A Small (58-nm) Attached Sphere Perturbs the Sieving of 40–80-Kilobase DNA in 0.2–2.5% Agarose Gels: Analysis of Bacteriophage T7 Capsid–DNA Complexes by Use of Pulsed Field Electrophoresis†

Philip Serwer,* Shirley J. Hayes, Elena T. Moreno, and Christopher Y. Park‡

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284-7760

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ABSTRACT: Although the icosahedral bacteriophage T7 capsid has a diameter (58 nm) that is 234-fold smaller than the length of the linear, double-stranded T7 DNA, binding of a T7 capsid to T7 DNA is found here to have dramatic effects on the migration of the DNA during both pulsed field agarose gel electrophoresis (PFGE; the field inversion mode is used) and constant field agarose gel electrophoresis (CFGE). For these studies, capsid–DNA complexes were obtained by expelling DNA from mature bacteriophage T7; this procedure yields DNA with capsids bound at a variable position on the DNA. When subjected to CFGE at 2–6 V/cm in 0.20–2.5% agarose gels, capsid–DNA complexes arrest at the electrophoretic origin. Progressively lowering the electrical potential gradient to 0.5 V/cm results in migration; most complexes form a single band. The elevated electrical potential gradient (3 V/cm) induced arrest of capsid–DNA complexes is reversed when PFGE is used instead of CFGE. For some conditions of PFGE, the mobility of capsid–DNA complexes is a function of the position of the capsid on the DNA. During either CFGE (0.5 V/cm) or PFGE, capsid–DNA complexes increasingly separate from capsid-free DNA as the percentage of agarose increases. During these studies, capsid–DNA complexes are identified by electron microscopy of enzymatically-digested pieces of agarose gel; this is apparently the first successful electron microscopy of DNA from an agarose gel. The data are explained by the following assumptions: (1) The capsid–DNA complexes are sterically arrested at 2–6 V/cm when DNA passes through pores that are too small to allow passage of the capsid; either Brownian motion (if the electrical potential gradient is low enough) or reverse pulsing prevents arrest. (2) The electrical potential gradient necessary to produce arrest decreases as the distance of the capsid from the most distal DNA end increases. Use of PFGE for isolating capsid–DNA complexes from lysates of T7-infected cells is demonstrated; a complex with dimeric DNA and a complex with trimeric DNA are detected.

Bacteriophage T7 capsid–DNA complexes are fractionated to determine the pathway of the packaging of DNA in the T7 capsid; some of these complexes consist of a mature T7 capsid bound to a linear, double-stranded, 39.936-kilobase (kb) pair DNA that is outside of the capsid [reviewed in Serwer (1989)]. However, during attempts to fractionate T7 capsid–DNA complexes by use of constant field agarose gel electrophoresis (CFGE), sharp bands were not reliably obtained (Serwer & Watson, 1981; P. Serwer and S. J. Hayes, unpublished data). This problem contrasts the numerous successful separations of some smaller protein–DNA complexes by use of polyacrylamide gel electrophoresis [reviewed in Crothers (1987), Ceglarek and Revzin (1989), and Fried (1989)].

During analysis of the structure of T7, bands formed by a T7 capsid–DNA complex were previously observed during pulsed field gel electrophoresis (PFGE) (Serwer et al., 1992). Analysis of the behavior of the T7 capsid-DNA complexes during both CFGE and PFGE should (1) provide a foundation for PFGE-based fractionation of protein–DNA complexes and (2) assist in understanding the sieving of protein-free DNA during PFGE. Thus, a systematic study was undertaken of the effect of both pulsed and constant fields on the migration of T7 capsid–DNA complexes during agarose gel electrophoresis. The results, some of which are summarized in a preliminary report (Serwer & Hayes, 1989a), are described here. Implications of the results are discussed for understanding sieving, analyzing viral assembly intermediates, and improving genomic DNA analyses.

