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STUDIES ON THE POLYPEPTIDES OF POXVIRUS
I. COMPARISON OF STRUCTURAL POLYPEPTIDES IN VACCINIA, COWPOX AND SHOPE FIBROMA VIRUSES

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SUMMARY Radioactively labeled vaccinia, cowpox and Shope fibroma virions free from any detectable contamination with host cell protein, were dissociated into their constituent polypeptides, and these were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The profiles of constituent polypeptide bands of four strains of vaccinia virus (IHD-W, IHD-J, Lister and DIs) were almost the same, except that a polypeptide of about 41,000 daltons was not detectable in the autoradiogram of strain IHD-W which has no hemagglutinin. The profile of polypeptide bands of cowpox virions was also almost the same as that of vaccinia virions, except for several polypeptides of about 40,000 to 50,000 daltons, but the profile of Shope fibroma virions differed considerably from that of vaccinia or cowpox virions.

INTRODUCTION

Procedures have been established for purification of pox viruses, principally by zonal sedimentation in a sucrose density gradient (Joklik, 1962a, b; Zwartouw et al., 1962) and equilibrium centrifugation in a gradient of cesium chloride (Planterose et al., 1962) or potassium tartrate (Pfau and McCrea, 1963). Joklik (1962a) purified four strains of poxvirus belonging to the vaccinia-variola subgroup (vaccinia, rabbitpox, cowpox and ectromelia) from infected chorioallantoic membranes by sucrose gradient centrifugation.

Significant progress in defining the polypeptide components of virions was achieved after introduction of the procedure of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Summers et al., 1965), and using this technique up to 30 bands can be resolved from radioactively labeled vaccinia virions (Holowczak and Joklik, 1967; Moss and Salzman, 1968; Sarov and Joklik, 1972). Arita and Tagaya (1977) compared the structural polypeptides of the virions of variola, monkeypox, cowpox and vaccinia viruses by SDS-PAGE and then staining with Coomassie blue.

We are attempting to identify poxvirus antigens in terms of virus-induced polypeptides. In this paper, we have initiated to undertake in an attempt to compare the structural polypeptides in radioactively labeled vaccinia,

cowpox and Shope fibroma virions.

MATERIALS AND METHODS

1. *Cells and viruses*

HeLa S3 and RK_{13} cells, purchased from Flow Laboratories, were propagated as monolayers in Eagle's minimum essential medium (MEM) (Eagle, 1959) supplemented with 10% calf serum. Primary chick embryo fibroblasts (CEF) were propagated in MEM supplemented with 5% calf serum.

The IHD-W and IHD-J strains of vaccinia virus, which were kindly supplied by Dr. Y. Ichihashi, Niigata University, were grown on monolayers of HeLa S3 or RK_{13} cells. The Lister strain of vaccinia virus, cowpox virus (LB red strain) and Shope fibroma virus (OA strain) were grown on RK_{13} cells. The DIs strain of vaccinia virus, isolated by Tagaya et al. (1961) and kindly supplied by Dr. I. Tagaya, National Institute of Health, Tokyo, was grown on CEF.

Viruses were purified by the method of Joklik (1962a) with slight modifications, and were used as stock viruses. Infected cells were scraped off petri dishes and centrifuged with culture fluid at 750×g for 10 min at 4 C. The supernatant was separated and the cell pellet was resuspended in 10 mM tris (hydroxymethyl) amino methane (Tris)-HCl buffer, pH 9.0, sonicated for 3 min in an ice cold bath and centrifuged at 750×g for 10 min at 4 C to remove cell debris. The resulting supernatant and previous supernatant were combined and centrifuged at 15,000×g for 30 min at 4 C, and the precipitate was resuspended in 1 mM Tris-HCl buffer, pH 9.0. The suspension was layered on 36% sucrose in Tris buffer, and centrifuged at 23,000×g for 80 min at 4 C in a Beckman SW 25.1 rotor. The pellet was suspended in a small volume of the same Tris buffer, layered on a sucrose gradient (25–40% sucrose in 1 mM Tris buffer) and centrifuged at 23,000×g for 45 min at 4 C in a Beckman SW 25.1 rotor. The virus band was collected by side-puncture with a needle, diluted with 1 mM Tris buffer and centrifuged at 23,000×g for 80 min at 4 C. The resulting pellet was suspended in phosphate-buffered saline, pH 7.4 (PBS), and stored at −20 C until use.

