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Structural Proteins of Polyoma Virus: Proteolytic Degradation of Virion Proteins by Exogenous and by Virion-Associated Proteases

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A model has previously been proposed for the genetic relatedness of the structural proteins of polyoma virus, based upon similarities in the peptide maps of the major capsid protein VP1 with the virion proteins VP2 and VP3. Newer evidence suggests that this model is incorrect, and that protein VP1 is a product of one viral gene and that the multiple components of VP2 and VP3 are products of a second viral gene. Two-dimensional peptide maps of several preparations of polyoma purified separately from four separate infected-cell lysates has shown a variable content of VP1 peptides in proteins VP2 and VP3, with some preparations being free of detectable VP1 material in VP2 and VP3. An alternative explanation for the presence of VP1 peptides in the regions of VP2 and VP3 of some polyoma preparations involves the cleavage of proteins of polyoma virions during exposure to proteolytic enzymes in lysates of infected cells or to endogenous proteolytic activity of virions. Prolonged incubation of infected-cell lysates at 37°C leads to an increase in the amount of 86,000-dalton dimer of VP1, a decrease in the relative amount of VP1, a decrease in or a loss of the lower band of VP2, and the appearance of a new major protein band of approximately 29,000 daltons. Two-dimensional peptide maps of the new 29,000-dalton protein show that it contains some VP1 peptides, indicating that this protein is derived from proteolytic cleavage of VP1. In addition, extensively purified polyoma virus contains a proteolytic activity that can be activated during disruption of the virus with 0.2 M Na_2CO_3-$NaHCO_3$ (pH 10.6) in the presence of 5×10^{-3} M dithiothreitol.

A previous report from this laboratory has suggested, on the basis of peptide mapping data, that the major capsid protein VP1 of polyoma virus and the two minor groups of proteins VP2 and VP3 are genetically related to one another (1). Contrary data from several laboratories have been presented, showing that peptide maps of the major capsid protein are different from those for the minor components (2-4), indicating that these proteins represent distinct gene products. The studies reported here show that proteins with molecular weights similar to authentic virion VP2 and VP3 can be generated from virions purified from cell lysates after prolonged exposure at 37°C, or by exposure of purified virions to pH 10.6 carbonate in the presence of the reducing agent dithiothreitol. The new 29,000-dalton protein found in virions purified after prolonged incubation of lysate at 37°C contains many VP1 tryptic peptides. These data indicate that VP1 peptides might be present in smaller-molecular-weight proteins of purified polyoma virus as a result of proteolysis during preparation of the virus. The most compelling evidence now suggests that VP1 is indeed a unique gene product distinguishable from VP2 and VP3, and that the latter minor proteins constitute families of molecules of a second gene product. The previously proposed model for polyoma protein processing, therefore, cannot be supported on the basis of currently available evidence.

MATERIALS AND METHODS

Wild-type polyoma virus was prepared in primary baby mouse kidney cells and purified by methods reported previously (5, 6). A second wild-type virus stock was obtained from W. Gibson of the Salk Institute for Biological Studies in La Jolla, Calif. Purified virus was stored for up to 3 months in 0.05 M Tris buffer, (pH 8.0) at 4°C or frozen in TD buffer (0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 25 mM Tris-hydrochloride, pH 7.4) in the presence of 50 μg of bovine serum albumin (BSA) per ml, obtained as fraction V from Pentex Corp.

Gel electrophoresis and peptide mapping. Analytical and preparative sodium dodecyl sulfate

(SDS)-polyacrylamide gel electrophoresis of purified virus was performed in slabs of 8 to 15% polyacrylamide gradients and 4% bisacrylamide as previously described (7), and the viral proteins were identified by the Fairbanks staining method using Coomassie blue (8). Proteins were eluted and prepared for two-dimensional tryptic peptide maps as reported elsewhere (7).