MATERIALS AND METHODS

Bacteriophages and Their Components. Unless radiolabeled, bacteriophages T7, ϕII, and T3, all received from Dr. F. W. Studier (Studier, 1979), were grown in 2× LB medium (20 g of Bacto-tryptone, 10 g of yeast extract, 5 g of NaCl/Liter of H_2O) and subsequently purified by centrifugation in cesium chloride density gradients (Serwer & Hayes, 1989b). T7 with ^{3}H-labeled DNA was prepared by procedures previously described (Serwer et al., 1992). *Escherichia coli* BB/1 was the host. Bacteriophages T4 and T5 were prepared by use of procedures previously described (Serwer & Hayes, 1989b). DNA length standards were linear DNAs from mature bacteriophage T7 (39.936 kb; Dunn & Studier, 1983), T5 (121 kb; Rhoades, 1982), T4 (170 kb; Kutter and Rüger, 1983). These DNAs were obtained by expulsion from bacteriophages at elevated temperature (75 °C); procedures are described in Serwer and Hayes (1989b). A mixture of T7 capsid-free DNA and T7 capsid–DNA complexes was obtained by expulsion of DNA from T7 at 51 °C (Serwer, 1974). The capsid–DNA complex produced by this type of procedure is known to have capsids bound at a variable distance from the nearest end (Serwer, 1974). Bacteriophage T3 and ϕII capsid–DNA complexes were obtained by expulsion of DNA at 60

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* Author to whom correspondence should be addressed.

‡ Current address: Yale University, New Haven, CT.

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°C and 51 °C, respectively, by use of the procedure used for T7.

Buoyant Density Centrifugation. To separate from each other capsid–DNA complexes, capsid-free DNA, and intact bacteriophage, buoyant density centrifugation was performed in a Beckman SW55 rotor, at 15 °C, by use of density gradients of either Nycodenz (Rickwood et al., 1982; Ford & Rickwood, 1984) or the closely related, sodium iothalamate (Hinton & Mullock, 1976). The buffer for Nycodenz was 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.005 M EDTA (standard buffer); the buffer for sodium iothalamate was 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA. Nycodenz was obtained from Accurate Chemical Co. (Hicksville, NY); iothalamic acid was obtained from Mallinckrodt Chemical Co. (St. Louis, MO). The initial density, volume of the gradient, time of centrifugation, and speed of centrifugation are indicated in the figure legends. Densities were determined by measurement of refractive index (Rickwood et al., 1982; Hinton & Mullock, 1976). When gradients contained less than 5 mL, the empty space in the centrifuge tube was filled with mineral oil.

DNA from Lysates of T7-Infected E. coli. T7-infected cell lysates that had ^{3}H-labeled T7 DNA were prepared by both labeling with [^{3}H]thymidine (14–22 min after infection) and lysing with the nonionic detergent Brij^{58} (Serwer, 1974; Serwer & Watson, 1981). DNA that had a sedimentation coefficient less than 100 was fractionated by rate zonal centrifugation in a sucrose density gradient (Serwer & Watson, 1981).

Agarose Gel Electrophoresis. To prepare a sample for electrophoresis, a solution of DNA was brought to 3–5% sucrose and 50–100 μg/mL bromphenol blue. The sample was layered in the sample wells of a horizontal slab gel that was submerged beneath electrophoresis buffer. Concentration of DNA is expressed in micrograms per square centimeter of gel surface at the plane of entry into the gel. For both CFGE and PFGE, Seakem LE agarose (Lot 61569; FMC Bioproducts, Rockland, ME) was used, unless otherwise indicated. Unless otherwise indicated, the electrophoresis buffer for both CFGE and PFGE was 0.01 M sodium phosphate, pH 7.4, 0.001 M EDTA. After the samples were layered, CFGE was performed at the indicated electrical potential gradient; buffer was circulated at either 100 mL/min or a greater speed. Unless otherwise indicated, the temperature was controlled to 15 ± 2 °C by circulation through a glass heat exchanger that was in a constant temperature water bath. PFGE (field inversion mode; Anand, 1986; Birren et al., 1989; Cantor et al., 1988; Olson, 1989; Nordén et al., 1991) was performed by use of either a four-port field inversion device from DNAStar (Madison, WI) or a comparable, one-port device from Hoefer Scientific (San Francisco, CA). The electrical field was periodically inverted, without having its magnitude altered. The time of each forward pulse was always three times larger than the time of each reverse pulse; the pulse time used to describe the field inversion is always the forward pulse time. After electrophoresis, unlabeled DNA was detected by either staining with ethidium (Serwer & Hayes, 1989b) or autoradiography after in-gel DNA–DNA hybridization to T7 DNA ^{32}P-labeled by use of nick translation (Serwer et al., 1992). To quantify ^{32}P-labeled DNA in agarose gels, a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was used for direct assay of ^{32}P decay. Programs that accompany the PhosphorImager were used to integrate the number of ^{32}P decays associated with a DNA band. Characteristics of this method for detection of ^{32}P are described in Johnston et al., (1990).