2. *Infectivity titration of inoculum*

The infectivity of all viruses, except Shope fibroma virus, was assayed by plaque-formation on the same cells as those in which the viruses had been grown. The infectivity of Shope fibroma virus was assayed by focus-formation (Mantani et al., 1970).

3. *Preparation of radioactive virions*

HeLa S3, RK_{13} cells or CEF were infected at a multiplicity of 10 plaque-forming, or focus-forming, units in PBS (1 ml per petri dish of 90 mm diameter).

After adsorption for 1 hr at 37 C, infected cells were washed three times with PBS and incubated for 12 hr in MEM supplemented with 2% calf serum. The culture medium was then replaced by radioactive medium, and incubation was continued for another 24 hr at 37 C. Two kinds of radioactive medium were used: one consisted of MEM containing one-tenth the normal concentration of leucine, 2% dialyzed calf serum and 1 μCi/ml of [^{14}C]-leucine (297 mCi/mmole); the other consisted of MEM containing one-tenth the normal concentration of methionine, 2% dialyzed calf serum and 2 μCi/ml of [^{35}S]-methionine (462.95 Ci/mmole).

After labeling, infected cells and culture fluid were collected and used for purification of virions. Radioactive virions were purified by the procedure described above, except that banding by centrifugation in a sucrose gradient was carried out twice in a Beckman SW 41 rotor.

Radioisotopes were purchased from the New England Nuclear.

4. *SDS-PAGE*

SDS-PAGE was conducted in 13% polyacrylamide slab gels (1 mm thick, 100 mm long) as described by Maizel (1971). The ratio of acrylamide to bisacrylamide was 30 to 0.8.

Radioactive virions purified by sucrose density gradient were collected by centrifugation, suspended in "sample buffer" (1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.001% phenol red, and 50 mM Tris-HCl buffer, pH 8.2) and solubilized by heating at 100 C for 90 sec. Electrophoresis was carried out at a constant voltage of 100 V, and continued until the phenol red marker reached the anodal end of the gel.

After electrophoresis, the gel was stained with Coomassie blue R250 and destained as described by Stern and Dales (1976). Kodak "X-Omat" R film was used for autoradiography.

RESULTS

1. *Demonstration of absence of contaminating host cell protein*

For confirmation that the purified virion fraction was not contaminated with host cell protein, the following two virion samples were prepared: Sample "A" was purified virions prepared from infected cells labeled with [^{14}C]-leucine or [^{35}S]-methionine as described in the Materials and Methods; Sample " B " was purified virions prepared from a mixture of unlabeled infected cells and an equal numbers of uninfected cells labeled with [^{14}C]-leucine or [^{35}S]-methionine. Sample " B " consistently contained slight radioactivity (usually less than 0.5% of the radioactivity of sample "A"). Samples "A" and " B " were then analyzed by SDS-PAGE, and their stained bands and labeled bands were compared.

If the purified virion fraction contained host cell proteins, bands of the latter should be detected in the autoradiogram of sample " B ". Figure 1 shows the polypeptide patterns of samples "A" and " B " from four strains of vaccinia, and from cowpox and Shope fibroma viruses. In gels stained with Coomassie blue the relative amounts of individual polypeptides in samples "A" and " B " were constant, irrespective of the cell source or the radioactive precursor. Autoradiograms prepared from the same gel, showed many polypeptide bands from sample "A" 's that were not obtained from sample " B " 's.

This result clearly eliminates the possibility that appreciable contaminating host cell protein is present in the purified virion fraction.

2. *Structural polypeptides of vaccinia viruses*

The strains of vaccinia virus examined were IHD-W, IHD-J, Lister and DIs, and the conditions used for virus infection and radioactive labeling are described in the Materials and Methods.

