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Synthesis and Processing of the Precursor to the Major Core Protein of Adenovirus Type 2

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An isopycnic Metrizamide-detergent gradient system was developed in which the newly synthesized precursor (polypeptide P-VII) to the major core protein of adenovirus type 2 (polypeptide VII) was confined to a spectrum of complexes with densities equal to or higher than that of adenovirions. The majority of the newly synthesized P-VII was, at the beginning of the logarithmic period of virus production, present as an entity of protein density. This pool of P-VII was efficiently depleted. P-VII was also associated with high-molecular-weight structures of intermediate density, sharing some properties with empty capsids or incomplete particles. The transfer of P-VII from the intermediate-density region was not quantitative, and only particles of true virion density subsequently contained polypeptide VII. No structures equivalent to the core structure of disrupted virions or identical to incomplete particles were detected in this system. A temperature-dependent transition of radioactivity from polypeptide P-VII into polypeptide VII was also detectable after in vitro incubation of P-VII-containing complexes. Addition of Ad2-infected cell extracts was required for processing of complexes derived from regions of protein density, whereas P-VII was processed spontaneously upon incubation in complexes of virion density.

The structural organization and topography of the adenovirus type 2 (Ad2) polypeptides have been studied extensively (4, 8). Two to five different classes of incomplete particles (16, 19, 21) or top components (TCs) (12, 14) have also been identified with the aid of equilibrium density centrifugation in CsCl. Some of these particles have been shown to contain fragments of viral DNA (6). Examination of the total polypeptide content of incomplete particles reveals (i) the presence of polypeptides not found in mature virions, and (ii) the absence of the internally located virion polypeptide VI and the two core polypeptides, V and VII (12, 19, 21). Data from protein pulse-chase experiments have suggested a precursor-product relationship between some classes of incomplete particles and virions (12, 19, 21).

In this study we describe the identification of an efficiently depleted "free pool" of newly synthesized precursor (polypeptide P-VII) to the major core protein of Ad2. A spectrum of complexes with densities between those of protein and virions also contain P-VII. The precursor-product relationship between the 20,000-molecular-weight polypeptide P-VII and the core protein (polypeptide VII; 18,500 molecular weight) is well established from studies on protein synthesis both in vivo (1) and in vitro (2, 7, 15). Therefore, we suggest that the complexes identified here are related to mature adenovirions in a precursor-product relationship.

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MATERIALS AND METHODS

Cells and virus. KB cells were cultivated in suspension cultures in Eagle minimal essential medium (Eagle MEM) (Flow Laboratories, Inc., Rockville, Md.) supplemented with 5% horse serum (Flow Laboratories, Inc., Rockville, Md.). Cells were routinely cultured for mycoplasmas.

Stock preparations of Ad2 were prepared as previously described (10). To increase the virus yield, a solubilization step was included in which 1% (vol/vol) *n*-butanol was added to the ultrasonically treated cells. After 1 h of incubation on ice with frequent mixing, the cell extract was centrifuged at 500 × *g* for 20 min. The supernatant was processed further as described (10). Purified virus was dialyzed extensively against 10% (vol/vol) glycerol-1 mM $MgCl_2$ in 0.01 M Tris-hydrochloride buffer, pH 7.5, frozen in a dry ice-ethanol bath, and stored at −70°C.

Pulse-chase isotope labeling of protein. Before pulse-labeling, KB cells productively infected with 2,000 particles of Ad2 per cell were sedimented and washed once in Earle salt solution containing 5% horse serum. The cells were resuspended and permitted to equilibrate for 10 min at a density of 2 × 10^7 cells/ml in Earle salt solution with $^1/_{20}$ volume of

Eagle MEM and fortified with 5% horse serum. At 14 or 16.5 h postinfection (p.i.), [^{35}S]methionine was added (400 μCi/ml; 276 Ci/mmol) to the cell suspension and permitted to incorporate for 5 or 20 min, respectively. After the pulse, the first cell sample was removed and diluted in ice-cold phosphate-buffered saline containing 215 μg (140 times the concentration during isotope labeling) of methionine per ml. After sedimentation the cells were resuspended in 0.01 M Tris-hydrochloride (pH 8.1)-1 mM EDTA-1 mM phenylmethane sulfonyl fluoride (TEP buffer) and frozen in a dry ice-ethanol bath. Unlabeled methionine was added to the remaining cells to give a concentration of 215 μg/ml; these cells were sedimented, washed once in chase medium (Eagle MEM containing 215 μg of methionine per ml), diluted, and further incubated in chase medium at a cell concentration of 3×10^5 cells/ml.

Pulse-chase labeling of DNA. The "temperature-shift" procedure described by Schilling et al. (20) was applied. KB cells productively infected with 2,000 Ad2 particles/cell were sedimented at 4°C and resuspended in cold Eagle MEM at a cell density of 10^7 cells/ml. After temperature equilibration at 4°C for 5 min, [^{3}H]thymidine (100 μCi/ml; 53 Ci/mmol) was added to the cells at 16.5 h p.i. The isotope was permitted to incorporate for 15 min at 4°C, after which the cells were sedimented at 4°C and resuspended in prewarmed medium containing the same concentration of isotope as before. After further incubation at 37°C for 15 min, the first cell sample was removed and diluted in ice-cold phosphate-buffered saline containing 2×10^{-2} M thymidine, sedimented, resuspended in TEP buffer, and frozen. The remaining cells were sedimented, washed, and chased at a concentration of 3×10^5 cells/ml in Eagle MEM containing 2×10^{-4} M thymidine. Throughout the isotope incubation and chase, samples were removed, precipitated, and monitored for incorporated isotope. During the period of isotope equilibration and phosphorylation at 4°C, a linear increase in isotope uptake was demonstrated (from 100 to 200 cpm/10^5 cells). After the temperature shift, [^{3}H]thymidine was incorporated at a 50- to 100-fold greater rate (yielding approximately 20,000 cpm/10^5 cells). No net increase of trichloroacetic acid-precipitable radioactivity was detected during the chase.

Preparation of samples for Metrizamide-Triton density centrifugation. Infected cells were frozen in TEP buffer at −70°C. After rapid thawing the cells were ultrasonically treated four times, 15 s each, at 0°C, Triton X-100 was added to 0.5% (vol/vol), and the preparation was incubated for 1 h with frequent mixing. After centrifugation at $300 \times g$ for 10 min, the extract was analyzed on preparative Metrizamide-detergent gradients.

Equilibrium density centrifugation. Preparative gradients of Metrizamide (Nyegaard and Co., A/S, Oslo, Norway) were made by mixing 6.5 ml each of 20 and 60% (wt/vol) Metrizamide in 0.01 M Tris-hydrochloride buffer (pH 8.1), 1 mM EDTA, and 0.5% (vol/vol) Triton X-100 (20 to 60% MAT gradients). Metrizamide solutions were freshly made, and the gradients were stored overnight at 4°C, shielded from light, and used the next day. All samples were made up to 3 ml, to which was added 1 ml of 80% (wt/vol) MAT, and centrifuged in an SW27.1 rotor at 25,000 rpm for 62 to 67 h at 4°C. Fractions were collected from the bottom of the tubes. Analytical gradients in MAT were made by mixing 2.2 ml each of 40 and 48% (wt/vol) MAT, and centrifugations were performed in an SW50.1 rotor at 38,000 rpm for 62 to 67 h at 4°C.

Preparation of density markers. Virus from ultrasonically treated and *n*-butanol-extracted cells was concentrated by sedimentation on to a double-layered cushion of CsCl (ρ = 1.32 and 1.40 g/cm^3). The virus material floating on the denser part of the cushion was removed, diluted in 0.05 M Tris-hydrochloride buffer (pH 8.1), and centrifuged on a preformed CsCl gradient (1.32 to 1.36 g/cm^3) in an SW50.1 rotor at 30,000 rpm for 10 h. The virus band was dialyzed against 10% (vol/vol) glycerol in 50 mM Tris-hydrochloride buffer (pH 8.1) and sedimented in an SW40 rotor through 10 ml of 30% (wt/vol) sucrose onto a cushion of CsCl (ρ = 1.40 g/cm^3). The sedimented virus material was finally diluted in CsCl (ρ = 1.345 g/cm^3) and banded on a self-generating CsCl gradient in a fixed-angle Ti50 rotor centrifuged at 30,000 rpm for 10 h. The virus banded in a narrow and symmetric peak with a mean density of 1.345 g/cm^3. The recovered virus was dialyzed and stored as described under the section "Cells and virus." Highly purified Ad2 virions, labeled with ^{14}C-labeled amino acids, were added to a [^{3}H]thymidine-labeled extract of virus-infected cells. The extract was subjected to equilibrium density centrifugation in the preparative MAT system. The ^{14}C radioactivity banded at the same density as the visible ^{3}H-labeled virus material, thus indicating that extensive purification in the CsCl system did not alter the buoyant density of virions when compared with the virus material transferred directly from the cellular environment into the MAT system.

