

Induced Extrusion of DNA from the Capsid of Herpes Simplex Virus Type 1

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DNA-filled capsids (C capsids) of herpes simplex virus type 1 were treated in vitro with guanidine-HCl (GuHCl) and analyzed for DNA loss by sucrose density gradient ultracentrifugation and electron microscopy. DNA was found to be lost quantitatively from virtually all capsids treated with GuHCl at concentrations of 0.5 M or higher, while 0.1 M GuHCl had little or no effect. DNA removal from 0.5 M GuHCl-treated capsids was effected without significant change in the capsid protein composition, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, or in its structure, as judged by electron microscopy. Electron microscopic examination of capsids in the process of emptying showed that DNA was extruded from multiple, discrete sites which appeared to coincide with capsid vertices. DNA exited the capsid in the form of thick strands or fibers that varied in diameter from approximately 4 to 13 nm with preferred diameters of 7 and 11 nm. The fibers most probably correspond to multiple, laterally aligned DNA segments, as their diameters are nearly all greater than that of a single DNA double helix. The results suggest that GuHCl treatment promotes an alteration in the capsid pentons which allows DNA to escape locally. Hexons must be more resistant to this change, since DNA loss appears to be restricted to the pentons. The ability of GuHCl to cause loss of DNA from C capsids with no accompanying change in capsid morphology or protein composition suggests that penton sites may open transiently to permit DNA exit and then return to their original state.

Herpes simplex virus type 1 (HSV-1) consists of an icosahedral capsid which contains the viral DNA and is surrounded by a membrane envelope. Between the capsid and the membrane is a layer of protein called the tegument (7, 12). DNA is thought to be the only component present inside the capsid cavity, where it occupies all of the available space (6). Infection of a host cell by HSV-1 begins when the virus membrane fuses with the cell plasma membrane, a process that results in deposition of the DNA-filled capsid (the nucleocapsid) into the cell cytoplasm (25). Once inside the cytoplasm, the nucleocapsid migrates to the cell nucleus, where it injects its DNA into the nucleoplasm, probably through a nuclear pore. Parental capsids are thought to inject their DNA without themselves entering the nucleus; parental capsids lacking DNA are found to accumulate at the periphery of the nucleus shortly after virus-cell fusion (2, 30).

Later in the infection cycle, DNA-free progeny capsids, called B capsids, self-assemble in the cell nucleus and are packaged with DNA. The resulting DNA-containing or C capsids can acquire tegument and an envelope and mature into complete virions. Capsid morphogenesis and packaging with DNA are accompanied by formation of a small number of DNA-free capsids, called A capsids, that cannot be packaged with DNA and therefore cannot mature into infectious virions (10, 22). A capsids are thought to arise after DNA loss from partially packaged B capsids or from C capsids that have lost their DNA prematurely (22, 23, 29). The structure of the icosahedral capsid shell is indistinguishable in A, B, and C capsids (1, 6, 26, 31). The protein compositions of the three capsid types are also closely similar, except for the fact that B capsids contain, inside the capsid cavity, the scaffolding or

assembly protein (VP22a), which is not found in A capsids, C capsids, or mature virions (6, 10, 18, 19).

To study DNA exit from the HSV-1 capsid, as observed in vivo in the situations described above, we have developed an in vitro model for the overall process. Purified C capsids were treated with guanidine-HCl (GuHCl) under conditions that do not cause disruption of the capsid icosahedron (18, 20); treated capsids were then analyzed for DNA loss by biochemical and electron microscopic methods. Our results obtained with this system, as described below, show that DNA can be extruded in vitro from intact capsids, and examination of capsids in the process of losing their DNA has clarified some details of how DNA exit takes place.

MATERIALS AND METHODS

Virus growth and capsid purification. All experiments were carried out with the 17MP strain of HSV-1, which was grown on monolayer cultures of BHK-21 cells as previously described (17). Capsids were isolated from infected cells by the method of Perdue et al. (21) with modifications adopted in our laboratory (17, 18). Preparations were carried out, beginning with 20 to 30 roller bottles (850-cm² surface area each) of cells that had been infected for 18 to 24 h. Yields were in the range of 0.5 to 1.0 mg for C capsids and 5 to 12 mg for B capsids. Electron microscopic analysis showed that C capsid fractions contained less than 15% B and less than 10% A capsids.

