Differences in the Subpopulations of the Structural Proteins of Polyoma Virions and Capsids: Biological Functions of the Multiple VP₁ Species†

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The structural proteins of polyoma virions and capsids were analyzed by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyoma virion VP₁ was found to be composed of six distinct species which had pI's between pH 6.75 and 5.75. Polyoma capsid VP₁ was found to contain four species with pI's between pH 6.60 and 5.75. The different forms of virion and capsid VP₁ appeared to be generated by modifications (phosphorylation and acetylation) of the initial translation product. The most basic of the virion VP₁ species (pI, pH 6.75) was absent in capsids and was found to be exclusively associated with the viral nucleoprotein complex. Three of the virion VP₁ species and three of the capsid VP₁ species were found in capsomere preparations enriched for hexon subunits. Two VP₁ species were specifically immune precipitated from virions with hemagglutination-inhibiting antibodies. These two VP₁ species were common to both virions and capsids. Polyoma virions, but not capsids, possessed a single VP₁ species which was immune precipitated with neutralizing antibodies. Both virion and capsid VP₂ were found to have pI's of approximately pH 5.50. Virion VP₃ had a pI of approximately pH 7.00, whereas capsid VP₃ had a pI of approximately pH 6.50.

The late region of polyoma virus DNA consists of 2,366 base pairs which code for the virus structural proteins VP₁, VP₂, and VP₃ (9). The major capsid protein VP₁ is encoded at the 3' end of the late region and is translated from a 16S mRNA, whereas the minor capsid proteins VP₂ and VP₃ are encoded at the 5' end of the late region and are translated from 19S and 18S mRNA's (10). These three proteins comprise the structural units which are responsible for maintaining the structural integrity of the virus, but they are also essential as receptor sites for infection of host cells and as hemagglutinins for agglutination of erythrocytes and are important in DNA packaging and virus assembly.

In a previous report from this laboratory it was demonstrated that polyoma capsids, which are devoid of DNA and histones, did not compete with polyoma virions for specific binding sites on the surface of mouse kidney cells (MKC) and were unable to inhibit virion infection of MKC. Capsids could, however, block virion adsorption to guinea pig erythrocytes (GPE). Virion adsorption to and infection of MKC were found to be independent of the ability of virions to agglutinate GPE (2). More recently, we have been able to separate neutralizing antibodies

from hemagglutination-inhibiting antibodies in antisera directed against the major structural protein VP₁ (3).

It is clear that the major virus protein VP₁ of papovaviruses does not represent a single polypeptide but rather is composed of a number of polypeptide species which have different pI's (11, 16, 17). However, the functions of the various VP₁ subspecies have remained obscure. Through the use of the antisera described above, along with our understanding of the biological and biochemical differences between polyoma virions and capsids, we have now been able to assign functions to the different species of polyoma VP₁.

MATERIALS AND METHODS

Cell and virus propagation. The preparation of primary cultures of MKC has been described (18). Wild-type large-plaque polyoma virus was used to infect cells at a multiplicity of infection of 10. Infected cultures were maintained in serum-free Dulbecco-modified Eagle medium (13).

Virus purification. Virions were purified from the infected-cell lysate as described previously (13). CsCl gradients used to purify the virus were prepared as described by Brunck and Leick (8) and were described in greater detail previously (6, 7). Polyoma capsids were purified from infected-cell lysates as described previously (3).

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Preparation of radioactively labeled virus. Purified polyoma virions or capsids were labeled in vitro with ^{125}I , using chloramine-T or the Enzymobead method as described previously (3). The preparation of ^3H -amino acid-labeled polyoma virus has been described previously (14), as has the preparation of ^{32}P -labeled virus (4). [^{14}C]acetate-labeled virus was prepared by maintaining the infected cell cultures in serum-free culture media with 5 μCi of [^{14}C]acetate per ml. *Methyl*- ^3H -labeled virus was prepared by maintaining the infected cell cultures in serum-free, methionine-free Eagle medium with 20 μCi of *S*-[*methyl*- ^3H]adenosyl-L-methionine.

Isolation of 48S DNA-protein complex and 18S, 12S, and 5S capsomere subunits. Radioactively labeled virions or capsids were incubated with ethylene glycol-bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) and dithiothreitol (DTT), and the virus core complex and capsomere subunits were recovered from 5 to 20% or 10 to 30% sucrose gradients as described by Brady et al. (7).

GuHCl chromatography. Radioactively labeled polyoma virions were treated with 6 M guanidine-hydrochloride (GuHCl) and 0.01 M mercaptoethanol as described previously (4). The virion proteins were then separated by gel filtration through Sephacryl S-300 at pH 5.0.

Immune precipitation. ^{125}I -labeled, GuHCl-purified VP₁ (100 μl) was incubated with 25 μl of anti-hemagglutinin, antireceptor, or normal rabbit immunoglobulin G, all at 10 $\mu\text{g}/\text{ml}$, for 4 h at room temperature. After incubation, 25 μl of normal rabbit immunoglobulin G (10 mg/ml) was added, followed by 50 μl of goat (immunoglobulin G fraction) anti-rabbit immunoglobulin G (20 mg/ml). The mixtures were then incubated overnight on ice, and the immune precipitates were pelleted by centrifugation and washed three times in 10 mM Tris buffer containing 0.25% Triton X-100 and 0.15 M NaCl (pH 7.8). This protocol was developed for immune precipitation reactions in which both the antigen and antibody are present in low quantities and avoids the use of *Staphylococcus aureus* protein A, which leads to large amounts of nonspecific precipitation of polyoma VP₁ (J.B.B., unpublished data).