Virion protease. Polyoma virus purified by polyethylene glycol precipitation and CsCl gradient centrifugation was further purified by sedimentation centrifugation in an SW41 Spinco rotor at 35,000 rpm for 45 min in a gradient of 10 to 30% glycerol in TD buffer containing 25 μg of BSA per ml. Purified virus was stored frozen at -20°C and, before protease assay, was freed of BSA by centrifugation as described above in a 10 to 30% glycerol gradient in TD without BSA. After dialysis against 0.05 M NH_4HCO_3 or 0.05 M Tris, both at pH 7.8, portions of the virus were: (i) lyophilized, (ii) freeze-thawed three times, (iii) exposed to 0.05% and 1.0% SDS, (iv) added to an equal amount of 0.625 M Tris (pH 6.8)-1% SDS, (v) exposed to 9 M urea with freshly dissolved ultrapure urea (Schwarz/Mann), (vi) dialyzed against 1,000 volumes 0.2 M Na_2CO_3-$NaHCO_3$-10 mM dithiothreitol (DTT) (pH 10.6) for 2 h at 0°C followed by dialysis against 0.05 M Tris (pH 7.8, and (vii) heated to 75°C for 1 min. All preparations were incubated overnight at 37°C and then precipitated with 16% cold trichloroacetic acid, washed twice each with cold 5% trichloroacetic acid and acetone, and air dried. The samples were then examined in SDS-8 to 15% gradient slab polyacrylamide gels containing 4% bisacrylamide, according to the method of Laemmli (9). Electrophoresis, staining, and autoradiography were as described previously (2).

RESULTS

A series of tryptic peptide maps of the isolated and purified viral proteins prepared from a variety of virus isolations showed that the amount of VP1 peptides present in proteins eluted from positions VP2 and VP3 was highly variable. Identical results are obtained in preparations containing little or no core material as is present in this preparation. However, several experiments gave results that are described in Fig. 1. Major capsid protein VP1 and the high-molecular-weight (86,000) protein gave indistinguishable peptide maps, as has been previously described (1). The two separated bands of the VP2 region gave peptide maps that were very similar to each other but quite different from VP1. Likewise, the separated bands of the VP3 region gave peptide maps identical to each other but again very different from VP1. The two components of VP2 were similar to both components of VP3, and differed most strikingly in the presence in VP2 of a major peptide that moved very little during electrophoresis, but which moved with the chromatography solvent front (arrows). Both components of VP2 contained this peptide, whereas both components of VP3 were missing this presumably hydrophobic peptide. There are also several minor peptides of VP2 missing in VP3. The bottom component of VP3 in most virus preparations showed several basic, nonhydrophobic peptides similar or identical to a group of VP1 peptides.

Since other preparations of similarly purified virus did contain more VP1 peptides in the VP2 and VP3 regions, proteolytic cleavage of the virion proteins during virus preparation or purification seemed a likely explanation for the variable results. Such a protease could be an integral part of the virion or a cell or serum contaminant. To examine the possibility of a virion-associated protease, I exposed purified polyoma virus to conditions used during the purification of the virus, and to conditions known or suspected to permit protease activity.

Figures 2 and 3 show the results of these treatments of polyoma virus. Exposure of virus to freeze drying, freeze thawing, 9 M urea, and heating to 75°C for 1 min, or to 0.05% SDS failed to produce any changes in the protein pattern of the virus. When purified virions were exposed either to gel sample buffer containing 0.5% SDS or to 0.5% SDS alone and subsequently incubated overnight, there was a marked and preferential loss of proteins VP2 and VP3 and the histone-like proteins, but no major new bands appeared. Figure 3 shows in addition that purified virus that was dialyzed against the pH 10.6 carbonate buffer in the presence of the reducing agent DTT, conditions which are known to disrupt polyoma virus (10), showed the preferential loss of the lower band of VP2 and the appearance of many major new protein bands between VP2 and VP3 and in the low-molecular-weight region of the gel. The shorter exposure period makes these changes after pH 10.6-DTT disruption inapparent in the gel shown in Fig. 2.

To detect a possible proteolytic activity in infected-cell lysates, a sample of stock infected-cell lysate was divided into two portions. From one-half of the preparation, virus was purified immediately by the standard methods of polyethylene glycol precipitation followed by CsCl gradient centrifugation (6). The remaining half was incubated for an additional 5 days at 37°C, and virus was isolated by the same method. The yields of labeled virus and the purification characteristics of the two preparations were similar. The purified virus preparations were examined in analytical SDS-polyacrylamide gels, as shown in Fig. 4. When compared with

