The Conformation of DNA Packaged in Bacteriophage G

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ABSTRACT When packaged in a bacteriophage capsid, double-stranded DNA occupies a cavity whose volume is roughly twice the volume of the DNA double helix. The data thus far have not revealed whether the compactness of packaged bacteriophage DNA is achieved by folding of the DNA, unidirectional winding of the DNA, or a combination of both folding and winding. To assist in discriminating among these possibilities, the present study uses electron microscopy, together with ultraviolet light-induced DNA-DNA cross-linking, to obtain the following information about the conformation of DNA packaged in the comparatively large bacteriophage, G: 1) At the periphery of some negatively stained particles of bacteriophage G, electron microscopy reveals strands of DNA that are both parallel to each other and parallel to the polyhedral bacteriophage G capsid. However, these strands are not visible toward the center of the zone of packaged DNA. 2) Within some positively stained particles, electron microscopy reveals DNA-associated stain in relatively high concentration at corners of the polyhedral bacteriophage G capsid. 3) When cross-linked DNA is expelled from its capsid during preparation for electron microscopy, some DNA molecules consist primarily of a compacted central region, surrounded by DNA strands that appear to be unraveling at multiple positions uniformly distributed around the compacted DNA region. The above results are explained by a previously presented model in which DNA is compacted by folding to form 12 icosahedrally arranged pear-shaped rings.

INTRODUCTION

Of the studied double-stranded DNA bacteriophages, all have a DNA genome packaged in a cavity with a volume that is twice the volume of the packaged DNA double helix (reviews: Earnshaw and Casjens, 1980; Casjens, 1985a; Serwer, 1989). This degree of compaction might be achieved by unidirectional winding of the DNA, folding of the DNA, or a combination of both winding and folding (Earnshaw et al., 1978; Harrison, 1983; Black et al., 1985; Witkiewicz and Schweiger, 1985; Serwer, 1989; Hud, 1995). However, thus far, discrimination among these possibilities has not been made. The following observations constrain models (reviews: Earnshaw and Casjens, 1980; Welsh and Cantor, 1987; Black, 1989; Serwer, 1989; Wurtz, 1992; Hud, 1995): 1) Both low-angle x-ray scattering and cryoelectron microscopy reveal that packaged DNA forms domains of parallel double-helical segments. 2) Probing with reagents specific for DNA secondary structure reveals regions of perturbed DNA duplex. 3) Both DNA-capsid and DNA-DNA cross-linking reveal the absence of strict inside-to-outside order, such as that expected of simple concentric winding on a spool; however, one end of the packaged DNA is, for some bacteriophages, found preferentially in the outer region of the cavity in which DNA is packaged. Furthermore, electron microscopy of a negatively stained specimen reveals DNA strands that form circular patterns when in an early stage of expulsion from several double-stranded DNA bacteriophages (λ, P2, T4, T5, and T7; Richards et al., 1973). This type of pattern has also been observed by cryoelectron microscopy of intact bacteriophage T7 (Booy et al., 1992; Cerritelli et al., 1996). Unidirectional winding of packaged DNA is a possible explanation. However, as clearly shown experimentally by observation of DNA compacted by ethanol (Eickbush and Moudrianakis, 1978), unidirectional winding is not the only possible explanation. For example, a rod formed by folded double-helical DNA segments will yield such a pattern if the rod cyclizes by the joining of its two ends smoothly enough so that the joint cannot be resolved. Thus the constraints of previous studies are not sufficient to propose a detailed model for the conformation of any packaged bacteriophage DNA.

In the present study, electron microscopy has been performed of the DNA packaged in bacteriophage G, a bacteriophage that has a DNA at least three times longer than the DNA genomes of previously studied bacteriophages. Bacteriophage G has both a polyhedral outer shell, approximately 80 nm in radius (Ageno et al., 1973; both icosahedral and octahedral outer shells were observed), and a double-stranded linear DNA, 670 kb long (Hutson et al., 1995). Genetic analysis of bacteriophage G has not yet been performed. However, the comparatively large size of bacteriophage G could potentially simplify the search for additional information about the conformation of packaged bacteriophage DNA.