Figure 2 shows the polypeptide components of virions labeled with [^{14}C]-leucine. At least 35 polypeptide bands were separated from vaccinia virions. The profiles of the polypeptide bands of three strains of vaccinia virus (IHD-J, Lister and DIs) were similar to each other. These profiles are also similar to those reported by others (Sarov and Joklik, 1972). The molecular weights (MW) of these polypeptides, calculated by comparison of mobilities with those of standard proteins, as described in the legend to Fig. 2, ranged from about 250,000 to about 14,000. These polypeptides were tentatively designated as VV (vaccinia virion polypeptide) 250K to VV14K. The main components of vaccinia virus were VV69K and VV61K polypeptides, which may correspond to VP4 described by Sarov and Joklik (1972). Considerable amounts of VV43K, VV42K, VV41K, VV34K, VV23K, VV18K and VV14K polypeptides were also found.

The profile of polypeptide bands of strain IHD-W was similar to those of the three strains described above, but VV41K polypeptide, which might correspond to VP6b described by Sarov and Joklik (1972), was not detectable in virions of this strain grown on HeLa S3 or RK_{13} cells. Since strain IHD-W was shown not to produce hemagglutinin (HA) (Kaku and Kamahora, 1964; Ichihashi and Matsumoto, 1969), VV41K polypeptide may be a component of HA.

Figure 3 shows the differences between the structural polypeptide components of [^{14}C]-leucine labeled virions and [^{35}S]-methionine labeled virions from the Lister strain. The two profiles were essentially similar, but the intensities of bands VV41K and VV20K of [^{35}S]-labeled virions were much less than those of [^{14}C]-labeled virions, whereas the intensities of bands VV35K, VV22K and VV19K of [^{35}S]-labeled virions were more than those of [^{14}C]-labeled virions.

3. *Structural polypeptides of cowpox virus*

Figure 4 shows the polypeptide components of cowpox virions labeled with [^{14}C]-leucine or [^{35}S]-methionine. At least 36 polypeptide bands were detected, and tentatively named CV (cowpox virion polypeptide) 250K to