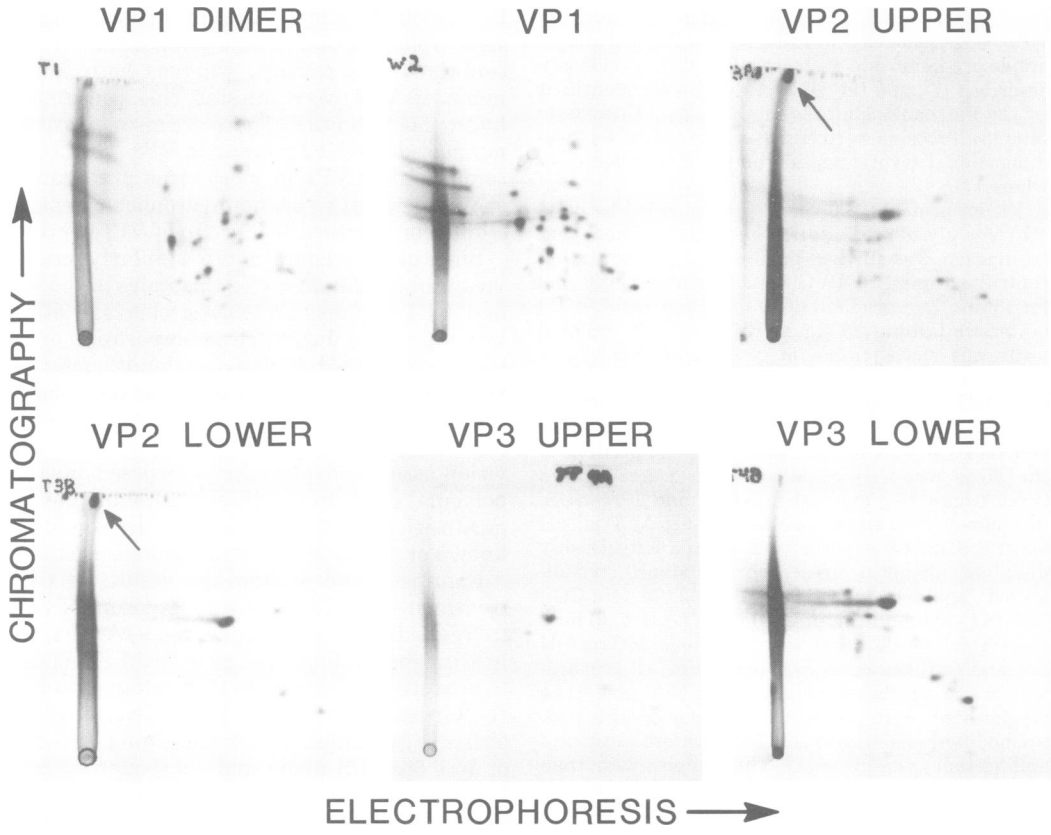


FIG. 1. *Two-dimensional tryptic peptide maps of the purified, separated proteins of polyoma virus, after SDS-polyacrylamide gel electrophoresis, elution, and trypsinization as described in Materials and Methods. Samples are: (a) 86,000-dalton VP1 dimer, (b) VP1, (c) upper band of VP2 doublet, (d) lower band of VP2 doublet, (e) upper band of VP3 doublet, and (f) lower band of VP3 doublet. Two-dimensional separation and autoradiography were performed as described previously (7).*

virus from unincubated lysate, virus purified from the incubated lysate was relatively deficient in VP1, had preferentially lost the lower band of VP2, and showed at least one new protein band between VP2 and VP3 with a molecular weight approximately 29,000. There was also a marked enrichment of the 86,000-dalton dimer of VP1. The separated proteins isolated from unincubated and from incubated and degraded virus were eluted and prepared for tryptic peptide mapping as described above. The control virus preparations gave results as described in Fig. 1, with distinctly different maps for VP1 and for VP2 and VP3. In the protiens prepared from virus purified from incubated lysate, the new protein band at 29,000 daltons showed peptides characteristic for VP1, whereas the upper band of VP2 and the components of VP3 continued to show a pattern different from VP1.

DISCUSSION

This model of polyoma protein processing previously suggested from this laboratory was based on the presence of peptides characteristic for VP1 in isolated proteins derived from VP2 and VP3 regions of purified virus. The preparations of purified polyoma were derived from a single high-titer stock of plaque-purified virus, from which was purified separately on several occasions. Since this model has not been confirmed in other laboratories, and since contrary evidence has come from several laboratories, we have attempted to characterize mechanisms by which proteins containing VP1 peptides can be elaborated during the production and purification of polyoma virus.