Ad2 virion core structures, virion group of nine hexon structures, and virion DNA was prepared as described by Prage et al. (18), Maizel et al. (14), and Tibbetts et al. (22), respectively. The buoyant density of these structures was established after centrifugation, individually and in mixtures, in the preparative and analytical MAT density gradient system. The same buoyant densities were obtained irrespective of the type of gradient and sample mixture used. The buoyant densities obtained in the MAT system were: 1.27 to 1.30 g/cm^3 for group of nine hexon structures, 1.245 g/cm^3 for Ad2 virions, 1.225 g/cm^3 for virion core structures and 1.13 g/cm^3 for virion DNA.

Polyacrylamide-gel electrophoresis. Samples were recovered from MAT gradients after precipitation with 20 volumes of 95% ethanol and incubated overnight at 4°C. After two washes with 90% ethanol and one with ethanol-ether (1:1), the samples were air dried overnight at room temperature and subsequently dissolved as described elsewhere (11). Analysis of the samples was carried out on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (13%; 1.5 mm thick, 70 mm wide, and 70 mm long), which were subjected to electrophoresis, stained, and processed for autoradiography as previously described (9).

Liquid scintillation spectrometry. Samples ob-

tained from MAT gradients were collected and dried on filters of Whatman no. 1 paper, precipitated with 10% trichloroacetic acid, washed twice each in 5% trichloroacetic acid and 70% ethanol, and washed once in ethanol-ether (1:1). Thoroughly dried filters were counted in 5 ml of toluene-based Spectrafluor (Amersham/Searle, Ill.). Gel slices obtained from electrophoresis experiments were hydrolyzed in 0.5 ml of 2 M NaOH at 65°C overnight. Ten milliliters of Aquasol (New England Nuclear Corp., Boston, Mass.) containing 10% water was added to the hydrolysates. A Beckman LS-250 liquid scintillation counter with automatic quench correction was employed for determination of radioactivity. Radioisotopes were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Isopycnic density centrifugation of virus-specific DNA and protein. We first analyzed the density distribution in MAT gradients of Ad2-specific DNA and protein, radioactively labeled late in the productive cycle. Approximately 25% of the [^{3}H]thymidine-labeled material was confined to a region coinciding with the density of virions (Fig. 1A). The virus-specific proteins banded in the virion region and at a lower density in a distinct peak (Fig. 1B). The radioactivity profile of an extract pulse labeled for protein and continuously labeled for DNA is shown in Fig. 1C. The newly synthesized proteins banded in two major density regions, one obviously denser than the region containing the majority of continuously labeled DNA. Fractions from the MAT gradients were pooled and analyzed on SDS-polyacrylamide slab gels. The stained gel revealed that late in infection, the extractable cell content of polypeptide P-VII-containing complexes was confined to a region with densities equal to or higher than that of virions (Fig. 2A). Although purified Ad2 virions normally contain low concentrations of presumably unprocessed P-VII (11), some regions of the MAT gradients contain equivalent amounts of P-VII and VII, indicating that P-VII may be confined to complexes distinct from virions. This was confirmed by analysis of the pulse-labeled material (Fig. 1C), in which most of P-VII is confined to a density region denser than those of virions and virus-specific DNA-protein complexes (Fig. 2B).

Pulse-chase isotope labeling of protein. A pulse-chase experiment was carried out to determine the fate of newly synthesized P-VII.

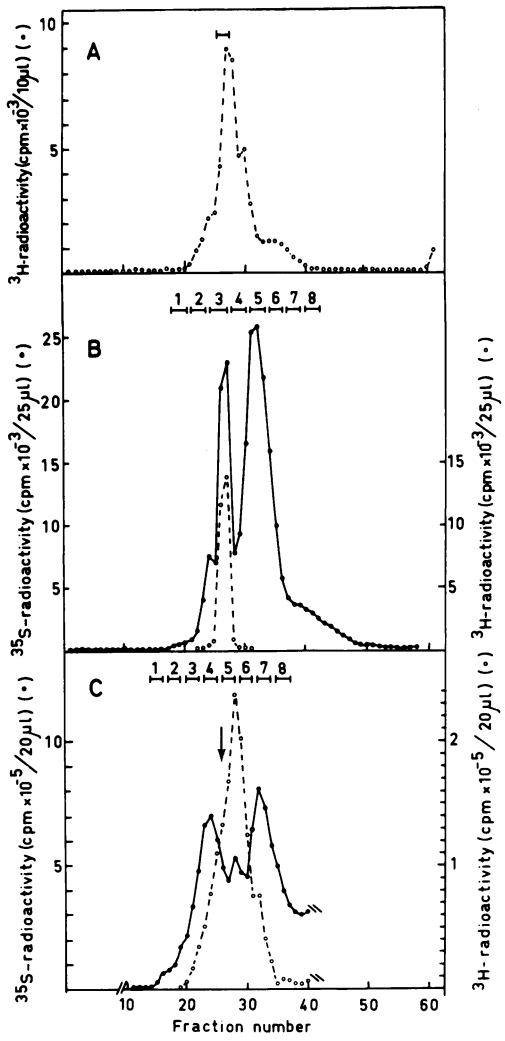


FIG. 1. *Isopycnic MAT centrifugation of isotope-labeled material in infected cells. Two spinner cultures of KB cells synchronously infected with 2,000 particles of Ad2 per cell were separately labeled with [^{3}H]thymidine (1 µCi/ml, 53 Ci/mmol) at 17 h p.i. or with [^{35}S]methionine (3.3 µCi/ml, 276 Ci/mmol) at 20 h p.i. At 48 h p.i. the cells were harvested by centrifugation, resuspended in TEP buffer, and processed for preparative MAT density gradient centrifugation as described in Materials and Methods. (A) ^{3}H radioactivity profile after isopycnic centrifugation of 6 × 10^7 cell equivalents. The horizontal bar indicates the position of the visible virus band. (B) Density distribution of ^{35}S-labeled virus-specific proteins. As an internal density marker, 7.4 × 10^5 cpm of highly purified [^{3}H]thymidine-labeled Ad2 virions (1.6 × 10^6 cpm/10^{12} particles) were included. (C) Radioactivity profiles after isopycnic banding of 10^7 cells labeled in the same experiment with [^{3}H]thymidine (10 µCi/ml, 50 Ci/mmol) between 14 and 18.5 h p.i., and with [^{35}S]methionine (30 µCi/ml, 265 Ci/mmol) between 16.5 and 18.5 h p.i. The arrow indicates the position of the visible virus band. The density increases to the left (from higher to lower fraction numbers). Fractions indicated by horizontal bars in panels (B) and (C) were subjected to electrophoresis (see Fig. 2).*

