000 molecules of polypeptide VII per virion (8), the number of copies of polypeptide $\mu$ was computed to be 125.

**DISCUSSION**

It has been reported that there is an arginine-requiring step for the propagation of adenoviruses (31, 33). Arginine deprivation reduced the yield of virus by several orders of magnitude. Under the condition of arginine starvation, components of the virus are still synthesized. However, they fail to assemble into mature virions (9, 31–33, 41). It has been suggested that the synthesis of the assembly factor is dependent upon the presence of arginine in the medium (26). The nature of this factor remains obscure. The existence of a new arginine-rich $\mu$ protein raises the possibility that the synthesis of this protein could be a rate-limiting step to the assembly of adenoviruses.

The chemical composition of polypeptide $\mu$ is reminiscent of the sperm-specific protein protamine. It is also very small, with a molecular weight of only 5,000, and about two-thirds of its total amino acid residues are arginine. Ling et al. (19) showed that protamine is synthesized via the classical pathway of protein synthesis—mRNA transcription and translation from diribosomes. We have not studied the synthesis of the $\mu$ protein; however, we have shown that polypeptide $\mu$ appears at about 16 to 20 h after infection (Fig. 5). This time course of appearance of polypeptide $\mu$, together with its unique amino acid composition, would argue strongly against its being a degradation product of a host histone. However, it is likely that $\mu$ protein may be the processed product of the

---

**Fig. 3.** SDS-gel electrophoresis of virion proteins isolated by phosphocellulose column chromatography. Aliquots of 25 $\mu$l from protein peaks 1 and 2 (arrows in Fig. 2) and of 50 $\mu$l from protein peaks 3 through 12 were subjected to SDS-gel electrophoresis as described in the text. Left to right: Whole virion protein from adenovirus type 5; protein peaks 1 through 12 (as indicated by arrows in Fig. 2).
viral precursor polypeptides and especially of polypeptide P-VII. If the $\mu$ protein is not a processed product, then, by analogy to protamine, a DNA-combining protein, $\mu$ is probably a component of the viral DNA core. In support of this contention, polypeptide $\mu$ is not detected in the "top components" as shown by the guanidinium-sensitive staining method (sample 5, Fig. 1A).

Polypeptide $\mu$ migrates as the fastest band in SDS-polyacrylamide gel (Fig. 3). This mobility is similar to the X-XII protein group of adenovirus type 2 as described by Anderson et al. (1). The reported molecular weight ranges from 5,000 to 6,500 (1), which is also in reasonable agreement with our molecular weight determination for polypeptide $\mu$ by Sephadex G-75 chromatography. It is possible that polypeptide $\mu$ bears the same identity as one of the polypeptide group X-XII; further study is needed to substantiate this. Alternatively, other investigators may not have observed polypeptide $\mu$; this may be attributed to its unique physical and chemical characteristics. Since it is a minor protein component of the adenovirus (5%), is extremely basic, and is low in molecular weight, the $\mu$ protein would tend to be lost during dialysis or by adsorption to the glassware and/or
Time course of appearance of the $\mu$ protein in adenovirus-infected KB cells. A suspension culture of KB cells in a total volume of 4 liters was infected with adenovirus type 5 as described in the text. An aliquot from a 400-ml infected culture was harvested at 0, 4, 8, 10, 12, 16, 20, 24, 36, and 48 h after infection. The pelleted cells, approximately 1.2 ml each, were stored at $-70^\circ$C until the last sample was harvested. The frozen cells were extracted with 15 volumes of 0.3 N HCl-4 M urea solution by stirring for 3 h at 4°C. The mixtures were allowed to stand overnight at 4°C and centrifuged at 30,000 $\times$ g for 10 min. The slightly turbid extracts were subjected to analysis by aluminum-lactate-starch-gel electrophoresis and guanidinium-sensitive stain as described in the text. Sample 1 through 10 contained (in a total volume of 50 µl) 33 µl of the virus-infected cell extract harvested at 0, 4, 8, 10, 12, 16, 20, 24, 36, and 48 h postinfection, respectively, 0.02 M β-mercaptoethanol, 10 ng of methyl green, and water. The sample in slot 11 contained 0.15 $A_{\text{abs}}$ unit of adenovirus type 5 and HCl, at a final concentration of 0.2 N, in place of the extract in the above mixture. 

