JOURNAL OF VIROLOGY, June 1984, p. 929–938
0022-538X/84/060929-10$02.00/0

Vol. 50, No. 3

Location of DNA-Binding Proteins and Disulfide-Linked Proteins in Vaccinia Virus Structural Elements

YASUO ICHIHASHI,[1]* MASAYASU OIE,[1] AND TAKASHI TSURUHARA[2]

Department of Virology, Faculty of Medicine, Niigata University, Asahimachi, Niigata 951,[1] and Department of Microbiology, Niigata College of Pharmacy, Kamishineicho 5827, Niigata 950-21,[2] Japan

Received 6 April 1983/Accepted 28 November 1983

Treatment with sodium dodecyl sulfate (SDS) converted the vaccinia virus strain IHD-J into particles of two types: (i) ghosts which possessed a thin-membrane vesicle derived from basement part of the virus membrane with attached lateral bodies and a membranous structure derived from the core wall and (ii) aggregates of a DNA-nucleoprotein eluted from the core. These particles lacked lipids, and all the viral phospholipids were detected in the SDS-soluble fraction. The viral membrane was composed of an SDS-soluble coat layer and the basement membrane, and the basement membrane was maintained by a mechanism other than the lipid bilayer. By comparisons of protein species in morphologically distinct subviral particles prepared by several solubilizing methods, protein compositions of viral structural elements were suggested as follows: 25,000-molecular-weight viral protein–17,000-molecular-weight viral protein (VP25K-VP17K), viral basement membrane; VP13.8K, major component of the lateral body; VP70K, VP69K, VP66K, and VP64K, minor components of the lateral body; VP61K, outer layer of core wall; VP57K-VP22K, inner layer of core wall; and VP27K-VP13K, nucleoprotein. These structural elements found in the SDS-insoluble particles dissolved in the same SDS solution under reducing conditions, indicating that the disulfide linkages seem to have a principal role in maintaining their morphological integrity. VP57K, VP27K, VP13.8K, and VP13K were revealed to possess affinity for DNA. Denatured calf thymus DNA and viral DNA in double- or single-stranded form associated equally well with these proteins, but RNA did not bind. Therefore, it was strongly suggested that disulfide-linked VP27K-VP13K represented the nucleoproteins of vaccinia virus. A structural model of vaccinia virus is proposed and discussed.

The morphology of vaccinia virus has been well documented (5, 18, 19, 23, 30), and overall classification of viral outer-region proteins and core components has been established based on differential solubilization of viral membrane with Nonidet P-40 (NP-40) plus 2-mercaptoethanol (2-ME) or digestion with enzymes (2, 7, 8, 17, 22, 25). However, except for the surface tubule, which has been reported to be composed of 58,000-molecular-weight (58K) protein (29), components of vaccinia virus structural elements, even the nucleoprotein, have not been identified.

We recently found that some viral proteins, which are not soluble in sodium dodecyl sulfate (SDS) solution but are soluble in the same SDS solution under reducing conditions, are probably linked with disulfide bonds (10, 22). The high molecular weights of the disulfide-linked proteins suggested a role for the disulfide bonds in assembly or maintenance of viral substructural elements. To identify which of these disulfide-linked complexes constructs which structural elements of the virus, the virus was degraded under conditions that produce particles constituted of different subviral structures, followed by electrophoretic analyses of the proteins. A staining method for detection of DNA-binding activity was used to identify nucleoproteins of vaccinia virus.

MATERIALS AND METHODS

Cells and virus. KB cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Vaccinia virus strain IHD-J was propagated in KB cells as described previously (11, 12) and was purified by the surcrose gradient centrifugation method (14) without treatment with proteases. The virus titer was assayed in Vero cells.

Treatment of virus with detergents. Purified vaccinia virus (200 μg of protein per ml, 10^8 PFU/ml) was mixed with an equal volume of SDS solution (1% SDS, 50 mM Tris-hydrochloride buffer, pH 7.4) or NP-40 + 2-ME solution (1% NP-40, 2% 2-ME, 50 mM Tris-hydrochloride buffer, pH 7.4) and incubated at 37°C for 30 min. Samples were layered onto 36% sucrose and centrifuged at 25,000 rpm for 30 min. The supernatant above the sucrose layer was passed through a membrane filter (Millipore Corp.; 0.22 μm) and designated as the soluble fraction. The pellet beneath the sucrose layer was resuspended in Tris-hydrochloride buffer (50 mM, pH 7.4) by gentle pipetting and was pelleted again by centrifugation. Particles that were pelleted by centrifugation after treatment with NP-40 + 2-ME (core fraction; 60-μg portions) were treated with trypsin (Worthington Diagnostics; tolylsulfonyl phenolalanyl chloromethyl ketone-trypsin, 20 μg/ml, 37°C, 15 min), followed by adjustment of pH to 2.0 with 0.1 N HCl, and were centrifuged at 25,000 rpm for 30 min. The pellet was subjected to electrophoresis. The NP-40 + 2-ME-treated core fraction was further treated with 0.2% acetic acid solution (37°C, 15 min) and centrifuged at 25,000 rpm for 30 min. The supernatant and pellet were lyophilized and subjected to electrophoresis. Samples for electron microscopic analysis were prepared by standard methods.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by O'Farrell (21) for the second dimension of two-dimensional electrophoresis with 12.5% gels. Samples were dissociated in lysis buffer (2.3%

* Corresponding author.

