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Three Structural Polypeptides Coded for by Minute Virus of Mice, a Parvovirus

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Purified full and empty virions of minute virus of mice were separated on CsCl gradients, and their polypeptides were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The empty particle contains two polypeptides, A (83,300 daltons) and B (64,300 daltons), which are 15 to 18% and 82 to 85%, respectively, of the virion mass. The full particle contains the single-stranded DNA genome, proteins A and B, and a third polypeptide, C (61,400 daltons). Again A is 15 to 18% of the protein mass, but the amounts of B and C vary inversely in different preparations of full particles. These polypeptides comprise >99.6% of the protein in either virion, and their molecular weights and molar ratios are independent of the species of host cell on which the virus is propagated. They are not found in uninfected cells, and no protein component of uninfected cells copurifies with either virion under our conditions. Pulse-chase experiments show that the three proteins are synthesized only after virus infection and are therefore probably virus coded. Sequential harvesting from the nuclei of cells infected under one cycle growth conditions shows an increase in the proportion of C in full particles as infection progresses, suggesting that C is derived from B in a late maturation step.

The parvoviruses are among the smallest DNA-containing vertebrate viruses, being icosahedral particles 18 to 30 nm in diameter (31). They are divided into two groups, namely the adenovirus-associated viruses, which are dependent upon adenovirus for replication, and those which, like minute virus of mice (MVM), are capable of autonomous replication. Cells infected with either the nondefective viruses alone or with adenovirus-associated virus and an adenovirus helper produce two types of virus particle. One type, the "full" particle, contains the single-stranded DNA genome, whereas the other, the "empty" particle, is apparently devoid of DNA.

The structural polypeptides of full virions have been examined for a number of serologically distinct parvoviruses and, in general, three polypeptides have been demonstrated (8, 19, 20, 22, 32, 33), with molecular weights ranging between 92,000 and 55,000. As the information required to encode the structural polypeptides of MVM excedes the genetic capacity of the genome by some 30%, we have examined the genetic origin of these three proteins. The possibility that the virus incorporates preexisting or induced host proteins, as do the papovaviruses (15, 24), has been investigated, as have possible precursor-product relationships between the virion polypeptides. For this we have developed procedures for the large-scale growth and controlled purification of full and empty virions, under conditions where artefactual proteolysis is minimal, and have analyzed their constituent proteins by high-resolution sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Cells and virus. Monolayers of BHK cells (28) and RL5E (3) were grown in 90-mm plastic tissue culture dishes in D medium (Dulbecco modification of Eagle minimal medium) supplemented with 10% fetal calf serum. The A9 strain of L cells (26) and the strain of Ehrlich ascites (EA) cells described by van Venrooij et al. (40) were grown in Spinner culture in DP medium (calcium-free D medium with 10× phosphate) supplemented with 5% fetal calf serum (GIBCO) as described previously (38). Cell stocks were routinely maintained in the absence of antibiotics, but media for virus production were supplemented with 10 μg of gentamicin (Schering Diag-

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nostics) per ml and 10 U of mycostatin (GIBCO) per ml. The plaque-purified strain of MVM, MVM (T) (38), was used in these experiments. Virus infectivity was determined by plaque assay on monolayers of A9 cells as previously described (38) with the following modification. Plastic tissue culture dishes (50-mm) were seeded with 2.6×10^5 A9 cells, from Spinner culture, and incubated at 37°C for 20 h. The medium was then removed, and 0.5 ml of virus, diluted in phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (11), was added to the sparse monolayer. After 60 min of incubation at 37°C, with occasional gentle shaking, the inoculum was removed, the cells were overlaid with agar medium, and the assay was continued as originally described (38). Viral hemagglutinin was estimated as before (38).

Virus production. Stocks of low-multiplicity-derived virus were obtained by adding 500 PFU of virus to a 25-ml Spinner culture of EA cells at 2×10^5 cells/ml. The culture was then maintained at 37°C between 2×10^5 and 8×10^5 cells/ml by dilution with fresh medium. Usually the culture stopped growing, at a volume of 3 to 4 liters, by 5 days after infection. Seven days after infection the cells were harvested by low-speed centrifugation. Virus in the cell pellet was extracted and purified as described below. The medium was centrifuged at 10,000 rpm in a Sorvall SS-34 rotor for 10 min at 5°C to remove cell debris. The supernatant was adjusted to pH 7.3 with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH buffer at a final concentration of 25 mM. This was then filter sterilized (0.2-μm porosity), assayed for infectivity, and stored at 5°C. These titered virus stocks were used to infect cells at high multiplicity for producing labeled virus in a single cycle of infection.

For single-cycle infections, rapidly dividing EA cells were pelleted and resuspended in virus stock to give a multiplicity of infection of 5 PFU/cell (usually at about 10^7 cells/ml). After mixing for 30 min the cells were recentrifuged and resuspended in fresh medium at 5×10^5 cells/ml. All operations were performed at 37°C. To obtain virus labeled in the capsid proteins, ^{3}H- or ^{14}C-amino acid mix (5 μCi/ml or 0.2 μCi/ml, respectively) was added at this time. Virus differentially labeled in its coat protein and DNA was grown in the presence of ^{14}C-amino acid mix (0.2 μCi/ml) and [*methyl*-^{3}H]thymidine (1 μCi/ml, 2.5×10^{-6} M). For labeling with [^{35}S]methionine, the infected cells were resuspended in low methionine medium (6 μg/ml), and [^{35}S]methionine was added (2.5 μCi/ml). Isotopes were obtained from New England Nuclear. Except where otherwise noted, cells infected at a high multiplicity of infection were usually harvested at 48 h after infection by low-speed centrifugation.

Extraction and purification of virus. The purification scheme employed is outlined in Fig. 1. All operations were carried out as rapidly as possible, at 5°C or on ice, unless otherwise stated. Infected cell pellets were washed in TNE buffer (0.15 M NaCl-50 mM Tris-hydrochloride-0.5 mM EDTA, pH 7.5). The cells were then resuspended at 0.5×10^7 to 2.5×10^7/ml in TE buffer (50 mM Tris-hydrochloride-0.5 mM EDTA, pH 8.7). A 0.01 volume of 0.1 M phenylmethylsulfonylfluoride (PMSF) in dry isopropanol was added with thorough stirring. The suspension was immediately homogenized at medium speed in a Polytron homogenizer for a total of 1 min in short bursts. The resulting homogenate (fraction 1) was cleared of nuclei by low-speed centrifugation and then of cell organelles and debris by centrifuging at 15,000 rpm for 30 min in a Sorvall SS34 rotor. The supernatant (fraction 2) was made 25 mM $CaCl_2$, and the virus was allowed to precipitate for 30 min. The precipitate was collected by centrifugation at 10,000 rpm for 10 min in the same rotor and suspended by gentle sonic treatment in a small volume of 50 mM Tris-hydrochloride-20 mM EDTA, pH 8.7. After another centrifugation (10,000 rpm for 10 min) to remove insoluble material, the supernatant (fraction 3) was placed, in 10-ml amounts, in 17-ml cellulose nitrate centrifuge tubes and underlayered successively with 2 ml of 1.0 M sucrose in TE buffer and 5 ml of CsCl (density, 1.40 g/cm^3) in the same buffer. Virus was sedimented to equilibrium in a Beckman SW27.1 rotor at 25,000 rpm for 20 h. Two bands of virus particles were observed in the CsCl layer: a sharp band of empty particles at a density of 1.32 g/cm^3 (fraction 4e) and a heterogeneous band of DNA-containing full particles between 1.41 and 1.46 g/cm^3 (fraction 4f). Occasionally this fraction was resolved as a doublet with individual bands at these two density extremes. Fractions 4e and 4f were dialyzed separately against TE buffer. $CaCl_2$ was added to 5 mM, micrococcal nuclease (Worthington) was added to 20 μg/ml, and the fractions were incubated at 37°C for 15 min. The virus suspensions were then adjusted to 5 mM EDTA, layered on 10-ml 5 to 20% sucrose gradients (in TE buffer), and centrifuged at 41,000 rpm for 2 h in a Beckman SW41 rotor. Each new fraction (5e and 5f) was then dialyzed against TE buffer and stored at 5°C.

Virus was also extracted from infected cell nuclei prepared by a modification of the method of Schubert (35). Cells (5×10^7) were washed in phosphate-buffered saline and suspended in 5 ml of 0.25 M sucrose-75 mM NaCl-10 mM $MgCl_2$-25 mM sodium phosphate buffer, pH 6.7. PMSF was added as above, and the cells were lysed by the addition of a 0.1 volume of 10% Nonidet P-40 (Shell Chemical Co.). After 2 min, nuclei were pelleted by low-speed centrifugation, suspended in a further 5 ml of 1% Nonidet P-40 in the same buffer, layered on 25 ml of 1 M sucrose-75 mM NaCl-10 mM $MgCl_2$-25 mM sodium phosphate buffer, pH 6.7, and centrifuged at $500 \times g$ for 10 min at 5°C in a Sorvall HB4 rotor. Virus was extracted from the nuclear pellet by resuspension in 10 mM Tris-hydrochloride-0.5 mM EDTA, pH 8.5. After standing for 2 min the nuclei were pelleted at $500 \times g$ for 2 min, and the extraction was repeated. The combined supernatants were then centrifuged at 15,000 rpm for 30 min in a Sorvall SS34 rotor. Virus was purified from this supernatant by sedimentation to equilibrium on sucrose-CsCl step gradients as described above.

