

Procedure for Purification of Intact DNA from Vaccinia Virus

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A procedure for the isolation of intact vaccinia DNA molecules by chromatography on hydroxyapatite in the presence of 6 M urea is described. When lysates of virions containing 0.5 to 10 μg of DNA were employed, over 95% of the viral DNA could be recovered free of proteins. Vaccinia DNA molecules isolated in this manner sedimented at 68S in neutral sucrose gradients and had an average contour length of 62.3 μm when examined in an electron microscope, and the DNA could be cleaved with the restriction endonucleases *EcoRI* and *BamHI*. The results of these analyses showed that intact vaccinia DNA molecules of 120×10^6 to 130×10^6 molecular weight could be obtained by the procedures described.

Digestion of viral DNAs with restriction endonucleases, followed by analysis of the fragments by electrophoresis in agarose gels, offers a method of identifying viruses on the basis of their unique restriction enzyme "fingerprints" (20). Further, *in situ* hybridization techniques are now available that permit hybridization of mRNA species to restriction enzyme fragments transferred from agarose gels to cellulose-nitrate filters (15, 26). These techniques offer the opportunity to study the mode of transcription of viral DNA and the genetic relatedness of viral strains assigned to a particular group. We would like to apply these methods for studying viruses of the *Poxviridae* family (7). As an initial step in our studies, we have examined a number of procedures for the isolation of vaccinia virus DNA, our aim being the development of a general method for the rapid isolation of intact DNA in high yields. A number of methods have been used to isolate DNA from poxviruses (8, 12, 14, 21-24). Their main disadvantages have been in the relatively low yields of DNA obtained and/or the extensive fragmentation of the DNA during isolation. We report here a method for isolation of intact DNA molecules from poxviruses that appears to be generally useful for the purification of large DNA molecules from virus particles. In 1969, Bernardi described the isolation of intact T-even phage and other phage DNAs by hydroxyapatite chromatography (1).

The method to be described is based on the binding properties of double-stranded DNA to hydroxyapatite (HAP; 1, 2). Fragments of DNA-DNA and DNA-RNA hybrids were separated from single-stranded DNA, free RNA, and pro-

teins by chromatography on HAP in the presence of a urea-phosphate solution (16, 18, 25). By this method, protein-free intact DNA molecules from vaccinia virus were obtained when detergent-treated lysates of purified virions were applied to the HAP columns. The viral DNA molecules isolated in this manner were characterized by their sedimentation behavior in neutral sucrose gradients, by contour length measurements in the electron microscope, and by analysis of the fragments by electrophoresis in agarose gels.

The growth of mouse L-cells in suspension culture, infection of the cells, and purification of vaccinia virus strain WR from mouse L-cells have been described previously (6, 11-13). The elementary body/PFU ratio was about 20 to 50:1 when titrated on Vero cells. Virions labeled in their DNA and proteins were prepared by labeling infected cells at 10^6 /ml with 2 μCi of [^3H]thymidine (50 Ci/mmol) per ml and 5 μCi of [^{35}S]methionine (153 Ci/mmol) per ml, added 2 h after virus infection. The labeled virus particles were purified 24 to 28 h later as previously described (11-13). Infected cells were labeled in medium containing 1/5 normal concentration of methionine and supplemented with 5% dialyzed fetal calf serum. The specific activities of the DNAs obtained from purified virion preparations were 1.7×10^6 ^3H cpm/ μg of viral DNA and 1×10^5 ^{35}S cpm/ μg of viral proteins. Virus preparations stored at -20°C in 30% sucrose were frozen and thawed only once before use.

Purified virions were diluted 1:10 (vol/vol) with 0.001 M sodium phosphate buffer (pH 7.0) and collected by centrifugation at 15,000 rpm for 30 min (Sorvall, SS-34 rotor). The pelleted