Treatment of capsids with GuHCl and biochemical analysis of treated capsids. Purified capsids or capsid mixtures to be treated with GuHCl were suspended in TNE-D buffer (20 mM Tris-HCl [pH 7.5], 0.5 M NaCl, 1 mM EDTA, 50 mM dithiothreitol) at a concentration of 1.5 to 2.0 mg of protein per ml and adjusted to 200 µg of protein per ml at the desired GuHCl concentration by addition of TNE-D buffer and TNE-D containing 6.0 M GuHCl. These preparations were used within 1 to 10 min for electron microscopic analysis of

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capsids in the process of losing their DNA. In all other cases, the capsid-GuHCl suspensions were incubated for 1 h at room temperature before further operations were performed. Sucrose density gradient analyses were carried out with 300 to 600 μ l of GuHCl-treated capsids, which were layered on top of a linear 20 to 40% sucrose gradient prepared in TNE-D buffer (no GuHCl in the gradient) and centrifuged for 45 min at 24,000 rpm in a Beckman SW50.1 rotor at 4°C. The resulting gradients were photographed with overhead illumination so that capsid bands were visualized by scattered light. The amount of capsid material present in individual bands was determined quantitatively by densitometric scanning of the photographic negatives in an LKB Ultrosan XL laser densitometer. Data were integrated by use of the LKB 2400 Gelscan program (version 1.2). GuHCl-treated capsids to be employed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopic analyses were recovered by centrifugation through a 150- μ l layer of 20% sucrose in a tube (10 by 41 mm) with a capacity of 0.8 ml. Centrifugation was for 90 min at 22,000 rpm in a Beckman SW50.1 rotor. Previously described procedures were employed for solubilization of capsid proteins and their analysis by SDS-PAGE (18, 20).

Electron microscopy. All electron microscopy was performed with capsids that were adsorbed to carbon-Formvar-coated copper electron microscope grids, fixed with 1% glutaraldehyde for 1 h at room temperature, critical point dried, and shadowed with Pt-C (13° angle) as described previously (18, 20). All microscopic observations were made with a JEOL 100CX transmission electron microscope operated at 80 keV. Capsid surface architecture was examined in samples that were treated with GuHCl for 1 h at room temperature and collected by centrifugation through a 20% sucrose layer as described above. Electron micrographs of C capsids in the process of extruding their DNA were prepared by treating purified C capsids with 0.2 to 0.5 M GuHCl for 1 to 10 min at room temperature as described above and then adsorbing them immediately to electron microscope grids. Specimens were then washed by floating grids briefly (30 s) on a drop of TNE-D buffer before fixation, critical-point drying, and shadowing as usual. DNase treatment, which was performed on selected samples (see Results), was carried out with GuHCl-treated capsids after they were adsorbed to electron microscope grids but before fixation or drying. Capsid-containing grids were floated on a drop of 2 mM MgSO₄-10 mM acetate buffer (pH 6) containing 50 μ g of DNase I per ml for 30 min at room temperature, washed briefly in TNE-D, and then processed further for electron microscopy. DNA strand diameters were measured in a data set consisting of 20 positive prints made from electron microscope negatives. The total magnification in all cases was at least \times 80,000. The diameters reported are average values obtained from several (at least five) measurements made at regular intervals along DNA fibers that appeared to be reasonably uniform in thickness.

RESULTS

Sucrose density gradient analysis of control and GuHCl-treated capsids. The ability of GuHCl to promote DNA loss from HSV-1 capsids was tested by treating C capsids in vitro with GuHCl and analyzing them for the presence of DNA by sucrose density gradient ultracentrifugation. Purified B capsids were added to the experimental sample (1:2 molar ratio of B-C capsids) as an internal control, since B capsids are converted to A capsids at a known GuHCl concentration (\sim 1.0 M [18]). Figure 1 shows the sucrose gradients obtained when capsid

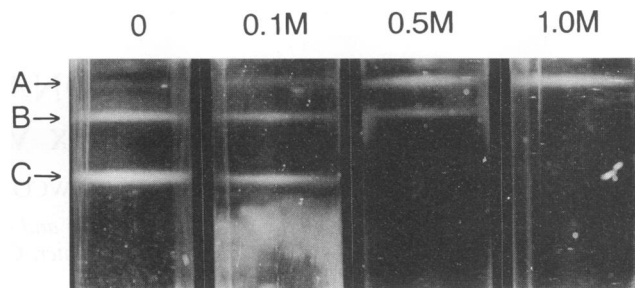


FIG. 1. Sucrose density gradient analysis of C-B capsid mixtures (2:1 molar ratio) after treatment in vitro with GuHCl at the concentrations indicated. Capsids were prepared, treated with GuHCl, and centrifuged as described in Materials and Methods. The resulting gradients were photographed with overhead illumination so that capsid bands could be visualized by scattered light.

mixtures were exposed to GuHCl at concentrations of 1.0 M or lower. The gradients demonstrate that whereas only the input capsid types were observed in untreated and 0.1 M GuHCl-treated samples, C capsids were lost quantitatively after treatment with GuHCl at concentrations of 0.5 M or higher (Fig. 1). Depletion of the C capsid population was accompanied by a corresponding increase in the amount of material sedimenting in the A (DNA-free) capsid region (see Table 1 for quantitation), suggesting that 0.5 M GuHCl caused loss of DNA from C capsids and thereby converted them to A capsids. All capsid material was found to sediment in regions of the gradient corresponding to either A, B, or C capsids; there was no evidence for the presence of bands in other regions of the gradient (e.g., between the B and C capsid regions).