Polyacrylamide gel electrophoresis. The SDS-Tris-glycine system of Laemmli (23) as modified for slab gels by Anderson et al. (2) was used. The SDS-phosphate system of Shapiro et al. (36) was modified

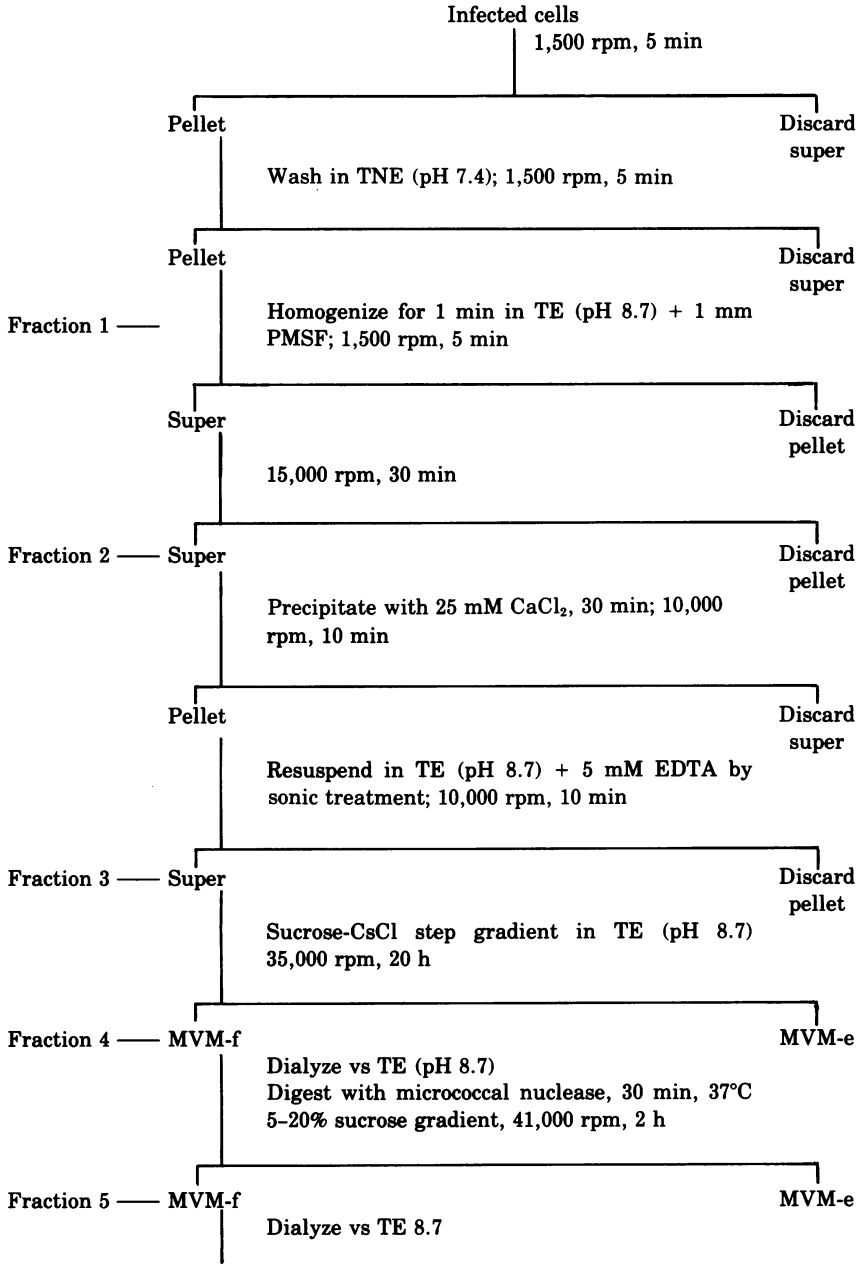

FIG. 1. *Purification of MVM particles.*

for slab gel operation. For molecular weight estimations, the stacking gel in the SDS-Tris-glycine system was omitted and sample wells were cast directly in the resolving gel. Samples were prepared in several ways for electrophoresis. In some cases virus was precipitated directly from CsCl by the addition of 10 volumes of 90% methanol. After standing for 30 min on ice the virus was centrifuged at 10,000 rpm for 20 min in a Sorvall HB4 rotor. The pellet was washed with cold methanol and resuspended in sample buffer (2% SDS–10% glycerol–0.012% bromophenol blue–0.1 M dithiothreitol–0.08 M Tris-hydrochloride, pH 6.8). Alternatively, samples were dialyzed against 10 mM triethylammonium bicarbonate buffer, pH 8.3, lyophilized, and taken up in sample buffer. In some cases concentrated samples in dilute buffer were added to an equal volume of 2× sample buffer. All samples were disrupted by heating at 95°C for 5 min immediately before application to the gel. For molecular weight measurements,

virus and marker proteins were placed in alternate sample wells.

SDS-Tris-glycine exponential gradient gels were prepared as follows. One gel volume of 15% acrylamide solution (15% acrylamide-0.173% bisacrylamide-15% glycerol, in resolving gel buffer with catalyst added) was pumped into the bottom of the gel apparatus from a closed chamber fitted with a stirring bar. The volume in this chamber was kept constant by drawing into it, from a separate reservoir, a solution of 7.5% acrylamide (7.5% acrylamide, 0.193% bisacrylamide, resolving gel buffer, and catalyst). A stacking gel containing sample wells was cast on the top of this gel as before.

Transverse gradient gels were poured at 90° to the direction of electrophoresis using a linear gradient former with 15% acrylamide-0.39% bisacrylamide-15% sucrose in a mixing chamber and 5% acrylamide-0.13% bisacrylamide-5% sucrose in the reservoir. Thus the acrylamide-bisacrylamide ratio was constant across the gradient. Each gel solution was in SDS-Tris resolving gel buffer as before and was supplemented with catalyst immediately before pouring. After polymerization the slab was rotated 90°, and a stacking gel, with a single sample well, was cast on the top edge of the resolving gel.

Slabs with stacking gels were initially subjected to electrophoresis at 60 V (<25 mA) until the sample entered the resolving gel (approximately 90 min) and then at 150 V (constant voltage) until the dye front was within 5 mm of the bottom of the slab (3.5 to 4 h for a 125-mm SDS-Tris-glycine gel). After electrophoresis gels were stained for 90 min in 0.1% Coomassie blue-25% isopropanol-10% acetic acid (13), destained overnight in 15% methanol-7.5% acetic acid, and photographed with a Polaroid Land camera by transmitted light. For autoradiography, gels were dried under vacuum and exposed to Kodak RP/R54 double-sided X-ray film. Gels were prepared for fluorography by the method of Bonner and Laskey (4) and exposed to presensitized film (25) at −70°C. Images produced by either method were quantitated using a Joyce-Lobel scanning densitometer. SDS was obtained from Serva, and acrylamide and bisacrylamide were from Eastman Kodak.

RESULTS

Virion purification and characterization. During our studies on the structural components of MVM, we have developed several procedures for large-scale growth and purification of the various forms of the virion. In previously described isolation procedures (9, 38) crude cell lysates were incubated under conditions where extensive proteolysis could occur. To eliminate such possible artefacts, we have devised a rapid isolation procedure, outlined in Fig. 1 and above, which employs low temperature and includes the serine protease inhibitor PMSF (12). The polypeptides present at each stage of the purification procedure are shown in Fig. 2. This

