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Characterization of Supercoiled Nucleoprotein Complexes Released from Detergent-Treated Vaccinia Virions

M. J. SOLOSKI AND J. A. HOLOWCZAK*

Department of Microbiology, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, New Jersey 08854

Treatment of vaccinia virions with 1% sodium dodecyl sulfate in the absence of reducing agents resulted in the release of subviral particles termed "subnucleoids," which contained viral DNA in combination with four polypeptides with molecular weights of 90,000, 68,000, 58,000, and 10,000. Biochemical and electron microscopic studies showed that viral DNA in combination with these polypeptides was maintained in a superhelical configuration. When subnucleoids were "fixed" with glutaraldehyde and formaldehyde and then examined by electron microscopy, spherical particles were observed, in which the supercoiled DNA was folded into globular structures that were 20 to 60 nm in diameter and were interconnected by DNA-protein fibers resembling the nucleosome structures described for eucaryotic chromatin.

In an electron microscope, negatively stained vaccinia virions appear as large, brick-shaped or oval structures with dimensions of 270 by 108 nm (59). The surfaces of the virions, as revealed by freeze-etching and negative staining, consist of smooth surfaces upon which parallel ridges of 5-nm globular subunits (or surface tubules) are superimposed (37, 38, 59). Recently, these surface tubules have been isolated and found to consist of a single viral structural polypeptide (molecular weight, 58,000), which is capable of eliciting antibodies that can neutralize infectivity and suppress cell-cell fusion after infection (52). The outer viral membrane, upon which these surface tubules reside, appears to have a typical bilayer structure. Unlike other enveloped virus membranes, poxvirus membranes are synthesized de novo rather than being derived from cellular membranes (14, 51). Boulter and Appleyard (7) demonstrated that vaccinia virus which is released from infected cells (extracellular virus) acquires an additional outer coat. Electron microscopy of negatively stained extracellular virus has shown a well-defined envelope surrounding the extracellular viral particles.

The internal structure of vaccinia virions can be observed when thin sections of virus are examined by electron microscopy. Electron photomicrographs show a dense biconcave central core and two oval lateral bodies residing in the core concavities. This structure is surrounded by a lipoprotein envelope (13). Treatment of virus with nonionic detergents and 2-mercaptoethanol, followed by gentle sonication, removes the outer envelope and lateral bodies and releases a brick-shaped core (15), which is covered by cylindrical pegs (palisade layer) imposed upon a smooth surface (13, 15, 37).

The internal structure of the vaccinia virion DNA-containing core has been studied by electron microscopy after negative staining of thin sections of vaccinia virions. This technique has revealed that the internal structure is a triad of oblong elements which are approximately 50 nm thick (41). Using specific staining procedures and electron microscopy, Hyde and Peters (25) observed a tubular "flower-like" structure in fowlpox virions; they suggested that this structure represented highly coiled viral DNA or DNA-protein complexes. Vreeswijk et al. (55) examined tilted serial sections of the *Molluscum contagiosum* poxvirus by electron microscopy and provided evidence that the internal core (or nucleoid) structure consists of tightly coiled DNA, which is folded into a spool and twisted to give a dumbbell shape.

When vaccinia virus is treated with sodium dodecyl sulfate (SDS), 100- to 150-nm spherical, subviral particles (called nucleoids) are generated, which retain 50 to 60% of the viral structural polypeptides (25). Thin sections of these particles reveal a dense inner structure surrounded by DNA (or DNA-protein) fibers. Using controlled protease digestion followed by NaCl-EDTA treatment, McCrae and Szilagyi (35) obtained subviral paticles which contained viral DNA associated with 30 to 35% of the viral structural proteins. An electron microscopic analysis of the subviral particles prepared in this way revealed brick-shaped particles with smooth, featureless surfaces (35). Studies on the configuration of the vaccinia viral chromosome

when it is released from virions and the proteins associated with such structures have not yet been reported.

We report here our studies on the controlled degradation of vaccinia virions by ionic detergent. DNA-protein complexes, which we call subnucleoids, were isolated and characterized by using a combination of biochemical and electron microscopic techniques. The results of the experiments reported here provide important insights into the structure and composition of the vaccinia chromosome as it may exist in mature virions.

(The results were taken, in part, from a thesis submitted by M.J.S. to Rutgers University. A preliminary presentation of the results was made at the 43rd Cold Spring Harbor Symposium, 1978.)

MATERIALS AND METHODS

Virus and cells. Vaccinia virus strain WR was propagated in mouse L-cells. Growth of cells, infection of cells, and purification of virus have been described previously (4, 23, 26).

Vaccinia virus labeled with ^{32}P was prepared by infecting cultures of mouse L-cells in phosphate-deficient medium. Shortly after infection, $^{32}PO_4$ was added to a concentration of 10 μCi/10^6 cells (27). Vaccinia virions labeled in their DNA and proteins were prepared by labeling infected cells with 1 to 2 μCi of [^{3}H]thymidine per ml and 2.0 to 2.5 μCi of [^{35}S]methionine per ml in a medium containing 25% of the normal concentration of cold methionine. Isotopes were added 20 min postinfection. Labeled virus particles were harvested 24 h postinfection and were purified as described by Joklik (27).

Preparation of subviral particles: subnucleoids. Purified vaccinia virus was concentrated by centrifugation at 15,000 rpm for 50 min at 4°C with a Sorvall RC-2 centrifuge and an SS-34 rotor. The pelleted virus was suspended in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA (TE buffer) with the aid of sonication to a concentration of 10^{11} elementary particles per ml. SDS (final concentration, 1.0%) was then added, and the mixture was incubated at room temperature for 15 min (24). The resulting preparation was then carefully layered onto the appropriate gradient for analysis, as described below. To minimize mechanical disruption of particles, samples were transferred by using pipettes whose tips were widened to have a diameter of 2 to 3 mm.

Sedimentation analysis in neutral sucrose gradients. We prepared 15 to 30% (wt/vol) linear sucrose gradients in 10 mM Tris-hydrochloride buffer (pH 8.0)–1 mM EDTA. Samples (1.0 ml) contained no more than 2 μg of viral DNA in order to avoid overloading the gradients. The gradients were centrifuged with a Spinco SW41 rotor for 3 h at 39,000 rpm and 20°C and fractionated, and a portion of each fraction was removed for an analysis of the distribution of trichloroacetic acid-precipitable radioactivity. The extent of supercoiling was estimated by determining the effects of different concentrations of ethidium bromide (EtBr) on the sedimentation properties of the subviral particles (2, 3). Linear, 11-ml, 15 to 30% sucrose gradients containing 0, 1, 2, 4, 6, and 10 μg of EtBr per ml (in the buffer described above) were prepared and overlaid with sample. The centrifugation conditions, fractionation of gradients, and determination of the distribution of radioactivity were as described above. Routinely, ^{14}C-labeled 33S lambda phage DNA (8) and ^{14}C- or ^{3}H-labeled 68S vaccinia DNA (16, 17, 20) were added to experimental samples or analyzed in parallel gradients and served as sedimentation reference markers.

Analysis of subviral particles by sedimentation in 20 to 60% sucrose-D_2O gradients. Gradients containing 35 ml of 20 to 60% (wt/wt) sucrose-D_2O in 10 mM Tris-hydrochloride buffer (pH 8.0)–1 mM EDTA were overlaid with 3.5 to 4.0 ml of sample. Gradients were centrifuged with a Spinco SW27 rotor at 20,000 rpm for 18 h at 20°C and then fractionated. We determined the refractive index (at 20°C) of every fifth fraction, beginning with fraction 2. We prepared a standard curve relating the refractive indexes of the sucrose solutions prepared in D_2O to the densities of such solutions (determined by weighing measured samples of such solutions). This permitted the refractive index measurements to be converted to their equivalent densities (in grams per cubic centimeter).