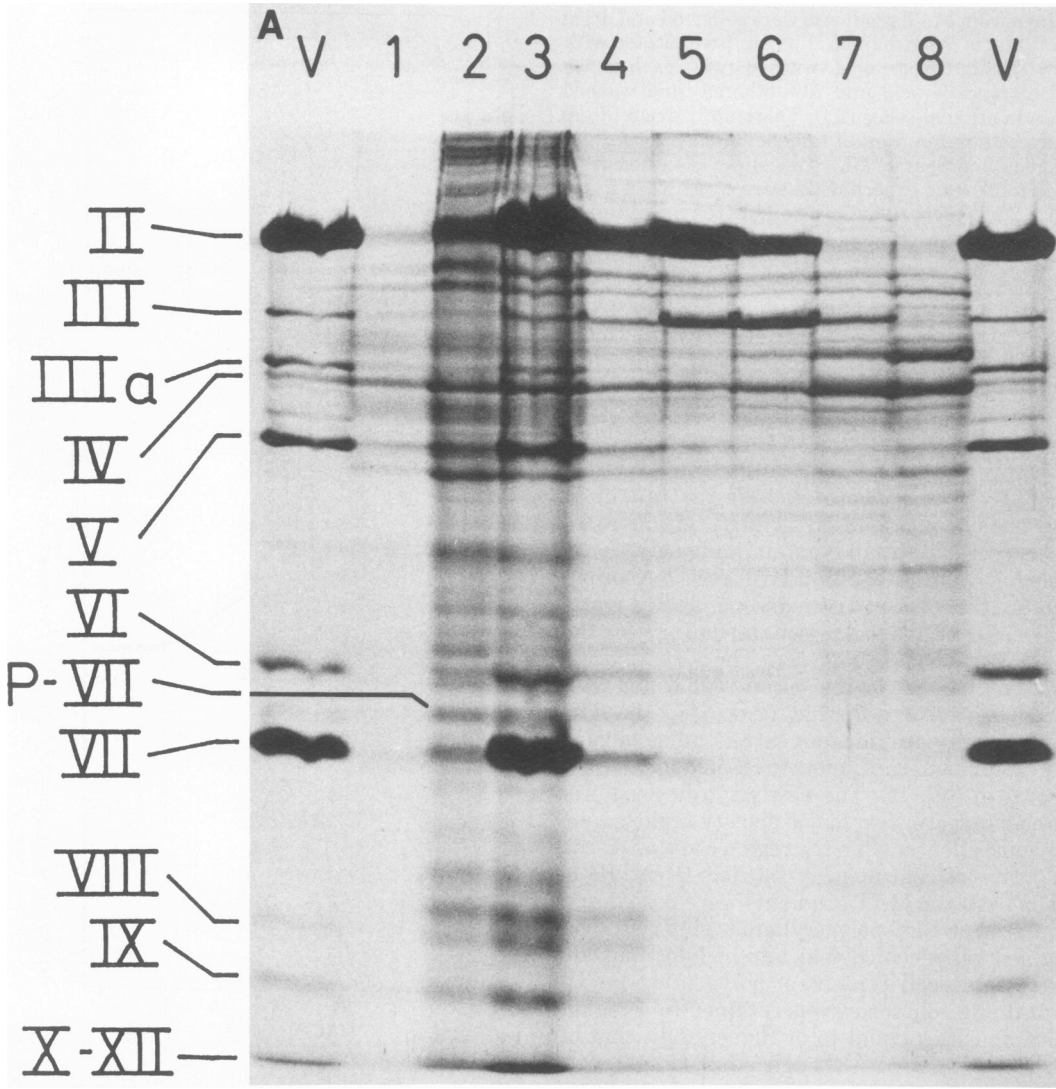


FIG. 2. *SDS-polyacrylamide gel electrophoresis of proteins recovered after MAT density centrifugation. (A) Stained electropherogram of proteins recovered from fractions indicated in Fig. 1B. (B) Autoradiogram of isotope-labeled proteins recovered from fractions indicated in Fig. 1C. Marker virions are indicated by (V), and virion polypeptides are denoted as described by Maizel et al. (13) and Everitt et al. (11). The virus-induced polypeptides are denoted by their molecular weights* $\times 10^{-3}$, *as introduced by Anderson et al. (1).*

Cell samples were obtained after a 20-min pulse with [^{35}S]methionine at 16.5 h p.i. and after a subsequent 8-h chase (see Materials and Methods). The cells were subjected to detergent extraction and isopycnic centrifugation in the preparative MAT system (Fig. 3). After the pulse, most of the labeled proteins were confined to complexes with low density, but also to a small peak in the high-density region. After the chase, a shift in the overall profile of radioactivity was apparent, with a peak in the position of the visible virion band. The pattern of radioactivity in Fig. 3B resembles the profile obtained after 2 h of protein labeling demonstrated in Fig. 1C. Fractions from both gradients (pulse and chase) were precipitated as indicated and analyzed for stainable and isotope-labeled polypeptide content on SDS-polyacrylamide slab gels (Fig. 4). The stained gels, the autoradiograms, and the subsequent removal and counting of specific polypeptides from these same gels reveal that the majority (65%) of newly synthesized poly-

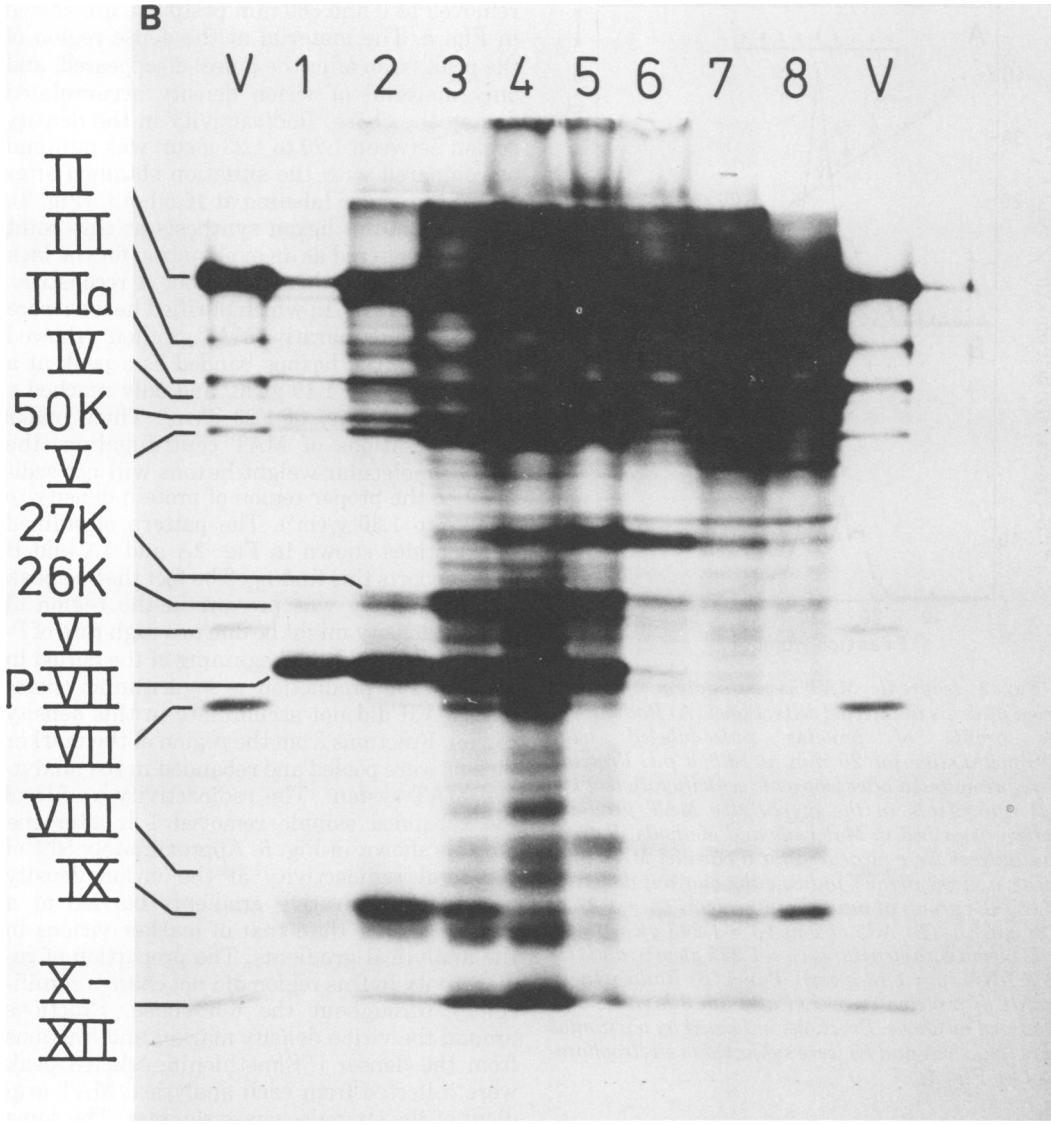


FIG. 2B

peptide P-VII was confined to a denser region than that of virions (ρ = 1.245 g/cm^3). After the 8-h chase, 40% of the total radioactivity in polypeptide P-VII throughout the gradient remained in the dense region (ρ = 1.27 to 1.30 g/cm^3), and 80% of the total radioactivity in polypeptide VII throughout the gradient was present in complexes with a mean density of 1.25 g/cm^3. After the chase, 55% of the total radioactivity in polypeptides P-VII plus VII throughout the gradient remained in polypeptide P-VII, indicating that the transfer of P-VII was not quantitative. A comparison of the processing of P-VII confined to two density regions (1.25 and 1.28 g/cm^3) is demonstrated in Table 1. The radioactivity in the region of protein density (ρ = 1.28 g/cm^3) decreased by 60% after the chase, whereas P-VII obtained from a region with a mean density of 1.25 g/cm^3 revealed an 8% increase after the chase. The radioactivity of polypeptide VII from the high-density region decreased by 40% after the chase, indicating the lack of accumulation of this polypeptide in this region. Polypeptide VII, derived from the region of virion density, increased its label by almost 500%.

Pulse-chase of P-VII-containing complexes of virion density. The maximum rate of syn-