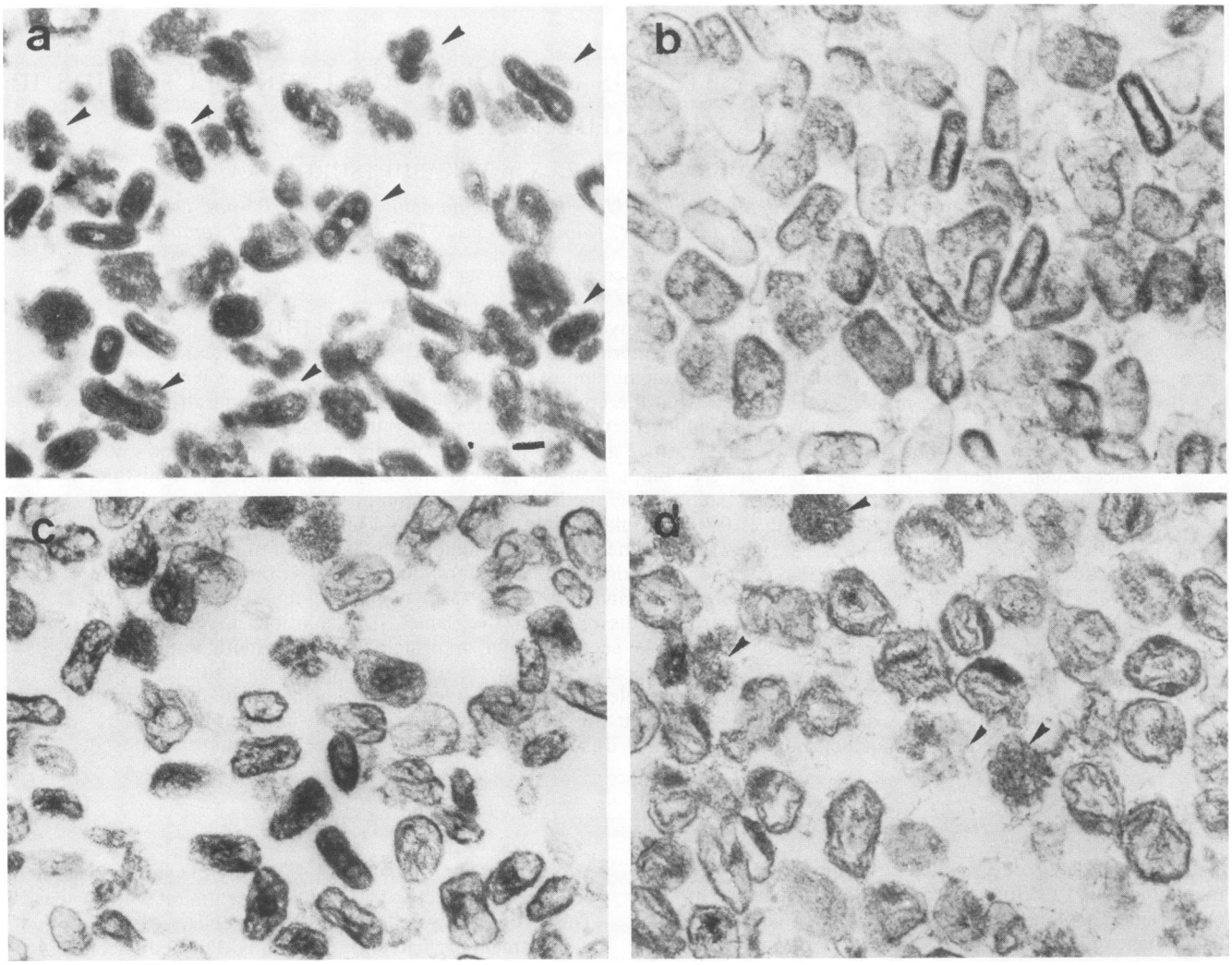

FIG. 1. Thin-section electron micrographs of virus treated with detergents. (a) Virus treated with NP-40 + 2-ME. Arrows indicate cores with attached lateral bodies. Bar, 100 nm. (b) NP-40 + 2-ME core fraction treated with trypsin. (c) NP-40 + 2-ME core fraction treated with acetic acid. (d) Virus treated with SDS. Arrows indicate filamentous aggregates.

SDS, 5% 2-ME, 10% glycerol, 60 mM Tris-hydrochloride buffer, pH 6.8) and heated at 100°C for 1 min.

Detection of DNA-binding proteins. DNA-binding activity of vaccinia virus structural proteins was detected by a staining method basically similar to that reported by Bowen et al. (3). Viral polypeptides were electrophoresed in a 12.5% polyacrylamide gel slab, and the proteins were transferred onto a nitrocellulose sheet (Toyo Roshi; TM-2, 0.45 μm) as described previously (13). The blots were soaked for 5 min in 1× SSC solution (SSC is 0.15 NaCl plus 0.015 M sodium citrate, pH 6.5) containing 0.1% bovine serum albumin and then incubated in denatured DNA solution (50 μg of calf thymus DNA per ml, 0.1% bovine serum albumin, 10 mM EDTA, 0.02% sodium azide, 1× SSC) for 30 min at room temperature. Calf thymus DNA (Sigma Chemical Co.; type I) was dissolved at 100 μg/ml in 1× SSC containing 10 mM EDTA and denatured by 20 min of incubation at 100°C, followed by quenching in ice. In some experiments vaccinia virus DNA, yeast RNA (Sigma; 100 μg/ml), or synthetic polyadenylate-polyuridylate in helical form (Sigma; 100 μg/ml) was used instead of calf thymus DNA. Vaccinia virus DNA was isolated from ^{32}P-labeled virus by treatment with proteinase K (Merck & Co., Inc.; 20 μg/ml) in the presence of 0.5% SDS, followed by extraction with phenol-chloroform, and was used with or without heat denaturation at a concentration of about 5 μg/ml (10^5 dpm/ml). After soaking in either of the above DNA solutions, the blot was rinsed briefly with 1× SSC, fixed with 10% trichloroacetic acid for 10 min, rinsed, and then stained for 5 min with ethidium bromide (0.02% in 1× SSC). The blot was photographed under short-wave UV light through a gelatin filter (Kodak; no. 22), or autoradiographed by using X-ray film (Kodak; X-Omat AR). KB cell nuclei, processed in parallel, served as control samples.

Thin-layer chromatography. Lipids in both detergent-treated virus and control virus were examined by thin-layer chromatography (28) with precoated plates (Merck; silica plate 60).

RESULTS

Structural elements of detergent-treated virus. Studies on the solubility characteristics of vaccinia virus have shown

that the pellet fraction obtained by treatment with SDS under nonreducing condition contains particles composed of 10 proteins and DNA, and the particles were thought to be "nucleoids" (9, 26, 27). However, in our repeated experiments, virus particles pelleted after treatment with SDS appeared to be of two types when negatively stained; aggregates of filamentous material and empty bags with partially bulging walls. Thin-sectioned profiles revealed the former structure to consist of an aggregate of 2-nm-wide electron-dense filaments without a limiting membrane. Similar-sized filaments were observed in some of the treated viruses (Fig. 1d and Fig. 2f and g). The latter structure was a virus ghost particle which possessed a thin-membrane vesicle together with attached lateral bodies and part of the core wall, and a large part of its DNA-nucleoprotein filament was eluted from the core chamber. The attached lateral bodies indicated that the membrane of the ghost particle was derived from a basement part of the virus membrane. The thickness of the membrane was reduced from 20 nm (intact virus membrane) to 5 to 7 nm. The viral membrane was composed of a SDS-resistant membrane (virus basement membrane) seen in the ghost particles and coat layer soluble in the SDS solution under nonreducing conditions. Treatment with higher SDS concentrations (up to 2%) produced similar particles.

When the SDS-insoluble fraction was treated with DNase, empty bags composed of viral basement membrane and lateral bodies remained, but filamentous aggregates disappeared (Fig. 3). The filaments eluted from the viral core chamber were identified as DNA, probably complexed with nucleoproteins. Residual core wall which was seen in some of SDS-treated ghost particles disappeared, which suggested association between the DNA filament and the core wall components.

Viral proteins soluble in NP-40 + 2-ME have been known to constitute the virus membrane (7, 22, 25); therefore, the component proteins of the ghost membrane could be determined by comparisons of proteins soluble in SDS and those soluble in NP-40 + 2-ME. The facts reported by Easterbrook (7) were confirmed again. Treatment of vaccinia virus with NP-40 + 2-ME solubilizes its membrane; particles pelleted after such treatment consist of the viral core and two attached lateral bodies (core fraction), and additional treatment of the core fraction with trypsin removes lateral bodies. Furthermore, the core fraction was treated with acetic acid to obtain particles of different structural elements. The treatment liberated the lateral bodies, and the pellet contained particles which had a thin membrane derived from the core wall and debris probably derived from lateral bodies (Fig. 1 and 2). Treatment with DNase reduced electron density of the core chamber (Fig. 2d).