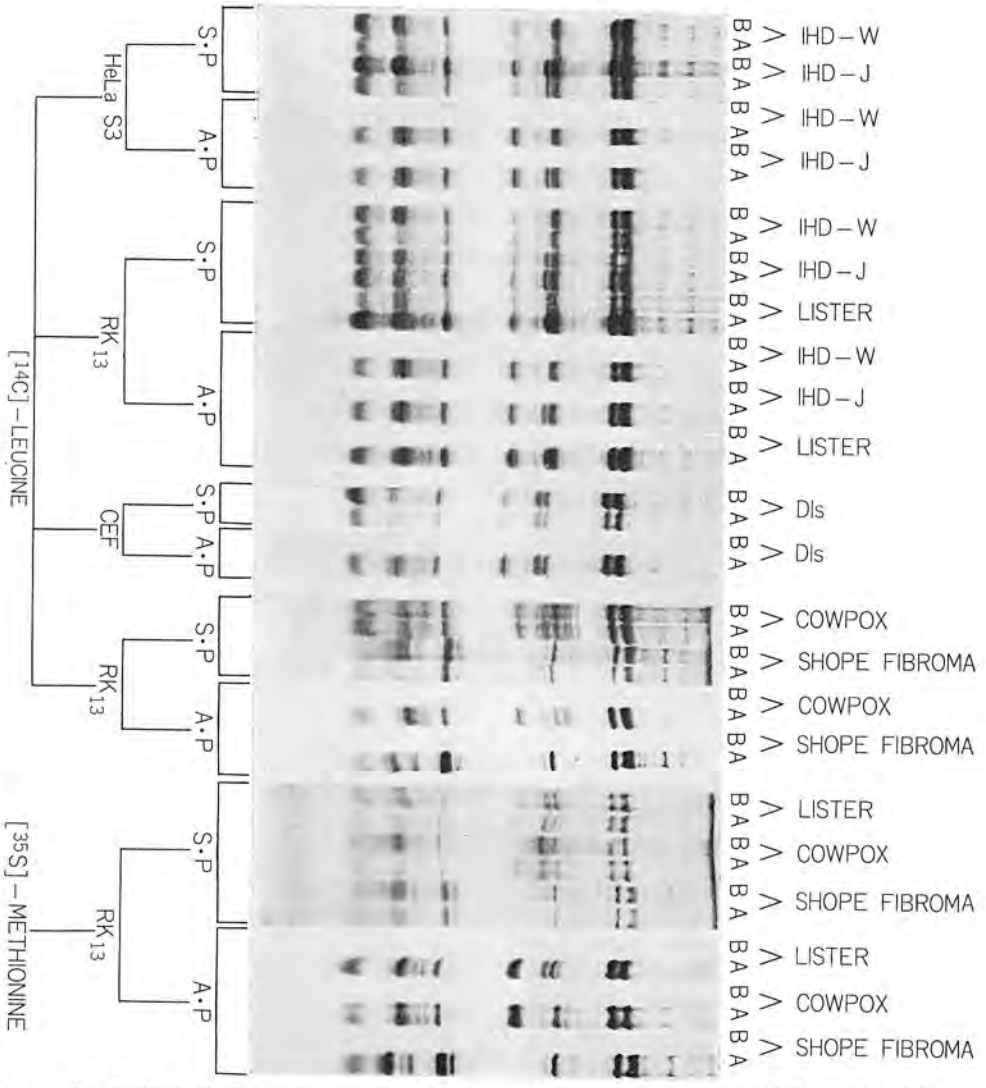

FIGURE 1. Comparison of electropherograms of virion polypeptides in samples "A" and " B " by staining with Coomassie blue and by autoradiography. Sample "A" was purified from radioactively labeled infected cells. Sample " B " was purified from a mixture of radioactively labeled uninfected cells and unlabeled infected cells. The following [^{14}C]-leucine labeled virion samples were used: IHD-W and IHD-J strains of vaccinia virus purified from HeLa S3 cells infected with stock viruses grown on HeLa S3 cells; IHD-W, IHD-J and Lister strains of vaccinia virus, cowpox virus and Shope fibroma virus purified from RK_{13} cells infected with stock viruses grown on RK_{13} cells; and DIs strain of vaccinia virus purified from CEF infected with stock virus grown on CEF. The following [^{35}S]-methionine labeled virion samples were used: Lister strain of vaccinia virus, cowpox virus and Shope fibroma virus purified from RK_{13} cells infected with stock viruses grown on RK_{13} cells. Virus infection, labeling and SDS-PAGE were done as described in the Materials and Methods. In this and all succeeding electropherograms, the bottom of the figure corresponds to the anodal end of the gel. The following nomenclatures were used: Staining profiles, S·P; autoradiographic profiles, A·P; "A" sample, A; and " B " sample, B.