Two-dimensional IEF and SDS-PAGE. Electrophoresis was done by the method of O'Farrell (15). Radioactively labeled virions or capsids were disrupted in 2% sodium dodecyl sulfate (SDS) and 5% mercaptoethanol for 2 min at 90°C. After cooling to room temperature, solid ultrapure urea was added to 10 M, Nonidet P-40 was added to 2%, pH 3.5 to 10 ampholines were added to 2%, and mercaptoethanol was added to 5%. The ampholines used for the isoelectric focusing (IEF; combining pH 3.5 to 10 and pH 5 to 8 ampholines) yielded very shallow pH gradients between pH 5 and 8. pI's were determined by slicing the focusing gel immediately after electrophoresis and measuring the pH of the individual gel segments in distilled water. For the SDS-polyacrylamide slab gel electrophoresis (PAGE) (when second-dimensional analysis was required), the equilibrated IEF tube gel was embedded in agarose above the stacking gel. Alternatively, the focusing gels containing ^{125}I -labeled samples were first sliced into 2-mm segments, the

radioactivity of each segment was determined by counting in a gamma counter, and the peak segments were then placed in wells of a conventional SDS-polyacrylamide slab gel as described previously (7).

Quantitative assays. Cesium chloride densities were determined by the refractive index with a refractometer and calculated by using the equation of Vinograd and Hearst (19). Protein concentrations were determined by the Bio-Rad protein assay, using bovine serum albumin as a standard. Radioactivity was quantitated in a toluene-Triton (3:1) scintillation fluid with a Beckman LS-233 liquid scintillation counter. When ^3H and ^{14}C or ^3H and ^{32}P isotopes were counted in the same sample, the results were corrected for the overlap of ^{14}C and ^{32}P into the ^3H channel and for quenching. Samples labeled with β -emitters were prepared for counting by incubating the slices with Protosol (New England Nuclear Corp.) scintillation fluid (1 liter of toluene, 4 g of Omnifluor, 30 ml of Protosol; 5 ml per vial) for 24 h at 37°C. The vials were cooled to room temperature before counting.

RESULTS

IEF of ^{125}I -labeled virions and capsids.

IEF of radioactively labeled polyoma virions and simian virus 40 (SV40) virions has been reported (11, 16, 17). However, in these reports the virus proteins were labeled by allowing infected cells to incubate in the presence of ^{14}C - or ^3H -amino acids, ^{32}P , or [^{35}S]methionine. We were interested in determining the pattern of IEF of in vitro chloramine-T- ^{125}I -labeled polyoma virions and comparing the polypeptide distribution of the virions with similarly labeled polyoma capsids. This type of in vitro labeling, which labels all of the virion and capsid polypeptides, yielded specific activities generally in the range of 5×10^6 to 10×10^6 cpm/ μg of virus protein using liquid scintillation spectroscopy and 1×10^8 to 5×10^8 cpm/ μg of virus protein using a gamma counter to quantitate the radioactivity. Figure 1A shows the IEF distribution of ^{125}I -labeled virions. A total of eight polypeptide species were resolved by slicing the IEF tube gels, counting the segments in a gamma counter, and resolving the portions corresponding to the peaks in an SDS-polyacrylamide slab gel. Virion VP₃ was found to have pI of pH 7.0, and virion VP₂ had a pI of 5.5. Six species with molecular weights in the VP₁ range (42,000 to 45,000 [42K to 45K]) were found and were assigned letter designations A through F. The most abundant virion VP₁ species (C) had a pI of pH 6.3, which is in agreement with the results of Hewick et al. (11). Our study revealed that the pI's of the other VP₁ species were as follows: A, pH 6.75; B, pH 6.5; D, pH 6.1; E, pH 5.9; F, pH 5.75. Figure 1B shows the IEF distribution of ^{125}I -labeled capsids. Only five polypeptides species were resolved. Two-dimensional analysis of these polypeptides revealed that capsid VP₂ had a pI iden-

FIG. 1. IEF of ^{125}I -chloramine-T-labeled virions and capsids. Equal concentrations of iodinated polyoma virions (A) or capsids (B) were subjected to IEF as described in the text. The gels were sliced into 2-mm segments, and the radioactivity in each slice was quantitated in a gamma counter. The molecular weights of each of the peak fractions were estimated by subsequent electrophoresis in an SDS-polyacrylamide gel, using iodinated virion proteins as standards.

tical to that of virion VP₂, whereas capsid VP₃ had a pI of approximately 6.5. Four capsid VP₁ species were found (designated A' through F) with the following pI's: A', pH 6.6; B, pH 6.5; D, pH 6.1; and F, pH 5.75. The letter designations for the capsid VP₁ species were given as those corresponding to species found in virions. The only capsid VP₁ species which did not have a

corresponding virion VP₁ species was found at pH 6.6 and was designated A'. These results show that polyoma capsids lack three of the VP₁ species found in polyoma virions (A, C, and E) and possess a VP₃ with an altered pI. It should also be noted that under the pH ranges used for these experiments, polyoma histones did not enter the IEF gels.