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MATERIALS AND METHODS

Preparation of bacteriophage G and G DNA

Bacteriophage G was preparatively grown in agar overlays; bacteriophage particles were purified by rate zonal centrifugation in a sucrose gradient (Serwer et al., 1995). Purified bacteriophage G was stored in 0.01 M

Tris-Cl (pH 7.4), 0.01 M $MgSO_4$, 6% polyethylene glycol with a molecular weight of 3350. To expel DNA from bacteriophage G before preparation for microscopy, 1 μl of a bacteriophage preparation was diluted into 9.0 μl of 0.1 M NaCl, 0.01 M sodium phosphate (pH 7.4), 0.001 M EDTA (NPE buffer); this mixture (1 μg DNA/ml) was placed in a water bath for 30 min at 60°C (see Serwer et al., 1995). To expel G DNA before preparation for electrophoresis, this procedure was used with NPE buffer that had 1% Sarkosyl NL97. Slow pipetting in pipettes at least 1.5 mm in diameter was used to avoid hydrodynamic shear-induced breakage of G DNA (see Zimm and Reese, 1990).

Electron microscopy

Samples (10–18 μg DNA/ml) were prepared for electron microscopy by a procedure designed for negative staining with uranyl acetate. After preparation of support films, a sample was placed on the film, washed, and finally, negatively stained with uranyl acetate (Serwer, 1977). Unless otherwise indicated, the wash was performed with five drops of water; staining was performed with two drops of uranyl acetate in a time between 15 and 45 s. This type of procedure does not cause condensation of DNA; in contrast, this type of procedure decondenses DNA that had previously been condensed by binding to a polypeptide (Vengerov and Semenov, 1992). Electron microscopy was performed by the use of a Philips 301 transmission electron microscope. In control experiments, none of the conditions used for electron microscopy caused bacteriophage G DNA to adopt the condensed conformation described here, when un-cross-linked DNA expelled from its capsid was the sample. All magnification bars are 100 nm long. For reproduction, all images were first digitized and then reproduced photographically (Griess et al., 1992). Because of the comparatively large size of bacteriophage G, contrast must sometimes be suppressed to visualize the background, while also visualizing the interior of the bacteriophage. When contrast was not suppressed (Fig. 2, below), the background was often lost.

To test for rotational symmetry in the image of a bacteriophage capsid with packaged DNA, software for the following was both added to the previously used program, NIH Image (Griess et al., 1992), and applied to centered images of the DNA-filled capsid of bacteriophage G: 1) rotation of the image in steps separated by $2\pi/n$ radians (n is an integer); 2) creation of a stack of the n images thus obtained; and 3) averaging of the n images to form a single, averaged image. (This software was written by Dr. G. A. Griess; it is available from the authors on request.) As previously shown (Markham et al., 1963; Crowther and Amos, 1971), this type of procedure yields a reinforced image to the extent that n represents the symmetry present. Because of variability of staining of particles analyzed, rotational power spectra (Crowther and Amos, 1971) were not used to test for symmetry (see also Kocsis et al., 1995).

Ultraviolet light-induced cross-linking

By use of a germicidal lamp according to a procedure previously described (Serwer et al., 1992), bacteriophage G was exposed for 20–30 min to ultraviolet light that had previously been found to induce both DNA-capsid and DNA-DNA cross-linking of bacteriophage T7 (Serwer et al., 1992). Gel electrophoresis (next paragraph) revealed that cross-linking for a time greater than 30 min caused significant breakage of the DNA. Immediately before cross-linking, the bacteriophage was diluted 10-fold into 0.5 M NaCl, 0.01 M Tris-Cl (pH 7.4), 0.001 M $MgCl_2$.