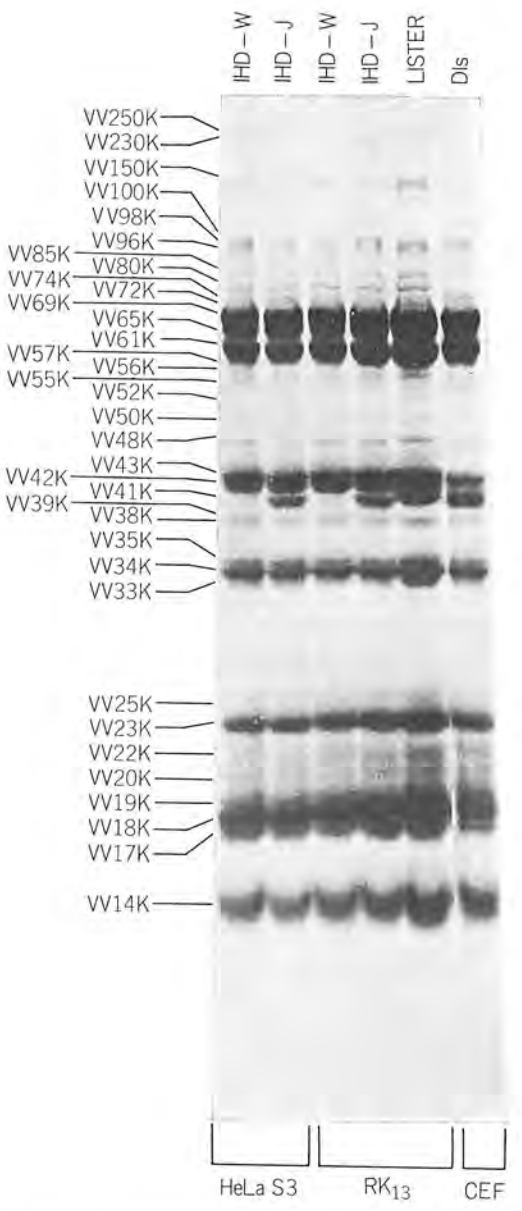

FIGURE 2. Autoradiographic comparison of the structural polypeptides in strains IHD-W, IHD-J, Lister and DIs of vaccinia virus labeled with [^{14}C]-leucine. The virion samples were the same as sample "A" shown in Fig. 1. Virions of IHD-W and IHD-J strains were prepared from both HeLa S3 and RK_{13} cells. Virions of the Lister strain were prepared from RK_{13} cells. Virions of the DIs strain were prepared from CEF. MW of polypeptides was calculated by comparison of mobilities with those of standard proteins [BDH MW marker mixtures (product No. 44223 2U, MW range 14,300–71,500, and 44230 2R, MW range 53,000–265,000) purchased from BDH Chemicals Ltd., England; and myosin, MW 215,000; bovine serum albumin, MW 67,000; ovalbumin, MW 43,500; and cytochrome c, MW 12,400].

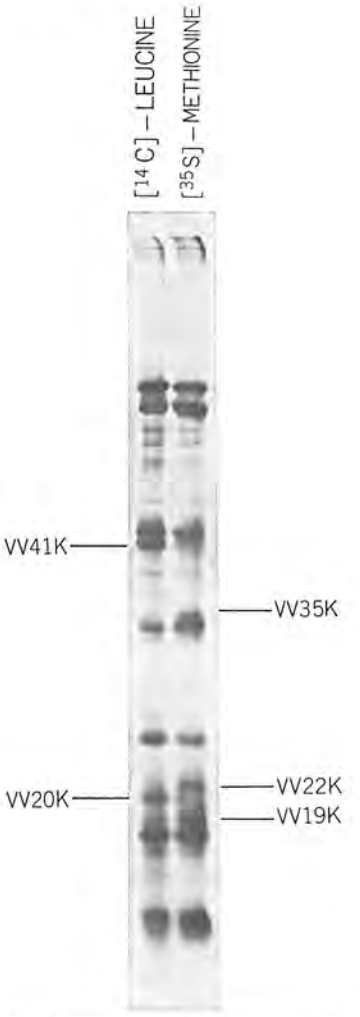

FIGURE 3. Autoradiographic comparison of structural polypeptides of the Lister strain of vaccinia virus prepared from RK_{13} cells, labeled with [^{14}C]-leucine or [^{35}S]-methionine. Both virion samples were the same as sample "A" in Fig. 1.

CV14K in order of their MW, calculated as described above. The main components of cowpox virus were CV69K and CV61K polypeptides, with considerable amounts of CV45K, CV43K, CV34K, CV23K, CV18K and CV14K polypeptides. The intensities of bands CV45K, CV43K, CV34K and CV22K polypeptides of [^{35}S]-labeled virions were more than those of [^{14}C]-labeled virions. Although CV41K polypeptide was present in cowpox virions, the intensity of its band was slightly less than that of VV41K from vaccinia virus.

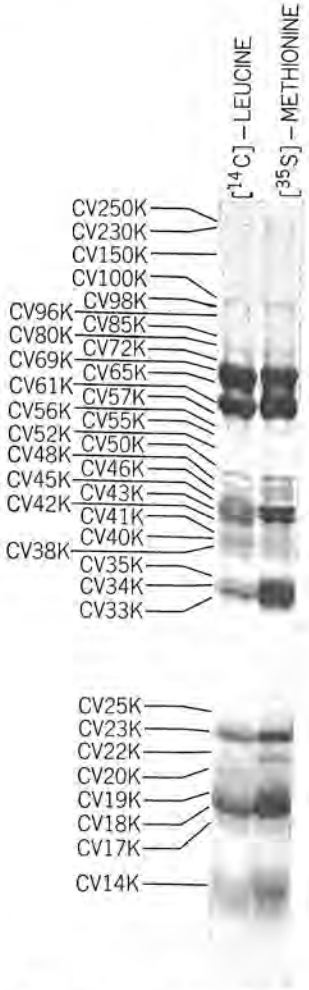

FIGURE 4. The structural polypeptides of cowpox virions prepared from RK_{13} cells, labeled with [^{14}C]-leucine or [^{35}S]-methionine. Both virion samples are the same as sample "A" in Fig. 1. MW of polypeptides was calculated by comparison of mobilities with those of standard proteins, as described in the legend to Fig. 2.