To perform two-dimensional electrophoresis, after electrophoresis in a first dimension, the direction of the electrical field was rotated by $\pi/2$ radians and, subsequently, a second-dimension electrophoresis was performed at 15 ± 2 °C. The direction of electrophoresis was changed by either rotating the gel or changing to an orthogonally oriented pair of electrodes. The conditions of electrophoresis for each dimension are indicated in the figure legends.

Measurement of Electrophoretic Mobility as a Function of Gel Concentration. To avoid fluctuations in electrical potential gradient among different gels used to determine electrophoretic mobility (μ) as a function of agarose percentage (A), all of these gels (running gels) were embedded in a single frame of agarose; the composite of running gels and frame is called a multigel (Serwer & Hayes, 1989b). Procedures of electrophoresis in multigels were the same as those used for gels of uniform concentration. To normalize μ (μ_n), division by the μ of another particle (normalizing μ), obtained from the same multigel, was performed. The normalizing μ was the μ of intact bacteriophage T3 in a 0.6% agarose gel; T3 is spherical, and its sieving is not altered by pulsing (Serwer & Hayes, 1989b). In photographs of multigels, the A value of a running gel is indicated above the running gel. The running gel that has intact T3 is indicated by ϕ. A second band in electrophoretic patterns of T3 is DNA spontaneously expelled from T3. The band formed by the bacteriophage was differentiated from the band formed by DNA by (1) the greater width of the former and (2) the increase in intensity of the former, but not the latter, during prolonged destaining (Serwer & Hayes, 1989b).

Electron Microscopy. To help identify particles that formed bands during gel electrophoresis, these particles were initially removed for electron microscopy by excising plugs of agarose from the gel, by use of a plastic soda straw. Because agarose forms fibers [see, for example, Serwer (1990)] that interfere with the observation of DNA during electron micrsocopy, agarose was digested before electron microscopy. To perform digestion, electrophoresis was conducted in a low melt agarose gel (SeaPlaque, FMC Bioproducts) and the gel plugs were melted at 65 °C for 10 min in plastic, conical, 1-mL centrifuge tubes. The temperature of the melted gel was lowered to 40 °C and 1 μL of GELase (1 unit/μL; Epicentre Technologies, Madison, WI)/100 μL of melted gel was added. Digestion was continued for 1 h. After digestion, samples of undiluted DNA from a digested agarose plug were prepared for electron microscopy by first adding 2 μL of sample to 1 μL of formamide and 0.5 μL of 500 μg/mL cytochrome *c* and, subsequently, negative staining with uranyl acetate (Serwer et al., 1992). This procedure reveals DNA-bound capsids by conventional negative staining [see, for example, Steven and Trus (1986)]; it reveals DNA by negative staining of DNA complexed with cytochrome *c*.

After scanning approximately 600 grid squares, arrays of fibers that appeared not to be DNA (they were presumably agarose) appeared on only two squares. Increasing the amount of GELase should further reduce the background of agarose fibers. When either capsid-free DNA or capsid–DNA complexes were subjected to a second PFGE after isolation from an agarose gel, the μ value was the same as that observed during a first PFGE (data not shown). Thus, the GELase used during preparation for electron microscopy was not degrading DNA.

Restriction Endonuclease Analysis. After preparative PFGE, restriction endonuclease analysis was performed on DNA in GELase-digested (liquefied) agarose plugs. To one