Figure 4 is a schematic drawing of vaccinia virus degradation by treatment with detergents, and Table 1 presents a summary of morphological findings.

Protein composition of viral structural elements. The proteins present in the various viral substructures described above were analyzed by SDS-PAGE. Figure 5 shows profiles of supernatant and pellet fractions of virus dissociated in SDS or NP-40 + 2-ME solution. Proteins existing in the soluble fraction of the treatments have been analyzed (22, 25), and present results coincidentally showed that the NP-40 + 2-ME-soluble fraction contained the 54K viral protein (VP54K), VP37K, VP34K, VP32K, VP29K, VP25K, VP21K, VP18K, VP17K, and VP16K. However, VP110K, VP88K, and VP37K were more prominent in the NP-40 + 2-ME precipitate than in the soluble fraction. These proteins

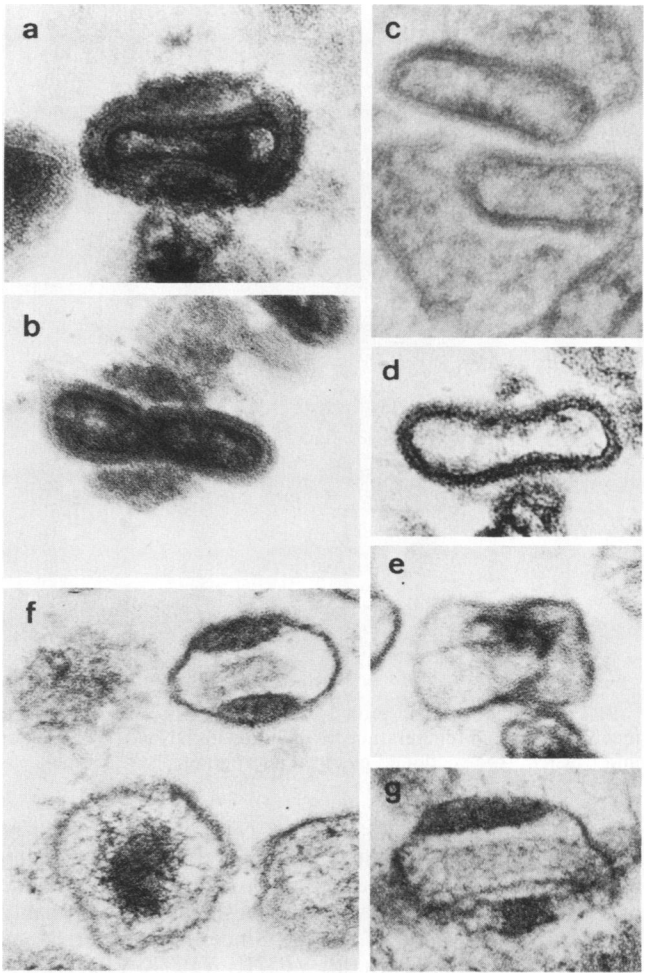

FIG. 2. High magnification electron micrographs of virus treated with detergents. (a) Control virus. (b) NP-40 + 2-ME core fraction. (c) NP-40 + 2-ME core fraction treated with trypsin. (d) NP-40 + 2-ME core fraction incubated with DNase. (e) NP-40 + 2-ME core fraction treated with acetic acid. (f and g) Virus treated with SDS. Bar, 100 nm.

may possibly be core components partially soluble in the NP-40 + 2-ME solution. But other explanations cannot be excluded; they may distribute both core and viral membrane, or different proteins of similar size may compose viral core and membrane structures. To determine which of these possibilities is the case, a new analysis method is required to overcome this limitation of the present method based on the solubility of proteins. As VP54K migrated with VP57K in 12.5% gel, they were distinguished by electrophoresis in a 8% gel and also by a staining method for DNA-binding protein which is described in the following section. These NP-40 + 2-ME-soluble proteins were components of the viral coat layer and basement membrane. Compared with SDS-soluble proteins, VP25K-VP17K was suggested to be the component of the viral basement membrane.

Protein composition of the core fraction was analyzed further by comparing the SDS-PAGE profile of the core fraction with those obtained after additional treatment with trypsin or acetic acid (Fig. 6). The major components in the core fraction (VP61K, VP57K, VP27K, and VP22K) corresponded to VP4a, VP4b, VP8, and VP9a of previous reports (22, 25). Core particles pelleted after treatment with trypsin

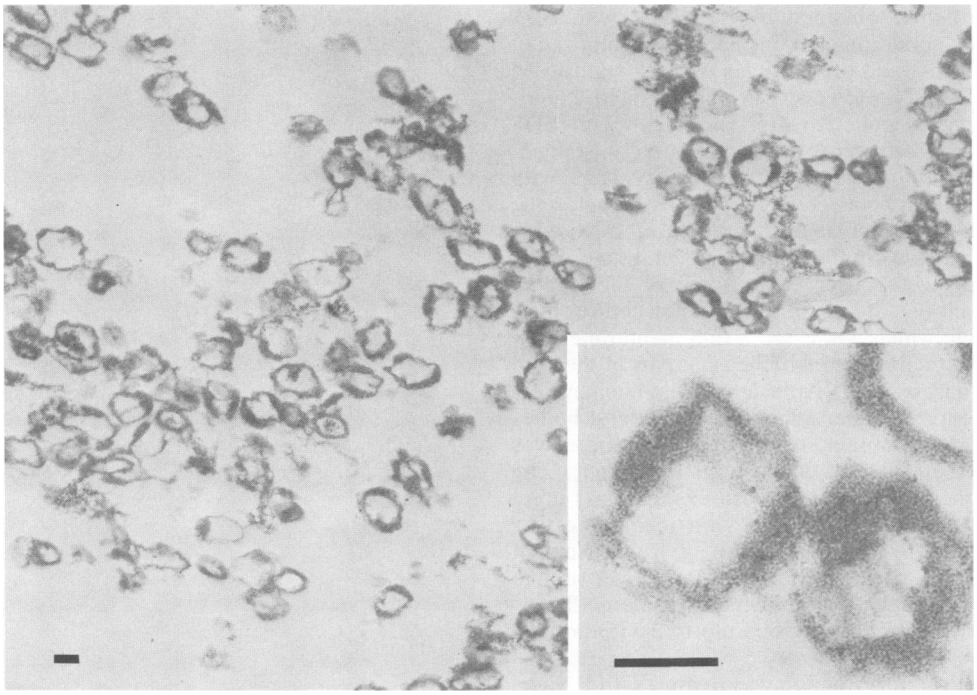

FIG. 3. Effect of DNase on SDS-treated virus. Virus (1 mg) suspended in a 1% SDS solution was centrifuged, and the precipitate was incubated at room temperature for 30 min with DNase (Worthington; DNase I, 100 U in 1 ml of 50 mM Tris-hydrochloride buffer [pH 7.4]–10 mM $CaCl_2$). Thin-sectioned electron micrograph. Bar, 100 nm.

contained VP57K, VP27K, VP22K, and a trace amount of VP13.8K, but other distinct proteins, such as that which appeared at 27.5K, were cleavage products. Disappearance of VP61K after treatment with trypsin shows that it probably occupies the surface of cores. Since VP13K exists as VP27K-VP13K complex (10), VP57K, VP27K-VP13K, and VP22K were suggested to be major components of the inner layer of core walls and of the nucleoprotein.