Electron microscopy. Samples containing purified vaccinia DNA or DNA-protein complexes were visualized by adsorbing them onto polylysine-coated grids, as described by Williams (60). Carbon-coated Formvar copper grids (75 by 300 mesh) were prepared and glow-discharged with aluminum. A 1-μg/ml solution of polylysine (type II; Sigma Chemical Co., St. Louis, Mo.) was applied to the grids for 30 s and then aspirated off by using a Pasteur pipette with a flared end, and the grids were allowed to air dry. Samples were dialyzed against 10 mM Tris-hydrochloride buffer (pH 8.0)–0.1 mM EDTA overnight at 4°C; 10 mM $MgCl_2$ was added, and a sample was applied to the polylysine-coated grids for 120 s. In some cases, the samples were pretreated with protease, DNase, or RNase as described below and then applied to the grids. In each case, buffers containing the appropriate enzymes but no DNA or DNA-protein complexes were processed in the same way for electron microscopy. After adsorption, the remaining sample was aspirated off, and the grid was washed by touching the surface to 3 successive drops of double-distilled water. Grids were then stained with 2% uranyl acetate for 30 s, washed three times with double-distilled water, air dried, and rotary shadowed with platinum-palladium at an angle of 7°. The grids bearing the samples were examined with a Phillips EM 300 electron microscope at 60 kV. All of the solutions used for electron microscopy were prepared in double-distilled water and passed through membrane filters (pore size, 0.22 μm). Polylysine solutions and grids were prepared on the same day that samples were applied.

Viral DNA-protein complexes were also examined by electron microscopy after negative staining. Samples were dialyzed overnight against 10 mM Tris-hydrochloride buffer (pH 8.0)–0.1 mM EDTA. Carbon-coated Formvar copper grids (75 by 300 mesh) were prepared, and samples were applied as described pre-

viously (28). Grids were stained with 2% potassium phosphotungstate (pH 7.0) and examined with a Phillips EM 300 electron microscope at 60 kV.

Enzymatic digestions. Unless otherwise noted, samples were prepared by dialyzing against a solution of 10 mM Tris-hydrochloride buffer (pH 8.0)–0.1 mM EDTA overnight at 4°C before digestion with an enzyme under the conditions described below. A 5-mg/ml solution of proteinase K (20 mAnson units per mg of protein) in sterile distilled water was "self-digested" for 30 min at 37°C. Experimental samples were digested with proteinase K (100 to 250 μg of enzyme per ml for 2 h at 37°C) and then processed for analysis. Pancreatic RNase A was prepared as a 1.0-mg/ml stock solution in sterile distilled water. Experimental samples were digested with pancreatic RNase (100 μg/ml; incubated for 2 h at 37°C) and then processed for analysis. Pancreatic DNase I (1 mg/ml) and micrococcal nuclease (10,000 U/ml) solutions were prepared in sterile distilled water. When samples were digested with micrococcal nuclease, 10 mM $CaCl_2$ and 100 to 500 U of enzyme per ml were added, and digestion was allowed to proceed at 37°C for 2 h. Pancreatic DNase I digestions were performed after 10 mM $MgCl_2$ and 50 to 100 μg of enzyme per ml were added; this was followed by incubation at 37°C for 2 h. Enzymatic treatments were terminated by adding trichloroacetic acid, by rapid cooling to 0°C, or by adding detergents, as indicated by the design of the experiments.

SDS-PAGE of proteins. For SDS-polyacrylamide gel electrophoresis (PAGE) the method of Laemmli (29) was modified. A 10% acrylamide resolving gel (0.375 M Tris [pH 8.9], 0.1% SDS) with a 3.5% stacking gel (0.125 M Tris-hydrochloride buffer [pH 6.7], 0.1% SDS) was prepared in a model 220 slab gel apparatus (Bio-Rad Laboratories, Richmond, Calif.). Samples were dialyzed extensively against distilled water at 4°C, lyophilized, dissolved by adding a solution containing 50 μl of 8.0 M urea, 5 μl of 10% SDS, and 1 μl of 2-mercaptoethanol (undiluted), and heated at 100°C for 2 min. Electrophoresis was carried out at 20 mA for 4 to 5 h. Gels were stained with Coomassie brilliant blue as described previously (18). For autoradiography, the gels were dried with a Hoefer model SE-540 gel dryer, and Kodak RP-Royal X-Omat film was exposed for 1 to 3 weeks, depending on the level of radioactivity present in the polypeptides resolved in the gels.

Molecular weights were determined by the technique of Weber and Osborn (58), using known protein standards. We used the following molecular weight markers, which were analyzed at the same time as the experimental samples: *Escherichia coli* RNA polymerase β subunit (molecular weight, 165,000), *E. coli* RNA polymerase β′ subunit (155,000), β-galactosidase (135,000), phosphorylase *a* (92,000), bovine serum albumin (68,000), pyruvate kinase (57,000), ovalbumin (41,000), *E. coli* RNA polymerase α subunit (39,000), trypsin inhibitor (21,000), and cytochrome *c* (11,500).

Agarose gel electrophoresis. Samples containing DNA or nuclease-digested DNA were analyzed by electrophoresis in horizontal agarose gels containing EtBr, as described previously (34, 36, 49). As markers, T7 DNA *Hap*I fragments were analyzed at the same time as the experimental sample. DNA bands were visualized by placing a gel on top of a UV Transilluminator (UV Products, Inc., San Gabriel, Calif.), and the gel was photographed and processed for autoradiography as described previously (34).

Reagents and isotopes. Urea (ultrapure grade) was purchased from Schwarz/Mann, Orangeburg, N.Y. Nonidet P-40 was obtained from BDH Chemicals, Ltd., Poole, England. Sarkosyl NL-97 was purchased from Chemical Additives Co., Farmingville, N.Y. Proteinase K was obtained from E. Merck AG, Darmstadt, Germany. Micrococcal nuclease, pancreatic DNase I and pancreatic RNase A were purchased from Worthington Biochemicals Corp., Freehold, N.J. D_2O, [^{35}S]methionine (specific activity, 283.8 to 596.0 Ci/mmol), [^{3}H]thymidine (specific activity, 42 to 50.8 Ci/mmol), and $^{32}PO_4$ were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Isolation and sedimentation properties of subnucleoids. An analysis of the SDS lysates of doubly labeled vaccinia virions in 20 to 60% sucrose–D_2O gradients resulted in the resolution of two types of subviral particles (Fig. 1A). Approximately 60 to 78% of the labeled virions were converted to particles which sedimented at a density of 1.26 to 1.27 g/cm^3 and contained viral DNA in association with 40 to 45% of the [^{35}S]methionine-labeled proteins found in whole virions. These particles, called nucleoids, have been described previously (24). A second subviral particle type, containing 10 to 25% of the total [^{3}H]thymidine-labeled DNA applied to the gradients in association with 12 to 15% of the [^{35}S]methionine-labeled proteins found in complete virions, sedimented at a density of 1.19 to 1.20 g/cm^3 (Fig. 1A). The characterization of these subviral particles, which we call subnucleoids, is described below. From the specific activities of the labeled, purified virions used in these studies (for example, the virions used in the experiments shown in Fig. 1 contained 149,390 cpm of [^{3}H]thymidine per μg of DNA and 2,400 cpm of [^{35}S]methionine per μg of protein), we calculated that virions contained 20 to 22 μg of protein per μg of DNA, nucleoids contained 10 to 11.5 μg of protein per μg of DNA, and subnucleoids contained 3.3 to 3.5 μg of protein per μg of DNA (average of four preparations).