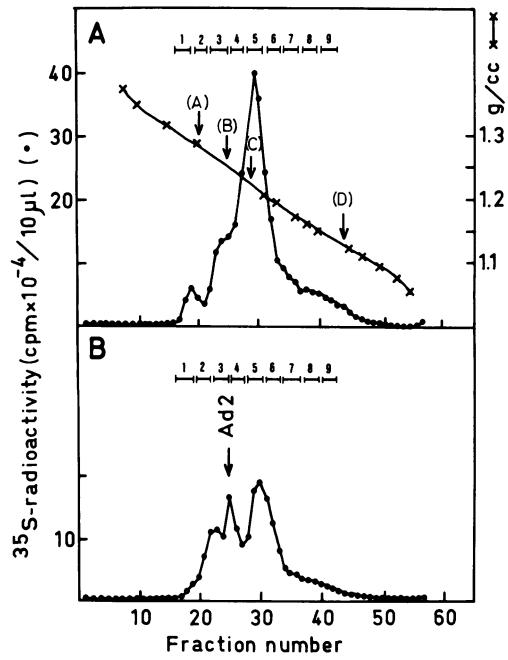
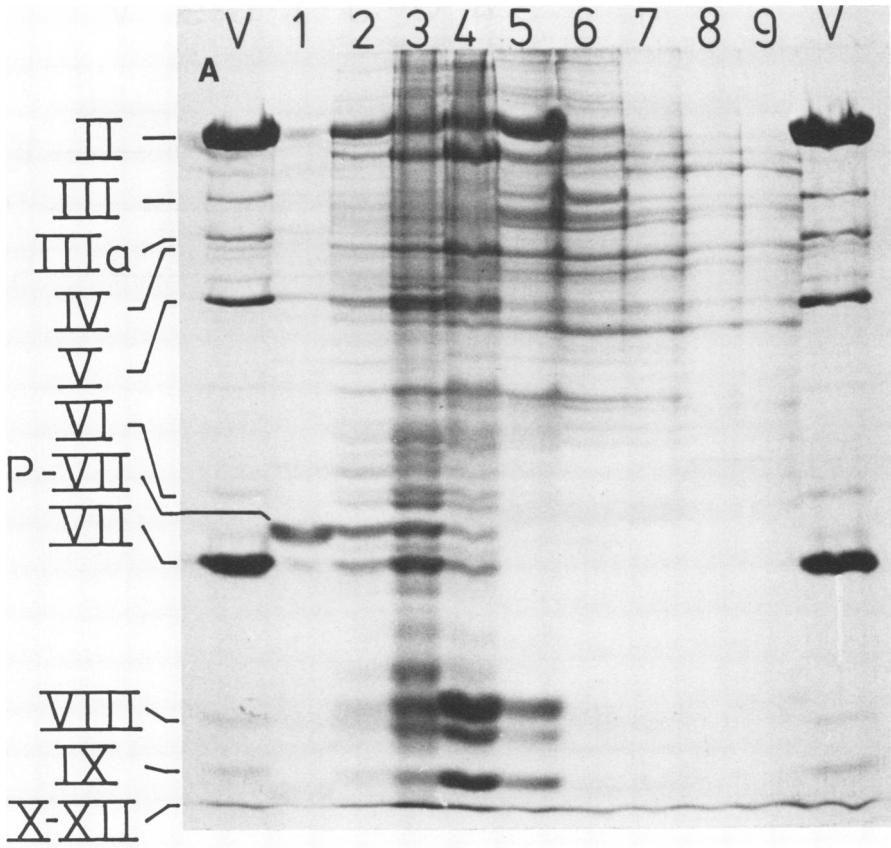


FIG. 3. *Isopycnic MAT centrifugation of ^{35}S-labeled proteins in infected cells. Panel (A) Radioactivity profile of proteins pulse-labeled with [^{35}S]methionine for 20 min at 16.5 h p.i. Proteins were precipitated after isopycnic centrifugation of 10^8 cell equivalents in the preparative MAT gradient system described in Materials and Methods. Refractive indexes were measured for a parallel MAT gradient, and the arrows indicate the buoyant densities of (A) the group of nine hexon proteins (ρ = 1.27 to 1.30 g/cm^3), (B) Ad2 virions (ρ = 1.245 g/cm^3), (C) Ad2 virion core structures (ρ = 1.225 g/cm^3), and (D) Ad2 DNA (ρ = 1.13 g/cm^3). Panel (B) Radioactivity profile of proteins recovered after an 8-h chase and analyzed as above. Fractions indicated by horizontal bars (panels A and B) were subjected to electrophoresis (see Fig. 4).*

thesis of polypeptide P-VII occurs at approximately 14 h p.i. (9). At this time cells were pulse labeled for 5 min with [^{35}S]methionine. Samples were removed at 0, 30, 60, 120, 270, and 360 min after the pulse, and all six samples were analyzed on preparative MAT gradients with [^{3}H]thymidine-labeled Ad2 virions as an internal density marker (see Materials and Methods). The radioactivity profiles of samples removed at 0 and 360 min postpulse are shown in Fig. 5. The material at the dense region of the peak (seen after the pulse) disappeared, and only material of virion density accumulated during the chase. Radioactivity in the density region between 1.20 to 1.23 g/cm^3 was minimal as compared with the situation obtained after the 20-min pulse labeling at 16.5 h p.i. (Fig. 3). The low rate of hexon synthesis at this point p.i. (10) is offered as an explanation for the lack of a peak in this density region. A reconstruction experiment, in which purified hexons were banded in a preparative MAT gradient, showed that 30% of the hexons banded as a peak at a mean density of 1.19 g/cm^3 and only reached a maximum density of 1.23 g/cm^3. Thus, under these conditions of MAT centrifugation, the 360,000-molecular-weight hexons will not sediment to the proper region of protein density (ρ = 1.27 to 1.30 g/cm^3). The pattern of stained polypeptides shown in Fig. 2A and 4A and B also supports this finding. The fact that no peak of radioactivity was present in the region of protein density might be due to a high rate of P-VII utilization at the beginning of the period in which virus production is logarithmic; therefore, P-VII did not accumulate in this density region. Fractions from the region of the marker virions were pooled and rebanded in the analytical MAT system. The radioactivity profile of the rebanded sample removed 1 h after the pulse is shown in Fig. 6. Approximately 50% of the total radioactivity at the virion density from the preparative gradients banded at a density higher than that of marker virions in the analytical gradients. The proportion of radioactivity in this region did not change significantly throughout the 6-h chase. Fractions around the virion density marker and fractions from the denser [^{35}S]methionine-labeled peak were collected from each analytical MAT gradient of the six pulse-chase samples. The same amount of radioactivity of all fractions was applied to SDS-polyacrylamide slab gels. The regions covering polypeptides P-VII and VII were cut out, hydrolyzed, and assayed for radioactivity as described in Materials and Methods. The total radioactivity of polypeptide VII recovered from the region of virion density increased by 100% after the chase (Fig. 7). The radioactivity of polypeptide P-VII (from the virion density

FIG. 4. *SDS-polyacrylamide gel electrophoresis of pulse-chase-labeled proteins recovered after isopycnic MAT centrifugation. MAT gradient fractions, as indicated in Fig. 3, were analyzed as described in Materials and Methods. Stained electropherograms of the pulse-labeled and chased protein series are shown in (A) and (B), respectively. Gels subjected to autoradiography were subjected to electrophoresis separately with one-fourth of the material used for the stained gels. The pulse-labeled and chased protein series are shown in (C) and (D), respectively. The Roman numerals for virion polypeptides are indicated to the left, and the virion marker is denoted by (V). The polypeptides are denoted as described in the legend of Fig. 2.*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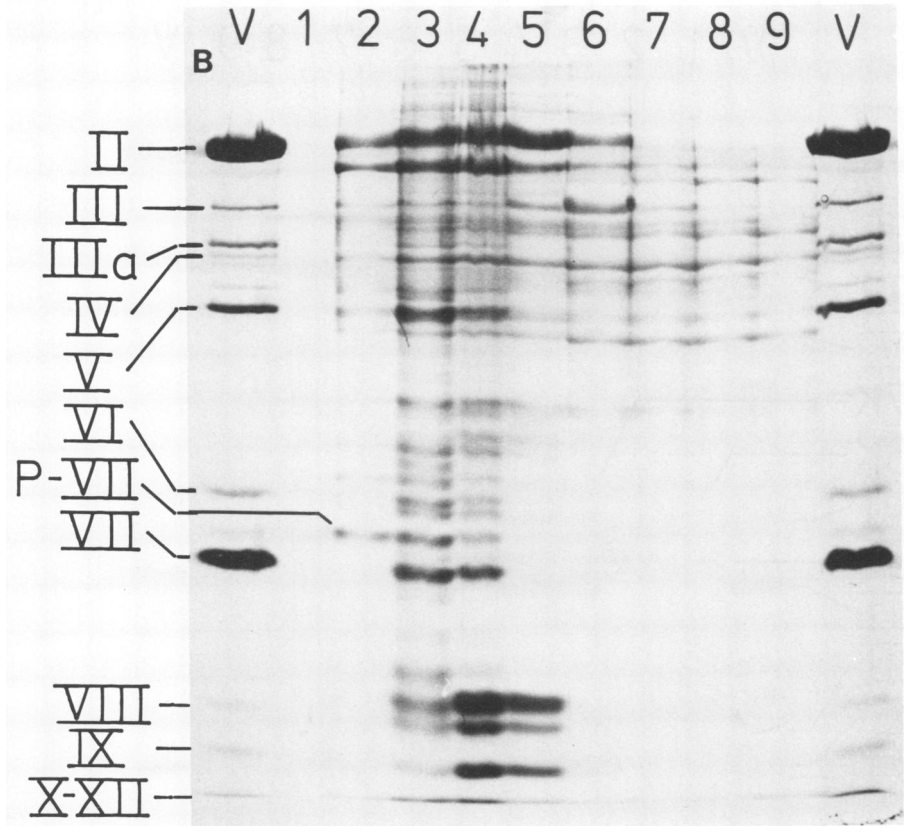
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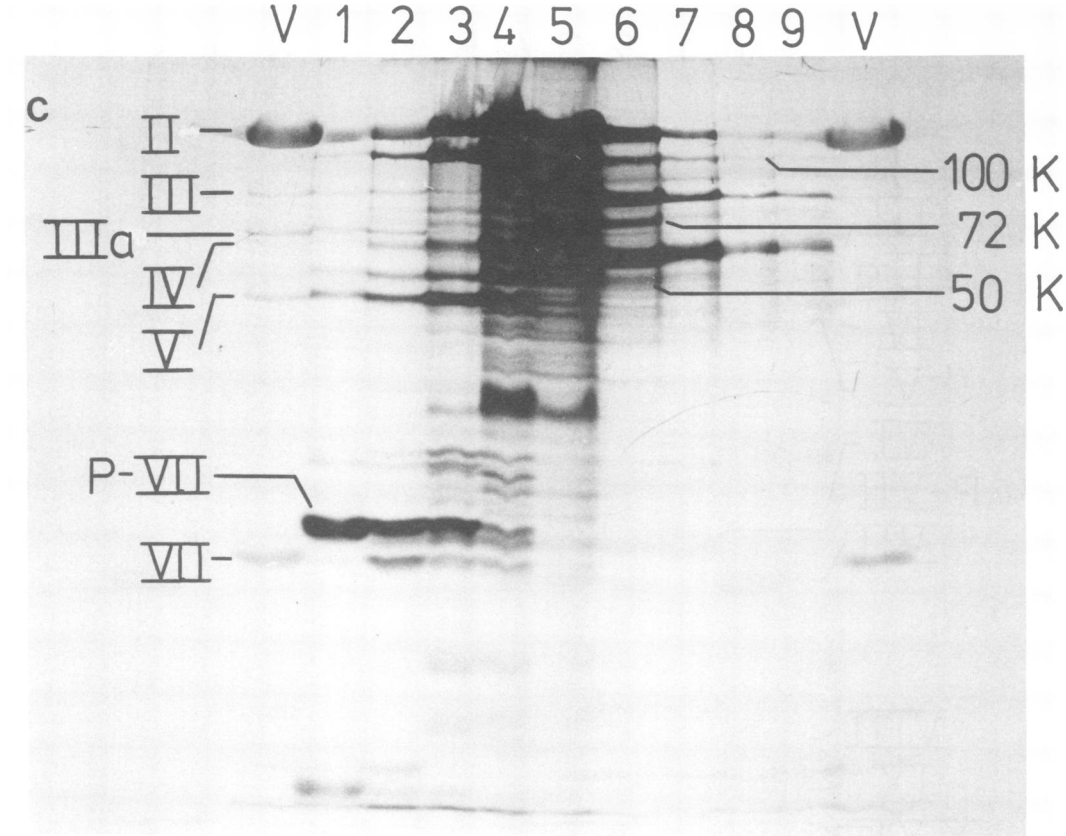
V 1 2 3 4 5 6 7 8 9 V
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100 K
72 K
50 K