Treatment of the core fraction with trypsin removed lateral bodies. The proteins that clearly were diminished or reduced in amount after treatment with trypsin were determined by comparison with the profile of the core fraction; these reduced proteins were VP110K, VP88K, VP79K, VP70K, VP69K, VP66K, VP64K, VP61K, VP37K, VP32K, and VP13.8K. Some of these proteins (VP110K, VP88K, VP79K, VP74K, VP37K, and VP32K) were considered to be components of the coat layer as mentioned above. Thus, the other trypsin-sensitive proteins (VP70K, VP69K, VP66K, VP64K, VP61K and VP13.8K) were suggested to be components of lateral bodies or the outer layer of the core wall.

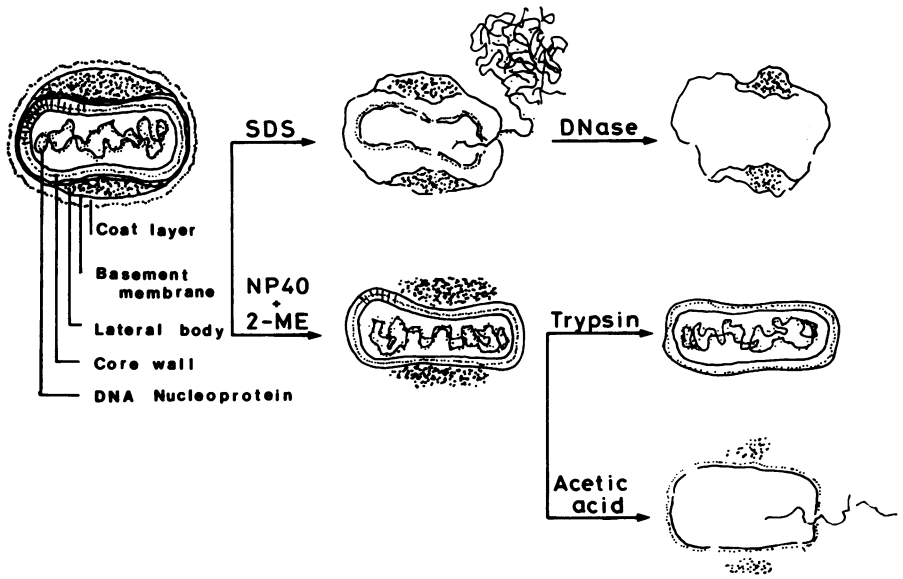

FIG. 4. Diagram of the controlled degradation of vaccinia virus by treatment with detergents.

TABLE 1. Summary of morphological studies of the effect of treatment with detergent on viral substructures

Virus substructure[a]	Presence (+) or absence (−) in pellet after treatment with[b]:				
	NP-40 + 2-ME	NP-40 + 2-ME		SDS	SDS + DNase
		+Trypsin	+Acetic acid		
CL	−	−	−	−	−
BM	−	−	−	+	+
L	+	−	±	+	+
OCW	+	±	−	−	−
ICW	+	+	+	+	+
NP	+	+	+	+	−

[a] Abbreviations: CL, coat layer; BM, basement membrane; L, lateral bodies; OCW, outer layer of core wall; ICW, inner layer of core wall; NP, DNA-nucleoprotein.

[b] ±, Degraded residual forms of the structure.

Treatment of the core fraction with 2% acetic acid liberated three proteins (VP61K, VP54K, and VP37K) and part of VP32K, VP27K, VP16K, VP13.8K, and VP13K. The pellet contained VP57K, VP32K, VP22K, VP13.8K and reduced amounts of VP61K and VP27K. Thus, the treatment with acetic acid did not clearly distinguish the location of these proteins; however, VP57K and VP22K were exceptional components; they were predominantly detectable in the acetic acid pellet fraction. As these two proteins appeared together in pelleted samples prepared by treatment of the virus with SDS, NP-40 + 2-ME, NP-40 + 2-ME + DNase, NP-40 + 2-ME + trypsin, or NP-40 + 2-ME + acetic acid, they probably form a substructure which is commonly detectable in these pellets, namely, the inner layer of the core wall (VP57K-VP22K complex).

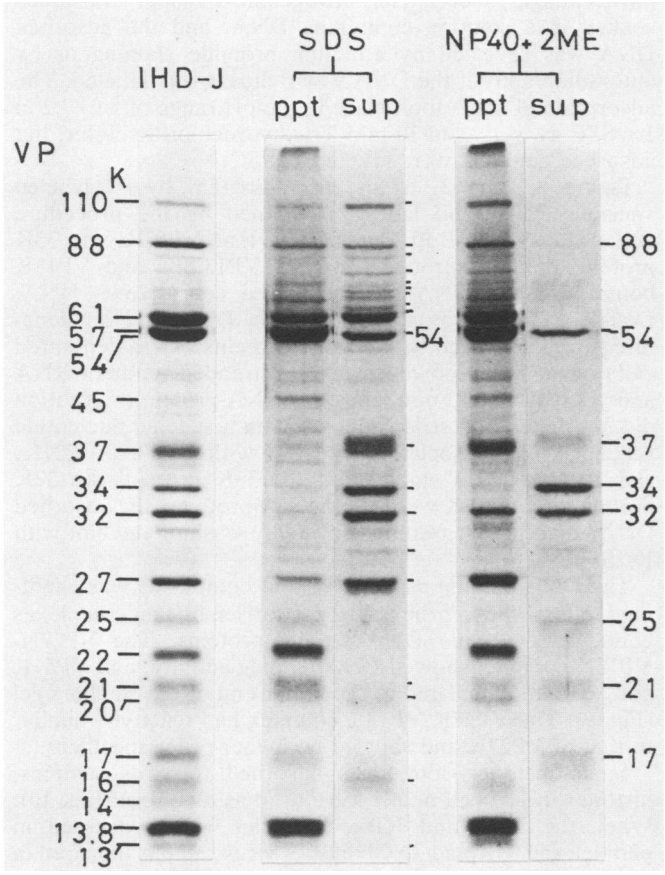

FIG. 5. Proteins in the supernatant and pellet after treatment with SDS or NP-40 + 2-ME. Virus was suspended in SDS (final concentration of 1%) or NP-40 + 2-ME (0.5% NP-40, 2% 2-ME, 50 mM Tris-hydrochloride buffer, pH 7.4) and centrifuged at 25,000 rpm for 30 min. Samples were electrophoresed in a 12.5% gel. VP54K in the supernatant solution was distinguished from VP57K by SDS-PAGE in a 8% gel and also by staining VP57K by the method to detect DNA-binding protein. ppt, Precipitated pellet; sup, supernatant.