Two-dimensional analysis of ^{125}I -labeled virions and capsids. Conventional O'Farrell (15) two-dimensional electrophoresis of ^{125}I -labeled virion and capsid preparations is shown in Fig. 2. When the autoradiographs were exposed for short periods (Fig. 2A and C), the VP₁ sub-species could be observed with some success. However, it is clear that during equilibration of the IEF gels, diffusion of the focused species occurred which prevented the observation of the distinct patterns observed when the single IEF dimension was analyzed. This problem was undoubtedly complicated by the ^{125}I labeling of the proteins, which tends to fog the film surrounding the actual polypeptide spot. Longer exposure of the autoradiographs clearly showed the migration of the minor proteins VP₂ and VP₃ (Fig. 2B and D). Both virion and capsid VP₂ and VP₃ migrated as doublets in the autoradiographs shown. However, the degree of separation of the VP₂ and VP₃ doublets varied considerably between experiments. Again, the alteration in the migration of capsid VP₃ in IEF dimension can be seen.

Differential labeling of virion and capsid proteins with ^{125}I . Experiments were performed to compare the IEF patterns of virions

and capsids labeled in vitro by the chloramine-T and Enzymobead techniques. In vitro iodination of virions and capsids with chloramine-T labels both internal and external proteins. In vitro iodination of these virus particles with Enzymobeads, however, should only label external proteins with exposed tyrosine residues. Indeed, preliminary experiments showed that iodination of virions with chloramine-T efficiently labeled virion capsomeres and the DNA-protein core, whereas iodination with Enzymobeads labeled only the virion capsomeres (data not shown). We were therefore interested to see what effect these two types of iodinations would have on the IEF distribution of virion and capsid proteins. Comparing Fig. 3A and B, it can be seen that virion VP₁ species A was not labeled with Enzymobeads. Note that in this figure only the distribution of VP₁ species is shown. Furthermore, the ratio of species C to the remaining species was drastically decreased after Enzymobead iodination, whereas the relative levels of species B, D, E, and F were not substantially altered. When the same experiments were conducted with capsids, only minor alterations in the ratios between the VP₁ species were observed (Fig. 3C and D). These results suggested

FIG. 2. IEF and SDS-PAGE of chloramine-T-iodinated virion and capsid proteins. Focusing was in the horizontal dimension, with acidity increasing from left to right. SDS-PAGE was in the vertical dimension. After the first-dimensional focusing, the gels were equilibrated and electrophoresed in the second dimension as described by O'Farrell (15). (A) Autoradiograph of polyoma capsids after a 4-h exposure; (B) autoradiograph of polyoma capsids after a 24-h exposure; (C) autoradiograph of polyoma virions after a 4-h exposure; (D) autoradiograph of polyoma virions after a 24-h exposure.

FIG. 3. IEF of chloramine-T- and Enzymobead-iodinated virions and capsids. Virions and capsids were labeled *in vitro* with either the chloramine-T or the Enzymobead technique. The samples were then analyzed by IEF and SDS-PAGE as described in the text. (A) Chloramine-T-iodinated virions; (B) Enzymobead-iodinated virions; (C) chloramine-T-iodinated capsids; (D) Enzymobead-iodinated capsids. Only the positions of virion and capsid VP₁ species are shown.

that virion VP₁ subspecies A and possibly some of species C were internal in the virion structure. Since such discrepancies were not observed with capsids, it was thought likely that polyoma capsids harbor no internal proteins. Capsid VP₂ and VP₃, likewise, exhibited no changes in their degree of iodination. (The extent of capsid VP₃ labeled with Enzymobeads was calculated from single-dimension SDS-polyacrylamide gels, and all structural protein ratios in virion and capsid experiments were calculated in an analogous fashion [data not shown].) In virions, however, we were able to calculate that approximately

20% of the virion VP₃ and 2 to 5% of the virion VP₂ was internal. These calculations were in agreement with the results of Brady et al. (7), who observed small amounts of VP₂ and VP₃ associated with the polyoma DNA-protein complex after virion dissociation with EGTA and DTT.

Isolation of virion DNA-protein complex; virion and capsid capsomere subunits. Since the prior experiments suggested that virion VP₁ species A and perhaps some of species C were internal, we were interested in determining which of the virion species were associated

with the DNA-protein complex. This complex was obtained by dissociation of ¹²⁵I-labeled (chloramine-T) virions with EGTA and DTT followed by velocity sedimentation of the dissociated material on 5 to 20% sucrose gradients to separate the core complex from the capsomeres (7). The iodinated cores were then analyzed by SDS-PAGE and by IEF. The results of IEF of the core are shown in Fig. 4. The only VP₁ species associated with the DNA-protein complex was species A. Small amounts of VP₂ and VP₃ were found when the SDS-polyacrylamide gels were autoradiographed for prolonged periods, but were not observed in the IEF gel due to their low concentration. These results suggested that a single species of polyoma VP₁ (species A) was associated with the virus DNA. Furthermore, these experiments led to the speculation that perhaps the tertiary structure of

FIG. 4. IEF of polyoma virion 48S DNA-protein complex. Chloramine-T-iodinated virions were dissociated *in vitro* by the EGTA-DTT method of Brady *et al.* (7), and the 48S DNA-protein complex was isolated after velocity sedimentation in 5 to 20% sucrose gradients. The complex was then analyzed by IEF and SDS-PAGE as described in the text.

virion VP₁ species C prevented the exposure of some of tyrosine residues of this polypeptide, thereby resulting in a lower degree of Enzymo-bead iodination since this species was clearly not associated with the core complex.