When capsid mixtures were treated with GuHCl at concentrations between 0.1 and 0.5 M, some but not all C capsids were converted to A capsids, the exact proportion varying from one preparation to another (data not shown). As with capsids treated with 0.5 M GuHCl, all capsid material sedimented in regions of the gradients corresponding to A, B, or C capsids.

Treatment of capsid mixtures with 0.5 M GuHCl caused no noticeable change in the sedimentation rate of B capsids (Fig. 1, gradient labeled 0.5 M). B capsids were, however, converted to A capsids when mixtures were exposed to 1.0 M GuHCl (Fig. 1, gradient labeled 1.0 M; Table 1). The presence of B capsids did not affect the GuHCl concentration required to promote DNA loss from C capsids. In highly purified C capsid preparations, 0.5 M GuHCl promoted DNA loss from 95% or more of the capsids, whereas little or no conversion to A capsids was observed after treatment with 0.1 M GuHCl (data not shown).

SDS-PAGE and electron microscopy of DNA⁺ and DNA⁻

TABLE 1. Capsid types present after treatment of C-B capsid mixtures (2:1 molar ratio) with GuHCl^a

GuHCl concn (M)	Fraction of total capsid population (%)		
	A	B	C
0 (control)	3	36	61
0.1	16	33	51
0.5	64	36	
1.0	100		

^a The photographic negatives employed to produce Fig. 1 were scanned in a laser densitometer as described in Materials and Methods. For each GuHCl concentration tested, the integrated density of each band was expressed as a fraction of the total density in all of the bands present.

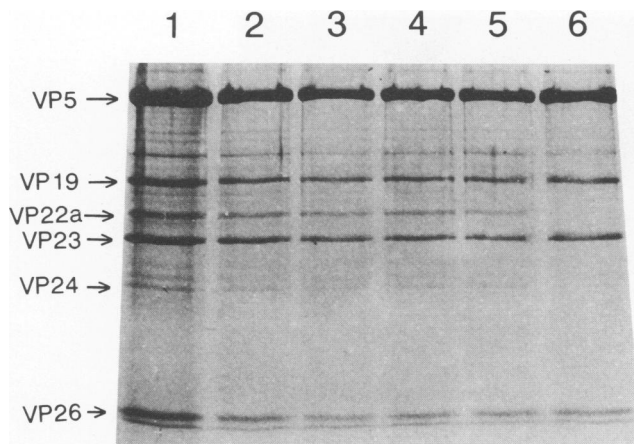


FIG. 2. SDS-PAGE analysis of C-B capsid mixtures (2:1 molar ratio) after treatment *in vitro* with GuHCl. Capsid mixtures were treated with TNE-D (lane 2) or TNE-D containing 0.1 M (lane 3), 0.2 M (lane 4), 0.5 M (lane 5), or 1.0 M (lane 6) GuHCl and recovered by centrifugation through a sucrose cushion as described in Materials and Methods. Lane 1 shows the beginning capsid mixture before any operations were performed. Protein bands were visualized by staining the gel with Coomassie blue (18).

capsids. Figure 2 shows the results of SDS-PAGE analyses carried out with the control and GuHCl-treated capsid mixtures described above. The gels demonstrate that GuHCl at concentrations of 0.5 M or lower had very little effect on capsid protein composition. In particular, no obvious change in protein composition correlated with DNA loss from C capsids (compare lanes 3 [DNA⁺ C capsids] and 5 [DNA⁻ capsids]). The predominant proteins present in both DNA⁺ and DNA⁻ preparations were VP5, VP19, VP23, and VP26. Quantitative analysis of stained bands indicated that the relative proportions among the four proteins did not differ to a greater extent in DNA⁺ compared with DNA⁻ preparations than in different specimens of DNA⁺ capsids (data not shown). As expected, 1.0 M GuHCl was found to solubilize VP22a from B capsids (compare lanes 5 and 6). This is consistent with the sucrose density gradient analyses (Fig. 1) showing that B capsids were converted to A capsids at 1.0 M GuHCl.