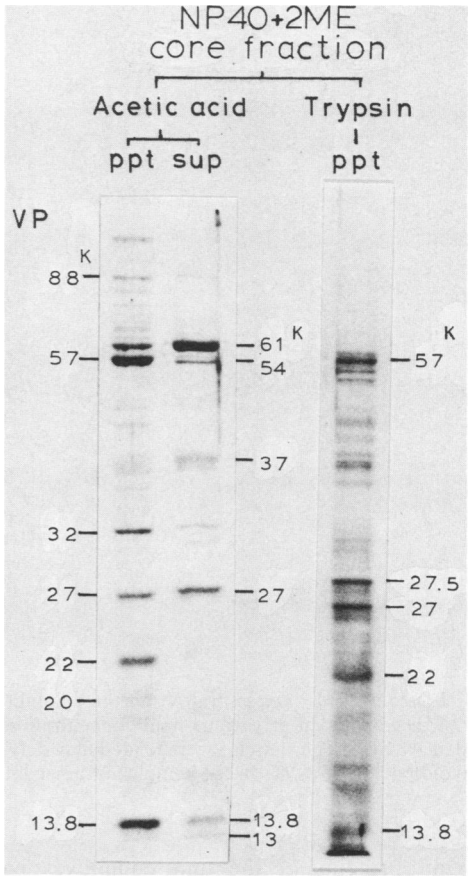

FIG. 6. SDS-PAGE profiles of viral core fractions treated with trypsin or acetic acid. The viral core fraction prepared by treatment with NP-40 + 2-ME was suspended in acetic acid (final concentration, 2%) or treated with trypsin (final concentration, 20 μg/ml; 37°C, 15 min) and centrifuged at 25,000 rpm for 30 min. The precipitated pellets (ppt) of the trypsin-treated cores and the ppt and supernatants (sup) of the lyophilized acetic acid-treated samples were subjected to SDS-PAGE in a 12.5% gel.

Disulfide-linked viral proteins. The virus that was pelleted after treatment with SDS contained no detectable amounts of lipids when examined by thin-layer chromatography; all viral lipids were found in the SDS-soluble fraction. The absence of lipids in the viral ghost particles and the existence of membrane structures in them indicated that the membranes were not maintained by a lipid bilayer but rather by linked proteins. The fact that the SDS-insoluble fraction can be dissolved in SDS + 2-ME solution strongly suggested that disulfide linkages in the proteins participate in maintaining the viral structures.

Figure 7 shows the effect of 2-ME concentration on the solubility of viral proteins which are suspended in SDS solution. SDS-soluble proteins, oligomers, and unit complexes appeared in the profile of the lane with SDS alone.

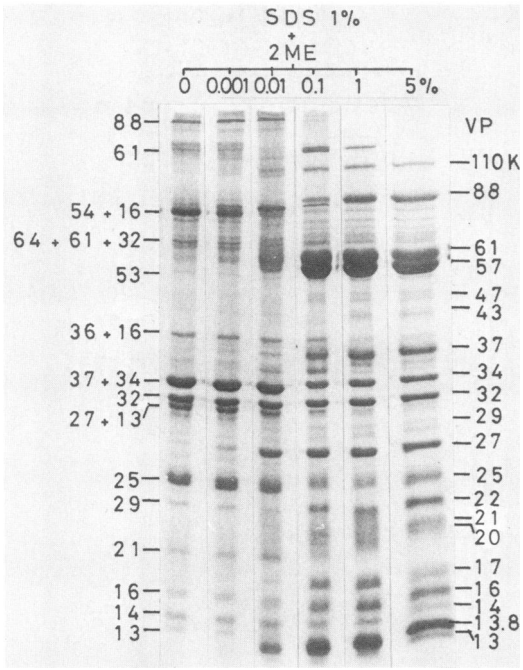

FIG. 7. Effect of 2-ME concentration on the solubility of virus proteins. 2-ME was added at various final concentrations to virus suspended in 1% SDS. The samples were loaded in widely separated lanes to avoid effects of 2-ME in the samples of adjacent lanes.

The subunit proteins of the unit complexes have been identified by the two-dimensional SDS-PAGE method, where the first dimension was performed under nonreducing conditions and the second dimension under reducing conditions. VP25K has been identified as the dimer form of VP17K (VP25K-VP17K), VP27K-VP13K exists as a complex, and some proteins (VP21K and VP29K) migrate with different speeds when they are solubilized in SDS or in SDS + 2-ME solutions (10). Results obtained by such analysis are presented at the left side of Fig. 7. In this experiment, the virus samples were loaded on the gel without separation of SDS-resistant particles to reveal all SDS-soluble components. If the particles were eliminated from the sample, VP25K, VP27K-VP13K, VP88K oligomer, VP61K oligomer, and VP70K to VP63K minor components were all reduced to trace amounts. The parallel lanes show that lower-molecular-weight proteins, such as VP14K, VP13.8K, and VP13K, migrated faster when virus was solubilized in 1% SDS containing 0.01 to 1% 2-ME than those that were solubilized in 1% SDS containing 5% 2-ME. Trace amounts of VP27K and VP13K were detectable in the lane with SDS alone; however, significant dissociation of the VP27K-VP13K complex into the subunits took place at a concentration of 0.01% 2-ME in 1% SDS solution, and complete separation of these subunit proteins occurred at a higher concentration of 2-ME (1%). VP13.8K was detectable in the lane where the sample was treated with 1% SDS + 0.01% 2-ME and seemed to be solubilized completely in 1% SDS + 0.1% 2-ME. Two major core components (VP61K and VP57K) appeared in the profile when the virus was treated with 1% SDS containing higher than 0.1% 2-ME. The VP54K-VP16K complex dissociated into subunits at 0.01% 2-ME, the VP37K-VP34K complexes dissociated into subunits at 0.1% 2-ME, and complete dissociation of VP133K required 5% 2-ME. Thus, each structural protein was affected differentially by the reducing conditions in SDS solution.

VP57K-VP22K, VP13.8K, and a large part of VP61K were barely soluble in SDS and did not migrate in the 12.5% gel. Since proteins smaller than about 2×10^5 daltons could be electrophoresed in the 12.5% polyacrylamide gel, all these proteins apparently existed in the virus particles in highly multiplied form, exceeding 2×10^5 daltons. Therefore, it was strongly suggested that the lateral bodies, core outer layer, and core inner layer, which remained after treatment with 1% SDS, were constructed by a bond highly sensitive to a reducing condition such as disulfide linkage. On the other hand, VP25K-VP17K and VP27K-VP13K complexes migrated in the gel. These two complexes also associated with SDS-insoluble particles; however, the association seemed to be weak and breakable by electrophoretic drive.

The results of SDS-PAGE analysis are summarized in Table 2.

Viral DNA-binding proteins. To identify vaccinia virus nucleoprotein, we employed the method for detecting proteins with DNA-binding capacity. This method is basically similar to that reported by Bowen et al. (3). Purified virus was electrophoresed in a slab gel by SDS-PAGE, and the viral proteins were electrophoretically transferred onto a nitrocellulose sheet. The nitrocellulose sheet was then soaked in a solution containing DNA, and the adsorbed DNA was revealed by ethidium bromide staining or by autoradiography if the DNA was radioactively labeled. The adsorption of DNA took place in the pH range of 5 to 9.2 in 1× SSC as well as in 10 mM Tris-hydrochloride buffer, but adsorbed amounts were low in 4× SSC.