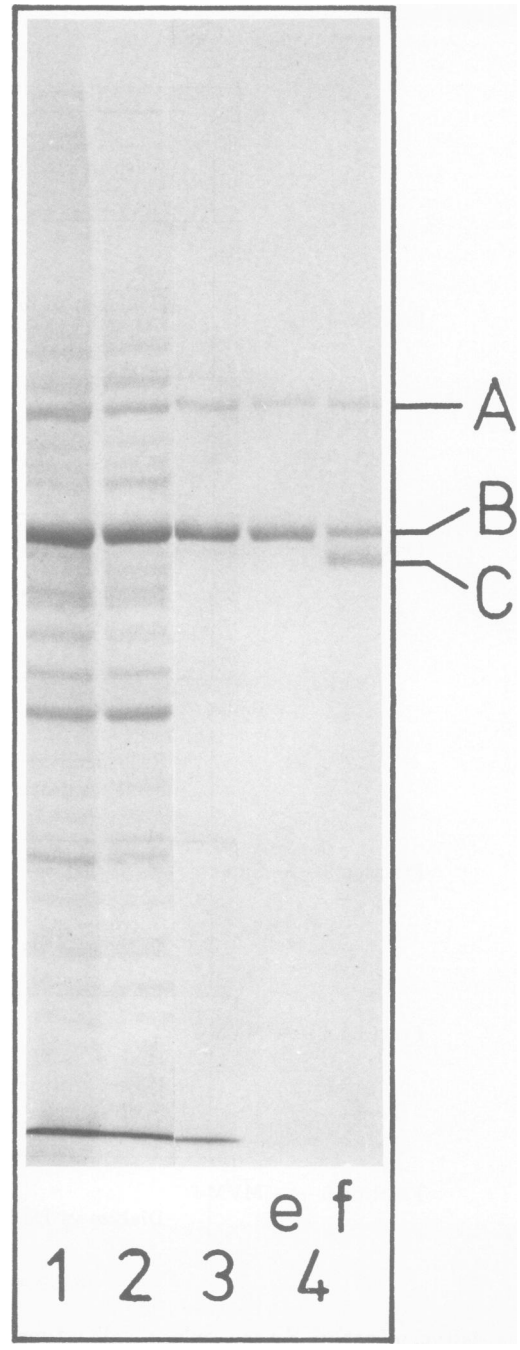

FIG. 2. *Purification of MVM virions from* ^{14}C-*amino acid-labeled, [*^{3}H*]thymidine-labeled, infected cells. An autoradiogram of an SDS-polyacrylamide gel containing samples of fractions 1 to 4 in the purification scheme. The sample of fraction 1, the whole-cell homogenate, was prepared by the addition of sodium deoxycholate to 0.5% followed by brief sonic treatment to reduce viscosity. The sample was then added to an equal volume of 2× sample buffer, as were samples from fractions 2 and 3. Samples from fractions 4f and 4e were precipitated from CsCl with methanol as described. The resolving gel contained 7.5% acrylamide and 0.193% bisacrylamide.*

method relies on the findings that MVM dissociates from cellular material at high pH and low ionic strength in the presence of EDTA, similar to the observations with H-1 virus (17) and that, in crude lysates at least, the virus precipitates in the presence of Ca^{2+}. As previously reported the virus separates into full and empty particles in CsCl gradients (9, 38). Figure 2 shows that, although the empty particle possesses only two polypeptide species, denoted A and B, the full particle contains a third polypeptide, C. Both particles were found to be essentially homogeneous when analyzed by velocity sedimentation and equilibrium density gradient centrifugation. Figure 3 shows the coincidence of protein label, DNA label, hemagglutinin, and infectivity for fraction 4f, analyzed by equilibrium sedimentation in CsCl (Fig. 3A) or velocity sedimentation in Sucrose (Fig. 3B). These results strongly suggest that the three polypeptides of the full particle are components of the infectious virion. This particle has a density of 1.41 g/cm^3 and sediments at 110S (10, 27; P. Tattersall, unpublished data). A similar analysis of empty virions (fraction 4e) shows that both protein label and hemagglutinin activity are coincident in a particle with a density of 1.32 g/cm^3 (Fig. 4A) and which sediments at approximately 70S (Fig. 4B). This fraction also contains micrococcal nuclease-resistant DNA label in particles with a heterogeneous density (1.32 to 1.38 g/cm^3) and which sediment predominantly on the leading edge of the main band. However, considering the scale changes between Fig. 3 and Fig. 4, it can be seen that the so-called "empty" particles contain, on the average, less than 1% of the DNA and less than 0.1% of the specific infectivity (PFU/unit of DNA) of full particles. The origin of the DNA in these particles is unclear, but consideration of the infectivity and sedimentation data in Fig. 4B indicates that it is not due

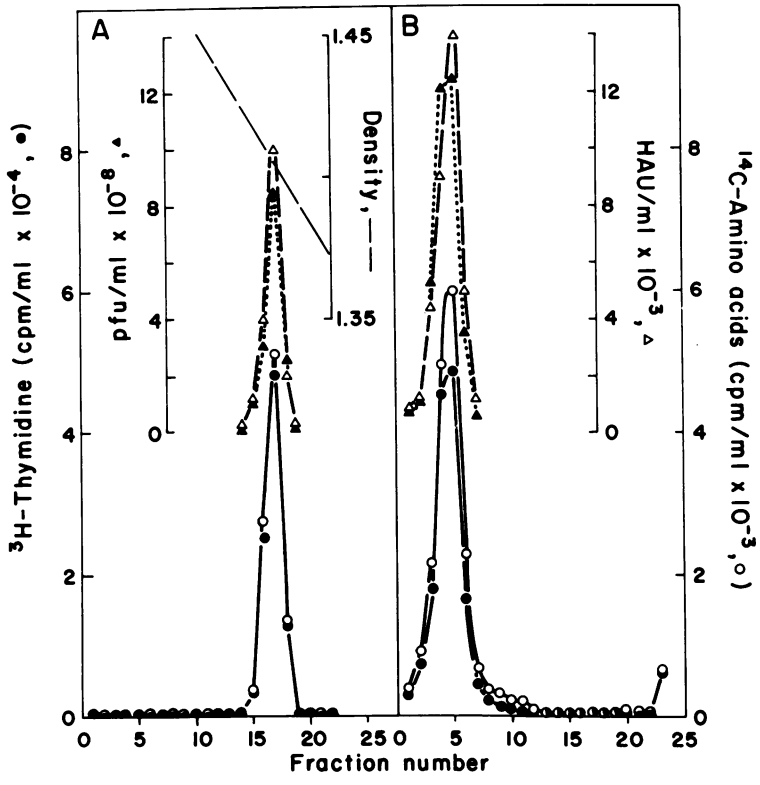

FIG. 3. *Analysis of full MVM particles (fraction 4f) by buoyant density in CsCl (A) and by sedimentation velocity in sucrose (B). Fraction 4f (see Fig. 2) was dialyzed and treated with micrococcal nuclease as described. (A) A sample was adjusted to a volume of 5 ml with 50 mM Tris-0.5 mM EDTA, pH 8.7, containing sufficient CsCl to give a final density of 1.43 g/cm^3. The sample was then centrifuged at 40,000 rpm for 20 h in an SW50.1 rotor at 5°C. Fractions were collected by bottom puncture, and samples were prepared for double-label counting as described previously (38). Density was determined by refractive index measurement. Samples were assayed for infectivity and hemagglutinin as described in the text. (B) A sample was layered on top of a 5-ml 5 to 20% sucrose gradient in 50 mM Tris-0.5 mM EDTA, pH 8.7, and centrifuged at 48,000 rpm for 1.5 h in an SW50.1 rotor at 5°C. Fractions were collected and assayed as above.*

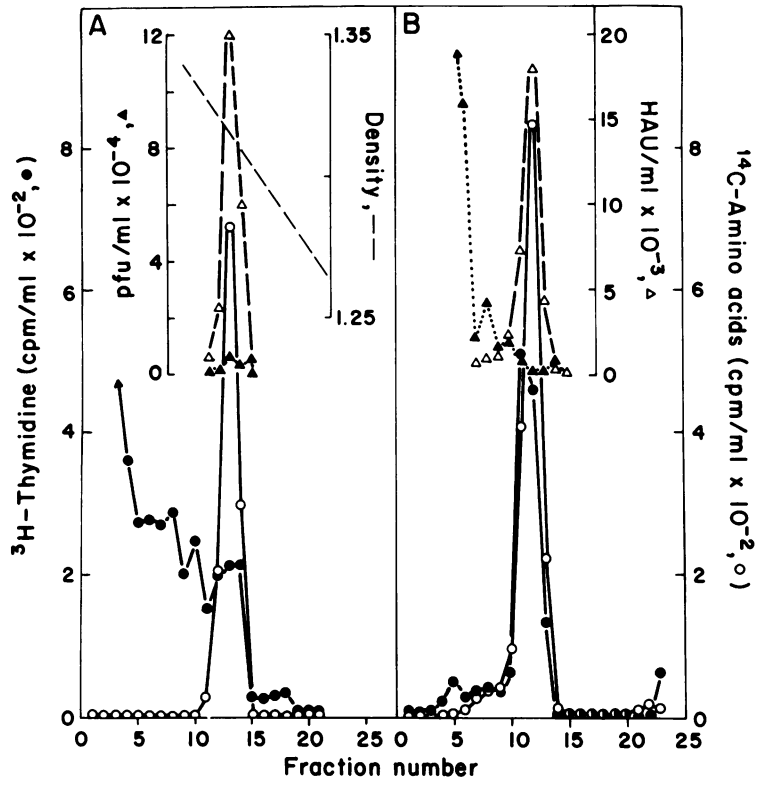

FIG. 4. *Analysis of empty MVM particles (fraction 4e) by buoyant density in CsCl (A) and by sedimentation velocity in sucrose (B). Fraction 4e was dialyzed and treated with micrococcal nuclease as described and analyzed on a CsCl equilibrium gradient (A) and by sucrose gradient velocity sedimentation (B) as described for Fig. 3 except that the CsCl solution in (A) had an initial density of 1.32 g/cm^3.*

to contamination by or aggregation with full particles.