by diffusion from the gel during destaining. Thus, visualization of the $\mu$ protein on a starch-gel is made possible by the fact that prolonged destaining is not required and that the stain is highly sensitive. 

Further investigations are in progress to de-
TABLE 1. Amino acid composition of the $\mu$ protein

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>1st Analysis</th>
<th>2nd Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol</td>
<td>mol/100 mol</td>
</tr>
<tr>
<td>Lys</td>
<td>0.348</td>
<td>2.2</td>
</tr>
<tr>
<td>His</td>
<td>1.590</td>
<td>10.2</td>
</tr>
<tr>
<td>Arg</td>
<td>4.137</td>
<td>26.6</td>
</tr>
<tr>
<td>Asp</td>
<td>0.687</td>
<td>4.4</td>
</tr>
<tr>
<td>Thr</td>
<td>0.353</td>
<td>2.3</td>
</tr>
<tr>
<td>Ser</td>
<td>1.598</td>
<td>10.3</td>
</tr>
<tr>
<td>Glu</td>
<td>0.861</td>
<td>5.5</td>
</tr>
<tr>
<td>Pro</td>
<td>0.322</td>
<td>2.1</td>
</tr>
<tr>
<td>Gly</td>
<td>2.308</td>
<td>14.8</td>
</tr>
<tr>
<td>Ala</td>
<td>1.505</td>
<td>9.7</td>
</tr>
<tr>
<td>Val</td>
<td>0.207</td>
<td>1.33</td>
</tr>
<tr>
<td>Met</td>
<td>0.716</td>
<td>4.6</td>
</tr>
<tr>
<td>Ile</td>
<td>0.228</td>
<td>1.5</td>
</tr>
<tr>
<td>Leu</td>
<td>0.396</td>
<td>2.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.187</td>
<td>1.2</td>
</tr>
<tr>
<td>Phe</td>
<td>0.111</td>
<td>0.7</td>
</tr>
<tr>
<td>Trp*</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Determined by methanesulfonic acid hydrolysis (24).

To determine the topographic localization of the $\mu$ protein in the virion particle, as well as its role during the assembly of adenovirus. It has been proposed that empty virus capsids are precursors of complete virions both in bacterial and animal viruses (6). In support of this hypothesis, several precursor polypeptides have been detected in the top components, whose pulse-label could be chased into complete virions (1, 14, 35, 40). We demonstrated that band e (Fig. 1) is present only in “top components” and in the adenovirus-infected KB cells but not in the complete virions. Furthermore, by SDS-gel electrophoresis, band e was found to correspond to P-VII of Anderson et al. (1). We have also been able to isolate polypeptide P-VII from virus-infected cells in an effort to study its possible relationships to polypeptide $\mu$.

ACKNOWLEDGMENTS

We are grateful to Loretta S.-Y. Lee and Roger Fallavollita for their excellent technical assistance in cell culturing and propagation of viruses. Thanks are also due to H. S. Ginsberg, Columbia University, E. Baril and B. Baril, Worcester Foundation, and B. W. Burge and V. Mautner, Massachusetts Institute of Technology, for critical reading of the manuscript. We thank M. Hatanaka, Flow Laboratories, for his kindness in screening our culture for mycoplasma contamination, and C. Hurley for amino acid analysis.

This work was supported by Public Health Service grant PO7-CA-12708 from the National Cancer Institute and by American Cancer Society grant NP-122.

LITERATURE CITED