Figure 8, lane 2, is an autoradiogram by ^{32}P-labeled vaccinia DNA, and lane 3 is stained by the procedure described above. Both lanes show that VP57K, the 33K protein, the 32K protein, VP27K, VP13.8K, and VP13K bound vaccinia DNA and denatured calf thymus DNA. Double-stranded ^{32}P-labeled vaccinia DNA and its denatured form adsorbed to the same proteins as did denatured calf thymus DNA; however, double-stranded synthetic RNA and yeast RNA did not. Thus the DNA-protein association did not depend on strandedness or on a specific nucleotide sequence. The association of DNA with VP57K, VP27K, and VP13.8K was stable, and the 33K protein, the 32K protein, and VP13K were low-affinity proteins; the adsorbed DNA could be released by repeatedly washing the blot with 1× SSC.

The DNA-binding capacity of unit complexes was examined to test whether the activity is carried by unit complexes containing vaccinia DNA-binding proteins. The VP27K-VP13K complex showed DNA-binding activity, and VP57K and VP13.8K appeared at the upper end of the stacking gel (Fig. 9). The VP27K-VP13K complex had reactivity similar to that of VP27K and showed no higher-order specificity.

Since histones sometimes adsorbed to virus samples, histones in KB cell nuclei were used as a control (Fig. 10). When the virus and KB cell nuclei were processed in parallel, the 33K and 32K bands of virus sample appeared in positions identical to those of the histone H1 bands, suggesting slight contamination by H1 of the virus sample, whereas other viral DNA-binding proteins migrated to positions different from those occupied by histones.

Model for vaccinia virus structure. The locations of viral proteins in vaccinia virus particles were determined by comparing the results presented in Table 1 and 2, and the locations are presented in the last column of Table 2.

NP-40 + 2-ME-soluble proteins have been classified as

TABLE 2. Protein constituents of virus fractions treated with detergents[a]

VP	NP-40 + 2-ME		NP-40 + 2-ME pellet			SDS		DNA binding	Location[b]	Unit complex
			+Trypsin (pellet)	+Acetic acid						
	sup	pellet		sup	pellet	sup	pellet			
110K	−	+	−	−	+	++	+			
88K	−	+	−	+	+	+	+			
80K	−	+	−	−	+	+	−			
79K	−	+	−	−	+	+	−			
74K	−	+	−	−	+	+	−			
70K	−	+	−	−	+	−	+		L	
69K	−	+	−	−	+	+	−		L	
66K	−	+	−	−	+	−	+		L	
64K	−	+	−	−		+	−		L	
61K	−	+	+	+++	+	+++	+		OCW	
57K	−	+++	++	−	+++	−	+++	+++	ICW	VP57K-VP22K
54K	++	+	−	+	−	+++	−		CL	
53K	−	+	−	−	+	+	−			
47K	−	+	−	−	+	−	+			
44K	−		−	−	+	−	+			
43K	−	+	−	−	−	−	−			
40K	−	+	−	−	−	−	+			
37K	+	+	−	++	+	++	−		CL	
36K	+	+	−	−	++	−	+			
35K	+	+	−	−	+	+	−			
34K	+++	−	−	−	−	+++	−		CL	
32K	+++	++	−	−	++	+++	−		CL	
31K	−	+	−	−	−	++	−			
29K	++	−	−	+	+	++	+		CL	
28.5K	+	−	−	−	−	+	+			
27K	−	++	++	++	+	+++	+	+++	NP	VP27K-VP13K
25K	++	−	−	−	−	−	++		BM	VP25K-VP17K
22K	−	+++	++	−	+++	−	+++		ICW	
21K	++	−	−	−	+	−	++		CL	
20K	−	+	−	−	+	+	−			
18K	+	−	−	−	−	−	−		CL	
17K	++	−	−	−	−	−	++		BM	
16K	+	++	−	++	−	++	−		CLL	
14.2K	+	−	−	−	−	+	−		CL	
13.8K	−	+++	−	+++	−	−	+++	+++	L	
13K	+	+	+	+	+	+	−	+	CL, NP	

[a] Relative amounts of viral proteins in the samples and intensity of DNA-binding capacity of proteins were measured with a densitometer and expressed as four grades (−, +, ++, +++). sup, Supernatant.

[b] Abbreviations are the same as those in Table 1, footnote *a*.

components of the virus membrane, which is composed of coat layer and virus basement membrane. The virus basement membrane seems to be composed of the VP25K-VP17K, and the other proteins in the NP-40 + 2-ME-soluble fraction are grouped to coat layer components. The NP-40 + 2-ME-soluble fraction formed a band at a density of 1.22 g/ml when it was centrifuged in 10 to 25% tartrate density gradient. The density was similar to that of surface tubule (29); however, the artificially aggregated material in the tartrate gradient had no tubular figure and contained VP54K, VP20K, and VP16K. Apparently, further studies are necessary to elucidate the organization of these coat layer components on the viral membrane or to identify the surface tubule components. By comparison of the core fraction before and after treatment with trypsin, the lateral bodies were suggested to be composed of VP13.8K, VP70K, VP69K, VP66K, and VP64K. Because of the appearance of VP57K-VP22K in all the examined pellet fractions which contained the inner layer of the core wall, VP61K was concluded to be a component of the outer layer of the core wall, and VP57K-VP22K was concluded to be a component of the inner layer of the core wall.

Among detected vaccinia DNA-binding proteins, the amounts of 33K and 32K proteins are too small to be viral nucleoprotein and are probably derived from host histone H1. VP13.8K is ascribed to be the lateral body component; thus, either VP27K-VP13K or VP57K-VP22K is the nucleoprotein of vaccinia virus. When the core fraction was treated

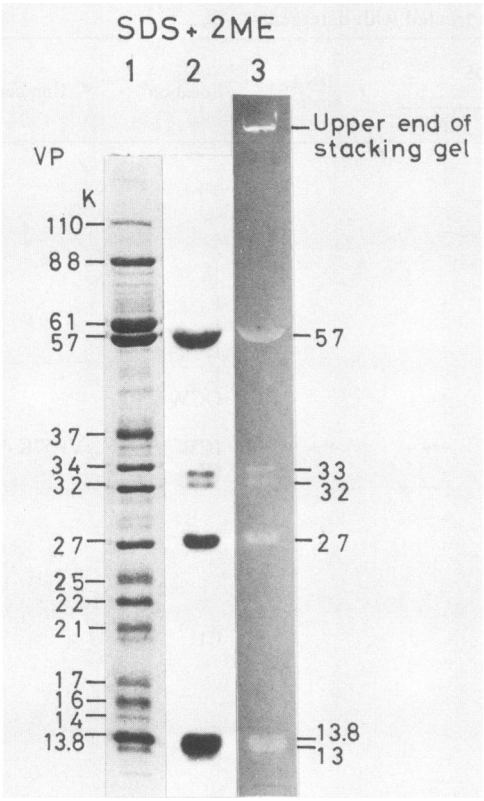

FIG. 8. DNA-binding proteins of vaccinia virus. Nitrocellulose sheets blotted with viral proteins were immersed for 30 min in denatured ^{32}P-labeled vaccinia virus DNA solution (10^5 dpm/ml, 0.01% bovine serum albumin, 0.02% sodium azide, 1× SSC) or in denatured calf thymus DNA solution (50 μg of DNA per ml, 0.01% bovine serum albumin, 0.02% sodium azide, 10 mM EDTA, 1× SSC). Bound DNA was detected by staining with ethidium bromide or autoradiographically by using Kodak X-Omat AR film. Lane 1, SDS-PAGE profile of control virus stained with Coomassie brilliant blue. Lane 2, autoradiogram of a nitrocellulose sheet processed as described in the text and soaked in ^{32}P-labeled vaccinia DNA. Lane 3, photograph of a nitrocellulose sheet processed as above and stained with denatured calf thymus DNA and ethidium bromide solutions. The bright band at the upper end of the stacking gel represents viral DNA in the electrophoresed sample.