To quantify cross-linking of G DNA, after release of the DNA from its capsid, G DNA (three parts) was mixed with a solution (one part) that contained 14% Nycodenz, 400 μg/ml bromophenol blue. Of this mixture, 30 μl was layered in the sample well of a 1.5% submerged, horizontal agarose (Seakem LE agarose, FMC Bioproducts) gel cast in electrophoresis buffer: 0.09 M Tris-acetate, pH 8.4, 0.001 M EDTA. After layering, the DNA was subjected to pulsed-field agarose gel electrophoresis (PFGE) in the field inversion mode. PFGE was performed at 3.0 V/cm, 15 ± 3°C for 30 h. Pulsing was performed by the use of a linear ramp of pulse times: forward pulse time = 12→39 s; reverse pulse time = 4→13 s. After PFGE, the DNA that formed a band was quantified by, first, staining with 1 μg/ml ethidium bromide for 2 h, followed by destaining with 0.001 M sodium EDTA, pH 7.4. Subsequently, quantification was performed by video fluorometry (Griess et al., 1995). To determine the number of DNA-DNA cross-links, the assumption was made that introducing either one cross-link or more in a G DNA molecule was sufficient to remove the molecule from the region of the band formed by un-cross-linked G DNA. By further assuming that the number of cross-links per molecule followed a Poisson distribution, the fraction of un-cross-linked DNA was used, together with the Poisson distribution (chapter 9 in Campbell, 1989), to determine the average number of (PFGE-detected) cross-links per DNA molecule.

RESULTS AND DISCUSSION

Electron microscopy of negatively stained bacteriophage G

In an attempt to observe domains of packaged G DNA, a specimen of bacteriophage G was prepared by negative staining. When observed by electron microscopy, negatively stained particles had a polyhedral outer shell that encompassed packaged DNA; the outer shell had an external projection (tail) attached. For most particles, the packaged DNA formed a comparatively electron-transparent zone that had no visible ultrastructure. In this respect, these particles have the appearance previously reported by Ageno et al. (1973). Examples are shown in Fig. 1, *a–d*. To conserve space in Fig. 1, only part of the external bacteriophage G tail is shown (*arrowheads*); this tail has previously been described by Donelli et al. (1972).

In contrast to the negatively stained particles in Fig. 1, *a–d*, a few informative negatively stained particles (<5%) had structural detail apparently associated with the packaged DNA. These informative particles had domains in which DNA strands were parallel to both each other and the outer shell of the capsid (indicated by *arrows* in Fig. 1, *e–h*). In any given particle, the domains occupied most of the particle's perimeter. However, for all particles observed, these domains were only at the outer rim of the packaged DNA; a typical domain occupied a band 13–22 nm in width. No domain extended to the center of the region of packaged DNA. The informative particles (Fig. 1, *e–h*) always ap-

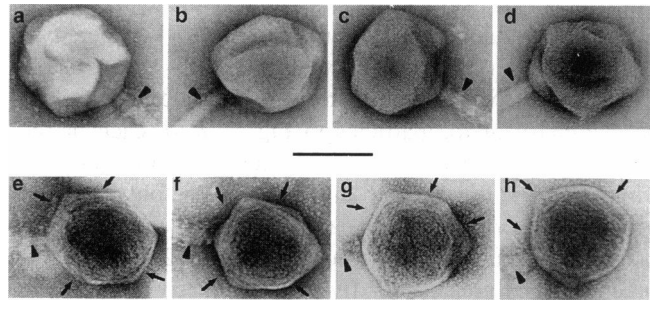

FIGURE 1 Electron microscopy of bacteriophage G. Images are presented of particles in a specimen prepared by negative staining with uranyl acetate. (*a–d*) Negatively stained particles that have a conventional appearance; (*e–h*) negatively stained particles that have visible strands of DNA near their periphery.

peared to have an interior darker than the interior of the comparatively uninformative particles (Fig. 1, *a–d*), as though the former had internalized more negative stain. For reasons not known, this internalization occurs at low frequency.

Electron microscopy of positively stained particles

On the same support films used to obtain the images of Fig. 1, positively stained particles were observed that had either no or comparatively little surrounding layer of negative stain. Usually no structural details were observed in the region of the positively stained DNA (not shown). For example, no evidence was observed for an internal aggregate of protein, such as that observed in bacteriophage T7 (reviews: Steven and Trus, 1986; Serwer, 1989). The positively stained particles appeared to be smaller than the negatively stained particles. For this difference in size, the cause has previously been shown, in the case of bacteriophage T7, to be flattening of the negatively stained, but not the positively stained, particles (Serwer, 1977). Positively stained particles often had either a hexagonal or pentagonal periphery, as though the outer shell were icosahedral.