For a series of purified preparations of polyoma, there are variable amounts of proteins in the region VP2 and VP3 which contain authen-

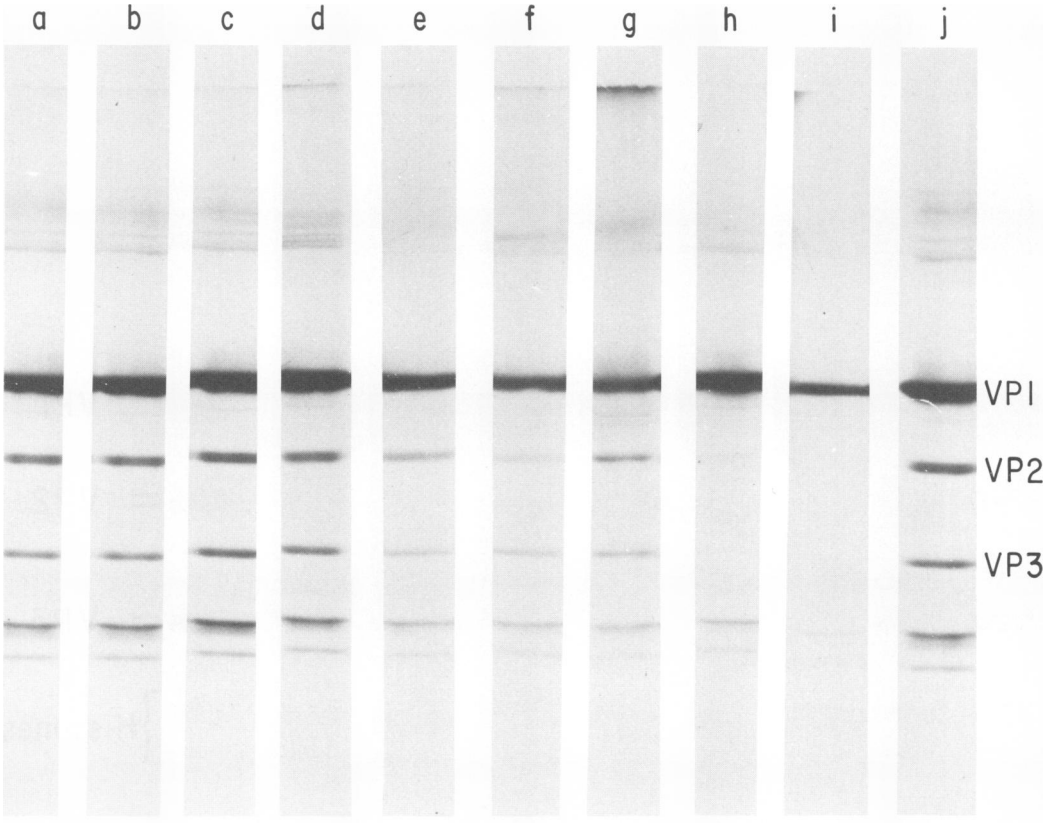


FIG. 2. *SDS-polyacrylamide gel electrophoresis of purified polyoma virus preparations after overnight exposure at 37°C to 0.05 M Tris, pH 7.8. The samples had previously been exposed to the following conditions, as described in Materials and Methods: (a) 0.05 M Tris, pH 7.8; (b) freeze-dried from 0.05 M Tris, pH 7.8; (c) freeze-thawed three times from 0.05 M Tris, pH 7.8; (d) 0.5% SDS; (e) 75°C for 1 min; (f) saturated (ca. 9.5 M) urea in 0.05 M Tris, pH 7.8; (g) 0.2 M Na_2CO_3-$NaHCO_3$, pH 10.6, in the presence of 10 mM DTT; (h) 1% SDS; (i) gel sample buffer, 0.31 M Tris (pH 6.8)-0.5% SDS; (j) 0.05 M Tris, pH 7.8. Samples were prepared for electrophoresis as described in Materials and Methods.*

tic VP1 peptides as judged by peptide mapping. However, in some preparations, including our wild-type virus as well as that from W. Gibson, there is little or no material in the region of the minor polyoma proteins which contains peptides derived from the major capsid protein VP1. The presence of variable amounts of material derived from VP1 in VP3 suggests proteolytic degradation of VP1, in agreement with the suggestions given by other workers (2–4).