with acetic acid, DNA and VP27K-VP13K were found in the supernatant, and VP57K-VP22K were recovered in the pellet. Based on the simultaneous solubilization of VP27K-VP13K and DNA, and on the existence of VP57K-VP22K in the core fraction treated with DNase, the VP27K-VP13K complex is concluded to be the nucleoprotein of vaccinia virus.

DISCUSSION

The viral membrane, which is composed of proteins and lipids, is assembled in the viroplasm according to a process that is quite different from that observed in cellular unit membrane formation. Morphologically, immature virus particles possess a membrane resembling a lipid bilayer unit membrane coated externally with a uniform spicule layer (6). The present results show that the unit membrane defined by electron microscopy corresponds to the membrane of the SDS-treated ghost particle, but it is not a lipid bilayer membrane. The virus membrane is stable in SDS even after complete removal of phospholipids, whereas the lipid bilayer of biological unit membranes can be dissociated easily by treatment with SDS. The absence of lipids in SDS-treated virus particles and the solubility of the viral ghost particles in SDS under reducing conditions indicate that disulfide bonds in the polymer of VP25K-VP17K complexes are the major bonds holding the viral membrane structure together. Based on these findings, we hypothesize that the morphogenesis of vaccinia virus membrane in the viroplasm starts by self-assembly of the basement membrane via interaction between VP25K-VP17K unit complexes. Spicules which may be composed of coat layer proteins then associate with the basement membrane, and lipids are incorporated into specific receptors of the membrane or coat proteins, or both. This hypothesis is consistent with the comment of Blough and Tiffany (1) that the amount of viral lipids is too small and that the lipids are of inadequate composition to form a continuous biological lipid bilayer membrane around the virus particle. Transfer of membrane phospholipids to purified virus (28) also supports this hypothesis. It is an interesting characteristic of the viral membrane that, despite differences in how it is constructed and what its components are, it can fuse with the cellular membrane; this has been shown by electron microscopic study of virus penetration (4).

In addition to the VP25K-VP17K complex located in the virus basement membrane, VP57K-VP22K (inner layer of the core wall), VP27K-VP13K (nucleoprotein), and VP13.8K (major component of the lateral body) were found

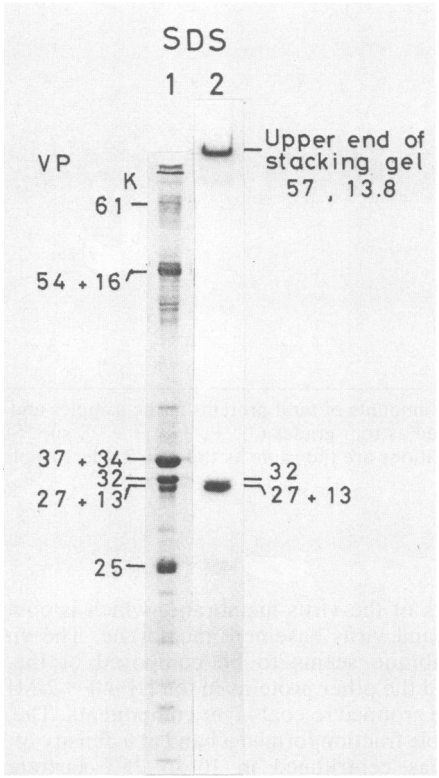

FIG. 9. DNA-binding activity of the VP27K-VP13K unit complex. Vaccinia virus suspended in 1% SDS solution (30 μg of protein in 20 μl of SDS solution) was electrophoresed in a 12.5% gel. Lane 1, profile by Coomassie blue staining. Lane 2, an autoradiograph. The proteins in the gel were transferred to a nitrocellulose sheet, and the sheet was incubated with ^{32}P-labeled vaccinia virus DNA following the procedure described in the text.

to exist as high-molecular-weight multiplicated proteins. The disulfide bonds may participate in formation of virus substructures by intramolecular or intermolecular bindings. The abundance of such proteins suggests that not only the virus membrane but also core morphogenesis is ascribable to self-assembly, which is largely dependent on sulfhydryl-disulfide exchange reactions. Polymerization coupled with cleavage processing of precursor protein is suggested in the finding that VP63K, which exists in the cytoplasm of the virus-infected cells in dissolved form, has a peptide composition similar to that of VP57K (13, 16). The enzymes catalizing the association of sulfhydryl proteins and dissociation of disulfide-bonded proteins may have important roles in morphogenesis of the virus particle and probably also in the uncoating process when virus infects cells.

We found that vaccinia basic proteins, namely, VP57K, VP27K, VP13.8K, and VP13K, possess affinity for DNA, but the other viral basic proteins, such as VP61K, VP32K, and VP25K-VP17K, did not show affinity for DNA. The binding occurs in solutions of wide pH and ionic-strength ranges, with no apparent specificity for strandedness or DNA sequence. However, since viral proteins had been solubilized in SDS + 2-ME (100°C, 1 min) and then transferred onto a nitrocellulose sheet in a electrophoretic buffer containing 20% methanol, such drastic conditions may destroy higher-order specificity. It also suggests that detected DNA-binding capacity itself could possibly be artifactual, but this possibility is thought to be less plausible because DNA binding capacity of histone and adenovirus nucleoprotein is specifically detected by this staining method.

Vaccinia virus DNA-binding proteins have been studied by column chromatography by using affinity to DNA-bound substrate. By comparison to the reported SDS-PAGE profiles of DNA-binding proteins, the molecular size of VP27K seems to correspond to FP14 of Nowakowski et al. (20). VP13K shares features such as DNA-binding capacity, basic charge, phosphorylation, and solubility in NP-40 with the previously reported arginine-rich basic protein of Pogo et al. (24) and the DNA-binding protein of Kao et al. (15). However, because the experimental conditions and procedures used by those workers were quite different from ours, direct comparison between their proteins and VP13K is not possible. And there seem to be several viral proteins of about 13K that have different isoelectric points (22). Nevertheless, we propose that their proteins are our VP13K, because the other DNA-binding protein of similar molecular size (VP13.8K) is different in two features: it is not soluble in NP-40 and lacks phosphate.

One can speculate on the function of the DNA-binding proteins by their location in the virion; the function of VP57K-VP22K core wall proteins may be to package the DNA nucleoprotein complex; the function of VP27K-VP13K may be that of nucleoprotein, to stabilize DNA and modulate gene function. Considering that the lateral bodies are released into the cytoplasm just after virus penetration, VP13.8K may play a role in the shut-down of host cell DNA synthesis. Apparently further studies are necessary to confirm these points.