Previous results from this laboratory have shown that, after EGTA-DTT dissociation of virions, three groups of capsomere subunits could be resolved by velocity sedimentation of the dissociated virus (7). These three groups had sedimentation values of 18S, 12S, and 5S. Similar treatment of capsids also yields similar capsomere sedimentation patterns (J.B.B., unpublished data). The interest in these virion and capsid capsomere subunits stems from the fact that the 18S and 12S capsomeres contain primarily VP₁. Electron microscopic analysis revealed distinct hexon arrangements (12S) and hexon aggregates (18S). No distinct structures could be observed in the virion or capsid 5S material, which contains primarily structural proteins VP₂ and VP₃ (7). The distribution of the various virion and capsid VP₁ species in the 18S, 12S, and 5S capsomeres is shown in Table 1. Note that in these experiments only the virion and capsid preparations labeled with ¹²⁵I *in vitro* that yielded protein distributions closely resembling *in vivo* ³H-amino acid-labeled virions and capsids were used (see below). Virion 12S capsomeres contained principally VP₁ species B, C, and D, and capsid 12S material was composed mostly of species A', B, and D. The same VP₁ species also made up the majority of virion and capsid 18S capsomeres, but were enriched in species B. Virion 5S capsomeres were composed principally of VP₁ species E and F, whereas capsid 5S capsomeres contained mostly species D and F. These results suggest that the hexon capsomeres of polyoma virions are made up of VP₁ and species B, C, and D and those of capsids are made up of VP₁ species A', B, and D. These experiments lead us to speculate that polyoma capsid species A' (pI, pH 6.6) plays a role equiv-

TABLE 1. Distribution of VP₁ species in capsomere subunits^a

Sample	Virions (%) ^b							Capsids (%) ^b					
	Total ^c	A	B	C	D	E	F	Total	A'	B	D	E	F
18S	21.3		48.2	30.1	20.0	0.8	0.9	20.5	35.2	56.3	18.5		
12S	66.3		30.4	40.0	27.6	0.5	1.5	65.3	48.3	26.1	22.3		3.3
5S	12.4	4.5	1.2	0.6		42.3	51.4	14.2	9.3		41.3		49.4

^a Capsomere subunits were isolated from sucrose gradients after EGTA-DTT dissociation of chloramine-T-iodinated virions and capsids.

^b The area of the peak for each VP₁ species is expressed as the percentage of the total area occupied by the species present in the sample. Each value is the average of a number of separate determinations of pooled samples.

^c The amount of radioactivity in each capsomere species is expressed as the percentage of the total VP₁ radioactivity recovered from the gradients.

alent to that of virion species C (pI, pH 6.3) since their relative distributions in the capsomere subunits were similar. The results may also shed some light on the nature of the polyoma penton. Since distinct penton capsomeres could not be observed in the 18S and 12S subunits, it has been suggested that the building blocks of the pentons reside in the 5S region (7). Thus, it is possible that virion VP₁ species E and F and capsid VP₁ species D and F are involved in the penton structure.

Post-translational modifications of the virion and capsid VP₁ species. We next sought to determine the nature of some of the modifications of the virion and capsid VP₁ species by labeling polyoma-infected cells with different radioactive precursors. The infected cells were labeled with ³²PO₄, [¹⁴C]acetate, or ³H-amino acids, and the virions and capsids were purified thereafter. Equal amounts of ³²P- or ¹⁴C-labeled virions and capsids and ³H-labeled virions and capsids were mixed, and the mixtures were examined by IEF, SDS-PAGE, and IEF-SDS-PAGE. The results of IEF of ³H-amino acid-labeled virions and capsids are shown in Fig. 5. It is important to note that the number of polypeptide species observed and their pI's were the same as the results obtained when chloramine-T-¹²⁵I-labeled virions and capsids were examined in this manner (see Fig. 1). These results suggested that in vitro iodination of polyoma virions and capsids did not appreciably alter the pI's of the virus proteins. However, these results also indicated that the relative amounts of the individual species could be altered to some extent during the in vitro labeling procedure. Thus, to quantitate the protein distribution, only in vivo ³H-amino acid-labeled virion and capsid proteins were used.

The results of the mixing experiments are shown in Table 2 and again point out the dramatic differences between polyoma virion and capsid proteins. Four of the polyoma virion VP₁ species (A, D, E, and F) and two of the capsid VP₁ species (D and F) were found to be phosphorylated. Of the VP₁ species common to both virions and capsids which were also phosphorylated, virion species D and F exhibited twice the level of ³²P incorporation as did capsid species D and F. Two of the virion VP₁ species (C and D) and three of the capsid VP₁ species (A', D, and F) were found to be acetylated. The minor structural proteins of both virions and capsids (VP₂ and VP₃) were found to be phosphorylated but not acetylated. Our attempts to label virions and capsids with *S*-[methyl-³H]adenosyl-L-methionine to determine whether any of the virus proteins were methylated have been unsuccessful.