part of liquefied plug (85–110 μL) was added 0.029 parts of 5 M NaCl, 0.047 parts of 1 M Tris-HCl, pH 7.4, 0.019 part of 0.2 M EDTA, and 0.024 part of H_2O. Subsequently, 1 part of this latter mixture was mixed with 2 parts of 0.021 M $MgCl_2$, 150 μg of bovine serum albumen/mL, and 0.007 M β-mercaptoethanol. Digestion was performed at 37 °C for 3 h by use of 30 units of restriction endonuclease *Xba*I (New England Biolabs, Beverly, MA). Digestion was terminated by adding 0.1 part of 0.2 M sodium EDTA, pH 7.4, and bringing the final volume to 600 μL by addition of standard buffer. After adding an additional 600 μL of Nycodenz in standard buffer (final density = 1.16 g/mL), the digests were subjected to buoyant centrifugation, followed by CFGE and in-gel hybridization-based detection of DNA. The *Xba*I fragments of T7 DNA are (in order of left to right placement, accompanied by the percentage of total nucleotides in a fragment; New England Biolabs, 1990–1991) A (32.13), C (25.28), B (28.47), and D (14.12). Positions on T7 DNA will be indicated by the percentage of the total DNA length from the left end of mature T7 DNA (Studier & Dunn, 1983).

Processing of Data. To perform measurement of band position, photographic negatives were digitized; measurements were made on the digitized image (O'Neill et al., 1989; Griess & Serwer, 1992). For figures that contain an image of a gel, the images were marked while digitized and, subsequently, reproduced photographically from a floppy disk (Griess & Serwer, 1992).

RESULTS

Detection of Capsid–DNA Complexes by Use of PFGE. To determine the behavior of T7 capsid–DNA complexes during agarose gel electrophoresis, a mixture of capsid–DNA complexes and capsid-free DNA was produced by expelling DNA from bacteriophage T7 at 51 °C. Before electrophoresis, capsid–DNA complexes were separated from capsid-free DNA by buoyant density centrifugation in a Nycodenz gradient. Analysis of fractions of this gradient by use of PFGE (3 V/cm, forward pulse time = 12 s) revealed two major ethidium-staining particles that formed bands in both the Nycodenz density gradient and the gel used for PFGE: capsid-free DNA (1.13 g/mL; marked DNA in Figure 1a) and a denser particle (1.17 g/mL) at the position previously found (Serwer et al., 1992) for a T7 capsid–DNA complex (marked C–DNA in Figure 1a). Some DNA in the C–DNA region of the Nycodenz gradient migrated more slowly than DNA that formed the C–DNA band during PFGE (bracketed region in Figure 1a.). In relation to DNA that formed the C–DNA band, the more slowly migrating DNA was more abundant at the higher Nycodenz densities than it was at the lower densities in Figure 1a. Because capsid protein is more dense than DNA in Nycodenz gradients (Serwer et al., 1992), the more slowly migrating DNA has a DNA/protein ratio lower than that of the band-forming capsid–DNA complex.

To test the conclusion that the C–DNA band of Figure 1a is a capsid–DNA complex, a preparative electrophoresis of the DNA expelled from T7 was performed, without buoyant density centrifugation; both the DNA and the C–DNA bands were observed after staining with ethidium (gel profiles are in the next section). Electron microscopy of particles from the C–DNA region revealed the following. Of 105 DNA-like strands observed, 6 had no capsid, 89 had one capsid, 7 had two capsids, and 3 had three capsids bound. The attachment of capsids to 94% of the strands observed confirms the conclusion that these strands are made of DNA. The predominance of monocapsid–DNA complexes confirms that these are the band-forming particles. Control preparations that had no DNA yielded no fibers comparable to those observed in the presence of DNA. The DNAs with multiple capsids bound are presumably contaminants that include at least some of the particles found in the bracketed region of Figure 1a. In Figure 2 is shown an electron micrograph of part of a capsid–DNA complex with one capsid (the capsid is indicated by an arrow); the insert is an enlarged image of the capsid.