Although most positively stained particles of bacteriophage G had no internal structure, some appeared to have the DNA-specific staining most intense in zones that were separated from each other by less intensely stained zones (each zone of comparatively intense staining will be called a stain-intense zone). Particles that had this appearance increased in frequency as the time of exposure to uranyl acetate solution increased to 5 min at the end of the preparation of the specimen. Some particles (5–15% of the total) had stain-intense zones (25 ± 3 nm in diameter) that appeared to be arranged with fivefold symmetry around a sixth, central stain-intense zone (29 ± 4 nm in diameter). The central stain-intense zone was always more intense than the peripheral stain-intense zones (Fig. 2, *a–d*). The peripheral stain-intense zones varied in intensity (compare Fig. 2 *a* with Fig. 2 *b*). Some of them formed a bulge at the corners of a rounded polyhedron-like border of the packaged DNA. For particles that had a visible tail (80% of the total), the tail was always attached to a corner that was between two corners occupied by stain-intense zones (tails are indicated by *arrowheads* in the top row of Fig. 2). To further explore the symmetry of the particles in Fig. 2, *a–d*, each of the particles was subjected to testing for fourfold rotational symmetry (*first row* beneath Fig. 2, *a–d*), fivefold rotational symmetry (*second row* beneath Fig. 2, *a–d*), or sixfold rotational symmetry (*third row* beneath Fig. 2, *a–d*). Reinforcement was greatest for fivefold symmetry, confirming the presence of the fivefold symmetry more qualitatively observed during inspection of the unprocessed images of Fig. 2, *a–d*.

The comparatively high intensity of the central stain-intense zone has two potential explanations: 1) The central

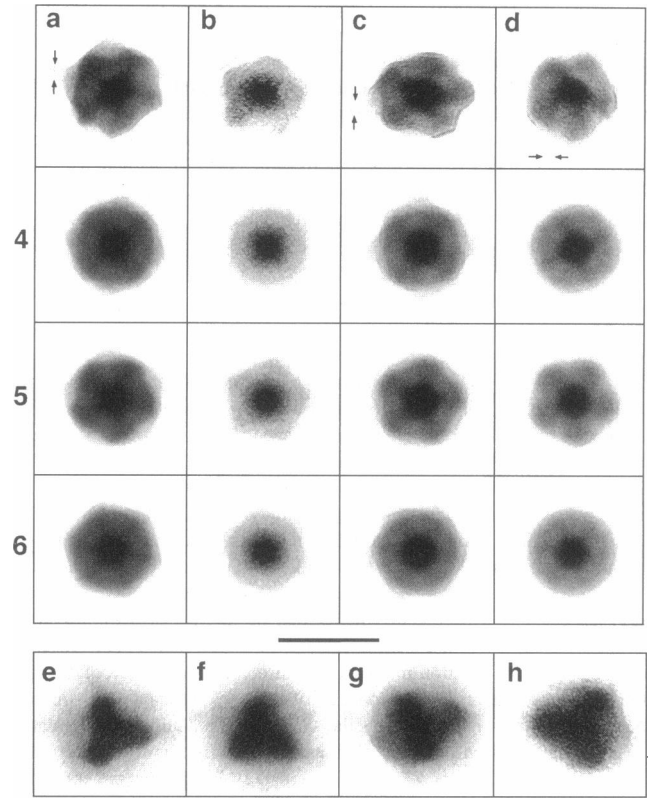

FIGURE 2 Informative positively stained particles. (*a–d*) Positively stained particles that have both a central stain-intense zone and five peripheral stain-intense zones. Beneath each original image presented in *a–d* is an image of the same particle after rotational averaging (fourfold, fivefold, and sixfold, as indicated in the figure). (*e–h*) Positively stained particles that have stain-intense zones, none of which is central.