Although a small amount of label has been found associated with purified virions and slightly more has been found associated with capsids, the degree of incorporation was sufficiently low that analysis by SDS-PAGE or IEF yielded inconclusive results.

Immune precipitation and IEF of GuHCl column-purified VP₁. In a previous report from this laboratory, polyoma virion proteins VP₁, VP₂, and VP₃ and histones were separated by using GuHCl gel filtration (4). We utilized this technique to isolate ¹²⁵I-labeled virion VP₁ to determine whether the GuHCl column-purified VP₁ showed an IEF pattern similar to that found previously with polyoma virions. The results shown in Fig. 6 clearly demonstrate that, indeed, all of the VP₁ species found during IEF of virions were found after IEF of the GuHCl column-purified virion VP₁. Samples of the same pooled VP₁ preparation were then used in immune precipitation experiments with rabbit anti-polyoma hemagglutinin and anti-polyoma receptor. In an earlier report (3), it was demonstrated that these two antibody activities could be separated by polyoma capsid affinity column chromatography from antiserum prepared against proteolytic cleavage products of the major structural protein VP₁ in SDS-polyacrylamide gels. In immune precipitation experiments, it was found that, after EGTA-DTT dissociation of polyoma virions (conditions known to lead to the breakdown of VP₁), the anti-polyoma hemagglutinin specifically precipitated a 16K-dalton VP₁ cleavage product, whereas the anti-polyoma receptor specifically precipitated a 14K-dalton VP₁ breakdown product. When the same virion preparation was dissociated under conditions which minimized VP₁ degradation, both anti-polyoma hemagglutinin and anti-receptor antibodies immune precipitated 44K-dalton VP₁. The goal of the experiments with GuHCl column-purified VP₁ was thus to determine whether any one or a group of the VP₁ species would be recognized by one or both of these antibody preparations. The results of these immune precipitation experiments are shown in Fig. 7. Two of the VP₁ species were specifically immune precipitated with anti-polyoma hemagglutinin (Fig. 7A). These two species corresponded to VP₁-D and VP₁-F. Only one of the VP₁ species (species E) was immune precipitated with anti-polyoma receptor immunoglobulin G (Fig. 7B). These results suggest that the VP₁ species associated with the hemagglutinating activity of polyoma virions consist of VP₁ species D and F. It is interesting that these two species are also found on polyoma capsids which have been shown to compete for the same cellular

FIG. 5. IEF of ^3H -amino acid-labeled virions and capsids. Polyoma virions and capsids were purified from the lysates of infected cells grown in the presence of ^3H -amino acids during the period of virus infection. ^3H -amino acid-labeled virions (A) and capsids (B) were then analyzed by IEF and SDS-PAGE as described in the text.

receptors as virions on the surface of GPE (2). These results further suggest that VP₁ species E is responsible for the virion-associated receptor activity of polyoma virions which is required for specific adsorption and infection of primary MKC since the anti-polyoma receptor antibodies specifically inhibited polyoma virion adsorption to MKC but not to GPE (3). Furthermore, VP₁-E is found only on polyoma virions and is absent in polyoma capsids which fail to compete with virions for receptor sites on the surface of MKC (2).

DISCUSSION

The central reason for comparing the IEF patterns of polyoma virion and capsid proteins stems from previous studies conducted in our laboratory which demonstrated distinct biological, biophysical, and biochemical differences between these two populations of virus particles (2, 3, 12, 20). Mackay and Consigli (12) reported that the fate of polyoma capsids within cells was distinct from the small population of polyoma virions which were responsible for cellular infec-

TABLE 2. IEF of polyoma virion and capsid structural proteins

Protein	Species	pI	% of total ^a	³² P/ ³ H ^b	¹⁴ C/ ³ H ^c
Virions					
VP ₁	A	6.75	8.6	0.2	
	B	6.50	18.0		
	C	6.30	37.8		0.05
	D	6.10	13.2	0.40	0.25
	E	5.90	6.6	0.60	
	F	5.75	5.8	0.80	
VP ₂		5.50	4.8	1.00	
VP ₃		7.00	5.2	1.10	
Capsids					
VP ₁	A	6.60	34.6		0.20
	B	6.50	18.5		
	D	6.10	20.9	0.20	0.40
	F	5.75	16.0	0.40	0.30
VP ₂		5.50	5.4	0.80	
VP ₃		6.50	4.6	1.00	

^a The area of the peak for each of the proteins and protein species is expressed as the percentage of the total area occupied by the protein or protein species present in the sample. Each value is the average of a number of separate determinations of pooled ³H-amino acid-labeled samples.

^b Ratio of ³²P-labeled proteins to ³H-amino acid-labeled proteins.

^c Ratio of [¹⁴C]acetate-labeled proteins to ³H-amino acid-labeled proteins.

FIG. 6. IEF of GuHCl-chromatographed virion VP₁. Chloramine-T-iodinated virion proteins were chromatographed on a GuHCl column as described in the text. The VP₁ fractions from the column effluent were pooled, dialyzed against Tris buffer (pH 7.4) to remove excess guanidine, and analyzed by IEF and SDS-PAGE as described in the text.