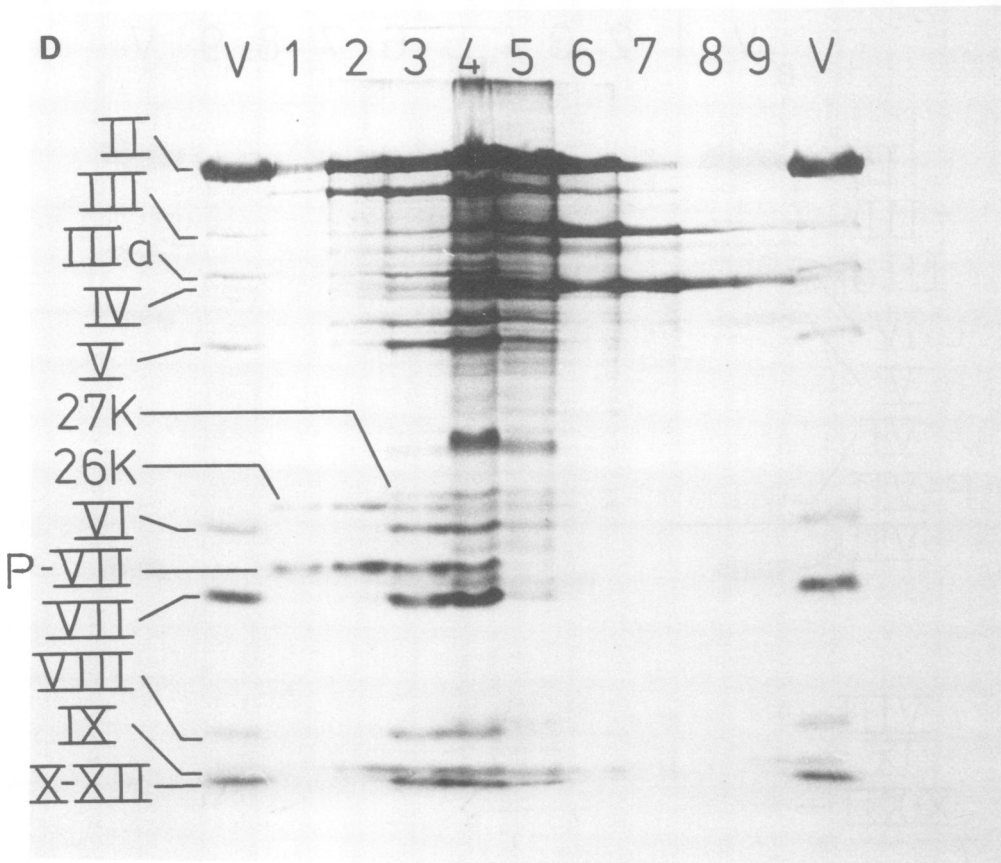
D
V 1 2 3 4 5 6 7 8 9 V
II
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FIG. 4C and D

TABLE 1. *Processing of dense and light P-VII in vivo*[a]

Mean density (g/cm³)	Polypeptide	Pulse (cpm)	Chase[b] (cpm)	Change[c] (%)
1.28	P-VII	25,338	10,167	−60
	VII	3,085	1,865	−40
1.25	P-VII	12,346	13,311	+8
	VII	2,897	16,533	+500

[a] Polypeptides were recovered from the SDS-polyacrylamide slab gels shown in Fig. 4, and the radioactivities were assayed as described in Materials and Methods. The dense material corresponds to the pooled fractions 1 + 2, and the light material of fractions 3 + 4, with mean densities of 1.28 and 1.25 g/cm³, respectively.

[b] Figures for the chase series were adjusted to compensate for the loss of material as revealed by the three-times-lower amount of total radioactivity recovered in the preparative MAT gradient of Fig. 3B.

[c] Change is expressed as the increase (+) or decrease (−) in radioactivity relative to the amounts obtained after the pulse.

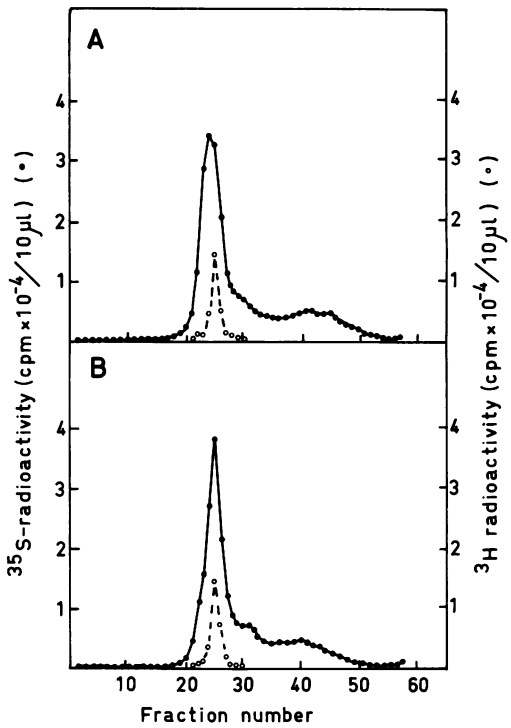

FIG. 5. *Isopycnic MAT centrifugation of ^{35}S-labeled proteins in infected cells. (A) Radioactivity profile of proteins pulse labeled with [^{35}S]methionine for 5 min at 14 h p.i. Approximately 4 × 10⁷ cell equivalents were centrifuged in the preparative isopycnic MAT system described in Materials and Methods. (B) Radioactivity profile of proteins recovered after a 6-h chase and analyzed as above. To each gradient was added 7.4 × 10⁵ cpm of highly purified [^{3}H]thymidine-labeled Ad2 virions as an internal density marker. The density increases to the left.*

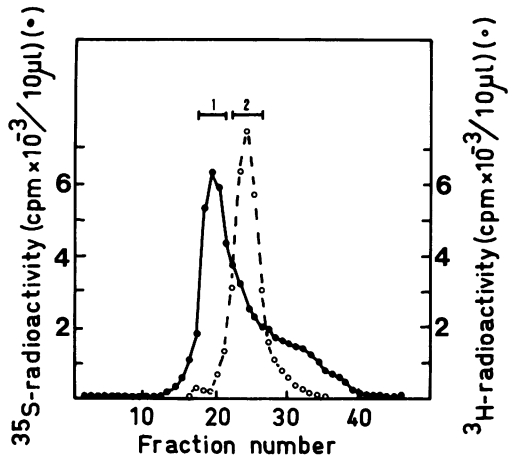

FIG. 6. *Analytical MAT density centrifugation of material recovered from a preparative MAT gradient. Material obtained from the region of virion density shown in Fig. 5 was rebanded in a narrow 40 to 48% MAT gradient as described in Materials and Methods. The sample was pulse labeled with [^{35}S]methionine for 5 min at 14 h p.i. and chased for 1 h. The density increases to the left. Purified [^{3}H]thymidine-labeled Ad2 virions were employed as a density marker.*

region) increased by around 10% after the first 2 h of chase, but then decreased gradually to a level 30% below the value obtained immediately after the pulse. Such a decrease in radioactivity was not as apparent for polypeptide P-VII recovered from the denser region of the analytical MAT gradients. The radioactivity of polypeptide VII obtained from the same region was constant.