Brief sonication (22) or vigorous pipetting of the SDS lysates or both resulted in a greater overall conversion of virions to subnucleoids (>60%). Similar treatment of the nucleoids also resulted in a further conversion of these particles to subnucleoids. These experiments showed that all of the virions in a preparation could be degraded to subnucleoids and that nucleoids may be an intermediate stage in this conversion. Whereas the procedures described above signifi-

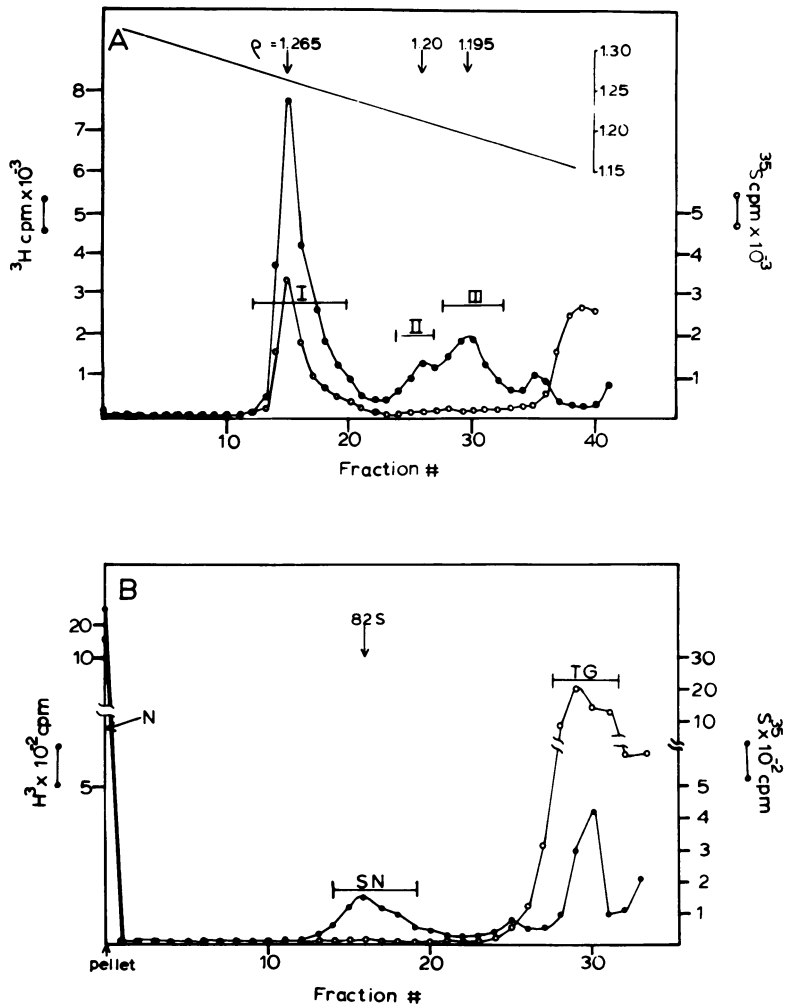

FIG. 1. *Analysis of particles released from vaccinia virions by treatment with 1% SDS. Purified vaccinia virus labeled with [^{35}S]methionine and [^{3}H]thymidine was suspended in TE buffer at a concentration of 10^{11} elementary particles per ml with the aid of sonication. SDS was added (final concentration, 1%), and the mixture was incubated at room temperature for 15 min. The incubation mixture was layered onto the appropriate neutral sucrose gradient for analysis. (A) Approximately 3×10^{11} to 4×10^{11} elementary, double-labeled, SDS-treated vaccinia virion particles were layered onto a 35-ml 20 to 60% (wt/wt) sucrose-D_2O gradient containing 10 mM Tris-hydrochloride (pH 8.0)-1 mM EDTA and then centrifuged in a Spinco SW27 rotor at 20,000 rpm and 20°C for 18 h. The gradients were fractionated, and the distribution of trichloroacetic acid-precipitable radioactivity and the sucrose-D_2O densities were determined as described in the text. ρ, Density (in grams per cubic centimeter). (B) Approximately 1×10^{10} to 2×10^{10} elementary, double-labeled, SDS-treated (see above) vaccinia virion particles were layered onto an 11-ml 15 to 30% (wt/vol) neutral sucrose gradient and then centrifuged in a Spinco SW41 rotor at 39,000 rpm and 20°C for 3 h. The gradients were fractionated, and the distribution of trichloroacetic acid-precipitable radioactivity was determined as described in the text. Symbols: ●, [^{3}H]thymidine counts per minute; ○, [^{35}S]methionine counts per minute.*

cantly increased the yield of subnucleoids, electron microscopic studies showed that such preparations contained variable amounts of fragmented particles. These fragments were recovered at a density of 1.19 to 1.20 g/cm^3 after centrifugation in sucrose-D_2O gradients, indicating that the ratio of protein to DNA in the fragments was similar to the ratio in the unfragmented particle population. The presence of fragmented particles complicated our electron microscopic studies and the sedimentation analysis of the particles in neutral sucrose gradients. To avoid these problems, subnucleoids that were released from virions after a single detergent

treatment and no further manipulation were examined in detail.

To examine the sedimentation properties of the subnucleoids, labeled virus was treated with 1% SDS, and the lysates were analyzed in 15 to 30% neutral sucrose gradients (Fig. 1B). Under the conditions of centrifugation employed, nucleoids were recovered in the pellet, whereas subnucleoids sedimented to a position of approximately 82S relative to the position of purified 68S vaccinia DNA (22).

To evaluate the role which protein played in determining the sedimentation properties of the subnucleoids, the following experiments were performed. Subnucleoids were prepared as described in the legend to Fig. 1, and the appropriate fractions were pooled and dialyzed overnight against TE buffer at 4°C. Samples (1.0 ml) of the dialyzed subnucleoids were removed, and each sample was treated in one of the following ways: (i) maintained at 4°C, (ii) incubated at 37°C for 1 h (mock-digested), or (iii) incubated at 37°C for 1 h with proteinase K (final concentration, 100 μg/ml). After treatment, each sample was analyzed in a 15 to 30% neutral sucrose gradient as described above. In each gradient, a known amount of purified 68S vaccinia virus DNA was included as a sedimentation marker. Figure 2 shows a summary of the results of these analyses. The subnucleoids which were incu-