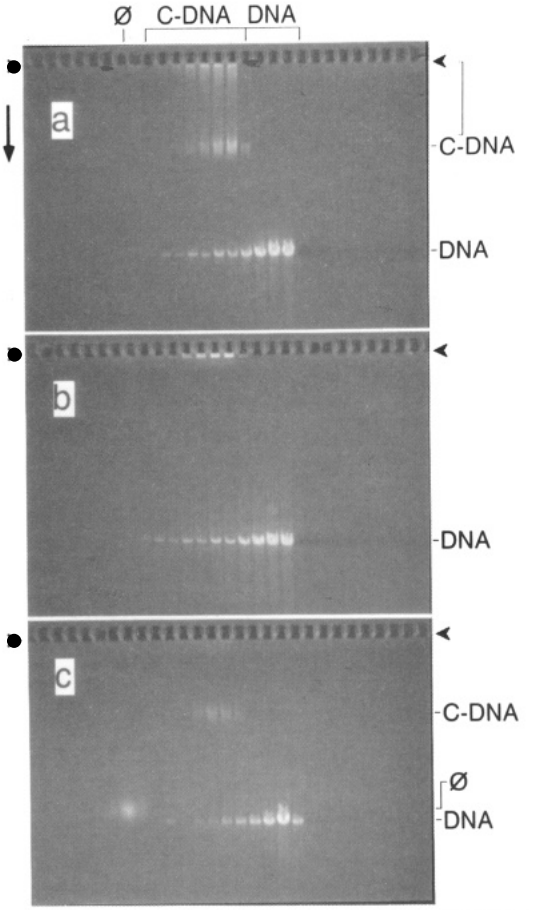

FIGURE 1: Detection of capsid–DNA complexes by PFGE. DNA was expelled from 100 μL of bacteriophage T7 (39.7 μg of DNA/mL) by raising the temperature to 51 °C; DNA was expelled from 100 μL of bacteriophage T3 (39.7 μg of DNA/mL) by raising the temperature to 60 °C. The products of expulsion were subjected to buoyant density centrifugation at 30 000 rpm for 80 h in 4.7 mL of Nycodenz, initial density = 1.173 g/mL, that had been layered above 0.3 mL of Nycodenz, density = 1.268 g/mL. After centrifugation, 10 μL of each fraction (200 μL) of a gradient was subjected to electrophoresis at 3 V/cm, for the time indicated, in a 1.5% agarose gel. The gel was stained with ethidium. (a) T7:PFGE, 26 h, forward pulse time = 12 s; (b) T7:CFGE, 13 h; (c) T3:PFGE, 26 h, forward pulse time = 12 s. The origins of electrophoresis are indicated by the arrowheads; the direction of electrophoresis is indicated by the arrow. The position in both the Nycodenz gradient and agarose gel is indicated for capsid–DNA complexes (C–DNA), capsid-free DNA (DNA), and, in the case of T3, intact bacteriophage (ϕ).

When the analysis of Figure 1a was performed at 3 V/cm by use of CFGE, instead of PFGE, the capsid-free DNA formed a band after migration into the gel (Figure 1b); the time of electrophoresis for Figure 1b was half of the time of electrophoresis for Figure 1a. The capsid–DNA complexes did not migrate into the gel; they arrested at the origin of electrophoresis (arrowheads in Figure 1b).

The PFGE of Figure 1a also yielded a C–DNA band when it was used to analyze DNA expelled (at 60 °C) from bacteriophage T3 (Figure 1c). Bacteriophage T3 is more stable

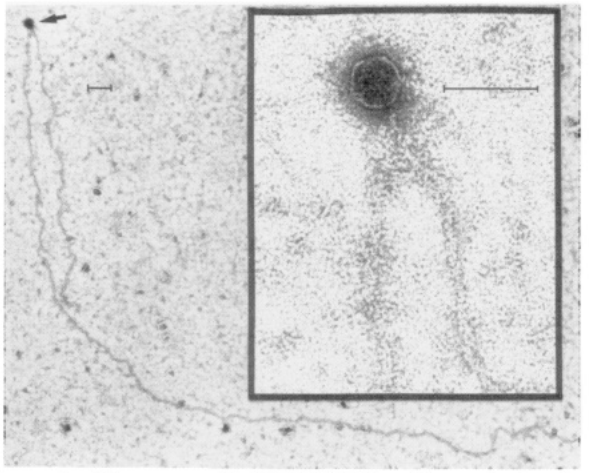

FIGURE 2: After preparative PFGE (3 V/cm, forward pulse time = either 6 s or 48 s), DNA that formed a C–DNA band was isolated and prepared for electron microscopy after digestion of agarose. Most DNA molecules from all C–DNA bands had one capsid bound. A capsid–DNA complex is shown at magnification (length of bar = 0.18 μM) low enough to observe most of the DNA. The capsid region (indicated by an arrow) is shown at a higher magnification (length of bar = 0.10 μM) in the inset.