FIG. 7. IEF of GuHCl column-purified VP₁ immune precipitation. Samples of the Chloramine-T-iodinated VP₁ isolated as described in the legend to Fig. 6 were used in immune precipitation experiments with antihemagglutinin and antireceptor antibodies as described in the text. The immune precipitates were analyzed by IEF. The figure shows the focusing pattern of GuHCl VP₁ plus antihemagglutinin (A) or antireceptor (B). For clarity, the background counts obtained by incubating normal rabbit immunoglobulin G with the GuHCl VP₁ have been subtracted.

tion. After virion adsorption to cells, a small percentage of the original inoculum was transported to the nucleus, where the virions were uncoated (12, 20), and early transcriptional activities were initiated (20). The majority of the original virions were found in cellular lysosomes, where they were degraded. After capsid absorption to cells, no significant nuclear transport of these "empty" virus particles was detected. Instead, almost the entire capsid population was sequestered into cellular lysosomes and degraded (12). Subsequently, it was reported that the reason for this differential fate of virions and capsids within cells resulted from the manner by which virions and capsids adsorbed to the cell surface (2). Although capsids efficiently competed with virions for receptor sites on the surface of GPE, capsids could not compete with virions for receptor sites on MKC, which were required for successful infection. This study also demonstrated that the reason only 7 to 10% of the input virions ever entered the nucleus was

because the number of specific surface receptors to which virions, but not capsids, could bind was limited. Recently, the maximum number of specific virion receptor sites on the surface of quiescent primary MKC has been estimated to be approximately 10,000 per cell (J.B.B., unpublished data). In the same report, it was reported that an adsorption mutant of polyoma (Py 235), originally described by Basilico and DiMayorca (1), which lacked the ability to adsorb to and agglutinate GPE, nevertheless possessed an equal ability to specifically adsorb to and infect MKC at 32°C as did wild-type virions. These results demonstrated that specific adsorption to MKC and infection of these mouse cells was independent of the ability of polyoma virions to adsorb to and agglutinate GPE. In a recent report (3), we were able to demonstrate the separation of neutralizing antibodies from hemagglutination-inhibiting antibodies in antisera directed against cleavage products of virion VP₁ which migrated in the histone region of SDS-polyacrylamide gels. It was found that the antigenic determinants responsible for hemagglutination-inhibiting activity of the serum were present on a 16K-dalton polypeptide, whereas those antigenic determinants responsible for neutralization activity of the serum were present on a 14K-dalton polypeptide. These two polypeptides were both derived from 44K-dalton virion VP₁. These results strongly suggested that the majority of the diverse biological functions observed were dependent on the major structural protein VP₁.

In this report we have shown distinct differences between the subpopulations of the structural proteins of polyoma virions and capsids. The results presented here demonstrate that polyoma virions possess six species of the major structural protein VP₁ in addition to the two minor structural proteins VP₂ and VP₃. Polyoma capsids were found to contain only four VP₁ species in addition to VP₂ and VP₃. Multiple species of polyoma virion VP₁ differing in their pI's have been reported previously (11, 17). However, the number of species and the functions of these multiple species were not clear. It is of considerable interest that O'Farrell and Goodman (16) reported that SV40 virions also possess six distinct VP₁ species. Such results suggest that this pattern of six VP₁ species (even though the pI's of these species vary considerably between polyoma and SV40) may be common to all papovaviruses. Our experiments have allowed us to tentatively assign functions to the various species of VP₁ and have aided in the comprehension of how VP₁ possesses the potential for such biological diversity.

The major structural protein (VP₁) of poly-

oma virions consists of six species which have pI's between pH 6.75 and 5.75. On two-dimensional analysis these species all had molecular weights in the 42K to 45K range. The molecular-weight diversity observed was not due to contamination of our standard wild-type virions with defective virus or deletion mutants, as freshly plaque-purified virus samples yielded identical electrophoretic patterns. The differences in pI's of the VP₁ species conceivably result from the different degrees and types of modifications observed (Table 2). Four of the VP₁ species were differentially phosphorylated (A, D, E, and F) and two of the species were acetylated (C and D). In our analysis to date, only species B appears to be unmodified. Similar experiments with capsids revealed only four VP₁ species with pI's between pH 6.60 and 5.75. Two of the capsid VP₁ species were phosphorylated (D and F). It is interesting that the degree of phosphorylation as determined by the ratio of ³²P/³H of these two capsid VP₁ species was only half that observed with virion species D and F, although the significance of this remains obscure. Similarly, the importance of three of the capsid VP₁ species A', C, and D being acetylated is also unclear. As with virions, capsid VP₁ species B does not appear to be modified via acetylation or phosphorylation. Analysis of the minor virion and capsid structural proteins VP₂ and VP₃ also revealed that differences exist. Virion VP₃ had a pI of pH 7.00, whereas capsid VP₃ had an apparent pI of pH 6.50. Again, the significance of this observation is not understood at the present time. Capsid and virion VP₂ had a common pI of pH 5.50. Further analysis of all of the virion and capsid VP₁ species, VP₂, and VP₃ using other analytical techniques, such as tryptic peptide mapping, and a wide variety of temperature-sensitive and deletion mutants is currently under way in this laboratory. It is anticipated that such an approach will be helpful in answering many of the questions raised in this investigation.