To assess the effect of DNA loss on capsid shell structure, control and 1.0 M GuHCl-treated C capsids were examined by electron microscopy of critical-point-dried and shadowed preparations. Purified C capsids were treated with either neutral buffer (TNE-D) or buffer containing 1.0 M GuHCl, reisolated by sucrose density gradient ultracentrifugation, and prepared for electron microscopy. Micrographs showed a close structural similarity between control and 1.0 M GuHCl-treated capsids (compare Fig. 3a and b). For example, there was no systematic difference in overall capsid diameter, in capsomer size or morphology, or in the spacing between capsomers. All capsomers, both hexons and pentons, were present in control and in 1.0 M GuHCl-treated capsids. By contrast, pentavalent capsomers were found to be lost selectively when C capsids were treated with 2.0 M rather than 1.0 M GuHCl (gaps in capsids shown in Fig. 3c). As in the case of 2.0 M GuHCl-treated B capsids (18, 20), penton loss appeared to be quantitative and highly selective for pentons as opposed to hexons. Wherever they could be clearly distinguished, gaps corresponding to missing capsomers were found to be surrounded by five rather than six capsomers (arrows in Fig. 3c).

Capsids in the process of losing their DNA. To visualize

capsids in the process of extruding DNA, purified C capsids were treated with 0.2 to 0.5 M GuHCl for 1 to 10 min at room temperature, adsorbed immediately to electron microscope grids, and processed for electron microscopy by critical-point drying and shadowing. The resulting images (Fig. 4 and 5) showed capsids connected by a network of fibers or strands, each of which was relatively uniform in diameter. Appearance of the fibers was dependent on GuHCl treatment; many fewer fibers, or none at all, were observed when capsids were not exposed to GuHCl (Fig. 4a). The fibers were assumed to be composed of DNA because they were lost selectively when preparations were treated with DNase I and because no comparable structures were observed after treatment of A or B capsids with GuHCl (data not shown). Fiber images did not differ greatly when C capsids were treated with GuHCl after adsorption to electron microscope grids rather than in solution as described above (data not shown).

In all preparations, fiber images were of two types: (i) darkly staining strands which nearly always extended in a straight line (Fig. 4b and c) and (ii) more lightly staining strands that could trace a more curvilinear course (Fig. 4d). All analyses were performed with the latter, more lightly staining fiber images, as these corresponded more closely to DNA images found in capsid-free DNA preparations (data not shown), and they predominated in regions of low capsid density, where fiber origins and ends could be more easily identified. We presume that the darkly stained fibers are straight because they shrink somewhat during preparation (by critical-point drying) for electron microscopy. Shrinkage may also cause the same fibers to be stretched slightly above the grid surface, where they would be shadowed on all sides and therefore appear darker than substrate-adherent fibers (the more lightly staining ones).

DNA fibers were found to arise from multiple, discrete sites on the capsid shell (see Fig. 4d and 5). In many cases, it was difficult to distinguish fiber origins from places where a fiber and a capsid were fortuitously juxtaposed on the grid. This situation was observed so often (Fig. 4b and c) as to suggest that capsid surfaces have an affinity for DNA. In other cases, however, fiber origins could be identified with greater confidence, particularly when the number of capsids on a grid was low or when a free fiber end could be traced to its capsid source (Fig. 5a and b). Examination of the most favorable images showed that an individual capsid could be the source of 0 to 7 DNA fibers. Nearly all capsids had at least one origin, and it was not unusual to find those with three or more, as shown in Fig. 4d and 5a and b. Origins were distributed on the capsid surface in such a way as to suggest that they coincide with capsid vertices. For instance, fiber origins were most often found at the vertices of the hexagonal profiles projected by capsids in electron micrographs (Fig. 4d and 5), and the closest unambiguous origins were nearly always found to be separated by at least the distance of a capsid edge.

Many DNA fiber ends could be identified in micrographs of GuHCl-treated C capsids, and in favorable instances the ends could be followed back to the capsid of origin. In some cases, more than two strand ends could be traced to the same capsid (see Fig. 5a and b), suggesting that at least some of the ends must correspond to looped DNA double helices since the HSV-1 genome contains only two physical ends (15, 25).