In several preparations, notably those derived from multiple-cycle, low-multiplicity infections, we have obtained an additional full virus band at the density of 1.46 g/cm^3. Consistent with the results of Clinton and Hayashi (8) we have been unable to detect any difference in the DNA content, DNA molecular weight, or particle molecular weight between these two virus bands. Most of the results reported here have been obtained with the 1.41-g/cm^3 full particle, although in some cases these two bands were not resolved in the CsCl-sucrose step gradient. In these cases, the full virus fractions contained both of these bands.

Figure 5 shows the UV spectra of full and empty particles purified to fraction 5. Full particles have a predominantly nucleic acid spectrum with a peak at 260 nm and an E_{260}/E_{280} ratio of 1.38. Empty particles, on the other hand, have a characteristic protein spectrum with a peak at 280 nm, an E_{260}/E_{280} ratio of 0.67, and a distinct shoulder at 290 nm. That this shoulder is due to a high percentage of tryptophan in viral protein is supported by the amino acid analyses presented in Table 1. Amino acid analysis using norleucine as an internal standard yield $E^{1\%}_{280\ \text{protein}}$ of 71.2 for full particles and 17.8 for empty particles. The DNA comprises 26.3% of the mass of the full virion as determined by amino acid and deoxyribose analyses (7) carried out on the same preparation of virus. Using this value and 1.48×10^6 for the molecular weight of the DNA (5) gives a particle molecular weight of 5.63×10^6 for the full virion, of which 4.15×10^6 is protein.

Protein molecular weight determinations. Polypeptide molecular weights were determined by electrophoresis on 10%, 8%, and 6% acrylamide gels in the presence of SDS. Figure 6 shows a typical 8% acrylamide slab gel of disrupted full and empty particles analyzed side by side with seven standard proteins as markers. No other polypeptides are apparent in the gels of viral proteins. The relative migration of each polypeptide was averaged for each gel concentration and plotted against log molec-

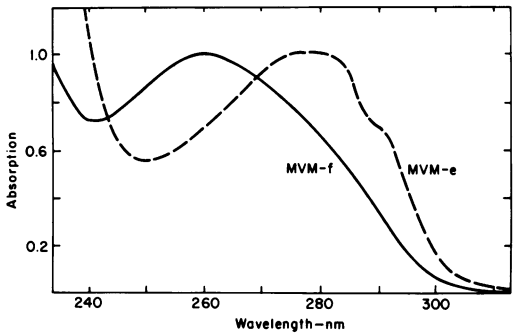

FIG. 5. *UV spectra of full and empty MVM particles. Suspensions of full and empty MVM virions purified to fraction 5 were dialyzed exhaustively against 10 mM triethylammonium bicarbonate, pH 8.3, and their UV spectra were determined in a Beckman 25 split-beam spectrophotometer against the same buffer as blank. The two spectra have been normalized to 1.0 optical density at λ_{max} for comparison.*

ular weight as described by Shapiro et al. (36). Figure 7 shows plots derived from four gels differing in buffer system or gel concentration, but of constant acrylamide-bisacrylamide ratio. The SDS-phosphate system of Shapiro et al. (36) gives a straight-line relationship between relative migration and log molecular weight but somewhat poorer resolution than an equivalent percentage gel in the Laemmli (23) buffer system. The latter gels, however, consistently gave a concave relationship, with the extent of nonlinearity increasing with molecular weight. Figure 6 and Table 2 show that the A polypeptides from full and empty particles comigrate with a mean apparent molecular weight (MW_{app}) of 83,300, as do the B polypeptides with a mean MW_{app} of 63,400. The C polypeptide was found to have a mean MW_{app} of 61,400. The mean molecular weight difference between polypeptides B and C, when measurements made in the same track were compared, was 2,900 (n = 11; range = 2,600 to 3,200).

To quantitate the number of copies of each polypeptide present per virion, it is necessary to show that the MW_{app}, as measured in SDS gels, is a reasonable estimate of the true polypeptide molecular weight. Many glycoproteins, for instance, show aberrant SDS binding (29) and consequently do not give correct molecular weights in terms of polypeptide chain length in SDS-acrylamide gels (6, 35). We have not been able to detect carbohydrate associated with any MVM polypeptide by direct periodic acid-Schiff staining. Furthermore, no [^{3}H]glucosamine was found in virions purified from infected A-9 cells (10^8) labeled with 0.2 mCi of [^{3}H]glucosamine (1 μCi/ml, 9 Ci/mmol) between 9 and 48 h postinfection. One characteristic of SDS-glycoprotein complexes is that their MW_{app} values are dependent upon gel concentration when measured against standard protein markers (6, 35, 42). Electrophoretic analyses using different gel concentrations do not show any significant differences in MW_{app} (Fig. 7). The mean MW_{app} values of polypeptide A were 84,400, 82,700, and 82,100 as measured in 10%, 8%, and 6%

TABLE 1. *Amino acid composition of full and empty MVM particles*

Amino acid	nmol/OD_{280}[a]		mol%	
	Full virions	Empty virions	Full virions[b]	Empty virions
AsX	130	535	11.84	12.33
Thr	87	358	7.92	8.25
Ser	56	221	5.10	5.09
GlX	93	373	8.47	8.60
Cys[c]		53	1.22	1.22
Pro	71	295	6.47	6.80
Gly	134	390	12.20[d]	9.00
Ala	81	322	7.38	7.42
Val	67	305	6.10	7.03
Met	18	88	1.64	2.03
Ile	36	147	3.28	3.39
Leu	65	271	5.92	6.25
Tyr	41	168	3.73	3.87
Phe	35	146	3.19	3.36
His	29	120	2.64	2.77
Lys	47	177	4.28	4.08
Arg	50	194	4.55	4.47
Trp[e]		176	4.06	4.06

[a] Suspensions of full and empty virions purified to fraction 5 were dialyzed against 10 mM triethylammonium bicarbonate buffer, pH 8.3, and their optical densities at 280 nm (OD_{280}) were determined. Samples were then mixed with a standard norleucine solution, lyophilized, resuspended in 6 N HCl plus 0.4% phenol, and hydrolyzed at 110°C for 24 h under vacuum. The hydrolysate was then analyzed on a Beckman automatic amino acid analyzer, and the yield of norleucine was used to correct for handling losses. Differential amino acid losses during hydrolysis were not determined, and values shown are the average of those obtained from three separate hydrolysates.

[b] The moles percent values for Cys and Trp determined for empty particles were used for the calculations of mole percent values of the other amino acids in full virions to allow comparisons to be made of the two particle types.

[c] Cys was determined as *S*-carboxymethyl Cys on a preparation of SDS-disrupted empty virions carboxymethylated essentially as described by Gurd (18).

[d] The high glycine level in the full virion probably reflects the glycine produced from acid hydrolysis of the viral DNA.

[e] Trp was determined by the fluorometric method of Sasaki et al. (34) using bovine pancreatic RNase A (Trp = 0.00%) and egg white lysozyme (Trp = 4.65%) as protein standards.

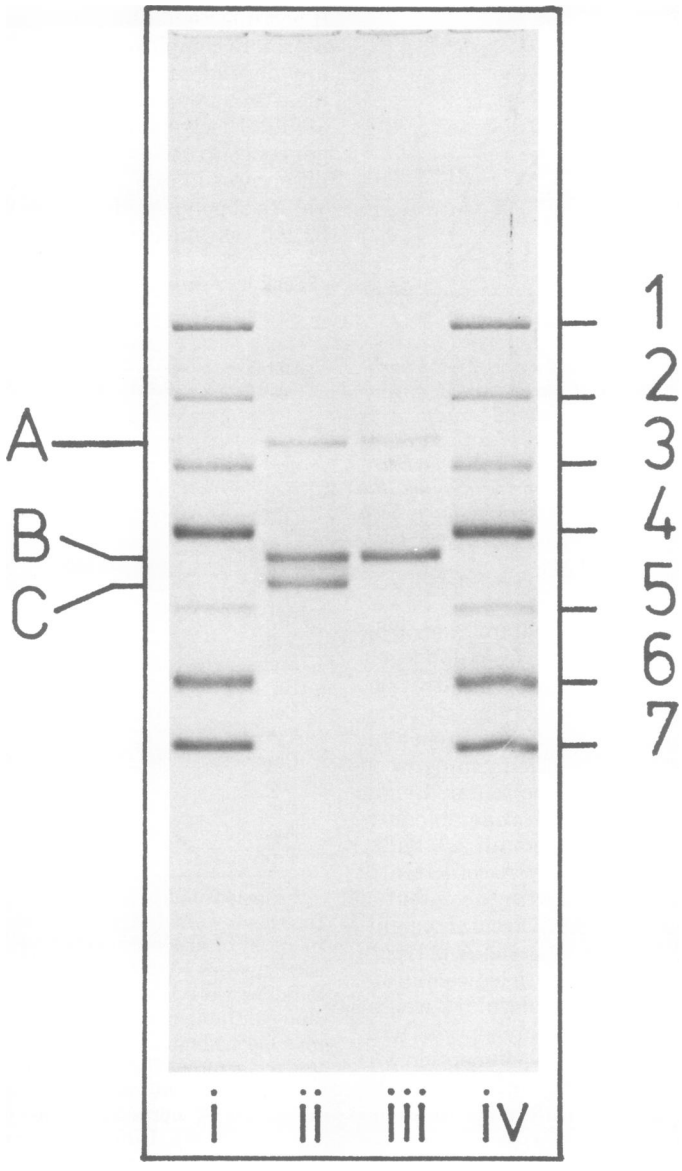

FIG. 6. *Coomassie blue-stained SDS-polyacrylamide gel of MVM full [track(ii)] and empty [track (iii)] particles purified to fraction 4 and run in parallel with standard molecular weight markers [tracks (i) and (iv)]. The gel contained 8% acrylamide and 0.205% bisacrylamide. The molecular weight markers were: (1) β-galactosidase (Escherichia coli), 130,000; (2) phosphorylase a (rabbit), 100,000; (3) transferrin (human), 76,600; (4) serum albumin (bovine), 68,000; (5) pyruvate kinase (rabbit), 57,000; (6) glutamate dehydrogenase (bovine), 53,000; (7) fumarase (porcine), 49,000. Molecular weights for marker proteins were taken from Weber et al. (42) and Fish et al. (14).*