The density of the [^{35}S]methionine-labeled peak obtained after analytical MAT centrifugation (Fig. 6) was determined next. Pooled peak fractions of [^{35}S]methionine radioactivity were rebanded in MAT together with [^{3}H]thymidine-labeled Ad2 marker virions. The newly labeled material was found to be of intermediate density between virions (ρ = 1.245 g/cm³) and protein (ρ = 1.27 to 1.30 g/cm³) (Fig. 8), indicating a complex of nucleic acid and protein. Upon fixation with glutaraldehyde and isopycnic centrifugation in a CsCl-detergent system, the [^{35}S]methionine-labeled material recovered from a MAT density of 1.26 g/cm³ revealed a density 15 mg/cm³, lighter than that of marker virions (not shown).

Polypeptide composition of P-VII-containing complexes of nonvirion density. The material that was denser than marker virions and enriched in P-VII (pool no. 2, Fig. 3A and 4C) was rebanded together with [^{3}H]thymidine-labeled Ad2 marker in an analytical MAT gra-

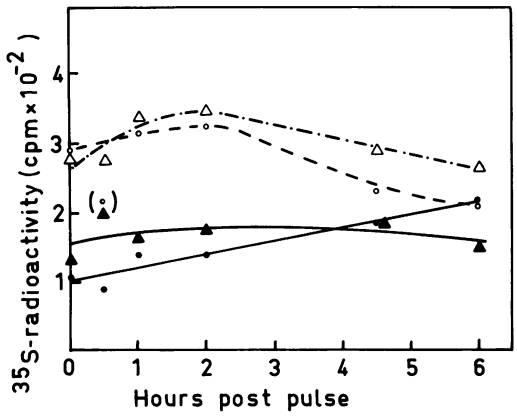

FIG. 7. *Quantitative kinetics of the transition of radioactivity from polypeptide P-VII into VII in vivo. Productively infected cells were pulse labeled with [35S]methionine for 5 min at 14 h p.i. and subsequently chased for a total period of 6 h. Six samples were removed at indicated intervals and subjected to detergent extraction and isopycnic centrifugation in the preparative MAT gradient system described in Materials and Methods (compare Fig. 5). Material from the density region including the internal virion marker was rebanded on analytical 40 to 48% MAT gradients as shown in Fig. 6. Material from the two density regions indicated in Fig. 6 by horizontal bars (1 and 2) was pooled and subjected to SDS-polyacrylamide gel electrophoresis. Stained polypeptide bands corresponding to polypeptides P-VII and VII were cut out from the gel, hydrolyzed, and assayed for radioactivity as described in Materials and Methods. Symbols: Polypeptide VII obtained from material of virion density, ●; polypeptide VII obtained from material of nonvirion density, ▲; polypeptide P-VII obtained from material of virion density, ○; and polypeptide P-VII obtained from material of nonvirion density, △. The mean values of two separate gel analyses are shown.*

dient. Peak fractions were pooled, precipitated, and analyzed on SDS-polyacrylamide gels. The polypeptide composition of [35S]methionine-labeled material denser than that of virions was thus compared with that of Ad2 virions and the material banding at virion density in analytical MAT gradients (equivalent to portion 2 of Fig. 6). The dense P-VII-containing complex revealed most of the polypeptides of matured virions by staining (Fig. 9A). Polypeptide III (penton base) and polypeptide IIIa were, however, present in lower amounts. The internal proteins corresponding to polypeptides V, VI, and VII were present together with unprocessed precursors of polypeptide VI (P-VI = 27K) and VII (P-VII). A polypeptide of unknown localization in the virion was detectable in the region between P-VII and VII of the dense material. The autoradiogram of the same gel was in accord with the stained protein pattern, although polypeptides III and IIIa could now be detected more easily. Moreover, a polypeptide in the tentative position of IVa_2 or 50K was present, with an apparently high specific radioactivity. Large quantities of labeled polypeptide P-VII were present in the dense material, and a significant degree of processing into polypeptide VII had occurred. Stainable and labeled quantities of two polypeptides in the 100,000-molecular-weight region were also detected.

Pulse-chase isotope labeling of DNA. To establish whether newly synthesized viral DNA became associated with polypeptide P-VII in any density region, a pulse-chase was carried out. At 16.5 h p.i. the majority of newly synthesized DNA was of viral specificity (5), and no attempts were made to quantitate the amount of host cell DNA synthesis possibly persisting. Four samples from a pulse-labeled and chased Ad2-infected cell culture were obtained. The cells were extracted and centrifuged in the isopycnic MAT system, and the trichloroacetic acid-precipitable radioactivity profiles are shown in Fig. 10. It is evident that the majority of the pulse-labeled DNA, present as DNA-protein complexes, banded in a region lighter than that of marker virions. After a subsequent chase, the radioactivity gradually merged into the density region of virions, and after 3 h of chase, a pronounced peak of [3H]thymidine radioactivity was confined to the same density

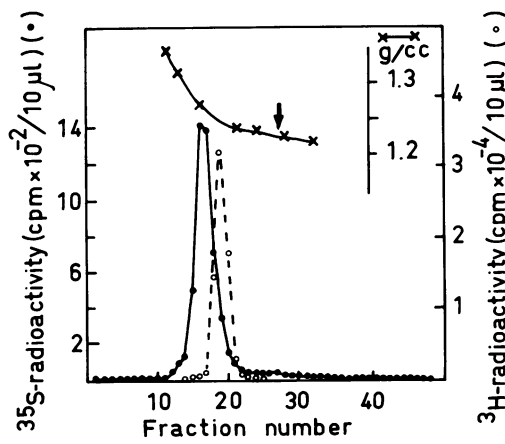

FIG. 8. *Rebanding of an [35S]methionine-labeled complex obtained after analytical MAT density centrifugation. The material denser than virions and indicated by the horizontal bar (1) in Fig. 6 was rebanded together with [3H]thymidine-labeled Ad2 marker virions in the analytical MAT system described in Materials and Methods. The density profile was obtained from the actual gradient and the arrow indicates the density position of core structures obtained from virions after pyridine treatment (18).*

region as that of the internal virion marker. No radioactivity was present in the region of the gradient with density higher than that of virions. This may indicate that the majority of newly synthesized P-VII is not associated with newly synthesized DNA, since the former appears in a density region that is, at the most, 50 mg/cm^3 denser than that of virions. However, the existence of minor quantities of P-VII in association with newly synthesized DNA cannot be ruled out (compare Fig. 1C and 2B).

In vitro processing of P-VII. Since a significant degree of spontaneous processing of polypeptide P-VII had been detected in complexes of intermediate density, processing in vitro was studied separately (Fig. 9). Polypeptide P-VII-containing complexes, confined to density regions ranging from protein density (ρ = 1.30 g/cm^3) to virion density (ρ = 1.245 g/cm^3) in the MAT gradients, were analyzed for processing of their polypeptide P-VII into polypeptide VII in vitro (Table 2). The mere storage of P-VII-containing complexes allowed for a significant processing when complexes were recovered from a region of mean density of 1.275 g/cm^3. No such processing was apparent for material in the protein density region (ρ = 1.30 g/cm^3). When four samples from the density region 1.245 to 1.30 g/cm^3 were incubated (i) in buffer at 37°C, (ii) in the presence of an Ad2-infected cell extract at 37°C or at 4°C, and (iii) in the presence of a mock-infected cell extract at 37°C, it became apparent that a virus-induced or virus-specified factor(s) was required for processing of P-VII-containing complexes of protein density. This processing is evidently temperature dependent since the transition was low at 4°C. Materials of intermediate and virion density processed the P-VII to some extent in the absence of exogenous factors.

DISCUSSION

Metrizamide (an iodinated benzamido derivative of glucose) is a versatile material for isopycnic density gradients (3), which may be needed to isolate labile intermediate structures during in vivo assembly of adenoviruses. CsCl density gradients may, because of the high salt concentrations, cause protein denaturation due to dehydration, but in Metrizamide high densities are obtained under conditions of low osmotic pressure and low ionic strength.