Although different extruded DNA fibers had different diameters, most individual fibers were quite uniform over long distances (300 nm or more). Diameters were found to be in the range of 4 to 13 nm, with preferred values of approximately 7 and 11 nm, as shown in Fig. 6. Occasionally branch points were observed at which one thick DNA fiber gave rise to two thinner ones. Examples of such branch points are shown in Fig. 5c and

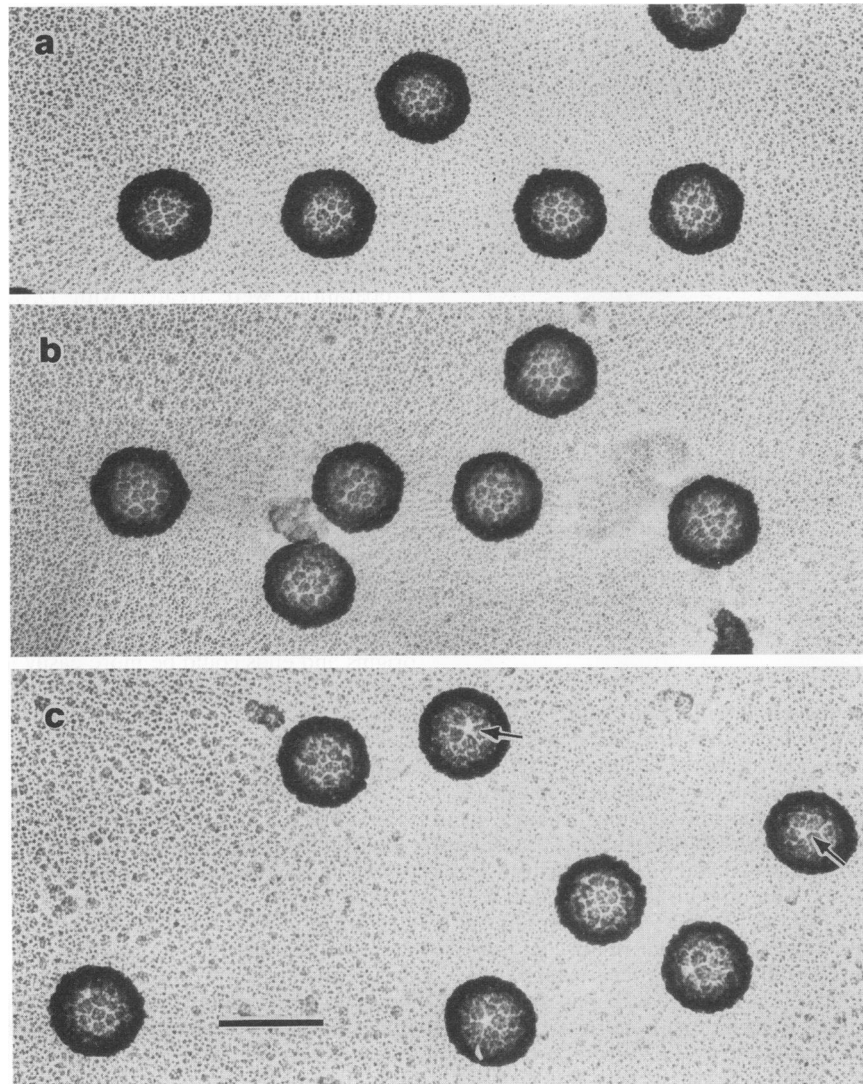


FIG. 3. Electron microscopy of control HSV-1 C capsids (a) and C capsids after treatment in vitro with 1.0 M (b) or 2.0 M (c) GuHCl. The arrows in panel c indicate the sites of representative missing pentons. Bar, 120 nm.

d (arrows). There was no evidence of supercoiling in the extruded DNA as found, for instance, in DNA lost from phage Mx416 after spreading on a 0.1 M NaCl hypophase (32). Absence of supercoiling may be due to the presence of single-strand breaks in the HSV-1 genome (9, 33).

DISCUSSION

HSV-1 DNA, as found in the virion and in the C capsid, is a single, linear molecule of double-stranded DNA approximately 153,000 bp long. Electron micrographs of C capsids preserved in the frozen-hydrated state (6) indicate that the DNA is condensed in the same liquid crystalline arrangement found in double-stranded DNA bacteriophage (14, 16). Strands are aligned (spacing, 2.6 nm) in local regions of the capsid cavity, but there appears to be no long-range packing order. The highly condensed DNA mass exerts outward pressure on the capsid shell (24), so it is expected that a relatively mild perturbation, such as that produced by the low GuHCl concentrations employed here, could cause DNA to be ex-

truded. In fact, the GuHCl concentration required to cause DNA loss from C capsids (0.2 to 0.5 M) is lower than that found to extract any other capsid component yet examined. For example, pentons, VP26, and VP22a are removed (from B capsids) at GuHCl concentrations of 1.5, 1.2, and 1.0 M, respectively (18). Similarly mild treatments have been found to cause extrusion of DNA from bacteriophages. Osmotic shock, for instance, is effective in promoting DNA loss from *Escherichia coli* phages T2 and T4 (5, 13); spreading on an H₂O or 0.1 M NaCl hypophase causes DNA extrusion from phages λ and Mx416 (27), and 0.1 M CaCl₂ causes DNA loss from phage ϕ X174 (34).