SDS-Tris-glycine gels, respectively. The corresponding mean MW_{app} values for polypeptide B were 63,000, 63,400, and 65,400, and for polypeptide C they were 60,700, 60,800, and 62,400.

We have also employed a novel form of SDS-acrylamide gel electrophoresis to compare the migration of the viral polypeptides A and B, relative to standard proteins, over a continuous range of gel concentration. Figure 8 shows that this technique generates a family of smooth curves for the polypeptides, which intersect only at the position of the dye marker, at which acrylamide concentration the SDS-polypeptide is no longer subject to the sieving ef-

fect of the gel. The shapes of these curves, and the curves in Fig. 7, are most probably a function of the extent of cross-linking in the gel, as discussed by Weber and Osborn (41). The significance of this technique is that any abnormalities in SDS binding or migration due to the presence of a side chain in a polypeptide species would result in a curve of different characteristics, which would be expected to intersect its neighboring curves. None of the polypeptides examined in Fig. 8 shows such behavior. This inability to detect any change in MW_{app} values with varying gel concentration strongly suggests that the MW_{app} values are a direct measure of polypeptide chain length.

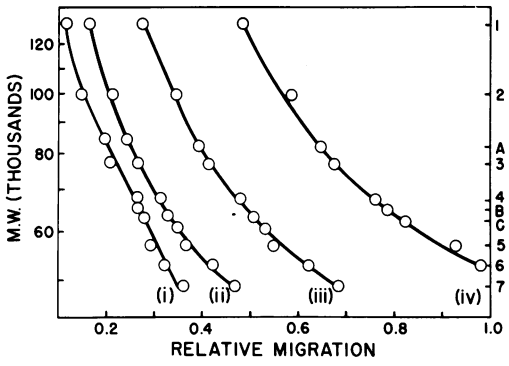

FIG. 7. *Semilog plot of molecular weight versus relative migration (36) for each gel system. (i) 8% acrylamide-SDS-phosphate, (ii) 10% acrylamide-SDS-Tris-glycine, (iii) 8% acrylamide-SDS-Tris-glycine, and (iv) 6% acrylamide-SDS-Tris-glycine. In each system the acrylamide-bisacrylamide ratio was 39:1. The standard curves were constructed with the average of at least three relative migration determinations per point and used to obtain molecular weight estimates for each of the viral polypeptides. The values quoted in the text are the means of 23 determinations each for A and B and 11 determinations for C (measured in the four gel systems described). All relative migrations were determined over a fourfold range of protein concentration to avoid sample overloading artefacts. No significant concentration-dependent differences in relative migration were observed over the range examined (0.05 to 0.2 μg/well). Numbers 1 to 7 refer to the standard proteins described in Fig. 6.*

Number of each polypeptide per virion. The proportion of the two polypeptides in the empty particles is constant from preparation to preparation, with A being 15 to 18% (Table 2). Although A again constitutes 16 to 18% of the protein mass in the full particle, the amounts of B and C vary widely from preparation to preparation. Carboxymethylation (18) of reduced, SDS-disrupted virions prior to electrophoresis does not alter their distribution or migration pattern (data not shown). Using these proportions and the molecular weights determined for the particle and its component proteins, the number of molecules of each protein per virion was calculated. The empty virions are composed of 8 to 9 molecules of A and 53 to 54 molecules of B. The full particles also contain 8 to 9 molecules of A and between 49 of B plus 4 of C and 10 of B plus 47 of C, depending upon the preparation.

Genetic origin of the structural proteins. The polypeptides of MVM grown in Syrian hamster and in rat cells are compared in Fig. 9. The molecular weights and distribution of the three polypeptides A, B, and C are independent of the species of host cell, suggesting that the information required to code for them is either carried by the virus or, if of host origin, has been stringently conserved during evolution. The full particles derived from rat cells contain a fourth polypeptide, D (MW_{app}, ~50,000), in addition to A, B, and C. This polypeptide is seen consistently in full virus derived from this cell line and may represent a specific cellular contaminant or the result of the action on viral polypeptides of a protease

TABLE 2. *Molecular weight and distribution of MVM structural polypeptides*

Polypeptide	Particles	Mol wt (10^{-3})			Weight % of total protein[a]		
		Mean	*n*	Range	Mean	*n*	Range
A	Full	83.1	11	81.1-84.7	16.3	4	15.1-17.1
	Empty	83.5	12	81.7-86.3	17.4	3	16.8-17.8
B	Full	64.4	11	63.1-67.0		4	15.3-76.6
	Empty	64.2	12	63.1-65.9	82.7	3	82.2-83.2
C	Full	61.4	11	60.1-63.1		4	6.5-69.6

[a] Weight percent of total protein was determined in gels of viral polypeptides labeled with radioactive amino acid mix by densitometer tracing of autoradiograms as described in the text or by fractionation of gels by the method described by Rose et al. (32). *n*, The number of determinations from which the mean was derived.

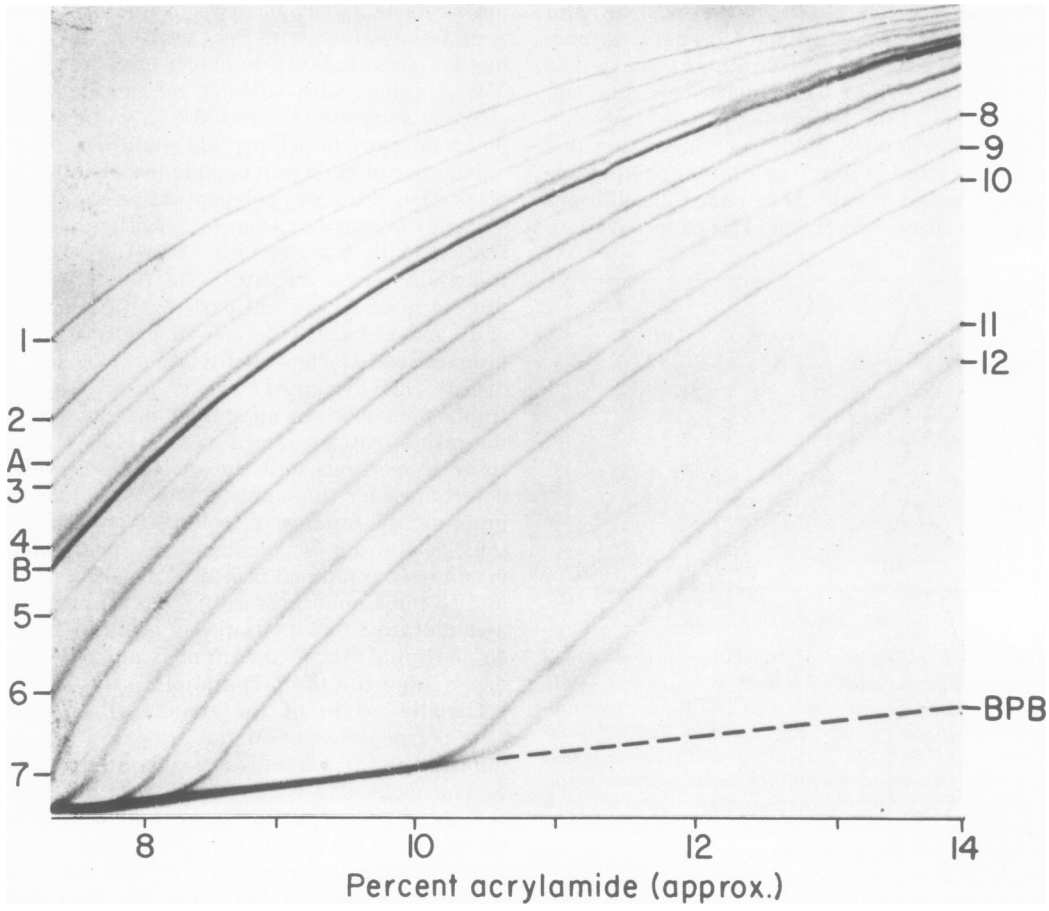

FIG. 8. *Coomassie blue-stained transverse gradient SDS-polyacrylamide gel (see text). Empty MVM virions purified to fraction 4e were subjected to electrophoresis in the presence of 12 marker proteins. Numbers 1 to 7 refer to the standard proteins described in the legend to Fig. 6. The remainder were: (8) aldolase (rabbit), 40,000; (9) lactate dehydrogenase (rabbit), 36,000; (10) carbonic anhydrase (bovine), 29,000; (11) myoglobin (equine), 17,200; (12) cytochrome c (equine), 12,400. The gradient is left to right, and the acrylamide concentration was estimated from the relative migration of markers at 8 and 10% using values obtained in Fig. 7 and assuming a linear gradient. The dotted line marked BPB (bromophenol blue) shows the position of the dye front.*

particularly abundant in these cells. Polypeptide D is rarely seen in virus grown in mouse or Syrian hamster cells.