virus was suspended at a final DNA concentration of 0.4 optical density unit at 260 nm (OD_{260}) per ml in freshly prepared 0.20 M sodium phosphate buffer (pH 6.8) containing 6 M urea (Ultrapure, Schwarz/Mann, Orangeburg, N.Y.), 1% Sarkosyl NL-97, 2% β -mercaptoethanol, and 10 mM EDTA (final concentrations). The preparations were gently mixed and incubated at 37°C for 10 min to complete lysis. HAP (Hypatite C, lot no. 6466; Clarkson Chemical Company, Williamsport, Pa.) suspended in 0.001 M sodium phosphate (pH 6.8) was used throughout. When 0.5 to 10.0 μ g of viral DNA was to be isolated, the HAP was poured into a 5-ml syringe barrel (1.1-cm diameter) onto a pad of Whatman no. 1 filter paper. To isolate 50 to 200 μ g of viral DNA, a 50-ml syringe barrel (2.8-cm diameter) was employed. The columns were equilibrated at room temperature with 0.24 M sodium phosphate buffer (pH 6.8)–8 M urea at a flow rate of 30 to 40 ml/h. One milliliter of HAP was used for each OD_{260} unit of native viral DNA calculated to be present in the viral lysates. The virus lysate was then applied to the surface of the HAP column, avoiding mixing, and the column was washed with 10 bed volumes of buffer containing 8 M urea–0.24 M sodium phosphate (pH 6.8) (step A), followed by 5 bed volumes of 0.14 M sodium phosphate buffer (pH 6.8) (step B). The viral DNA was then eluted with 2 to 3 bed volumes of 0.50 M sodium phosphate buffer (pH 6.8) (step C). The fractions containing the DNA were pooled and dialyzed against sterile 10 mM Tris-hydrochloride buffer (pH 7.6)–0.1 mM EDTA at room temperature for 2 h (to avoid crystallization of phosphate salts in the dialysis tubing) and then at 4°C for 14 h. The viral DNA was concentrated either by placing the dialysis tubing in contact with Aquacide II (CalBiochem, La Jolla, Calif.) or by ethanol precipitation. In the latter case, the solution was adjusted to 0.2 M with respect to NaCl, mixed with 2 volumes of cold ethanol, and stored at –20°C for 16 h. The precipitated DNA was then collected by centrifugation at 10,000 rpm for 15 min at 4°C. The pelleted viral DNA was air dried, resuspended in a small volume of sterile 10 mM Tris-hydrochloride buffer (pH 7.6)–0.1 mM EDTA, and stored at –20°C. Figure 1 shows the result obtained when lysates of doubly labeled vaccinia virus particles were applied to an HAP column and the column was developed as described above. All of the 35 S-labeled proteins present were eluted with the urea-sodium phosphate buffer (step A). Identical results were obtained when 6 M guanidine-hydrochloride was used in place of the urea. The column was then washed with 0.14 M sodium phosphate buffer

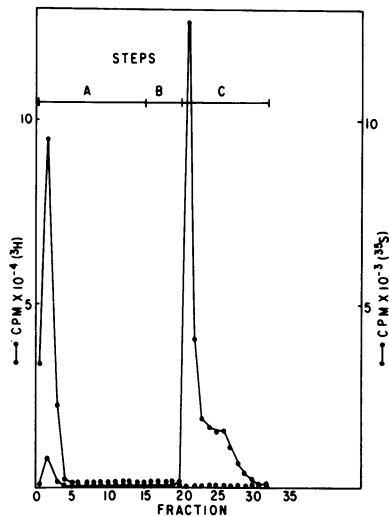


FIG. 1. Purification of vaccinia DNA on HAP columns. Doubly labeled vaccinia virions were lysed and processed for HAP chromatography as described in the text. Fractions of 1 ml were collected and 100- μ l portions were removed for determination of trichloroacetic acid-precipitable radioactivity. Symbols: (●) [3 H]thymidine-labeled DNA; (○) [35 S]-methionine-labeled proteins.

(pH 6.8) to remove all traces of urea. Analysis showed that during this procedure all of the [3 H]DNA remained bound to the column. Application of the eluting buffer (0.50 M sodium phosphate [pH 6.8]) resulted in elution of the viral DNA as a sharp peak (Fig. 1). When 0.5 to 10 μ g of viral DNA was present in the lysates and was analyzed in this manner, over 95% of the DNA was recovered. This high recovery made it possible to calculate the content of DNA per virus particle. Assuming that 1 OD_{260} unit is equivalent to 1.2×10^{10} virus particles (12, 14), the amount of DNA obtained from 2.1 OD_{260} units of purified virus (specific activity, 1.7×10^5 cpm/ μ g of DNA) was 7.35 μ g; thus, the content of DNA per vaccinia virus particle was determined to be 2.92×10^{-10} μ g. This result agreed with previously published calculations with quite different methods (13, 14, 24, 27). When higher concentrations (50 to 200 μ g) of DNA were isolated, recoveries ranged from 70 to 90% of the DNA applied to the columns.