stain-intense zone is formed by superposition of the images of two intensely stained regions, one at each of two corners of a polyhedron (presumably an icosahedron) that was viewed parallel to an axis of fivefold rotational symmetry (Caspar and Klug, 1962; Casjens, 1985b). 2) The intensity of staining varies with the distance of the intensely stained region from the support film; the central intensely stained region is either closer to or further from the support film than the peripheral intensely stained regions. These two explanations do not exclude each other. If the intensely stained regions were icosahedrally arranged and both sides of the particle were equally revealed, then a view along a fivefold axis would have 10 peripheral stain-intense zones. If, however, one side of the particle predominated, then this view would, as observed for bacteriophage G, have five peripheral stain-intense zones (Caspar and Klug, 1962; Casjens, 1985b). Therefore, the observation of five peripheral stain-intense zones suggests that the intense stain is predominantly on one side of the capsid. The observation of tails between stain-intense zones supports this assumption of one-sided contrast. If, as is likely, the tails are on the substrate-proximal side, then the stain-intense zones are produced by staining on the substrate-distal side.

If, as assumed here, the intensely stained regions were at the corners of a polyhedron, then images should exist in which all stain-intense zones are at positions other than the center of the image of packaged DNA. This type of image was observed (Fig. 2, *e–h*). The stain-intense zones of Fig. 2, *e–h*, are more intense than the five peripheral stain-intense zones of Fig. 2, *a–d*. This observation is explained by the above conclusion that the intensity of staining increases as the distance from the support film increases. That is, the intensely stained regions of Fig. 1, *e–h* are on the side of the particle distal from the support film and, therefore, farther from the support film than the peripheral intensely stained regions of Fig. 1, *a–d*. Because of variability of staining, a detailed interpretation has not been attempted for the arrangement of the intensely stained regions in Fig. 1, *e–h*.

A model for the conformation of packaged G DNA

For constraining models for the conformation of packaged G DNA, the stain-intense zones of positively stained bacteriophage G do not directly reveal DNA strands. Because the staining intensity between the stain-intense zones was less than the staining intensity of the more uniformly positively stained particles (not shown), the stain-intense zones appear to have been generated by either staining faster or destaining slower in the more intensely stained regions than it was in the other regions of the packaged DNA. The formation of a bulge at the position of some stain-intense zones indicates that the concentration of DNA in the more intensely stained regions is higher than it is in adjacent regions. If uranyl cations moved through the packaged DNA less rapidly as the density of the DNA increased (this relationship has been demonstrated for the movement of ethidium cations through packaged bacteriophage T7 DNA; Griess et al., 1986), then the differential staining must have been generated by comparatively slow destaining of the more concentrated DNA, when fluid was withdrawn from the support film at the end of staining. Thus the assumptions are made that 1) DNA has a comparatively high concentration in the more intensely stained regions of the packaged DNA, and 2) the intensely stained regions are at the corners of a polygon; the polygon is probably, but not necessarily, icosahedral.

Of the models that have been developed to describe the conformation of packaged bacteriophage DNA, the model of Witkiewitz and Schweiger (1985) best explains the data of Figs. 1 and 2. In this model, the conformation of the packaged DNA can be understood by imagining the following process (this process, however, does not necessarily represent the DNA packaging pathway that actually occurs): 1) DNA (Fig. 3 *a*) internally folds to form 12 multistranded bent rods (two bent rods are illustrated in Fig. 3 *b*; also, see figures 5 and 6 of Vengerov et al., 1985). 2) The ends of the bent rods join to form 12 pear-shaped rings, each similar to the single pear-shaped ring formed by a DNA molecule in figure 8 of Eickbush and Moudrianakis (1978) (two of these rings are shown in Fig. 3 *c*). 3) The 12 pear-shaped rings are placed in the corners of an icosahedron; the thin end of each pear points toward the center of the icosahedron (Fig. 3 *d*). By the model of Fig. 3, a pear-shaped ring forms each of the intensely stained regions observed in Fig. 2. In addition, this model explains parallel DNA strands present only at the periphery of the packaged DNA (Fig. 1, *e–h*). That is, flattening and shrinkage during negative staining would merge the DNA strands from the pear-shaped rings while maintaining them parallel to the capsid only in the outer region of the packaged DNA (illustrated in Fig. 3 *e*).