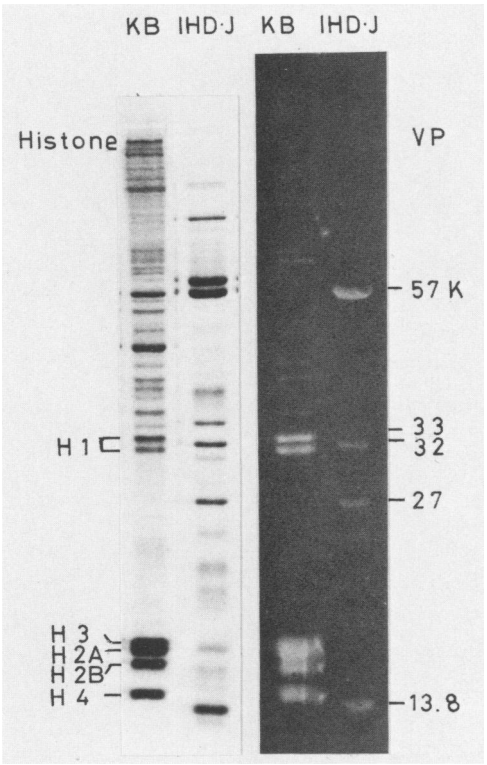

FIG. 10. Comparison of histones and vaccinia DNA-binding proteins. KB cell nuclei were prepared by treating KB cells with 0.02% NP-40 in 1× SSC. KB cell nuclei and vaccinia virus were electrophoresed in parallel, blotted, and stained as described in the text. KB cell histones were identified by comparison with standard histone samples.

ACKNOWLEDGMENTS

We thank W. K. Joklik and D. J. Pickup for critically reviewing this manuscript. Y.I. appreciates the continuous encouragement and support of the late S. Matsumoto.

LITERATURE CITED

1. **Blough, H. A., and I. M. Tiffany.** 1973. Lipid in viruses. Adv. Lipid Res. **11**:267–339.
2. **Boisvert, J., and T. Yamamoto.** 1977. Effect of detergents on purified vaccinia virus: analysis by SDS polyacrylamide gel electrophoresis and electron microscopy. Can. J. Microbiol. **23**:240–252.
3. **Bowen, B., J. Steinberg, U. K. Laemmli, and H. Weintraub.** 1980. The detection of DNA-binding proteins by protein blotting. Nucleic Acids Res. **8**:1–20.
4. **Chang, A., and D. H. Metz.** 1976. Further investigations on the mode of entry of vaccinia virus into cells. J. Gen. Virol. **32**:275–282.
5. **Dales, S.** 1963. The uptake and development of vaccinia virus in strain L cells followed with labeled viral deoxyribonucleic acid. J. Cell Biol. **18**:51–72.
6. **Dales, S., and E. H. Mosbach.** 1968. Vaccinia as a model for membrane biogenesis. Virology **35**:564–583.
7. **Easterbrook, K. B.** 1966. Controlled degradation of vaccinia virions in vitro: an electron microscopic study. J. Ultrastruct. Res. **14**:484–496.
8. **Holowczak, J. A., and W. K. Joklik.** 1967. Studies of the structural proteins of virions and cores. Virology **33**:717–725.
9. **Holowczak, J. A., V. L. Thomas, and L. Flores.** 1975. Isolation and characterization of vaccinia virus "nucleoids." Virology **67**:506–519.
10. **Ichihashi, Y.** 1981. Unit complex of vaccinia polypeptides linked by disulfide bridges. Virology **113**:277–284.
11. **Ichihashi, Y., and M. Oie.** 1980. Adsorption and penetration of the trypsinized vaccinia virion. Virology **101**:50–60.
12. **Ichihashi, Y., and M. Oie.** 1982. Proteolytic activation of vaccinia virus for the penetration phase of infection. Virology

116:297–305.
13. **Ichihashi, Y., T. Tsuruhara, and M. Oie.** 1982. The effect of proteolytic enzymes on the infectivity of vaccinia virus. Virology **122**:279–289.
14. **Joklik, W. K.** 1962. The purification of four strains of poxviruses. Virology **18**:9–18.
15. **Kao, S.-Y., E. Ressner, J. Kates, and W. R. Bauer.** 1981. Purification and characterization of a superhelix binding protein from vaccinia virus. Virology **111**:500–508.
16. **Kats, E., and B. Moss.** 1970. Formation of a vaccinia virus structural polypeptide form a high molecular weight precursor: inhibition by rifampicin. Proc. Natl. Acad. Sci. U.S.A. **66**:677–684.
17. **McCrae, M. A., and J. F. Szilagyi.** 1975. Preparation and characterization of a subviral particle of vaccinia virus containing the DNA-dependent RNA polymerase activity. Virology **68**:234–244.
18. **Medzon, E. L., and H. Bauer.** 1970. Structural features of vaccinia virus revealed by negative staining, sectioning and freeze-etching. Virology **40**:860–867.
19. **Morgan, C.** 1976. Vaccinia virus reexamined: development and release. Virology **73**:43–58.
20. **Nowakowski, M., J. Kates, and W. Bauer.** 1978. Isolation of two DNA-binding proteins from the intracellular replication complex of vaccinia virus. Virology **84**:260–267.
21. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
22. **Oie, M., and Y. Ichihashi.** 1981. Characterization of vaccinia polypeptides. Virology **113**:263–276.
23. **Peters, D., and G. Müller.** 1963. The fine structure of the DNA-containing core of vaccinia virus. Virology **21**:266–269.
24. **Pogo, B. G. T., J. R. Katz, and S. Dales.** 1975. Biogenesis of poxviruses: synthesis and phosphorylation of a basic protein associated with the DNA. Virology **64**:531–543.
25. **Sarov, I., and W. K. Joklik.** 1972. Studies on the nature and location of the capsid polypeptides of vaccinia virion. Virology **50**:579–592.
26. **Soloski, M. J., C. V. Cabrera, M. Esteban, and J. A. Holowczak.** 1979. Studies concerning the structure and organization of the vaccinia virus nucleoid. I. Isolation and characterization of subviral particles prepared by treating virions with guanidine-HCl, Nonidet P-40, and 2-mercaptoethanol. Virology **99**:209–217.
27. **Soloski, M. J., and J. A. Holowczak.** 1981. Characterization of supercoiled nucleoprotein complexes released from detergent-treated vaccinia virions. J. Virol. **37**:770–783.
28. **Stern, W., and S. Dales.** 1974. Biogenesis of vaccinia: concerning the origin of the envelope phospholipids. Virology **62**:293–306.
29. **Stern, W., and S. Dales.** Biogenesis of vaccinia: isolation and characterization of a surface component that elicit antibody suppressing infectivity and cell-cell fusion. Virology **75**:232–241.
30. **Westwood, J. C. N., W. J. Harris, H. T. Zwartouw, D. H. J. Titmuss, and G. Appleyard.** 1964. Studies on the structure of vaccinia virus. J. Gen. Microbiol. **34**:67–78.