The sedimentation behavior of the viral DNA obtained after elution from the HAP column was determined by analysis in neutral sucrose gradients. The viral DNA sedimented at about 68S relative to adenovirus 2 DNA (32S), regardless of whether the viral DNA was obtained from freshly prepared or frozen virus (Fig. 2). This sedimentation coefficient is appropriate for

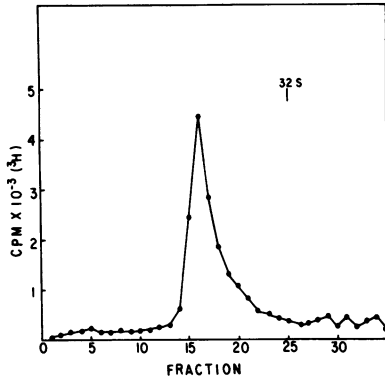


FIG. 2. Sedimentation analysis in neutral sucrose gradients of ^3H -labeled vaccinia DNA isolated by chromatography on a HAP column. A portion of viral DNA eluted from the HAP column was analyzed by sedimentation in a 15 to 30% linear sucrose gradient (Spinco SW41 rotor, 39,000 rpm, 20°C , 3 h) as described previously (5).

DNA molecules of 120×10^6 to 130×10^6 molecular weight (3, 5, 9, 10, 24). It should be pointed out that sedimentation analysis in alkaline sucrose gradients showed that when detergent-treated lysates of freshly prepared virions were employed (Method II, reference 10), over 70% of the viral DNA eluted from the HAP column, sedimented with unit-length and cross-linked mature viral DNA (5). However, when detergent-treated lysates from frozen virions were used, the viral DNA eluted from the HAP column contained a significant and variable number of nicks; this phenomenon may be related to the extensive sonic treatment used during virus purification or to the action of the virion-associated single-strand DNase that may be activated during storage and freeze-thawing of purified virions (10). Contour length measurements of the viral DNA were carried out in an electron microscope after the molecules were spread by the formamide technique of Davis et al. (4). The average length of the DNA molecules isolated by chromatography on HAP columns was $62.3 \mu\text{m}$ (Fig. 3). Relative to the length of adenovirus type 2 DNA ($10.75 \mu\text{m}$; molecular weight, 23×10^6 ; (19) used as an internal marker, the vaccinia DNA isolated by HAP chromatography was estimated to have a molecular weight of 132×10^6 .

Analysis of vaccinia DNA restriction endonuclease fragments generated after digestion with *EcoRI* and *BamHI* indicated that vaccinia DNA was cut into a discrete series of fragments by both *EcoRI* and *BamHI* when the cleavage fragments were analyzed in horizontal slab gels of 0.7% (wt/vol) agarose as described by McDonnell et al. (17; not shown).

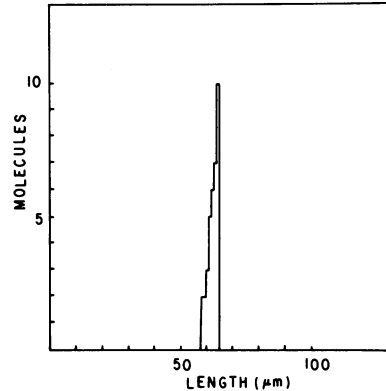


FIG. 3. Length distribution of vaccinia DNA molecules isolated by chromatography on a HAP column. A portion of viral DNA eluted from the HAP column was spread by the formamide technique (4). Molecules were photographed, slides were prepared from the micrograph plates, and DNA molecules, projected onto a screen, were traced and measured with a map-reading device (Derby, S. A., Switzerland). The majority ($\geq 90\%$) of the viral DNA molecules appeared intact when spread under these conditions.

The major advantage of the technique described here over one employing phenol extraction is that it minimizes the losses and fragmentation of the viral DNA molecules that can occur during the manipulations required for extraction with phenol (8, 12, 14, 21, 23). Lysis of virions on top of neutral sucrose gradients, followed by velocity sedimentation, is another effective method to isolate intact vaccinia DNA molecules (M. Esteban, L. Flores, and J. A. Holowczak, *Virology*, in press; 10, 22, 24); however, a limitation of this technique is that only relatively small numbers of virions can be effectively lysed on the sucrose gradients, limiting the amount of DNA that can be isolated.

In conclusion, we have described a method by which vaccinia DNA can be isolated with HAP columns. The method should be generally useful for purifying intact DNA from DNA-containing viruses.

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