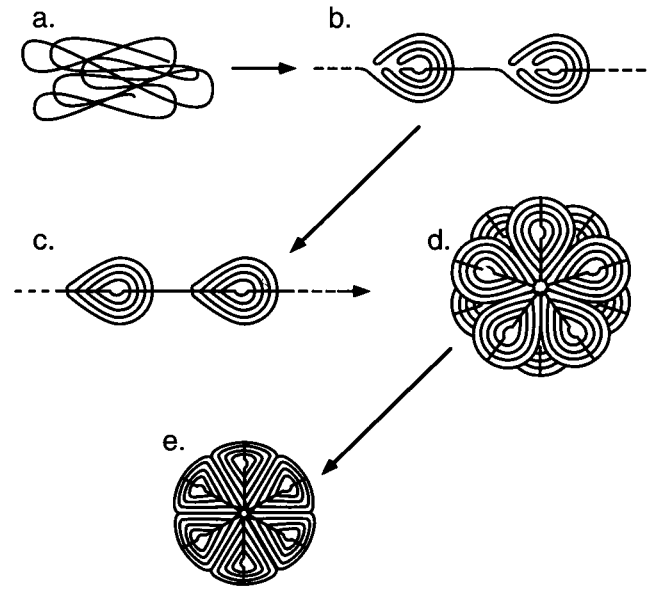

FIGURE 3 A model that accounts for the data. (*a–c*) Folding of DNA to yield (*d*) the conformation proposed by Witkiewicz and Schweiger (1985); (*e*) the change expected when bacteriophage G both shrinks and flattens during negative staining for electron microscopy. To avoid overlap of pear-shaped rings in *d* and *e*, one pear-shaped ring above and one pear-shaped ring below the plane of the image are not shown. If the number of pear-shaped rings were altered, this model would also apply to DNA packaged in a capsid that was not icosahedral.

Electron microscopy of partially unraveled DNA

A primary feature of the model in Fig. 3 is the absence of net winding of the DNA. Before an arc of 2π radians is achieved, all local winding is reversed by a fold. In the absence of net winding, a segment of compacted DNA can unravel, without disturbing neighboring segments along the double helix. The result would be uniformly distributed unraveling of independent DNA segments at multiple positions, even if the compacted DNA adhered to a support film either before or during unraveling. To determine the appearance of partially unraveled G DNA, packaged G DNA was cross-linked before its expulsion from a capsid. After cross-linking until PFGE revealed 1–2 DNA-DNA cross-links per DNA molecule without DNA breakage (Materials and

Methods), bacteriophage G was prepared for electron microscopy by a procedure of negative staining designed to expel DNA from bacteriophage already adsorbed to the support film. This procedure was that of the Materials and Methods, modified by washing of the support film-adsorbed bacteriophage with 5–7 drops of 0.001 M sodium EDTA, pH 7.4, instead of with water. The resultant specimens had capsids of primarily two types: 1) a DNA-filled capsid like those in Fig. 1, and 2) an empty capsid that was usually closely associated with partially unraveled DNA that had been expelled from the capsid (Fig. 4, *a–d*; the *arrows* indicate the capsids). This close association indicates that the DNA adhered to the support film either before or during unraveling; otherwise, the turbulence of washing and staining would have separated the DNA from its capsid. The fraction of empty capsids increased as the number of drops used to wash the support film was increased from 5 to 7. The unraveled portion of the DNA usually consisted of multiple overlapping DNA segments that were on all sides of the portion of the DNA that was still compacted (Fig. 4, *b–d*), as though unraveling had occurred en masse, rather than at the position of one segment of the DNA double helix. This aspect of the partially unraveled DNA confirms the above prediction of the model of Fig. 3. In contrast, if the DNA were unidirectionally wound, unraveling would occur at one point, thereby either 1) producing a single DNA segment that rotated around the compacted DNA region or 2) twisting DNA segments around each other, as they unraveled (see Bloomfield et al., 1974). Untwisting after unraveling appears unlikely because of adherence to the support film. Thus unraveling of unidirectionally wound DNA does not explain the appearance of partially unraveled DNA in Fig. 4, *b–d*.