Initially, we wanted to examine whether the precursor polypeptide (P-VII; 20,000 molecular weight) of the major arginine- and alanine-rich core protein ([17], polypeptide VII; 18,500 molecular weight), shortly after its synthesis, becomes associated with DNA of cellular or viral origin, thus forming a precursor core structure. Pilot experiments were performed to learn the conditions under which a maximum degree of separation between Ad2 virions and Ad2 virion core structures is achieved. Preformed Metrizamide gradients stored overnight at 4°C and centrifuged in swinging-bucket rotors provided the best approach for this purpose. We also determined initially that low concentrations of salt (e.g., NaCl) present in the samples and/or in the gradients caused a nonspecific precipitation of virus-specific and cellular nucleoprotein complexes. Minute concentrations of divalent cations (e.g., Mg^{2+}, Ca^{2+}) also caused significant aggregation. Using a Metrizamide, EDTA, and Tris-hydrochloride system, no visible aggregation occurred during isopycnic banding of total cellular extracts. However, it became apparent that upon rebanding this material in the same system, the results were often not reproducible. This difficulty was believed to be due to a small degree of aggregation, and the nonionic detergent Triton X-100 was therefore included in the system at a concentration of 0.5%. To minimize disaggregation of possible complexes between the basic precursor protein P-VII and any species of DNA, the pH was kept at 8.1 using the Tris-hydrochloride buffer system.

We first showed that late in the productive cycle, polypeptide P-VII was present in DNA-containing complexes of intermediate density and distinct from virions (Fig. 1 and 2). However, when introducing a protein pulse labeling at 16.5 h p.i. the majority of newly synthesized P-VII was confined to a density region significantly denser than that of Ad2 virions (ρ = 1.245 g/cm^3), and approaching the density region of proteins (ρ = 1.27 to 1.30 g/cm^3) (Fig. 1 to 4). We favor the idea that P-VII in the denser part of this region is present as a protein aggregate, thus constituting the "free pool" of newly synthesized P-VII. Subsequent chase studies revealed that P-VII disappeared efficiently from this region and could not easily be detected late in infection at this position (Fig. 1 to 4, Table 1). P-VII from the intermediate-density region between those of protein and virions was, however, less efficiently processed (Table 1, Fig. 7). Thus, we suggest that part of P-VII, present in complexes close to virion density, is poorly processed, whereas another portion is properly cleaved to increase the polypeptide VII content of the material confined to the position of true virion density. A nonquantitative or nonefficient transition of radioactivity from P-VII into VII has been demonstrated previously for total cellular and nuclear extracts (1, 9, 12), thus indicating that the entire pool of P-VII is not used up for virion assembly. The excess pool

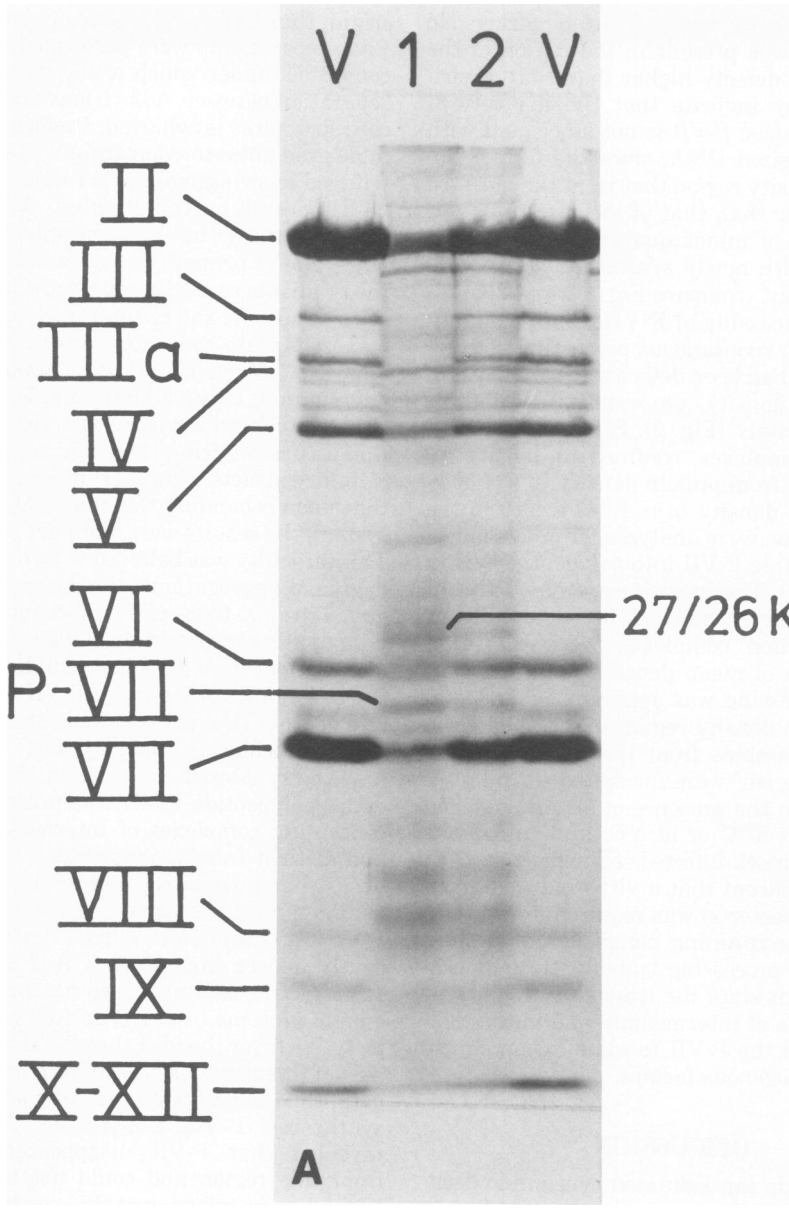

FIG. 9. *Comparison between the polypeptide composition of virions and complexes of intermediate density isolated after MAT density centrifugation. (A) Stained electropherogram of proteins recovered from virion and nonvirion density regions of an analytical MAT gradient as described in Fig. 6. The sample analyzed was obtained from cells pulse labeled for 20 min at 16.5 h p.i. After isopycnic centrifugation in the preparative MAT system, material including fractions of pool no. 2 as indicated in Fig. 3A and 4C was rebanded in an analytical MAT gradient. Fractions indicated in Fig. 6 by the horizontal bar (1) had a mean density of 1.265 g/cm³, and those indicated by (2) had a mean density of 1.245 g/cm³. Gel slots no. (1) and (2) refer to densities 1.265 and 1.245 g/cm³, respectively. (B) Same electropherogram as above subjected to autoradiography. Marker virions are indicated by (V), and virion and virus-specified polypeptides are denoted as described in the legend to Fig. 2.*

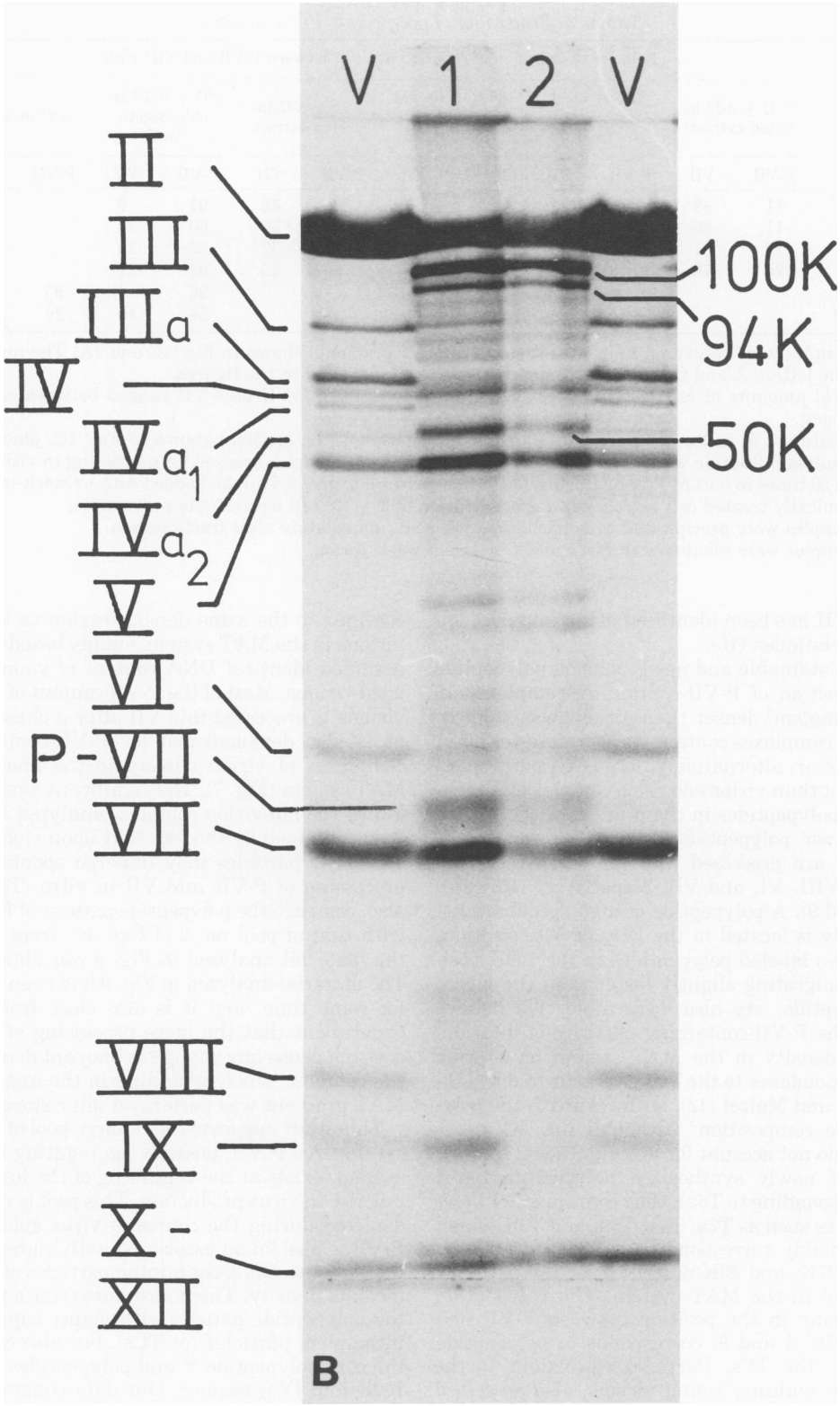
V 1 2 V
II
III
IIIa
IV
IVa1
IVa2
V
VI
P-VII
VII
VIII
IX
X-
XII
100K
94K
50K
B