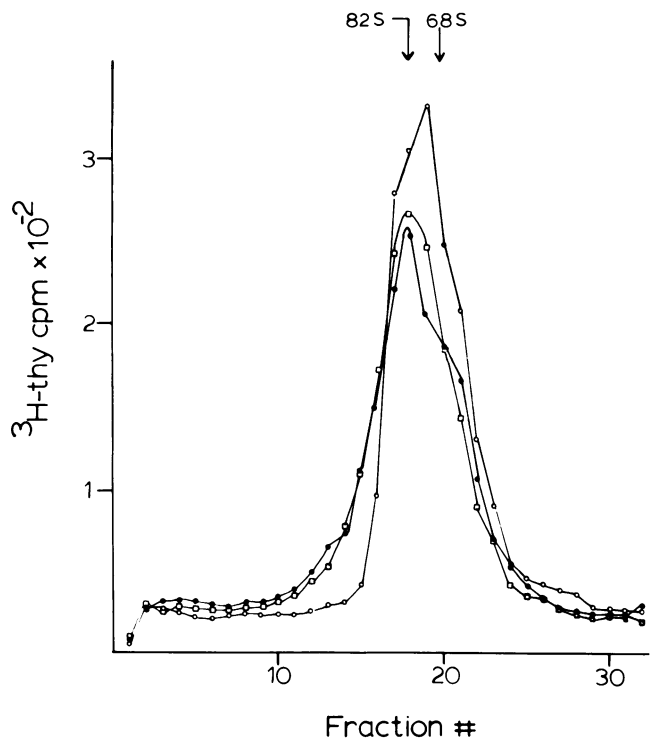

FIG. 2. *Effect of proteinase K digestion on the sedimentation properties of subnucleoids. Vaccinia virions labeled with [^{3}H]thymidine (^{3}H-thy) and [^{35}S]methionine were treated with 1% SDS and analyzed in 20 to 60% sucrose-D_2O gradients as described in the legend to Fig. 1. The subnucleoid peak was pooled and dialyzed overnight against TE buffer at 4°C. Samples (1.0 ml) of the dialyzed subnucleoids were removed, and each sample was treated in one of the following ways: maintained at 4°C (□); incubated at 37°C for 1 h (●); or incubated at 37°C for 1 h with 100 μg of proteinase K per ml (○). After treatment the samples were layered onto 11-ml, 15 to 30% neutral sucrose gradients containing 10 mM Tris-hydrochloride (pH 8.0)-1 mM EDTA and centrifuged in a Spinco SW41 rotor at 39,000 rpm and 20°C for 3 h. All gradients were centrifuged in parallel and fractionated, and the distribution of radioactivity was determined as described in the text. The results from several gradients have been plotted together relative to the position of purified, 68S vaccinia DNA, as detected by absorbance at 260 nm in each gradient (indicated by the arrow). The distribution of [^{35}S]methionine counts per minute in each gradient is not shown. In the gradients in which subnucleoids incubated at 4 or 37°C for 1 h were analyzed, 275 to 300 trichloroacetic acid-precipitable [^{35}S]methionine counts per minute was found associated with the [^{3}H]thymidine DNA peak. No detectable trichloroacetic acid-precipitable [^{35}S]methionine counts per minute were detected in the gradient in which subnucleoids treated with proteinase K were analyzed.*

bated at 4°C in the absence of proteinase sedimented 1.5 to 2.0 fractions faster than 68S virion DNA, confirming the observations shown in Fig. 1B. When the subnucleoids incubated at 37°C were analyzed, a prominent, slower-sedimenting "shoulder" was observed, which cosedimented with the 68S DNA marker. This shoulder was absent when subnucleoids maintained at 4°C were analyzed, suggesting that the subnucleoid structure may be unstable at elevated temperatures. When subnucleoids were treated with proteinase K and then analyzed in 15 to 30% neutral sucrose gradients, they sedimented more slowly than the mock-digested subnucleoids, implying that proteins were involved in maintaining the subnucleoid structure (Fig. 2). However, it should be noted that part of the proteinase K-digested subnucleoid population sedimented slightly faster than 68S virion DNA. This suggested that either some of the protein associated with the subnucleoid structure was not accessible to protease digestion or something other than protein was responsible, at least in part, for the sedimentation properties of the subnucleoid particles.

SDS-PAGE of the polypeptides associated with subnucleoids. To determine which virion polypeptides were associated with the nucleoid and subnucleoid particles, the appropriate fractions were pooled (Fig. 1A and B), and their contents were analyzed by SDS-PAGE. In some experiments, the subnucleoids were resolved into two species when they were analyzed in 20 to 60% sucrose-D_2O gradients (Fig. 1A). These two subnucleoid fractions were analyzed separately by SDS-PAGE.

The results of SDS-PAGE showed that the nucleoid fraction(s) (Fig. 3) contained several polypeptides which co-electrophoresed with the major and minor virion structural polypeptides (Fig. 3A, lanes V and I, and Fig. 3B, lanes V′ and N). This was in agreement with previously pub-

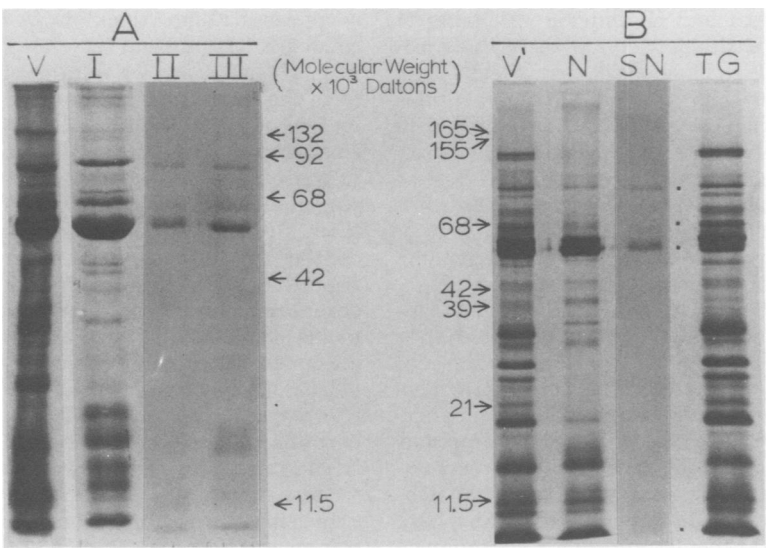

FIG. 3. *Autoradiograms of 10% polyacrylamide slab gels showing [^{35}S]methionine-labeled polypeptides associated with vaccinia virus nucleoids and subnucleoids. Purified [^{3}H]thymidine- and [^{35}S]methionine-labeled vaccinia virions were treated with 1% SDS and analyzed in either 20 to 60% sucrose-D_2O gradients or 15 to 30% neutral sucrose gradients as described in the legend to Fig. 1. The appropriate peaks were pooled as indicated by the bars in Fig. 1A and B; these peaks were dialyzed against distilled water at 4°C, lyophilized, and prepared for SDS-PAGE as described in the text. The conditions for PAGE and the processing of the gels for autoradiography were as described in the text. (A) Polypeptides associated with nucleoids and subnucleoids prepared by sedimentation in 20 to 60% sucrose-D_2O gradients. Lane V, [^{35}S]-methionine-labeled polypeptides associated with purified vaccinia virions; lane I, polypeptides associated with vaccinia virus nucleoids; lanes II and III, polypeptides associated with vaccinia virus subnucleoids. (B) Polypeptides associated with nucleoids and subnucleoids prepared by sedimentation in 15 to 30% neutral sucrose gradients. Lane V′, [^{35}S]methionine-labeled polypeptides associated with purified vaccinia virions; lane N, polypeptides associated with vaccinia virus nucleoids; lane SN, polypeptides associated with vaccinia virus subnucleoids; lane TG, polypeptides released from vaccinia virus after treatment with 1% SDS and remaining at the top of the gradient after centrifugation. The positions of the polypeptides of known molecular weights which were used as standards and were analyzed at the same time as the experimental samples are indicated by the arrows. The squares (in B) indicate the positions of the four major polypeptides.*

lished data (24).