If HSV-1 DNA is to enter the nucleus of an infected cell as a single molecule (i.e., without breaking into fragments), then it must leave the virus capsid through a single site. At the time of injection, therefore, a unique vertex must be defined. Packaging of DNA into the B capsid must also involve the functioning of a unique site (possibly also a vertex). The results described here, however, have provided no clues about the structure or functioning of a unique capsid vertex. All pentons

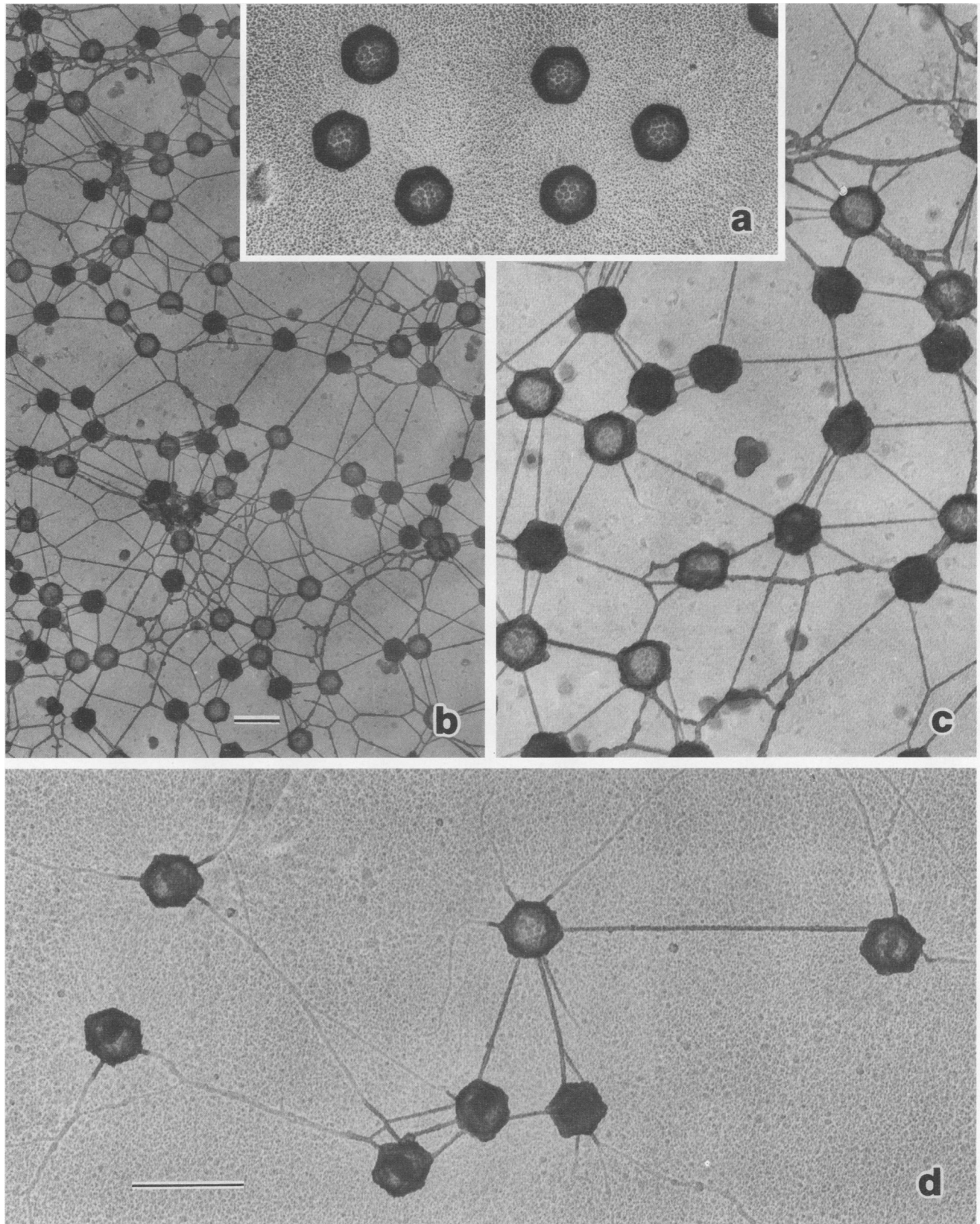


FIG. 4. Electron microscopy of control HSV-1 C capsids (a) and 0.5 M GuHCl-treated C capsids in the process of extruding their DNA (b to d). Purified C capsids were treated with 0.5 M GuHCl and prepared for electron microscopy by critical-point drying and shadowing as described in Materials and Methods. Panels b and c show representative fields where extruded DNA fibers are straight and darkly staining, while more curvilinear, lighter-staining fibers are shown in panel d. Bar, 200 nm. Panels a, c, and d are shown at the same magnification.