FIG. 9B

TABLE 2. *Processing of polypeptide P-VII in vitro*

Sample[a]	Relative distribution of radioactivity (%) between P-VII and VII[b] with:											
	37°C + Ad2-infected extract[c]		37°C + mock-infected extract		37°C + 10 mM Tris-hydrochloride, pH 7.5		4°C + Ad2-infected extract		4°C + 10%[d] trichloroacetic acid		4°C in MAT[e]	
	P-VII	VII	P-VII	VII	P-VII	VII	P-VII	VII	P-VII	VII	P-VII	VII
IC-1	11	89	89	11	94	6	52	48	91	9		
IC-2	11	89	88	12	90	10	50	50	90	10		
IC-3	19	81	70	30	76	24	68	32	86	14		
IC-4	24	76	74	26	75	25	75	25	83	17		
3A-1									96	4	95	5
3A-2									84	16	26	74

[a] Samples were recovered from the preparative MAT gradients shown in Fig. 1C and 3A. The numbers after the letters A and C refer to the pooled fractions as indicated in the figures.

[b] Total amounts of radioactivity recovered from polypeptides P-VII plus VII ranged between 600 and 50,000 cpm.

[c] Treatment of material; immediately upon fractionation of the gradient shown in Fig. 1C, phenylmethane sulfonyl fluoride was added at a concentration of 1 mM. Samples assayed for processing in vitro were diluted 50 times in 0.01 M Tris-hydrochloride buffer (pH 7.5), to give 0.5 ml, and added Ad2- or mock-infected ultrasonically treated cell extracts at a concentration of 3×10^5 cell equivalents per sample.

[d] Samples were precipitated with trichloroacetic acid immediately after fractionation.

[e] Samples were incubated in MAT at 4°C over a 4-week period.

of P-VII has been identified using immunological techniques (10).

The stainable and newly labeled polypeptide composition of P-VII-containing complexes 15 to 20 mg/cm^3 denser than virions was studied. These complexes contain more protein per DNA content or, alternatively, less DNA per protein content than virions do. This complex class contains polypeptides in the position of the known precursor polypeptides 26K, 27K, and P-VII, which are processed into the virion polypeptides VIII, VI, and VII, respectively (15) (Fig. 4D and 9). A polypeptide of high specific radioactivity is located in the IVa_2 or 50K position, and two labeled polypeptides in the 100K position, migrating slightly faster than the hexon polypeptide, are also discernible. We believe that the P-VII-containing particles of intermediate density in the MAT system have some correspondence to the TCs characterized by Ishibashi and Maizel (12), with regard to the polypeptide composition. Although the TC structures do not account for any significant quantities of newly synthesized polypeptide P-VII (corresponding to T6a), they contain other polypeptides such as T2a, T4a, T5a, and T5b, which presumably correspond to polypeptides 100K, IVa_2, 27K, and 26K described for the particles isolated in the MAT system. The polypeptide migrating in the position between P-VII and VII (Fig. 4 and 9) corresponds to polypeptide T6b in the TCs. Particles equivalent to the DNA-containing young virions, also described by Ishibashi and Maizel (12), are presumably confined to the same density region as that of virions in the MAT system, simply based on the assumed identical DNA content of young and aged virions. Most of the P-VII content of young virions is processed into VII after a chase (12), as is also demonstrated for P-VII-containing complexes of virion density in the analytical MAT system (Fig. 7). The significant amount of VII in the nonvirion complex, analyzed in Fig. 9, is explained by the fact that upon storage at 4°C these particles may undergo spontaneous processing of P-VII into VII in vitro (Table 2; also, compare the polypeptide pattern of Fig. 9B with that of pool no. 2 of Fig. 4C, from which the material analyzed in Fig. 9 was obtained). The material analyzed in Fig. 9 had been stored for some time, and it is also clear from this experiment that the mere processing of P-VII does not cause any change in buoyant density of the complex, since rebanding in the analytical MAT gradient was performed after storage.

This study suggests that a large pool of newly synthesized P-VII, possibly aggregating in this system, exists at the beginning of the logarithmic rise in virus production. This pool is rapidly depleted during the course of virus synthesis. P-VII is also found associated with high-molecular-weight, DNA-containing particles of intermediate density. These structures share part of the polypeptide pattern with empty capsids or incomplete particles (or TCs), but also contain the core polypeptide V and polypeptides in the 100K and IVa_2 regions. Our data suggest that there does not seem to be any temporal coordi-

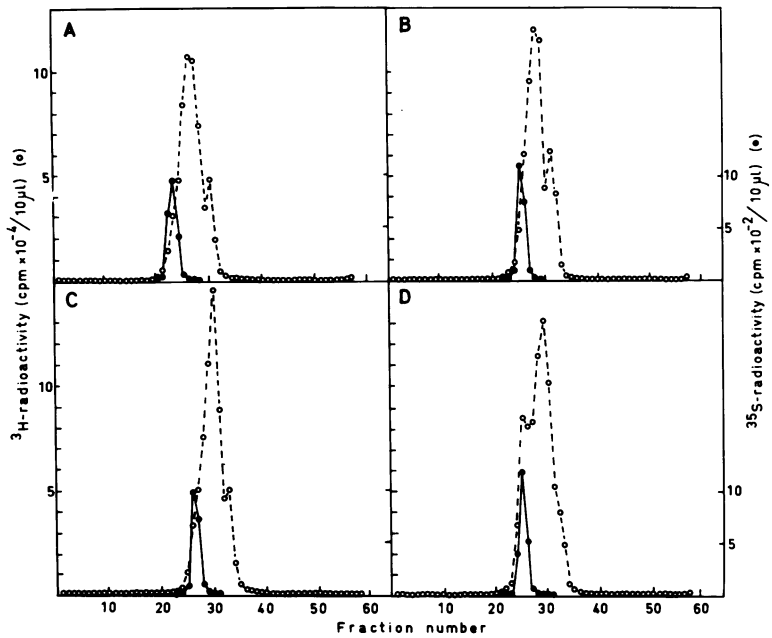

FIG. 10. *Isopycnic MAT centrifugation of ^{3}H-labeled DNA in infected cells. Radioactivity profiles of DNA-protein complexes pulse labeled with [^{3}H]thymidine for 15 min at 16.5 h p.i. and chased for 3 h (see Materials and Methods). Approximately 4 × 10^7 cells were centrifuged in the preparative isopycnic MAT system. The panels show the patterns of radioactivity from samples removed immediately after the pulse (A), and after 30 min (B), 90 min (C), and 180 min (D) of subsequent chase. An internal Ad2 virion density marker labeled with [^{35}S]methionine (1.3 × 10^7 cpm/10^{12} particles) was employed.*

nation between the synthesis of the majority of polypeptide P-VII and viral DNA to form the originally anticipated P-VII-DNA precursor core complex (Fig. 10).

The processing of polypeptide P-VII in vitro showed that this polypeptide is more likely to become spontaneously cleaved in complexes of relatively higher DNA content, whereas complexes presumably devoid of DNA required the addition of some virus-specified or virus-induced factor(s) (Table 2). Whether the mere presence of DNA is a prerequisite for a possible processing, or whether there are soluble factors, specific or nonspecific, present in the lighter MAT gradient fractions providing the spontaneous processing, is at present under investigation. Arginine starvation applied to the adenovirus system has shown that an early factor, possibly involved in DNA replication, is impaired (10). Presumably as a consequence of this, no processing of polypeptide P-VII is detected in such depleted cells (B. Edvardsson and E. Everitt, unpublished data). Another system of interest for further studies on the mechanism of protein processing is derived from the Ad2 *tsl* system, in which noninfectious DNA-containing particles of virion density with unprocessed precursors to the polypeptides VI, VII, and VIII will be assembled (23). This observation demonstrates that the mere processing is not by itself a prerequisite for virion assembly. Finally, the MAT system developed offers a simple basis for the isolation of a test substrate for an in vitro assay of proteolytic activities associated with the processing of virus-specified polypeptides in the adenovirus system.

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