The study of purified virions has suggested the presence of a proteolytic enzyme after exposure to one of the conditions normally used for virus purification and analysis, since exposure of purified virus to conditions similar to those found in gel sample buffer, i.e., high concentrations of SDS, leads to a loss of proteins VP2 and VP3 and virion histone-like proteins. At the present time, I cannot distinguish between selective losses and proteolysis as explanations for this loss of protein, although proteolysis is suggested by the relative sparing of only one of many proteins in the virion. When purified virus is dialyzed against pH 10.6 carbonate buffer with 10 mM DTT, and subsequently incubated at 37°C in Tris or NH_4HCO_3 buffers, not only does the lower band of VP2 decrease markedly, but several major new proteins appear in the regions of VP2 and VP3. This proteolysis is not prevented by incubation in the presence of 1 mM phenylmethyl sulfonyl fluoride (PMSF), suggesting that the protease is not a serine protease. The existence of a proteolytic enzyme activated by SDS or by the pH 10.6-DTT conditions may be related to the presumed proteolytic degradation in cell lysates or during virus purification, although no conditions of high pH or treatment with reducing

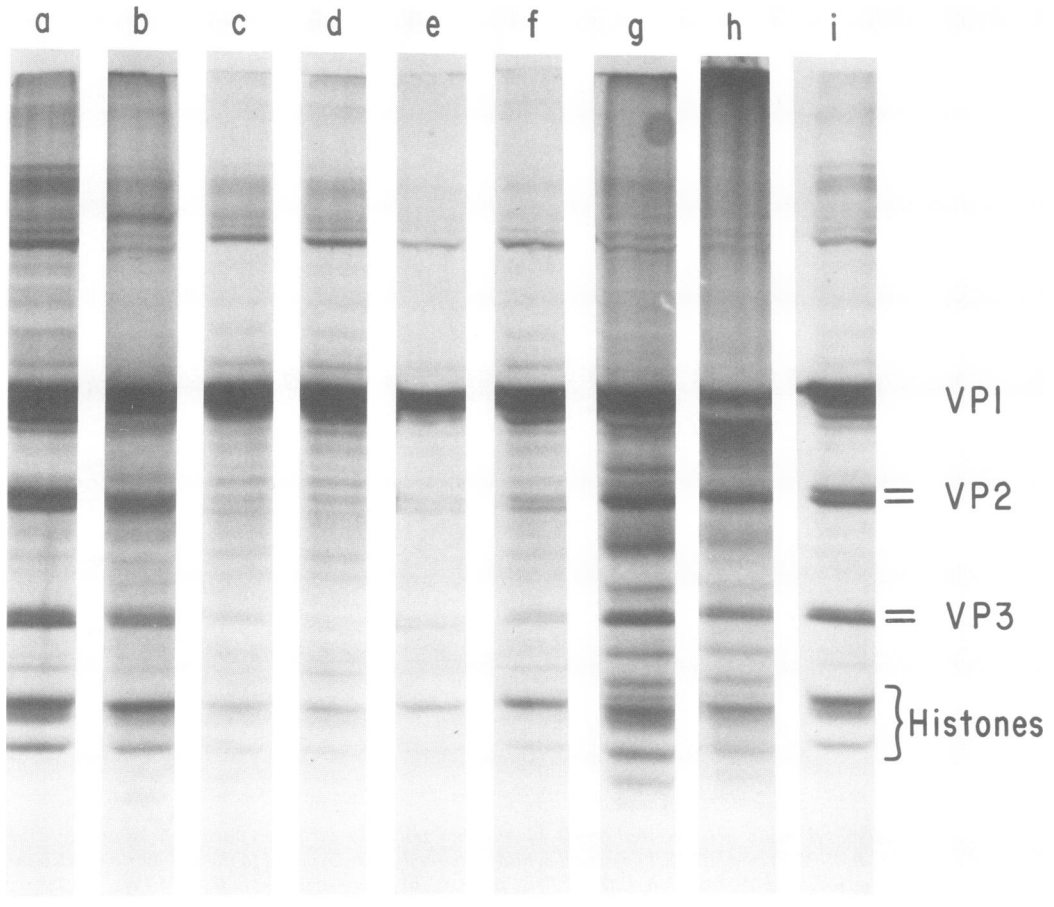


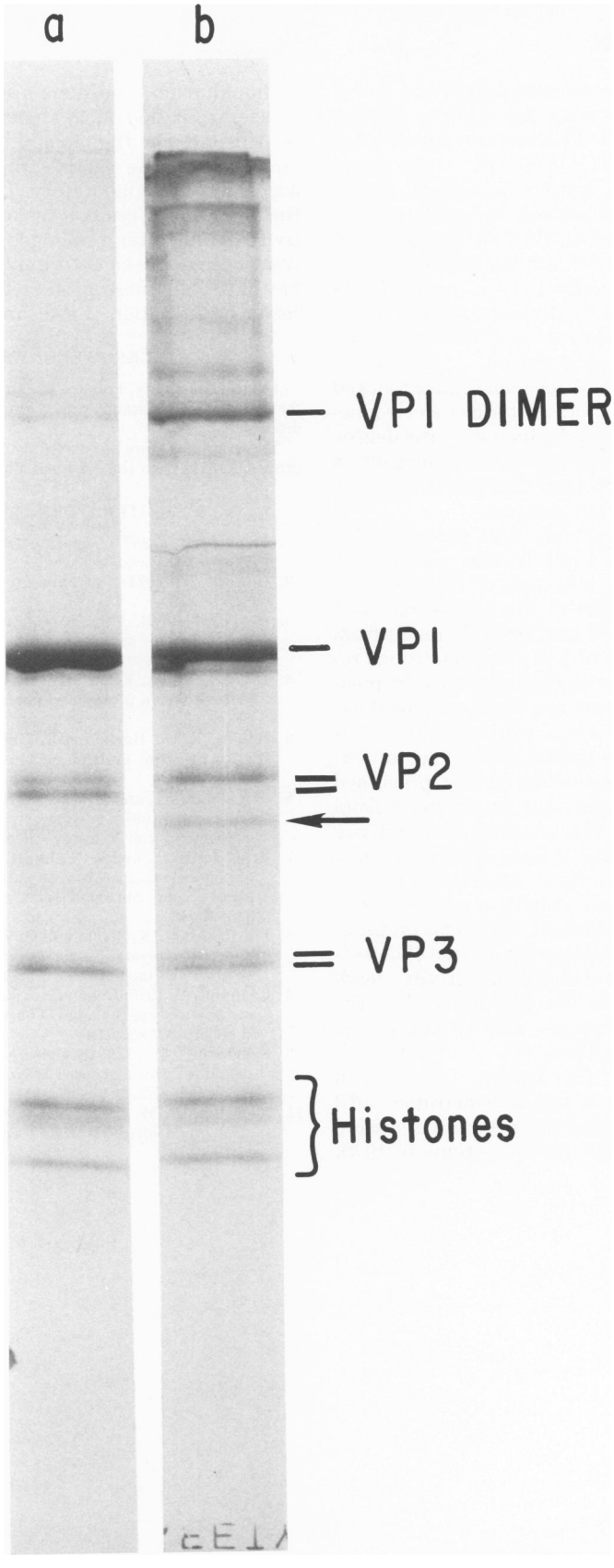
FIG. 3. *SDS-polyacrylamide gel electrophoresis, as in Fig. 2, of polyoma virus preparations after overnight exposure at 37°C to: (a) 0.05 M $NaHCO_3$, pH 7.8; (b) 0.05 M $NaHCO_3$, pH 7.8, with 9.5 M urea; (c) 0.05 M $NaHCO_3$, pH 7.8, and 1% SDS; (d) 0.05 M $NaHCO_3$, pH 7.8, 1% SDS, and 1 mM PMSF; (e) sample buffer, 0.31 M Tris (pH 6.8)-0.5% SDS; (f) 0.31 M Tris (pH 6.8), 0.5% SDS, and 1 mM PMSF; (g) 0.05 M $NaHCO_3$, pH 7.8, after disruption in 0.2 M Na_2CO_3-$NaHCO_3$, pH 10.6, with 10 mM DTT; (h) as in (g), plus 1 mM PMSF; (i) 0.05 M $NaHCO_3$, pH 7.8. Electrophoresis was as described in Materials and Methods.*

agents are included in virus purification.

Prolonged exposure of polyoma virus in infected-cell lysates can lead to changes in the electrophoretic pattern of the proteins. During infection of baby mouse kidney cells with low multiplicities of virus, there is opportunity for progeny virus to remain in contact with a putative proteolytic enzyme during the prolonged incubation time necessary until a full cytopathic effect is obtained.