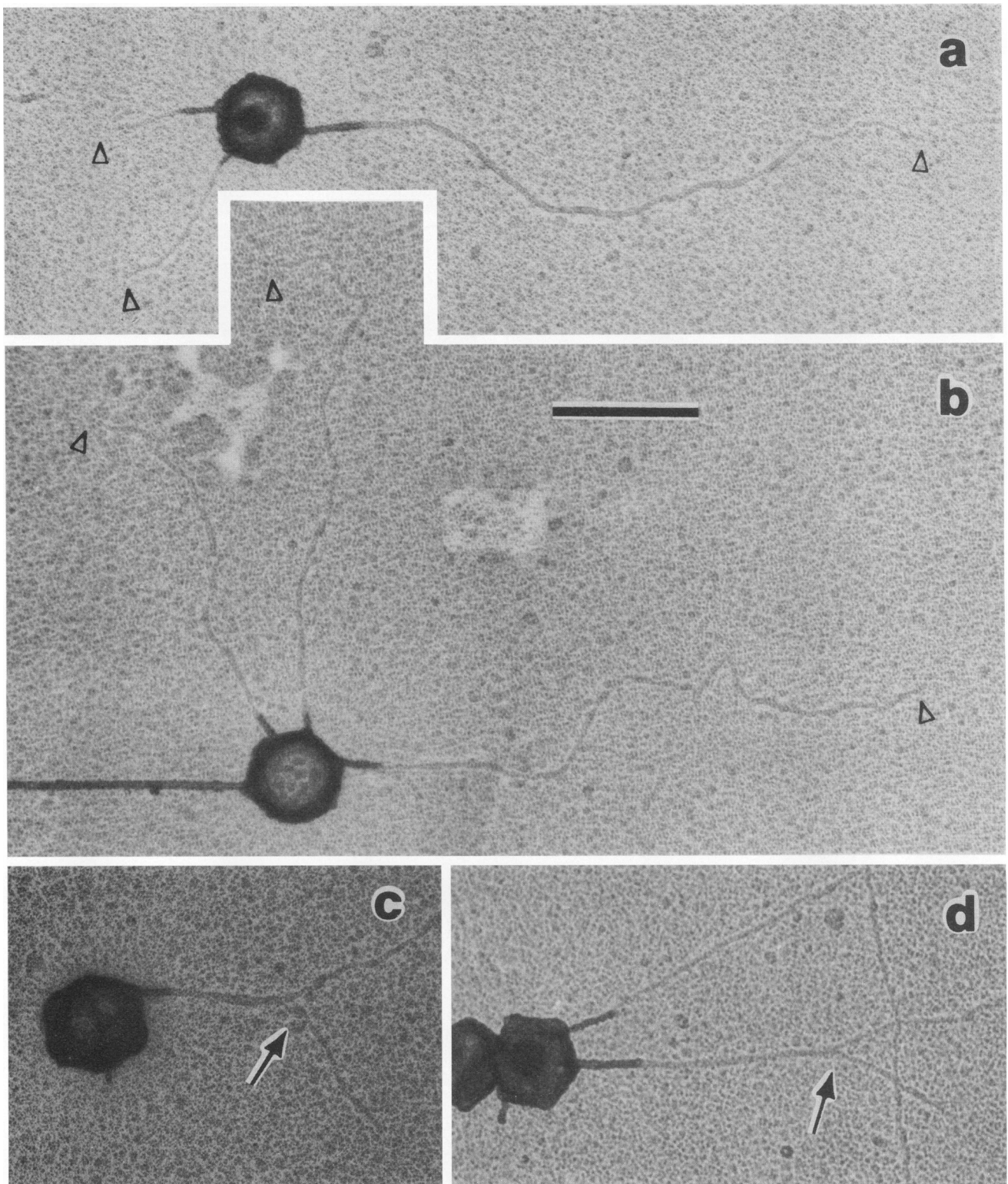


FIG. 5. Electron microscopy of 0.5 M GuHCl-treated C capsids in the process of extruding their DNA. Panels a and b show DNA fiber ends (arrowheads) that can be traced to the capsid of origin. DNA fiber branch points (arrows) are shown in panels c and d. Bar, 150 nm.