When the subnucleoid fractions were analyzed by SDS-PAGE (Fig. 3A, lanes II and III, and Fig. 3B, lane SN), four major polypeptides were detected. The molecular weights of these polypeptides were estimated to be 90,000, 69,000, 58,000, and 10,000. Exposure of the film for 40 to 60 days failed to reveal additional distinct polypeptide bands resolved in the gels. A densitometric analysis, as well as direct excision and determination of the radioactivity associated with the four major polypeptides, indicated that they accounted for 87 to 92% of the total radioactivity analyzed in each sample. The 58,000-dalton protein, which was the major polypeptide detected in subnucleoid fractions, comigrated with VP-4b, a major viral structural polypeptide which is located in the vaccinia virus core and represents 10% of the total protein found in mature virions (48). The remaining three subnucleoid polypeptides also comigrated with polypeptides present in virions and nucleoids (Fig. 3A, lanes I and II, and Fig. 3B, lanes N and SN). A densitometric analysis of the autoradiograms (Fig. 3) showed that the 90,000-, 68,000-, 58,000-, and 10,000-dalton polypeptides were present in a ratio of 4 to 5:1:15 to 20:6 to 10 (or a molar ratio of 3:1:20:40). The 58,000-dalton polypeptide constituted more than one-half of the protein in the particles. We calculated that the 82S particles contained 7×10^{-10} to 8×10^{-10} μg of protein associated with each viral genome or a total mass of about 1×10^{-11} μg.

A fifth polypeptide, which migrated at approximately 20,000 daltons, was detected after PAGE of the lower-density particles in the subnucleoid population (Fig. 3A, lane III). This polypeptide was not detected when the higher-density particles in the subnucleoid fractions were analyzed (Fig. 3A, lane II) or when subnucleoids isolated after analysis in 15 to 30% neutral gradients (Fig. 3B, lane SN) were analyzed. This polypeptide could represent a contaminating polypeptide species from the leading edge of the peak of [^{35}S]methionine-labeled polypeptides which were released from virions after SDS treatment and remained at the top of the gradient. No 20,000-dalton protein was associated with 82S subnucleoids when these particles were isolated after sedimentation in 15 to 30% sucrose gradients (Fig. 3B, lane SN). However, the fact that the particles present in the two subnucleoid fractions could be separated in the 20 to 60% sucrose-D_2O gradients suggested that the 20,000-dalton protein may also contribute in some way to the structure of the particles found in the lower-density side of the subnucleoid peak after analysis in sucrose-D_2O gradients.

Electron microscopy of Subnucleoids. Subnucleoids which banded at a density of 1.19 to 1.20 g/cm^3 in 20 to 60% sucrose-D_2O gradients were prepared and dialyzed overnight against 10 mM Tris-hydrochloride buffer (pH 8.0)-0.1 mM EDTA at 4°C, and 10 mM $MgCl_2$ was added to the dialyzed particles. Samples were applied to polylysine-coated grids and examined with an electron microscope, as described above. Structures representative of the structures that were typically observed in the electron microscope are shown in Fig. 4. The particles observed in the subnucleoid fractions that were isolated as described above appeared to consist of highly twisted and coiled DNA. Because of the highly twisted nature of the molecules and the presence of condensed regions of DNA or DNA in combination with protein, it was difficult to determine whether the observed structures contained one intact viral DNA molecule or whether complexes released from a number of virions contributed to the structures which we observed. In some cases 20-nm "thick" DNA fibers (Fig. 4B, small arrow) and 5-nm "extended" DNA strands (Fig. 4B, large arrow) were observed emerging from the same condensed region. A close examination of the 20-nm thick DNA fibers (Fig. 4B, small arrow) revealed that the subnucleoid DNA in these regions formed a repeating structure resembling a solenoid. The presence of thin and thick DNA fibers emerging from a single condensed structure suggests that there may be regions of DNA coiling in the vaccinia viral chromosome which perhaps resemble the domains of supercoiling present in the folded chromosomes which have been isolated from *E. coli* (42, 62), *Drosophila melanogaster* (5), and *Saccharomyces cerevisiae* (43). Figure 4C shows part of a subnucleoid structure in which several DNA strands are interwoven to form a complex tertiary structure.

In general, the subnucleoids observed by electron microscopy consisted of a complex array of intercoiled, possibly supercoiled, DNA strands. Some variation in the morphology of the subnucleoid particles was noted when samples from different preparations were examined. The possibility existed that the manipulations involved in processing the samples for electron microscopy disrupted the subnucleoid structure, yielding the observed coiled structure, and permitted interactions between structures released from single virions. To explore this possibility, subnucleoids were isolated from 20 to 60% sucrose-D_2O gradients as described in the legend to Fig. 1 and then immediately fixed with 1% formaldehyde-0.6% glutaraldehyde. After fixation, samples were dialyzed against 10 mM Tris-hy-

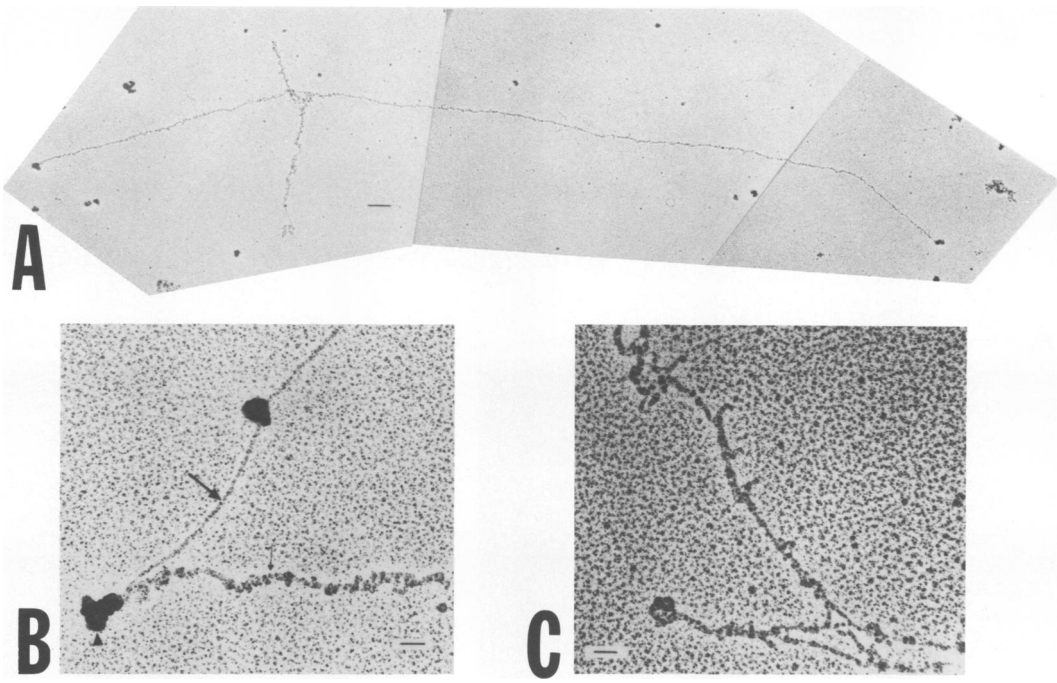

FIG. 4. *Electron photomicrographs of subnucleoids. Subnucleoids were prepared and isolated in 20 to 60% sucrose-D_2O gradients as described in the legend to Fig. 1. The subnucleoid fractions were pooled and dialyzed against 10 mM Tris-hydrochloride buffer (pH 8.0)-0.1 mM EDTA, and $MgCl_2$ was added (final concentration, 10 mM); these preparations were applied to polylysine-coated grids as described in the text. (A) Typical highly twisted and coiled structure of subnucleoids. Bar = 300 nm. (B) High magnification of a portion of a subnucleoid. The arrows indicate a linear or relaxed DNA strand (large arrow) and a coiled DNA strand (small arrow), both emerging from a condensed structure (arrowhead). Bar = 60 nm. (C) High magnification of a portion of a subnucleoid, demonstrating the complex, twisted DNA structure. Bar = 60 nm.*