The variety of presumably host-contributed post-translational modifications of the virus structural proteins, particularly VP₁, suggests that such modifications could allow a number of different functions to be expressed by a single protein. At the present time, this appears to be the case with polyoma virus and represents another example of the virus' ability to efficiently utilize and expand its limited genetic coding capacity. Our results suggest that polyoma virion VP₁ may serve at least four distinct functional roles. The first is a structural function. The experiments in which the various VP₁ species were differentially iodinated with Enzymobeads or chloramine-T (Fig. 3) demonstrated that VP₁

species A was an internal protein whereas the remaining VP₁ species were available for external labeling. Analysis of the EGTA-DTT-derived capsomere subunits (Table 1) demonstrated that virion VP₁ species B, C, and D were present in capsomere preparations enriched with hexon subunits, whereas species A, E, and F were absent. These results suggest that polyoma virion hexons are composed of VP₁ species B, C, and D. They also suggest that VP₁ species E and F may be involved in the penton capsomere subunits of the virion. Thus, potentially five of the six VP₁ species perform a structural function for the virus. Similar analysis of capsids suggested that VP₁ species A' in capsids likely performs the same function as virion VP₁ species C (Tables 1 and 2). In capsids, therefore, the hexons are apparently composed of VP₁ species A', B, and D. Since capsids lack a VP₁ species which physically corresponds to virion species E, it appears that capsid species D, along with F, might compensate structurally for the lack of E. Minor proteins VP₂ and VP₃ were found both in capsomere fractions being accessible for surface iodination with Enzymobeads and in association with the DNA-protein core (7; J.B.B., unpublished data). At this time, the roles of VP₂ and VP₃ as structural entities or regulatory proteins, or both, unfortunately remain unresolved.

The second role that polyoma VP₁ possesses is exemplified by virion VP₁ species A. This species, as noted above, could not be labeled *in vitro* by the Enzymobead technique (Fig. 3B) and was found to be the only VP₁ species firmly associated with the virion DNA-protein core (Fig. 4). Furthermore, VP₁ species A was not found in capsids (Fig. 1A, 3C and D, and 5; Tables 1 and 2) or in significant amounts in capsomere subunits (Table 1). The function which this DNA core-associated VP₁ species fulfills is not clear at this time. That VP₁ species A, the most basic (pI, pH 6.75) of the VP₁ species, is apparently exclusively associated with the DNA-protein complex of the virus implies that its role may not be structural in nature. One function VP₁ species A may serve is suggested by the recent experiments by Brady et al. (5; personal communication). They demonstrated that 110S SV40 nucleoprotein cores derived from intact 240S SV40 virions by EGTA-DTT dissociation contained VP₁ and a small amount of VP₂ in addition to the proteins normally found associated with the SV40 55S minichromosome (i.e., VP₃ and histones). In addition to keeping the 110S core compact, the associated VP₁-VP₂ significantly modified the transcriptional activity of the core in the presence of *Escherichia coli* RNA polymerase. The presence of the late

virus proteins resulted in transcription of 16 to 18S RNA which hybridized to the "early" SV40 DNA region. In the absence of VP₁-VP₂, the RNA transcription product was only 4 to 6S. These results lead to the speculation that the same events may take place with polyoma nucleoprotein complexes. Experiments to test this hypothesis are currently being conducted in our laboratory. Nevertheless, the experiments of Brady et al. clearly point out the probability that VP₁ and perhaps VP₂, normally considered only structural entities, also possess regulatory functions.

The remaining two functions which can be attributed to polyoma VP₁ deal with the virus' ability to adsorb to mammalian cells such as GPE and primary MKC or mouse embryo cells. The data presented in this report indicate that three of the virion VP₁ species (D, E, and F) are involved in such activities. VP₁ species D and F appear to be common to both virions and capsids, although their distribution among capsomere subunits, degree of modification, and absolute concentration differ to some extent (Tables 1 and 2). Our data suggest, however, that whereas the capsid D and F species appear to be slightly different from their virion counterparts, they may fulfill similar functions. These results also point out the possibility that some of the modifications observed may not necessarily dictate functional changes other than allowing conformational diversity of the VP₁ molecules required for assembly of virus particles. The additional biological functions attributed to some of these species could thus be dependent on the presence of other unidentified modifications or in many instances they could be independent of such alterations. The functional identity of virion species D and F was suggested by the immune precipitation experiments (Fig. 7). Antibodies whose only known function was to inhibit virion or capsid absorption to and agglutination of GPE could specifically recognize only VP₁ species D and F. Thus, it appears that virion VP₁ species D and F are involved with the virus' ability to adsorb to and agglutinate GPE. These results further suggest that capsid VP₁ species D and F probably fulfill the same function, since virions and capsids have an equal ability to cause hemagglutination which can be equally inhibited by the hemagglutination-inhibiting antisera (3). Virion VP₁ species E appears to represent the virion protein which is required for successful virus adsorption to specific cellular receptors, which results in infection of MKC and mouse embryo cells. Thus, VP₁ species E apparently fulfills the role of the polyoma virion-associated receptor. Two lines of evidence support this

contention. Primarily, VP₁ species E is found only on virions, never on capsids. Since, as discussed above, capsids do not compete with virions for specific cellular receptors (2), it seems logical that capsids would lack the corresponding protein present on virions. Similarly, since capsids and virions share the same cell-associated receptor on GPE (2), one would expect these two types of virus particles to share common hemagglutinins. As discussed above, this appears to be the case. Second, antibodies whose only known activity was to inhibit virion adsorption to mouse cells, but not to GPE, specifically recognized distinct antigenic determinants only on VP₁ species E in immune precipitation experiments (Fig. 7B).