To define more precisely the genetic origin of these virion-associated proteins, infected and uninfected cells were labeled with [^{35}S]methionine, and their polypeptide profiles were compared. Figure 10 shows that the polypeptides found in the empty particle, i.e., A and B, are readily detectable in infected cells. Although there are faintly labeled polypeptide species in the uninfected cell that correspond in molecular weight to A and B, they are obviously quite different quantitatively. In contrast to polypeptides A and B, polypeptide C is not readily detected in infected cells. This is not unexpected, as the majority of the label appearing in virus proteins in these cells can be isolated as intact virions, and the empty to full particle ratio is often as high as 50:1. Although most of the label in the B polypeptide can be isolated as virion protein, analysis by densitometry shows that the number of molecules of A relative to B is about 2.5 times greater in infected cells than in purified virions.

Additional experiments to test whether the virion-associated polypeptides are synthesized after infection are outlined in Fig. 11 and Table 3. Figure 11A, track (i), shows a fluorogram of the polypeptide profile, displayed on a gradient gel, of infected cells labeled with ^{3}H-amino acid mix. Bands corresponding to the virion proteins

A and B are the most prominently labeled species in the infected cell. This is compared, in Fig. 11A, track (ii), with the pattern obtained when infected, unlabeled cells were mixed with an equal number of uninfected cells that had been labeled for 24 h. The viral bands A and B are not detected, although they are clearly distinguishable as stained bands in the gel (not shown). Figure 11A, track (iii), shows the same population of labeled, uninfected cells as in track (ii), now harvested 45 h after subsequent infection in the absence of isotope. Each sample was adjusted so that approximately the same amount of viral protein (as measured by hemagglutinin and stained protein in the gel) was added in each track. As can be seen in track (iii) there is considerably less label in protein than in track (ii), although the samples represent approximately equivalent numbers of the same preparation of labeled cells. The decrease in protein specific activity implies that during the 45 h of infection considerable turnover of preexisting polypeptides has occurred, possibly leading to isotope reuse after dilution with exogenous cold amino acids. This might explain the light labeling, in this experiment, of viral protein bands in track (iii), which were not labeled in the uninfected cells in track (ii).

Figures 11B and C show the labeled polypeptide profiles of full and empty virions purified from the three batches of cells described above. Virions isolated from cells labeled after infection show (Fig. 11B and C, track i) in the resolving gel only the viral proteins already described, and these are labeled quite distinctly. Although these polypeptides are clearly visible after a 3-day exposure, no other protein species are detected in these tracts, even after the prolonged exposure time shown (32 days). The limit of detection was a species containing 0.1% of the total label, or <1 copy of a 5,000-dalton polypeptide per virion. A small amount of label is seen at the interface of the stacking and resolving gels in Fig. 11B, track (i). The nature of this label is unclear but may be either a small amount of undissociated virus or protein still complexed with the viral DNA. Adenovirus particles are known to contain a protein linked to the 5′ end of the viral DNA (30). Although we cannot rule out the existence of such a complex in MVM, the 5′-terminal nucleotide of MVM DNA can be labeled with $^{32}PO_4$ by polynucleotide kinase after phosphatase treatment (5), unlike the 5′ end of adenovirus DNA (R. Roberts and J. Arrand, personal communications).

Track (ii) of Fig. 11B and C shows that no component of labeled uninfected cells copurifies with either type of virion during our isolation procedure.

Virions purified from prelabeled cells do show incorporated label (Fig. 11B and C, track

TABLE 3. *Recovery of infectivity, hemagglutinin, and radioactivity during purification of MVM from ^{3}H-amino acid-labeled cells*

Expt[a]	Fraction	Infectivity		Hemagglutinin		Radioactivity		Sp act	
		Total PFU ($\times 10^{-8}$)	% Recovered	Total HAU[b] ($\times 10^{-3}$)	% Recovered	Total cpm ($\times 10^{-5}$)	% Recovered	cpm/HAU[b]	% of original
(i)	1[c]	33.0	100	200	100	82.0	100	41.0	100
	3	15.3	46	200	100	17.5	21	8.8	21
	4f	5.04	15	2.1	1.1	0.24	0.29	11.4	28
	4e	0.065	0.2	82	41	6.73	8.2	8.2	20
	5f	3.02	9.2	2.3	1.2	0.39	0.48	12.6	31
	5e	0.001	0.003	66	33	3.85	4.7	5.8	14
(ii)	1[c]	12.0	100	200	100	122.3	100	61.2	100
	3	7.6	63	200	100	27.2	22	13.6	22
	4f	7.4	62	3.2	1.6	0.14	0.11	4.4	7.2
	4e	0.041	0.34	170	85	0.50	0.41	0.3	0.49
	5f	3.93	33	4.0	2.0	<0.02	<0.016	<0.5	<0.82
	5e	0.001	0.008	65	33	<0.01	<0.008	<0.02	<0.03

[a] The experiment numbers refer to those described in the legend to Fig. 11; i.e., in experiment (i) the radioactivity is in infected cell protein, whereas in experiment (ii) the radioactivity is in uninfected cell protein.

[b] HAU, Hemagglutinin units.

[c] The infectivity, hemagglutinin, and radioactivity in whole cell homgenates were determined on samples disrupted with deoxycholate and sonically treated as described in the legend to Fig. 2. Radioactivity was determined by counting small samples as 10% (vol/vol) aqueous suspensions in Aquasol (New England Nuclear).

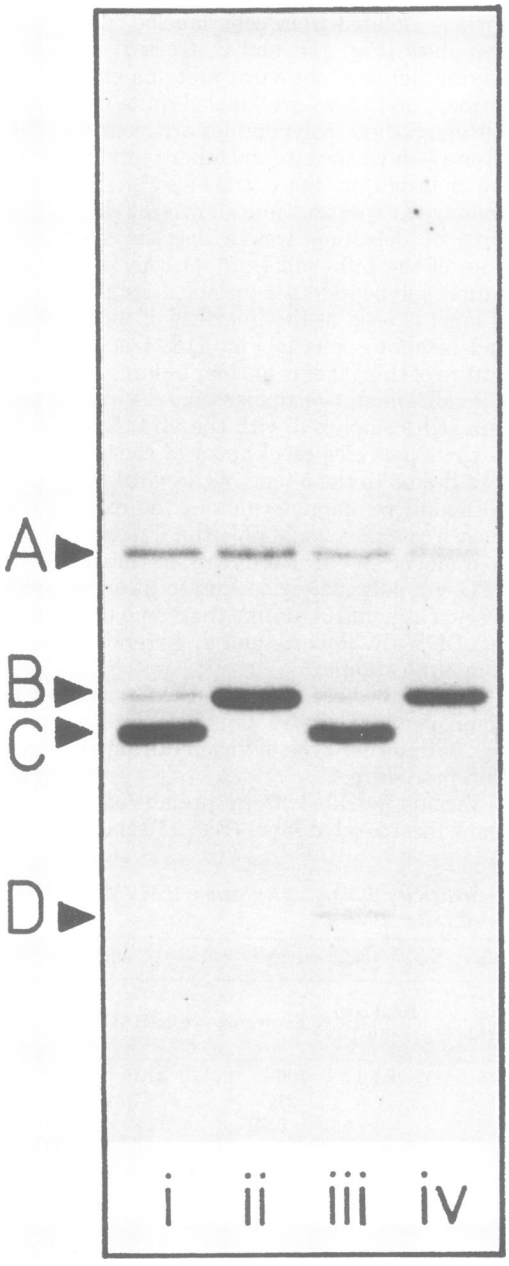

FIG. 9. *Fluorogram of an SDS-polyacrylamide gel of ^{14}C-amino acid-labeled MVM virions grown in two different host cell species and purified to fraction 5. Track (i), full virus; track (ii), empty virus; from BHK/C13, a baby Syrian hamster kidney cell line. Track (iii), full virus; track (iv), empty virus; from RL5E, a Moloney sarcoma virus-transformed rat liver cell line. The resolving gel contained 7.5% acrylamide and 0.193% bisacrylamide.*

iii); however, it is some 39-fold less than is present in virus labeled after infection. This suggests that the observed radioactivity is due to slow dilution of label out of intracellular pools, or the breakdown and reutilization mentioned above, rather than the preexistence of virion-associated polypeptides in the uninfected cell. This argument is supported by the finding that label enters all of the polypeptides in the same ratio whether from cells labeled before or after infection unlike, for instance, the preferential prelabeling of the histones found in polyoma virions (15).