drochloride buffer (pH 8.0)-0.1 mM EDTA and processed for electron microscopy as described above. Electron photomicrographs of fixed subnucleoids are shown in Fig. 5. The majority (>95%) of the subnucleoids observed resembled those shown in Fig. 5A and B and consisted of electron-dense globular structures which were interconnected by DNA fibers. Occasionally, we observed a subnucleoid (Fig. 5C) in which the globular structures were in close association, forming a spherical structure. It is known that the procedures used for direct mounting of DNA-protein complexes for electron microscopy subject such complexes to uncontrolled shearing forces which may distort the structure (21). Therefore, we suggest that the structures in Fig. 5A and B arose when the structures shown in Fig. 5C were "pulled apart" by such shearing forces.

The sizes of the globular structures within the complexes ranged from 20 to 70 nm. A close examination of the original electron photomicrographs of the fixed subnucleoids indicated that the smaller 20-nm globular structures were clustered together to form the larger (30- to 70-nm) subunits. This structural organization is most prominent in Fig. 5C. The 20-nm size of the small globular structure is identical to the size of the thick DNA fiber shown in Fig. 4B (small arrow). Measurements of the widths of the interconnecting DNA fibers ranged from 5 nm (the size of naked DNA [60]) to 20 nm. It is not clear whether these larger DNA fibers are composed of DNA complexed with protein or whether they represent several DNA strands which are oriented in parallel. It is interesting to note that the smaller globular structures observed (20 to 30 nm) had a similar size and bore a morphological resemblance to the globular structures observed in electron photomicrographs of histone H1-containing simian virus 40 minichromosomes (10, 39, 45).

When fixed subnucleoids were prepared and examined under the electron microscope at higher salt concentrations (0.1 M NaCl), structures identical to those shown in Figure 5 were observed.

The unfixed subnucleoids were incubated be-

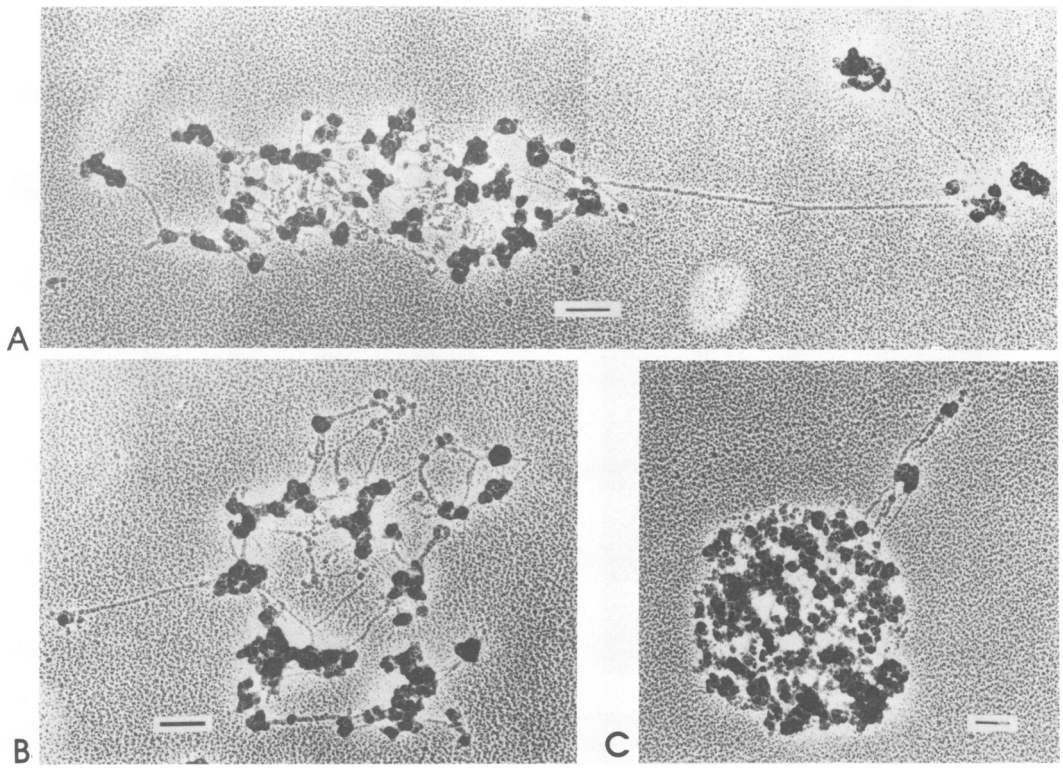

FIG. 5. *Electron photomicrographs of subnucleoids fixed with formaldehyde and glutaraldehyde. Subnucleoids were isolated by sedimentation in 20 to 60% sucrose-D_2O gradients as described in the legend to Fig. 1. Appropriate fractions were pooled, and samples (0.5 ml) were treated with formaldehyde (final concentration, 1%); this was followed by a 15-min incubation on ice. Glutaraldehyde (final concentration, 0.6%) was then added, and the sample was incubated for an additional 15 min on ice. The samples were then dialyzed against 10 mM Tris-hydrochloride buffer (pH 8.0)-0.1 mM EDTA overnight at 4°C to remove the aldehydes and sucrose. $MgCl_2$ (final concentration, 10 mM) was added to the dialyzed samples, and the samples were applied to the polylysine-coated grids as described in the text. Bar = 200 nm in all three panels.*

fore application to polylysine-coated grids with pancreatic DNase, RNase, and proteinase K, in order to probe the structure of the subnucleoids as observed by electron microscopy. When samples were pretreated with pancreatic RNase (37°C, 60 min, 100 μg of enzyme per ml), typical subnucleoid particles were observed, which had structures resembling those shown in Fig. 4. Pretreatment of subnucleoids with proteinase K (60 min, 37°C, 200 μg/ml) dramatically altered the subnucleoid structure. No coiled or twisted DNA structures were observed. Instead, linear DNA molecules containing little or no tertiary or higher-order structures were the predominant species observed. The structure of the proteinase K-treated subnucleoids as observed by electron microscopy closely resembled the structure of protein-free vaccinia virus strain WR DNA analyzed under identical conditions (16). When subnucleoids were pretreated with pancreatic DNase I (60 min, 37°C, 100 μg/ml) and applied to polylysine-coated grids, no particles were observed in the electron microscope. The enzymatic digestion data were consistent with the sedimentation analysis shown in Fig. 2 and provided additional evidence that proteins were involved in maintaining the structure of the subnucleoids.