In Fig. 4, *b* and *c*, the compacted portion of the DNA appears to have maintained some of the polygonal character of the capsid in which this DNA had been packaged. Because of the independence from each other of DNA segments separated by folds, this observation is also explained by the model of Fig. 3. This observation also requires inside-to-outside polarity during DNA packaging. Less direct procedures have suggested polarity of this type for the packaged DNA of bacteriophages λ, T4, and T7 (reviews: Welsh and Cantor, 1987; Serwer, 1989; Black, 1989).

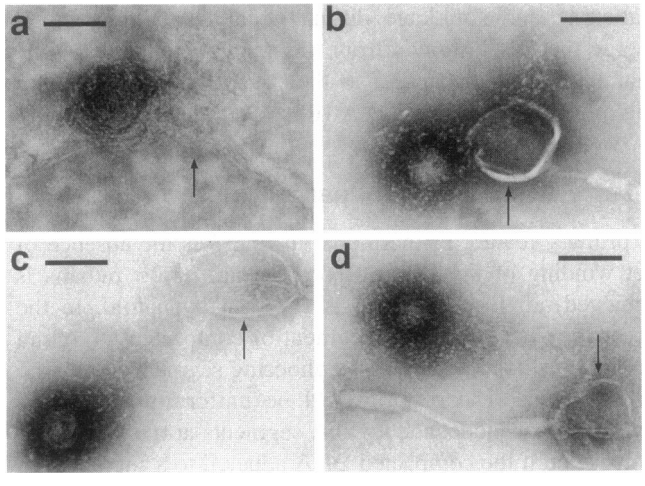

FIGURE 4 Electron microscopy of DNA expelled after cross-linking. After cross-linking, bacteriophage G was prepared for electron microscopy by a procedure that substituted washing with five to seven drops of 0.001 M sodium EDTA, pH 7.4, for washing with water. In *a–d*, four particles are presented that have expelled DNA from a nearby capsid (*arrows*).

CONCLUDING COMMENTS

In the present study, electron microscopy of bacteriophage G has yielded images that have informative characteristics; these characteristics were not present for all particles. The working assumption is made here that the DNA conformation of all bacteriophage G particles is constrained by the informative characteristics observed for some of the particles. If so, then the model of Fig. 3 has the features required by the informative characteristics of the images presented here. Clearly, related versions of this model would also account for the informative characteristics of these images. For example, small amounts of unidirectional winding beyond 2π radians could not have been detected by the procedures used here.

Witkiewicz and Schweiger (1985) presented the model of Fig. 3 for bacteriophages that had a DNA more than 10-fold shorter than bacteriophage G DNA. Twelve roughly spherical zones, like the 12 pear-shaped rings of this model, have previously been observed during electron microscopy of ion-etched adenovirus 2 (Newcomb et al., 1984; see also Brown et al., 1975). However, at least in the case of bacteriophage T7, cryoelectron microscopy has revealed circular patterns of parallel DNA that, in at least some particles, extended from the inner surface of the capsid to a position close to the center of the packaged DNA (Booy et al., 1992; Cerritelli et al., 1996). Thus the model of Fig. 3 is not in agreement with the data for T7. Possibly, in the case of T7, packaged DNA (the total length of T7 DNA is 39.936 kb; Dunn and Studier, 1983) compacts by forming one pear-shaped ring similar to each of the pear-shaped rings (56 kb each) that would be present in bacteriophage G, according to the model of Witkiewicz and Schwieger (1985). Similarly, elongated bacteriophage T4 variants (mature T4 DNA is 170 kb long) also appear to have packaged DNA in a single compartment (Earnshaw et al., 1978; Lepault et al., 1987). That is, the DNA compaction of T7 and, possibly, other bacteriophages may occur by a variation on the theme found here for packaged bacteriophage G DNA.

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