4. *Structural polypeptides of Shope fibroma virus*

Figure 5 shows the polypeptide components of Shope fibroma virions labeled with [^{14}C]-leucine or [^{35}S]-methionine. At least 37 polypeptide bands were detected, and tentatively named SV (Shope fibroma virion polypeptide) 250K to SV14K in order of their MW, calculated as described above. The main components of Shope fibroma virus were SV72K and SV64K polypeptides with considerable amounts of SV105K, SV43K, SV25K, SV23K, SV20K, SV17K and SV14K polypeptides. The intensities of bands of SV38K, SV37K, SV36K and SV30K polypeptides of [^{14}C]-labeled virions were slightly more than those of [^{35}S]-labeled virions.

5. *Comparison of structural polypeptides in vaccinia, cowpox and Shope fibroma viruses*

The structural polypeptides of the Lister strain of vaccinia virus, cowpox virus and Shope fibroma virus labeled with [^{14}C]-leucine were compared, and the results are shown in Fig. 6.

The polypeptides of vaccinia virus and cowpox virus were essentially similar, the main polypeptides of vaccinia virions (VV69K and VV61K) corresponding to those of cowpox virions (CV69K and CV61K). However the profile of the polypeptides of 40,000 to 50,000 daltons of cowpox virus was considerably different from that of vaccinia virus.

The polypeptide bands of Shope fibroma virions differed considerably from those of vaccinia or cowpox virions: the main polypeptide components were SV72K and SV64K, but these differed in MW from the main components of vaccinia virus (VV69K and VV61K) or cowpox virus (CV69K and CV61K).

The structural polypeptide components of

vaccinia, cowpox and Shope fibroma virions are summarized in Table 1. At least 33 polypeptides of vaccinia virions corresponded in MW to the polypeptides of cowpox virions, and at least 21 polypeptide bands were common to vaccinia, cowpox and Shope fibroma virions, judging by their MW, although the intensities of some of their bands were very different in vaccinia, cowpox and Shope fibroma virions, as seen in Fig. 6.

DISCUSSION

In the present work the protein components of vaccinia, cowpox and Shope fibroma viruses

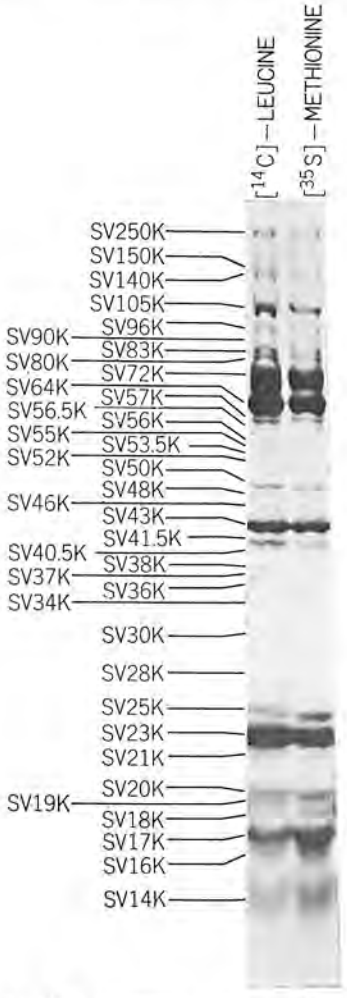

FIGURE 5. The structural polypeptides of Shope fibroma virions prepared from RK_{13} cells, labeled with [^{14}C]-leucine or [^{35}S]-methionine. Both virion samples were the same as sample "A" in Fig. 1. MW of polypeptides was calculated as described in the legend to Fig. 2.