Effect of EtBr on the sedimentation of subnucleoids. The electron microscopic observations summarized above suggested that the DNA contained in subnucleoids could be supercoiled (Fig. 4). To establish the presence of supercoiled DNA in the subnucleoid structure, we investigated the effects of different concentrations of EtBr on the sedimentation of subnucleoids in neutral sucrose velocity gradients (3). Vaccinia virions labeled with [^{3}H]thymidine were treated with 1% SDS and then analyzed in 15 to 30% neutral sucrose velocity gradients con-

taining varying concentrations of EtBr (Fig. 6). When purified, protein-free, 68S vaccinia DNA was analyzed in a similar fashion, we observed no change in its sedimentation behavior. Vaccinia virus DNA labeled with [^{14}C]thymidine was included as a sedimentation marker in each gradient when subnucleoids were analyzed. These analyses showed that the 82S subnucleoid fraction sedimented slower at low EtBr concentrations (0 to 2 μg/ml), appeared to reach a minimum rate at EtBr concentrations of 4 μg/ml, and then sedimented more rapidly at higher EtBr concentrations (6 to 10 μg/ml). This sedimentation behavior in the presence of EtBr is a

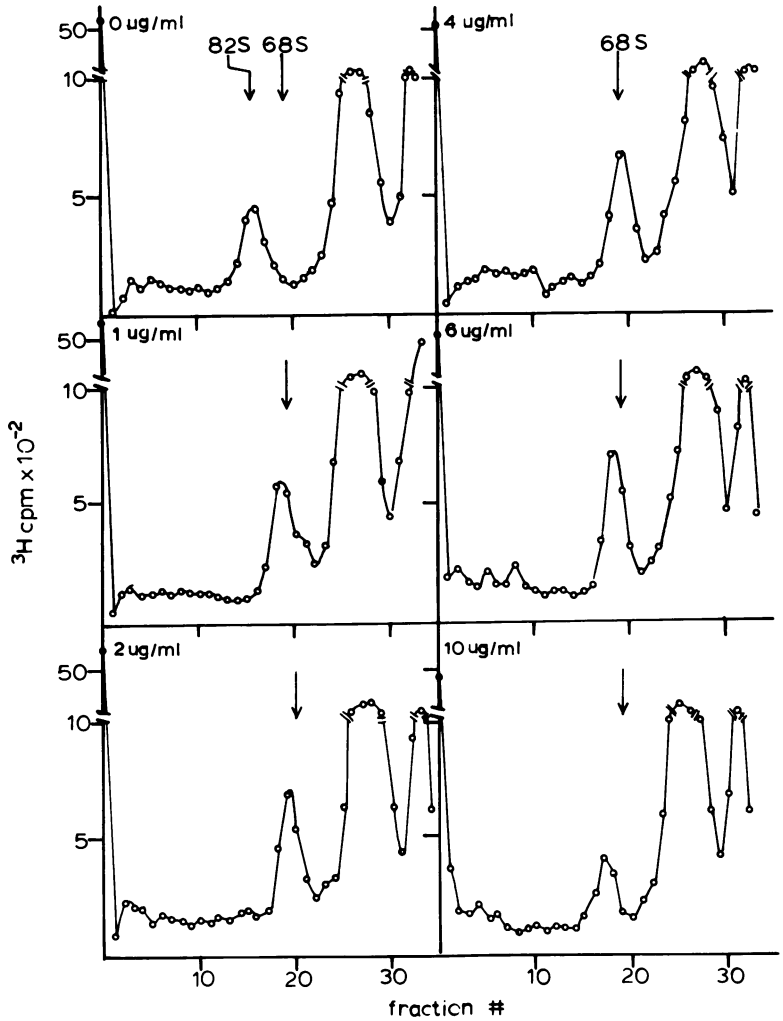

FIG. 6. *Sedimentation behavior of subnucleoids in the presence of EtBr. Vaccinia virus labeled with [^{3}H]thymidine was treated with 1% SDS as described in the text. Samples containing 2.1 × 10^9 elementary particles (or 0.5 μg of viral DNA) were layered onto 11-ml 15 to 30% sucrose velocity gradients in 10 mM Tris-hydrochloride buffer (pH 8.0)–1 mM EDTA. Gradients contained 0, 1, 2, 4, 6, or 10 μg of EtBr per ml, as indicated on the figure. Gradients were centrifuged in a Spinco SW41 rotor at 39,000 rpm and 20°C for 3 h and fractionated, and the distribution of trichloroacetic acid-precipitable radioactivity was determined as described in the text. In each gradient, ^{14}C-labeled, purified vaccinia DNA was included as a 68S marker; its position is indicated by an arrow. Under these conditions of centrifugation, the nucleoids were recovered in the pellet fraction, whereas the subnucleoids sedimented as an 82S species (Fig. 1B). When samples were predigested with micrococcal DNase or proteinase K, no 82S particles were detected. Near the top of each gradient [^{3}H]thymidine-labeled material sedimenting at 20 to 27S was observed. This represented DNA associated with the surface of virions and was removed by predigesting purified virions with DNase (22).*

property of covalently closed circular molecules (2, 56, 57) and of the more complex folded genomes which have been isolated from *E. coli* (62), *Drosophila* (5), and *Saccharomyces* (43), all of which contain supercoiled DNA.

To estimate the superhelical density (number of base pairs per turn) in the subnucleoids, it was necessary to determine the concentration of EtBr required to remove all of the superhelical turns. At this point (the equivalence point), the sedimentation of the superhelical molecules should be equal to 68S (12). The equivalence point for vaccinia virus subnucleoids was determined by plotting the calculated S values (relative to 68S marker DNA) of the subnucleoids against the EtBr concentrations (Fig. 7). This analysis indicated that the equivalence point occurred between 2 and 4 μg of EtBr per ml. A similar concentration of EtBr has been shown to relax superhelical DNAs from simian virus 40 (33), *E. coli* (62), *D. melanogaster* (5), and *S. cerevisiae* (43). An extrapolation of the data from these previous studies to the system under study here indicated a superhelical density of 200 base pairs per superhelical turn in vaccinia virus subnucleoids.

Digestion of subnucleoids with micrococcal nuclease. The electron photomicrographs of fixed subnucleoids revealed 20- to 60-nm condensed globular structures (Fig. 5). These structures may be similar to the large DNA fibers observed in electron photomicrographs of eucaryotic nuclei. The eucaryotic 20- to 30-nm DNA fibers are formed by an ordered coiling of the nucleosome 10-nm filaments (9, 19, 61). The size of the globular structure observed in the subnucleoids suggested that the vaccinia virion DNA may be packaged into nucleosome-like structures. Therefore, we decided to determine the accessibility of the vaccinia DNA in subnucleoids to micrococcal nuclease. Vaccinia virions labeled with ^{32}P were treated with SDS and analyzed on 20 to 60% sucrose–D_2O gradients (Fig. 1A), and the fractions containing subnucleoids were pooled. The subnucleoids were then digested or mock-digested with micrococcal nuclease, and the amount of DNase-resistant radioactivity was determined. Under conditions where >99.9% of the naked vaccinia virus DNA was rendered trichloroacetic acid soluble in 15 min at 37°C, 2.5% of the subnucleoid ^{32}P counts per minute was DNase resistant after 15 min and remained so throughout the 60-min incubation. To determine whether this DNase-resistant radioactivity represented a discrete size class of DNA, the micrococcal nuclease digestion products were further digested with proteinase K, concentrated by ethanol precipitation, and analyzed by agarose gel electrophoresis, as described above. We detected no DNase-resistant material migrating into the gel, indicating that the DNA in vaccinia subnucleoids was completely accessible to nuclease. This result implied that the DNA folding involved in the subnucleoid structure was fundamentally different than the DNA folding involved in eucaryotic nucleosomes. Additional experiments with a variety of nucleases under varying conditions must be carried out to confirm these observations. Moreover, because detergents were employed in preparing the complexes, some extra caution must be exercised in interpreting these results. Although the subnucleoids were relatively stable (that is, they could be collected after resolution in gradients, dialyzed, and rebanded without loss of protein material), it is possible that the interaction of protein with DNA was compromised in some way, making the DNA accessible to nuclease.