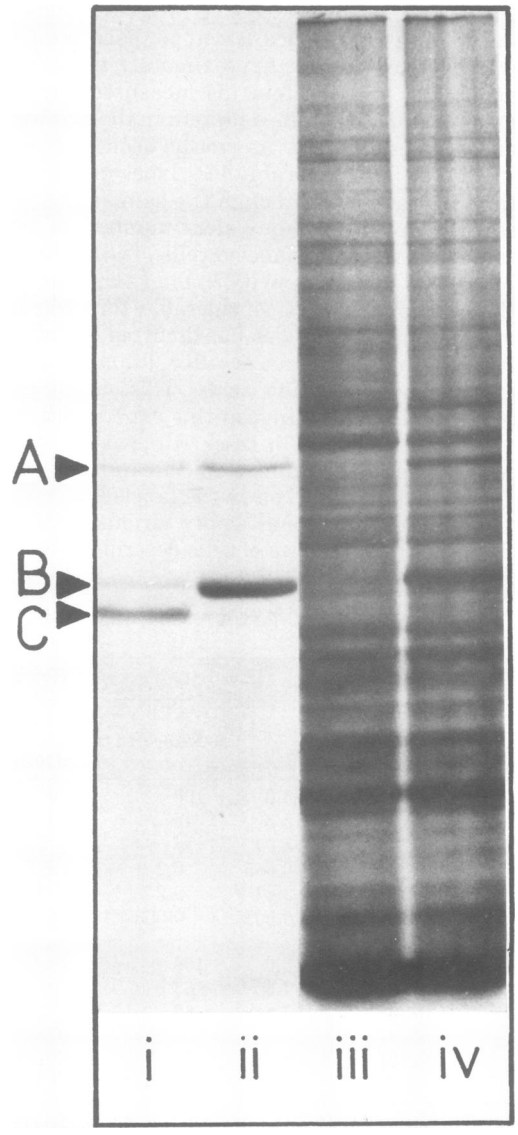

FIG. 10. *Autoradiogram of an SDS-polyacrylamide gel of [^{35}S]methionine-labeled infected (and mock-infected) cells and virions purified from them to fraction 4. Track (i), full virus; (ii), empty virus; (iii), mock-infected cells, fraction 1; and (iv), infected cells, fraction 1. EA cells were infected and labeled as described in the text. The resolving gel contained 7.5% acrylamide and 0.193% bisacrylamide.*

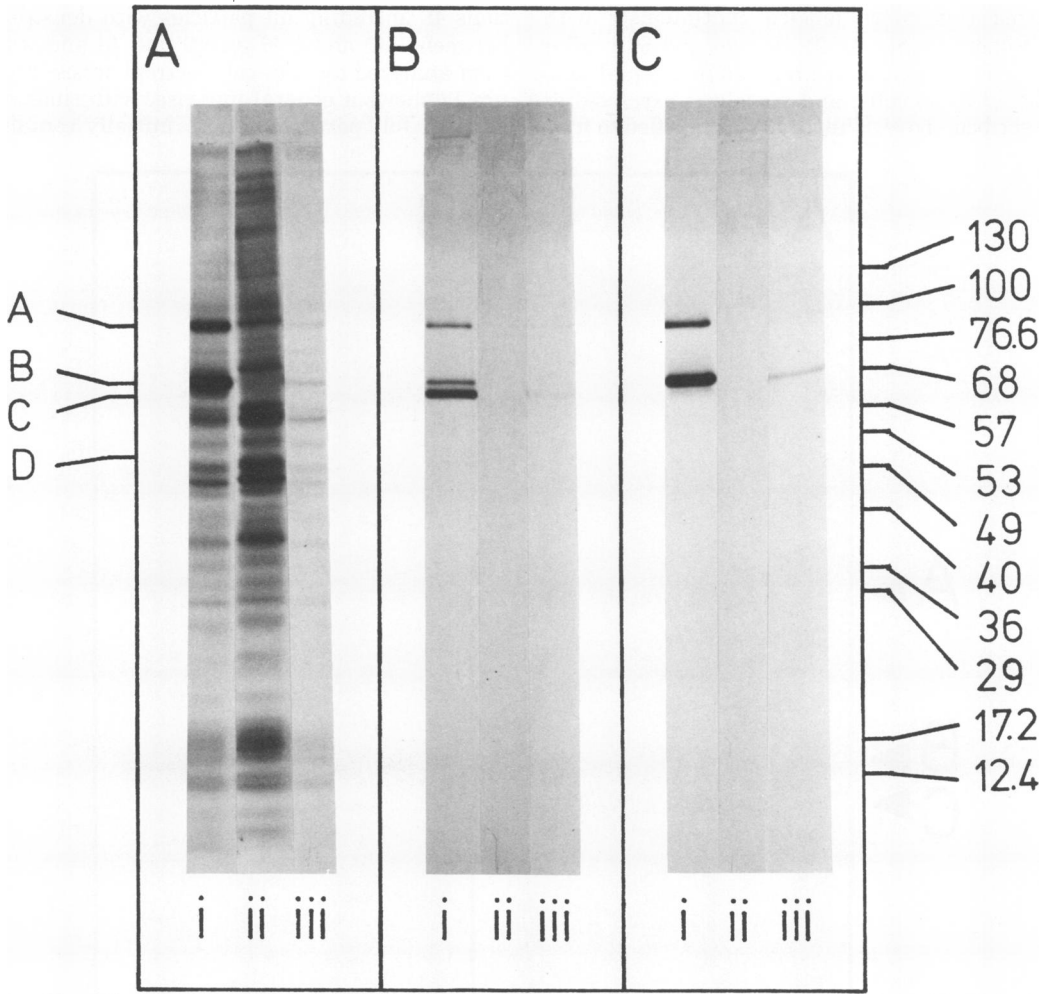

FIG. 11. *Fluorogram (32-day exposure) of an exponential gradient SDS-polyacrylamide gel, as described in the text, of ^{3}H-labeled protein. (A) Total labeled protein obtained from cells prepared in the following three experiments. Infected cells were labeled for 45 h after infection, fraction 1 was prepared, and a sample was run in track (i). Uninfected cells were labeled for 24 h. Half of these cells were mixed with an equal number of unlabeled cells harvested 45 h after infection, a combined fraction 1 was prepared, and a sample was run in track (ii). The other half of the labeled, uninfected cells were washed, infected, and incubated 45 h in the absence of label before harvesting. Again fraction 1 was prepared, and a sample was run in track (iii). (B) Total labeled protein obtained from full virus purified to fraction 5 from the fraction 1 homogenates described in (A). (C) Total labeled protein obtained from empty virus purified in parallel to the full virions in part B. The track numbers in (B) and (C) refer to the same experiments as described in (A). In each experiment the label was ^{3}H-amino acid mix at 5 μCi/ml and normal medium amino acid concentrations. All samples were run in the same gel, and each was adjusted to contain approximately the same amount of protein as estimated by hemagglutinin. This was confirmed by the similar quantities of viral polypeptide detected in each track by Coomassie blue staining (not shown). The positions, in this gel, of the marker proteins described in Fig. 8 are shown on the right-hand side of the figure (as their molecular weights $\times 10^{-3}$).*

The recoveries of infectivity, hemagglutinin, and radioactivity during the purification of virions from cells labeled postinfection (i) and from a mixture of infected and uninfected cells where the radioactivity is in uninfected cells (ii) are given in Table 3. The specific activity of viral protein (counts per minute/hemagglutinin unit) remains constant during purification, after fraction 3, in experiment (i). In experiment (ii), as expected, the specific activity of each type of virion drops over 100-fold during the same purification procedure.