These results suggest that the appearance of material with electrophoretic properties similar to the virion proteins VP2 and VP3 can result from the action of a cellular, serum, or virion enzyme that can act on virion proteins to degrade VP1 to forms with electrophoretic properties similar to authentic viral VP2. The data from peptide mapping now are consistent with the concept that purified virions of polyoma contain gene products of at least two separate and unique viral genes, namely, those for the major capsid protein VP1 and for the minor capsid proteins VP2 and VP3. Two-dimensional protein separations now make it clear, however, that VP2 exists not only as a doubtlet, but in fact as a series of five or six differently

FIG. 4. *SDS-polyacrylamide gel electrophoresis of polyoma virions purified (a) without and (b) after prolonged exposure to infected-cell lysate at 37°C. Electrophoresis was as described in Materials and Methods. The position of the new 29,000-dalton component is given by the arrow.*


a
b
VP1 DIMER
VP1
VP2
VP3
Histones

charged species, the relationship of any one of which to any other is at the moment unclear (A. Gonenne and T. Friedmann, unpublished data). Similarly, VP3 is seen still to exist in two and possibly three distinct isoelectric forms. Presumably, the differences between the proteins within VP2 and within VP3, as well as the differences between the families of molecules in VP2 and VP3, are reflections of post-translational modifications that might include phosphorylation, deamidation of glutamine or asparagine residues, acetylation, or glycosylation. All these modifications may cause marked alterations in the factors that affect electrophoretic mobility in SDS gels, including the degree of SDS binding, the effect of bound detergent on protein conformation, and changes in the frictional coefficient of the molecule.

These data demonstrate that proteases are available in infected-cell lysates and virions capable of causing cleavage of intact virion VP1, and lend support to the notion that VP1 peptides found in VP2 may possibly result from proteolytic cleavage of VP1. The conditions required to activate proteolytic activity in purified virions of polyoma are harsher than those employed here for virus purification, but it seems reasonable to presume that such an enzyme might be responsible for the presumed degradation in the previous observations from this laboratory (1). The presence of a protease in extensively purified virions of polyoma may indicate such an enzymic activity for a viral gene product or, just as likely, a tenacious cellular or serum contaminant. The virus preparations used in these studies are all free of detectable, cell-derived endonuclease activity, lending some support to the possibility that the virion-associated protease may be one of the virus structural proteins (11; A. Roman, J. Champoux, and T. Friedmann, unpublished data). This activity is not demonstrable until the virion is disrupted by alkaline reducing conditions or by high concentrations of SDS. Although such conditions are not used during virus purification, such an activity may still be responsible for the apparent degradation of viral VP1 in the peptide maps previously reported from this laboratory. The relationship of the endogenous proteolytic activity and the activity found after prolonged exposure to cell lysate has not been determined, and it is possible that both these proteolytic events are the result of the action of the same protease.

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LITERATURE CITED

1. **Friedmann, T.** 1974. Genetic economy of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. 71:257-259.
2. **Gibson, W.** 1974. Polyoma virus proteins. Virology 62:319-336.
3. **Fey, G., and B. Hirt.** 1974. Fingerprints of polyoma virus proteins and mouse histones. Cold Spring Harbor Symp. Quant. Biol. 39:234-241.
4. **Hewick, R., M. Fried, and M. Waterfield.** 1975. Non histone virion proteins of polyoma. Virology 66:408-419.
5. **Roblin, R., E. Härle and R. Dulbecco.** 1971. Polyoma virus proteins: multiple virion components. Virology 45:555-566.
6. **Friedmann, T., and M. Haas.** 1970. Rapid concentration and purification of polyoma virus and SV40 with polyethylene glycol. Virology 42:248-250.
7. **Friedmann, T., and W. Eckhart.** 1974. Virion proteins of polyoma temperature-sensitive mutants: late mutants. Cold Spring Harbor Symp. Quant. Biol. 39:243-246.
8. **Fairbanks, G., T. Steck, and D. Wallach.** 1971. Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606-2617.
9. **Laemmli, U.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) 227:680-684.
10. **Friedmann, T.** 1971. In vitro reassembly of shell-like particles from disrupted polyoma virus. Proc. Natl. Acad. Sci. U.S.A. 68:2574-2578.
11. **McMillen, J., M. Center and R. Consigli.** 1976. Origin of polyoma virus-associated endonuclease. J. Virol. 17:127-131.