An additional point of interest concerns the precursor-product relationship between the VP₁ species. The pulse-chase experiments performed by O'Farrell and Goodman (16) suggested that the most acidic of the SV40 VP₁ species acted as a precursor to two additional VP₁ species which focused immediately adjacent to the precursor. If the same relationship is also present with polyoma, then species F would represent the precursor for species D and E. Such a relationship would indicate that the VP₁ species involved in cellular adsorption activities are derived from a common precursor which is distinct from other VP₁ species, possibly through dephosphorylation of the precursor (Table 2).

This study has allowed us to assign functions to the VP₁ species of polyoma virus. In the future we anticipate being able to better understand the molecular interactions which result in the modified forms of VP₁ and to determine what other functions VP₁, VP₂, and VP₃ might express during the course of polyoma infection.

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LITERATURE CITED

1. Basilico, C., and G. DiMayorca. 1974. Mutant of polyoma virus with impaired adsorption to BHK cells. *J. Virol.* 13:931-934.
2. Bolen, J. B., and R. A. Consigli. 1979. Differential adsorption of polyoma virions and capsids to mouse kidney cells and guinea pig erythrocytes. *J. Virol.* 32: 679-683.
3. Bolen, J. B., and R. A. Consigli. 1980. Separation of neutralizing and hemagglutination-inhibiting antibody activities and specificity of antisera to sodium dodecyl sulfate-derived polypeptides of polyoma virions. *J. Virol.* 34:119-129.
4. Brady, J. N., and R. A. Consigli. 1978. Chromatographic separation of the polyoma virus proteins and renaturation of the isolated VP₁ major capsid protein. *J. Virol.* 27:436-442.
5. Brady, J. N., C. Lavialle, and N. P. Salzman. 1980. Efficient transcription of a compact nucleoprotein complex isolated from purified simian virus 40 virions. *J. Virol.* 35:371-381.
6. Brady, J. N., V. D. Winston, and R. A. Consigli. 1977. Dissociation of polyoma virus by the chelation of calcium ions found associated with purified virions. *J. Virol.* 23:717-724.
7. Brady, J. N., V. D. Winston, and R. A. Consigli. 1978. Characterization of a DNA-protein complex and capsomere subunits derived from polyoma virus by treatment with ethyleneglycol-bis-*N,N'*-tetraacetic acid and dithiothreitol. *J. Virol.* 27:193-204.
8. Brunck, C. F., and V. Leick. 1969. Rapid equilibrium isopycnic CaCl₂ gradients. *Biochim. Biophys. Acta* 179: 136-144.
9. Deininger, P., A. Esty, P. LaPorte, and T. Friedmann. 1979. Nucleotide sequence and genetic organization of the polyoma late region: features common to the polyoma early region and SV40. *Cell* 18:771-779.
10. Fried, M., and B. Griffin. 1977. Organization of the genomes of polyoma virus and SV40. *Adv. Cancer Res.* 24:67-114.
11. Hewick, R. M., M. D. Waterfield, L. K. Miller, and M. Fried. 1977. Correlation between genetic loci and structural differences in the capsid proteins of polyoma virus plaque morphology mutants. *Cell* 11:331-338.
12. Mackay, R. L., and R. A. Consigli. 1976. Early events in polyoma virus infection: attachment, penetration, and nuclear entry. *J. Virol.* 19:620-636.
13. McMillen, J., M. S. Center, and R. A. Consigli. 1976. Origin of polyoma virus-associated endonuclease. *J. Virol.* 17:127-131.
14. McMillen, J., and R. A. Consigli. 1974. In vitro radioisotopic labeling of proteins associated with purified polyoma virions. *J. Virol.* 14:1627-1629.
15. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
16. O'Farrell, P. Z., and H. M. Goodman. 1976. Resolution of simian virus 40 proteins in whole cell extracts by two-dimensional electrophoresis: heterogeneity of the major capsid protein. *Cell* 9:289-298.
17. Ponder, B. A. J., A. K. Robbins, and L. V. Crawford. 1977. Phosphorylation of polyoma and SV40 virus proteins. *J. Gen. Virol.* 37:75-83.
18. Smith, G. L., and R. A. Consigli. 1972. Transient inhibition of polyoma virus synthesis by Sendai virus (parainfluenza I). I. Demonstration and nature of the inhibition by inactivated virus. *J. Virol.* 10:1091-1097.
19. Vinograd, J., and J. E. Hearst. 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient. *Prog. Chem. Org. Nat. Prod.* 20:372-422.
20. Winston, V. D., J. B. Bolen, and R. A. Consigli. 1980. Isolation and characterization of polyoma uncoating intermediates from the nuclei of infected mouse cells. *J. Virol.* 33:1173-1181.