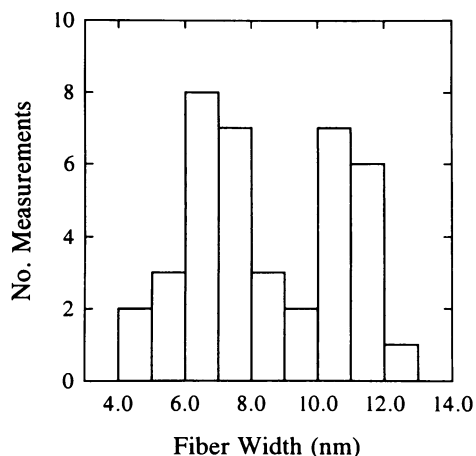


FIG. 6. Histogram of measured DNA fiber widths. Measurements were made on a total of 39 fibers in eight different micrographs.

were found to have the same morphology in electron micrographs of critical-point-dried and shadowed capsids (Fig. 3a and b; see also references 18 and 20). Similar analysis of capsids in the process of losing their DNA showed that DNA extrusion takes place at multiple discrete sites that appear to coincide with capsid vertices. As all 12 vertices have the same morphology and occupy equivalent positions in the capsid icosahedron, extrusion of DNA from several sites in the same capsid suggests that all 12 pentons have the potential to serve as DNA exit sites, at least in GuHCl-treated capsids *in vitro*. We found no evidence for preferential DNA exit through a unique vertex. As we have observed with HSV-1, DNA is found to be extruded from multiple capsid vertices when phage ϕ X-174 is treated with an inducing agent (0.1 M CaCl₂) *in vitro* (34).

How, then, can a unique vertex be defined? It is possible that one fivefold site is differentiated from the other 11 by the presence of a minor capsid protein and/or by a structural feature not resolved by the electron microscopic techniques employed here. Its functioning could require a specific tegument protein or possibly an interaction of the C capsid with a nuclear pore. In the absence of such an interaction, all capsid vertices may be able to serve as DNA exit sites, as observed in the present study.

A remarkable feature of the results described here is that DNA was found to leave the C capsid without producing any apparent damage to the capsid shell. For instance, although the diameter of the extruded DNA fibers (4 to 13 nm) is greater than the diameter (~3 nm) of the capsomer axial channels (the only holes in the capsid shell [1, 6, 18]), electron microscopic examination of capsids after DNA extrusion showed no change in the morphology of the pentons or any other capsid structure. Similarly, SDS-PAGE analysis showed no significant difference in the protein composition of C capsids before versus after induced DNA exit (Fig. 2). These findings suggest that the GuHCl-treated capsid may permit DNA extrusion by a "trapdoor" mechanism in which holes open transiently while DNA is leaving and close afterwards. This would be accomplished, for example, if the transpenton axial channels or other sites near the pentons were to open in a reversible manner during DNA extrusion. Opening would have to be restricted to the pentons, since DNA does not appear to be extruded at hexon sites. The capsids after extrusion would be expected to lack DNA but to have the same

morphology and protein composition as C capsids. These are the properties of A capsids (1, 6, 10), so it is expected that DNA-free C capsids will sediment during sucrose density gradient ultracentrifugation at the same rate as A capsids, as we have observed (Fig. 1). Our findings are, therefore, consistent with the idea that A capsids may originate *in vivo* by premature DNA loss from C capsids (22).

The DNA fibers extruded from C capsids were all found to be greater in diameter than a single DNA double helix (~2.0 nm). The thinnest (~4.0 nm in diameter) and thickest (~13.0 nm), for example, correspond in diameter to two and six or more double helices, respectively. It is most probable, therefore, that the extruded fibers correspond to multiple DNA strands that are aligned laterally. As more than two fiber ends are often traceable to the same capsid, it is likely that some of the extruded fibers correspond to DNA loops. No loops were observed in the extruded DNA fibers examined in the present study, but their absence may be due to the method (critical-point drying and Pt shadowing) employed to prepare specimens for electron microscopy. We are attempting to identify conditions of sample preparation that will permit loops to be visualized in DNA fibers extruded from C capsids.

The extrusion of HSV-1 DNA from multiple capsid vertices as described here helps clarify the issue of how DNA may be arranged in the C capsid shell. It is readily apparent that DNA could be extruded at multiple sites if it were arranged in the capsid in the form of folds or loops, as hypothesized in the spiral-fold and related models (3, 4, 27). As extrusion at multiple sites is more difficult to imagine if DNA is packed in accordance with spooled or concentric-shell schemes (8, 11), the present results favor DNA packing arrangements based on loops or folds.

We assume that DNA extrusion from GuHCl-treated capsids begins when the combination of internal DNA pressure and denaturant-induced modification of the capsid surface weakens a penton sufficiently to allow DNA to escape. The extruded DNA would, according to this assumption, arise from the local region of the capsid cavity closest to the vertex from which it projects. Since any region of the genome can lie beneath a capsid vertex (16, 28), the extruded DNA is expected to be nonspecific with respect to its location on the HSV-1 genetic map.

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