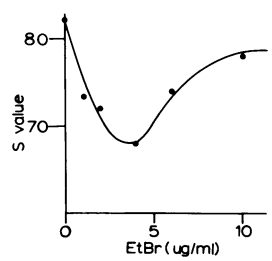

FIG. 7. *Change in sedimentation behavior of vaccinia virus subnucleoids as a function of EtBr concentration. Vaccinia virus subnucleoids were analyzed in 15 to 30% sucrose gradients containing varying concentrations of EtBr (see Fig. 6). The sedimentation of the subnucleoids relative to marker 68S vaccinia DNA was calculated (22) and expressed as a function of the total EtBr concentration in each gradient. Since only small amounts of vaccinia DNA were analyzed in each gradient (0.5 μg), the concentration of free EtBr was very near the indicated total EtBr concentration.*

DISCUSSION

We describe here the isolation and partial characterization of a unique nucleoprotein complex that is released from vaccinia virions when they are treated with ionic detergent. We postulate that this complex represents the supercoiled, vaccinia virus chromosome. Because an ionic detergent was used in the preparation of these particles, we realize that some components may have been removed which, when appropriate methods of preparation are available, will prove to be integral components of "native" viral chromosomes. For this reason, we refer to these complexes as subnucleoids, although on mor-

phological grounds, as revealed by electron microscopy, they clearly resemble the chromosomal elements isolated from eucaryotes (5, 40, 43), procaryotes (62), and certain animal viruses (39). Further support for the concept that subnucleoids represent a basic substructure of mature virions comes from our recent studies in which we isolated subviral particles by independent techniques (50, 50a); in particular, particles were released by treating virions with guanidine hydrochloride in the presence of Nonidet P-40 and β-mercaptoethanol (Gu-subviral particles) (50). The Gu-subviral particles had a more complex polypeptide composition than subnucleoids, but they had in common polypeptides with molecular weights of 90,000, 68,000, 58,000 and 10,000. More striking was the observation that after the Gu-subviral particles were fixed with glutaraldehyde and formaldehyde, the morphology of the highly coiled nucleoprotein complexes observed by electron microscopy on the surfaces of Gu-subviral particles resembled the morphology of the fixed subnucleoids described above.

The results presented here demonstrate that viral DNA in combination with four polypeptides (as resolved by single-dimension PAGE) can be supercoiled and even assume the higher order of folding described in this report. It has been shown previously that when viral cores are extracted with acid, two polypeptides with molecular weights of 58,000 and 10,000 to 15,000 solubilized in addition to polyamines (30). On the basis of their solubility properties, these polypeptides may be basic in nature. Although the identity of these polypeptides with the 58,000- and 10,000-dalton polypeptides found in subnucleoids remains to be established, such basic polypeptides (in combination with polyamines) could play an important role in neutralizing the charge on viral DNA and thus facilitating its supercoiling and higher-order folding.

When examined by electron microscopy, unfixed subnucleoids appeared as complex, twisted or supercoiled structures (Fig. 4A). The superhelical configuration of the DNA in the subnucleoids was confirmed by determining the effects of varying concentrations of EtBr on the sedimentation properties of subnucleoids (Fig. 6 and 7). Treatment of the subnucleoids with proteinase K yielded structures which sedimented more slowly (approximately 68 to 72S, compared with 82S for subnucleoids and 68S for vaccinia virus DNA) (Fig. 2) and, when examined by electron microscopy, lost their complex, supercoiled structure. These results indicated that the removal of the protein from subnucleoids resulted in the unfolding (or disruption) of the complexes and showed that the polypeptides found in subnucleoids were responsible for the superhelical configuration of the DNA.

We observed in the electron photomicrographs of the unfixed subnucleoids regions in the complexes where coiled and linear DNA could be seen originating from a single, condensed, globular structure (Fig. 4B). This observation suggested that vaccinia virion DNA may be organized into domains in much the same way as suggested for procaryotic (42, 62) and eucaryotic (5, 40, 43) chromosomes. The domains described previously (5, 40, 42, 43, 62) are stretches of DNA which are organized into loops by DNA-RNA or DNA-protein interactions. The interactions which stabilize the domains restrict the rotation of the DNA from one domain to the next. As a result, a nick introduced into one strand of the DNA in a domain allows the unwinding of the supercoiled DNA within that domain. However, because the interactions act as a barrier, adjacent domains are not affected. The fact that a linear DNA molecule, such as vaccinia virion DNA, can be isolated with a superhelical structure supports the hypothesis that domains are involved in the virion chromosome structure for the following reasons. First, in order for a linear DNA molecule to assume a superhelical configuration, the ends of the molecule must be restrained. Second, we have purified vaccinia virus by method II described by Holowczak (22), a procedure which in our hands yielded virus particles containing DNA bearing several nicks. This was confirmed by an analysis of the DNA from such preparations in alkaline sucrose gradients. When subnucleoids were prepared from such virus and examined by electron microscopy, supercoiled regions were clearly seen; these were much like those present in the electron photomicrographs shown in Fig. 4. If only the ends of the DNA molecule were restrained, then the viral chromosome would behave as a large closed circular DNA molecule, and the introduction of only one nick would necessarily relax the entire structure. This was not the case, and this result suggested that there must be a number of barriers along the vaccinia DNA chromosome that prevent the complete unwinding of the DNA. The interactions involved in the presumptive barriers are not clear, but it has been shown that DNA-RNA interactions stabilize the *E. coli* chromosome domains (42, 62), whereas both DNA-RNA and DNA-protein interactions can stabilize eucaryotic chromosome domains (5, 40, 43). In fact, the presence of RNA in highly purified vaccinia virions has been demonstrated (47), but the function of this RNA remains to be determined.

The observation that vaccinia virion DNA is maintained in a supercoiled configuration is con-

sistent with the presence within the virion of enzymes which function in the synthesis and processing of RNA transcripts. Bauer et al. (1) have shown that there is an enzymatic activity in vaccinia virions which is capable of relaxing superhelical DNA. Several previous studies have shown that superhelical DNA serves as a better template for *E. coli* RNA polymerase (32, 46, 57) and some eucaryotic RNA polymerases (6, 31) in vitro than relaxed DNA. In addition, Wang (57) demonstrated that superhelical *lac* operon DNA has a much higher affinity for repressor than relaxed *lac* operon DNA. Taken together, these results imply that it is biologically advantageous to have DNA assume a superhelical configuration.

Electron photomicrographs of subnucleoids which were fixed immediately after isolation revealed, instead of the complexed twisted structure present in unfixed subnucleoids (Fig. 4), particles which contained condensed globular structures bridged by DNA-protein fibers (Fig. 5). Globular structures with a similar morphology were occasionally observed in the unfixed subnucleoid population (Fig. 4A and B). The diameter of the globular structures ranged from 20 to 60 nm. The larger structures appeared to be composed of multiple aggregates of the 20-nm structures. This relationship is most obvious in Fig. 5C. The smaller structures (20 nm) have a similar size and bear a morphological resemblance to the 20-nm globular structures present in histone H1-containing simian virus 40 nucleoprotein complexes (10, 39, 54). These 20-nm structures are thought to represent a higher-order coiling of the 10-nm nucleosome-containing filament into the 20- to 25-nm fiber found in eucaryotic chromatin (39, 44, 53). Additional experiments with viral chromatin prepared from infected cells or virions without the use of ionic detergents are necessary in order to define completely the structure of vaccinia chromatin.

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