Time course of appearance of polypeptides in the virion. To determine whether there was any difference in the kinetics of appearance of polypeptides B and C in the full virion, in-

fected cells were labeled continuously with [^{35}S]methionine. At 24, 36, 48, and 60 h after infection, nuclei were prepared from equal samples of these cells, and virus was extracted as described above. The virus was purified to fractions 4f (including all particles with densities between 1.41 and 1.46 g/cm^3) and 4e and was then analyzed by SDS-gel electrophoresis. Figure 12 shows a general increase, with time, of label in full particles. These initially contain

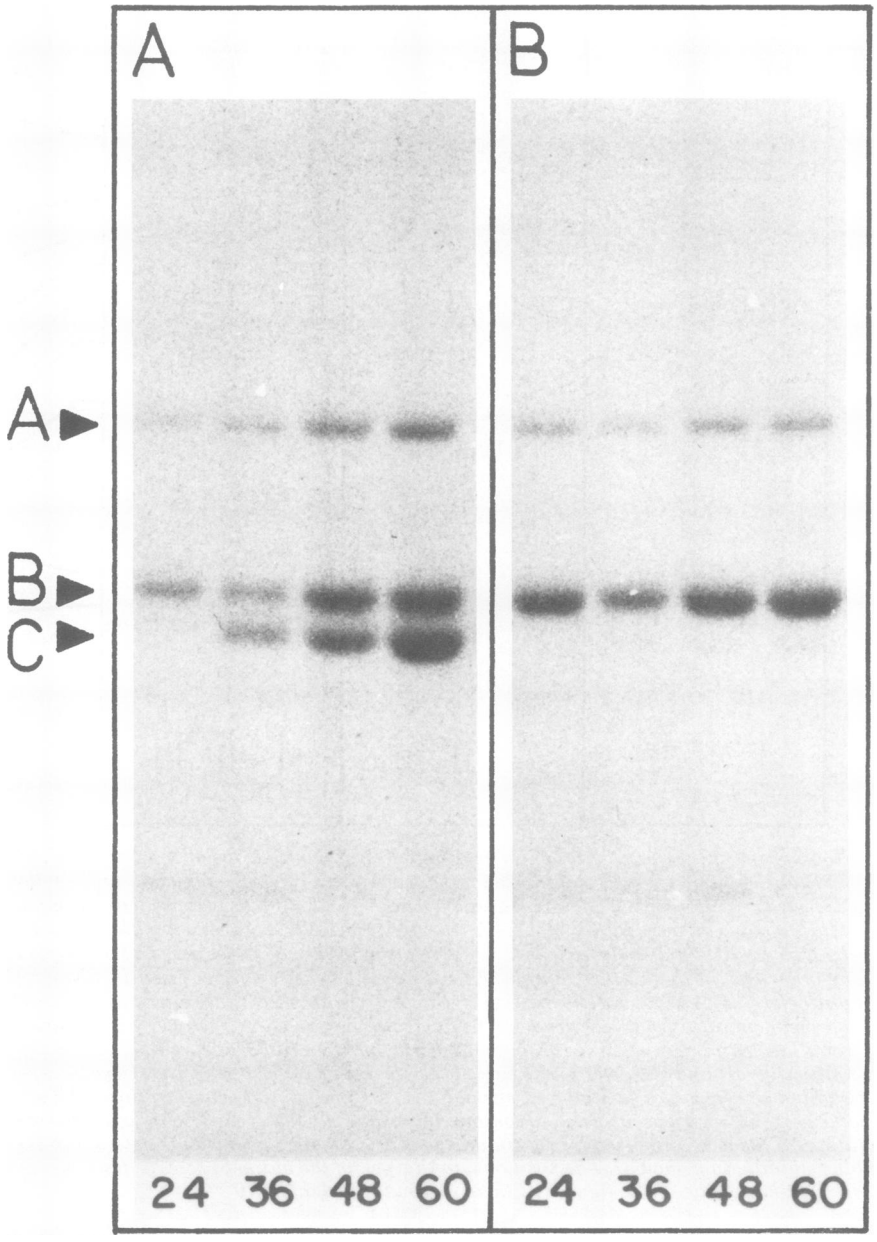

FIG. 12. *Protein composition of full and empty MVM particles as a function of time postinfection. Infected EA cells were labeled continuously with [^{35}S]methionine as described. Virus was extracted from equal cell samples at various times after infection, purified to the fraction 4f and 4e stage, and analyzed by SDS-acrylamide gel electrophoresis. (A) Autoradiogram of full virion polypeptides and (B) autoradiogram of empty virion polypeptides, run in the same gel. The values below each track show the time, in hours postinfection, of extraction of the virus sample. The resolving gel contained 7.5% acrylamide and 0.193% bisacrylamide.*

only the B polypeptide, but as infection progresses polypeptide C appears and, by 48 h, it is the major capsid protein. In contrast, no change was detected in the composition of empty particles during this time period. The cessation of incorporation of methionine into acid-precipitable material in these cells by 30 h after infection suggests that the replacement of B by C as major capsid protein is due to a precursor-product relationship rather than de novo synthesis.

DISCUSSION

Using the slab modification of the Laemmli gel system (2, 23), coupled with autoradiography or fluorography, we have demonstrated a doublet protein band, the B-C pair, in the polypeptide complement of MVM. The lower-molecular-weight protein of this pair is absent from empty virions and from progeny full virus harvested early in infection. Clinton and Hayashi (8) have shown that the separation of full virus into the 1.41- and 1.46-g/cm^3 species reveals a difference in polypeptide composition. The higher-density particle contains predominantly the B protein, and the lower-density particle has C as its major polypeptide. They have also shown that a pulse label initially labels the denser species and that the label chases into the less dense particle as infection proceeds. Our finding that particles containing the C polypeptide accumulate in the nucleus late in infection is in agreement with these results. Since the nucleus is the site of parvovirus assembly as detected by fluorescent anti-capsid antibody staining (31), the late appearance of polypeptide C suggests that it is derived from the B polypeptide by proteolytic cleavage during maturation. We will show in a subsequent communication that the amino acid sequences of B and C are almost identical as determined by peptide mapping. In addition, polypeptide B can be converted in vitro to a polypeptide which comigrates with C, by the action of trypsin on intact full but not empty virions, thus mimicking the in vivo observations. Maturation involving proteolytic cleavage has been reported for a number of viruses, for instance, bacteriophage T4 (23) and adenovirus type 2 (2). If the cleavage of B to C is sufficient for the large shift in density, its effect must be primarily upon the structure of the particle, since it produces a minimal (<3%) change in particle composition.

In some preparations of full virus, especially those grown in the RL5E line of rat cells, we detected a fourth polypeptide, D (molecular weight, ~50,000). This is equivalent in molecular weight and distribution to the polypeptide C reported in the nondefective parvoviruses KRV by Salzman and White (33) and H-1 by Kongsvik and Toolan (22). We have observed no significant difference in the specific infectivity of full virus containing D, such as in Fig. 9, and virus derived from EA cells where D is not detectable, as in Fig. 11B, track (i). Since the difference in content of polypeptide D between these two virus preparations is greater than 25-fold, we conclude that D is not an essential polypeptide. Indeed, Kongsvik et al. (21) have reported that H-1 full particles, purified after one cycle of infection in synchronized NB cells, do not contain this lower-molecular-weight polypeptide and that the major polypeptide can be resolved into doublet band, similar to our finding with MVM proteins. Gautschi and Siegl (16) have demonstrated two polypeptides in the infectious virion of LuIII, again with no species corresponding to D. Their use of the SDS-phosphate system (36) coupled with gel slicing may not have allowed them to resolve the major polypeptide band as a doublet. We have been unable to resolve the B-C pair of MVM proteins using these techniques. However, recent experiments employing the slab gel and detection system described in this article have shown that the molecular weights and molar distributions of the capsid proteins of several nondefective parvoviruses, namely, H-1, H-3, KRV, and LUIII, are very similar to those reported here for MVM (R. Karess, D. Leonard, and D. C. Ward, manuscript in preparation).

In contrast to a number of other small animal viruses, such as polyoma (15) and simian virus 40 (24), no histones could be detected in MVM virus particles purified from cells labeled prior to or after infection [Fig. 11B and C, tracks (ii) and (iii)]. The lack of virion-associated histones is of some interest, since the viral proteins A and B are the major polypeptides in chromatin fractions prepared from cells infected with LuIII (M. Gautschi, G. Siegl, and G. Kronauer, submitted for publication) or MVM (J. Peterson, P. Tattersall, and D. C. Ward, unpublished observations).

Although the ratio of full particles to empty particles varies from preparation to preparation, the empty particles are always present in excess, from 3- to 50-fold. The considerable stability of the full particles suggests that empty particles are unlikely to be derived by degradation of complete virions. In addition, thin sections of cells infected with H-1 show large numbers of empty particles, notably associated with the nucleolus (1). Attempts are currently being made to determine whether empty particles are themselves precursors for full particles or derived during isolation from unstable intermedi-

ates in full virus assembly or merely dead-end pathways of assembly due to overproduction of viral protein.

The relationship between polypeptides B and C suggests that the virus may only code for A and B, i.e., 147,000 daltons of polypeptide. Although this is marginally within the 160,000 daltons that could be coded for, it would appear that a much higher proportion of the genetic information of the virus is expended on coat protein than for most nondefective viruses. Recent results (P. Tattersall, A. J. Shatkin, and D. C. Ward, manuscript in preparation) from peptide mapping indicate, however, a considerable sequence overlap between polypeptides A and B, in addition to the expected overlap of B and C.

The evidence presented here shows that the three polypeptides found in infectious virions of MVM are synthesized after infection. They are only found in infected cells, and their molecular weights and distributions are independent of the species of the host cell. In the absence of coupled in vitro transcription and translation, or a suitable chain termination mutant system to test the hypothesis directly, we feel this is strong evidence that all three polypeptides are coded for by the viral genome.

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