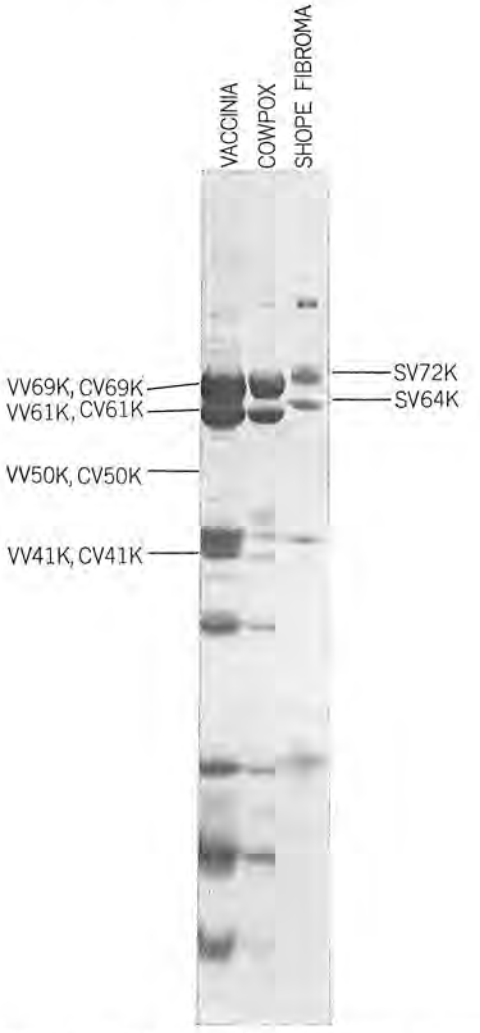

FIGURE 6. Autoradiographic comparison of the structural polypeptides in the Lister strain of vaccinia virus, cowpox virus and Shope fibroma virus, all prepared from RK_{13} cells labeled with [^{14}C]-leucine. All virion samples were the same as sample "A" in Fig. 1.

TABLE 1. *Structural polypeptides of vaccinia, cowpox and Shope fibroma viruses*

Vaccinia	Cowpox	Shope fibroma
V V250K	C V250K	S V250K
V V230K	C V230K	
V V150K	C V150K	S V150K
		S V140K
		S V105K
V V100K	C V100K	
V V98K	C V98K	
V V96K	C V96K	S V96K
		S V90K
V V85K	C V85K	
		S V83K
V V80K	C V80K	S V80K
V V74K		
V V72K	C V72K	S V72K
V V69K	C V69K	
V V65K	C V65K	
		S V64K
V V61K	C V61K	
V V57K	C V57K	S V57K
		S V56.5K
V V56K	C V56K	S V56K
V V55K	C V55K	S V55K
		S V53.5K
V V52K	C V52K	S V52K
V V50K	C V50K	S V50K
V V48K	C V48K	S V48K
	C V46K	S V46K
	C V45K[b]	
V V43K	C V43K[b]	S V43K
V V42K	C V42K	
		S V41.5K
V V41K[a]	C V41K	
		S V40.5K
	C V40K	
V V39K		
V V38K	C V38K	S V38K[a]
		S V37K[a]
		S V36K[a]
V V35K[b]	C V35K	
V V34K	C V34K[b]	S V34K
V V33K	C V33K	
		S V30K[a]
		S V28K
V V25K	C V25K	S V25K
V V23K	C V23K	S V23K
V V22K[b]	C V22K[b]	
		S V21K
V V20K[a]	C V20K	S V20K
V V19K[b]	C V19K	S V19K
V V18K	C V18K	S V18K
V V17K	C V17K	S V17K
		S V16K
V V14K	C V14K	S V14K

[a] Intensity of polypeptide; [^{14}C]-leucine > [^{35}S]-methionine.

[b] Intensity of polypeptide; [^{14}C]-leucine < [^{35}S]-methionine.

were compared. The possibility that the virion fraction was contaminated with host cell protein in the course of purification of the virion was ruled out by the results shown in Fig. 1, but if virions with host cell materials as integral parts are released from infected cells as mature virions, these host materials would not have been removed under the conditions used in this work. Joklik (1962a) tested for the presence of host cell materials in a virion fraction by serological procedure, and showed that a purified virus suspension contained very little material with host cell serological specificity. However, the possibility that mature virions are contaminated with some host cell material with very weak antigenicity cannot be ruled out by either our method or the serological procedure of Joklik, and this possibility remains to be tested.

The structural polypeptides of vaccinia virus have been analyzed by SDS-PAGE by several investigators (Holowczak and Joklik, 1967; Moss and Salzman, 1968; Sarov and Joklik, 1972), and at least 30 structural polypeptides have been separated. In the present study, we separated 35 polypeptides of vaccinia virions, 36 polypeptides of cowpox virions, and 37 polypeptides of Shope fibroma virions, naming them tentatively VV250K to VV14K, CV250K to CV14K, and SV250K to SV14K, respectively. The differences in reported values for MW of the polypeptides may be due to different conditions of electrophoresis. The vaccinia virus genome is large enough to code for between 200 and 400 average-sized proteins, the coding capacity expressed as the total MW of protein being between 8×10^6 and 16×10^6. The total MW of the 35 to 37 polypeptides of poxvirus detected in the present study was about 2×10^6. This large genome could code non-structural proteins as well. Using discontinuous SDS-PAGE, Pennington (1974) detected about 80 virus-induced polypeptides among the polypeptides of BSC_1 cells infected with vaccinia virus. Among these 80 polypeptides, about 50 were post-replicative polypeptides. He reported a total MW of

3×10^6 with about 80 virus-coded polypeptides. Greater numbers of polypeptide bands of poxvirus virions may be detected by methods with higher resolving power, such as two-dimensional PAGE.

The similarity of the IHD-W, IHD-J, Lister and DIs strains of vaccinia virions was shown by comparing their profiles on SDS-PAGE. Although VV41K, which may correspond to VP6b of Sarov and Joklik (1972), was detected in three strains of vaccinia virus (IHD-J, Lister and DIs), it was not detected in strain IHD-W. The production of HA during vaccinia virus infection was first described by Nagler (1942). The relationship of the HA to the infectious particle has been studied by numerous investigators, and the dissociation of HA from vaccinia virions has been demonstrated by the finding that the virions are not adsorbed to HA-sensitive chick red blood cells (Burnet and Stone, 1946; Blackman and Bubel, 1972). Physicochemical studies using differential centrifugation (Neff et al., 1965), column chromatography (McCrea and O'Loughlin, 1959) and PAGE (Weintraub and Dales, 1974) have all demonstrated that the HA is not a component of the vaccinia virion. In addition, the major glycoprotein, " J glycoprotein ", was detected in plasma membranes infected with strain IHD-J, but not strain IHD-W (Weintraub and Dales, 1974; Weintraub et al., 1977), and " J glycoprotein " was suggested to be a component of plasma membrane HA. Ichihashi (1977) also identified three HA specific glycoproteins (P1: MW, 150,000; P2: MW, 34,000; P3: MW, 12,000), derived from the membrane fraction of infected HeLa cells, by SDS-PAGE of HA bound to chick erythrocytes. On the other hand, there are a few reports that HA is associated with vaccinia virions (Schiek, 1974; Payne and Norrby, 1976). In the present studies, on comparison of the structural polypeptides of four strains of vaccnia virus, VV41K polypeptide was found in the virions of all the strains except strain IHD-W. Preliminary studies using immunoprecipitation revealed that VV41K polypeptide has the immunogenicity of HA (unpublished data), suggesting that VV41K polypeptide is a component of HA. Comparative studies on the biological characters of HA and VV41K polypeptide are now in progress.

The polypeptides of vaccinia and cowpox virions were also similar, differing only in polypeptides of 40,000 to 50,000 daltons and in the intensities of various polypeptides. This characteristic difference between vaccinia virions and cowpox virions may correspond to the results of Arita and Tagaya (1977), who detected characteristic polypeptides in comparative studies on the structural polypeptides in variola, monkeypox, cowpox and vaccinia virions by SDS-PAGE and staining with Coomassie blue.

The polypeptide bands of Shope fibroma virions differed considerably from those of vaccinia and cowpox virions in MW. The variola-vaccinia subgroup and the myxoma-fibroma subgroup differ biologically, although they share common antigens, " NP antigens ", detectable by a serological technique (Woodroofe and Fenner, 1962). Since adsorption of antisera produced against these " NP antigens " with purified virus failed to remove the precipitin, these " NP antigens " were suggested to be associated with internal protein, and not to be present on the surface of intact virus particles by the same authors. Sarov and Joklik (1972) detected 17 polypeptides in cores of vaccinia virus, but it is uncertain which of them are " NP antigens ". In the present studies, 21 polypeptide bands were found, on the basis of MW, to be common to vaccinia, cowpox and Shope fibroma virions, although the intensities of their polypeptide bands varied in the different virions. We are now trying to identify " NP antigens ".

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