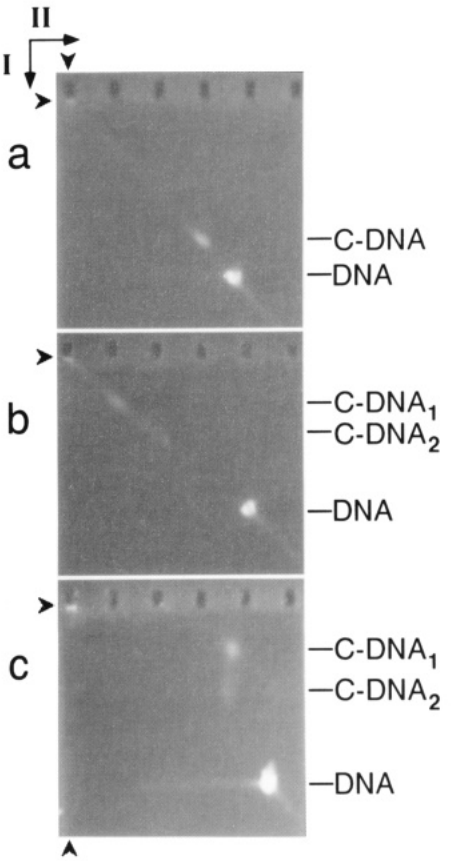

FIGURE 3: Two-dimensional PFGE. After DNA was expelled from bacteriophage T7, 10 μL of the products of expulsion (16 μg of DNA/mL) was subjected to two-dimensional PFGE in a 1.0% agarose gel; the time of electrophoresis was 11 h for each dimension. The forward pulse times (seconds) for the first and second dimensions were, respectively, (a) 6 and 6; (b) 48 and 48; (c) 48 and 6. The gel was stained with ethidium. The arrowheads indicate the origins of electrophoresis; the arrows indicate the directions of the first- (I) and second- (II) dimensional electrophoresis. The sample wells at the right of the origin were not used.

than T7 to elevated temperature; a band of residual, intact bacteriophage T3 was observed at a density of 1.27 g/mL (ϕ in Figure 1c). The stability of T3 has promoted its use as a normalizing particle (Serwer & Hayes, 1989b). When subjected to PFGE at 3 V/cm (forward pulse time = 6 s) in the same 1.5% agarose gel, the capsid–DNA complexes of T7, T3, and a third relative ϕII (Hyman et al., 1974; Studier, 1979; Serwer et al., 1983), all comigrated (data not shown). However, the solid support-free electrophoretic mobilities of the capsids of these bacteriophages differ by up to a factor of 1.7 (Serwer et al., 1983).

Tests for Effects Other Than Those of Sieving. To determine whether μ varied as a function of the time of electrophoresis for DNA expelled from T7, two-dimensional PFGE was performed in a 1.0% agarose gel; the conditions of the second electrophoresis were the same as the conditions for the first electrophoresis (3 V/cm, forward pulse time = 6 s). After the second dimension, both the C–DNA and DNA bands were on a line that passed through the electrophoretic origin (Figure 3a). With the exception of a comparatively small amount (about 10%) of the total DNA, the more origin-proximal DNA also migrated along this line; the remainder of the DNA did not migrate in the second dimension of Figure 3a. Thus, (1) the μ of the monomeric capsid–DNA complexes is independent of the time of electrophoresis; and (2) most of the more origin-proximal capsid–DNA complexes did not detectably adhere to the gel; however, a comparatively small number did adhere during electrophoresis in the first dimension.

To determine whether changing the concentration of DNA changes the position of either the DNA or the C–DNA bands during PFGE, μ was determined as a function of DNA concentration. A concentration-dependent change in band position was not detected until the concentration of DNA reached a limit that decreased as the A of gel decreased (data, not shown, were taken by use of 3 V/cm and a forward pulse time = 6 s). The approximate upper limits for DNA loading without distortion of band position were 0.3 μg/cm^2 for A = 0.2; 0.5 μg/cm^2 for A = 0.4; 1 μg/cm^2 for A = 0.6–1.2; 2 μg/cm^2 for A = 1.5–2.0, and 5 μg/cm^2 for A = 2.5. When distortion occurred, the leading edge of the band was at the position of an undistorted band formed by a lower concentration of DNA, if the concentration of DNA was either 10 μg/cm^2 or less. Thus, all quantitation was performed with samples at least this dilute. When band distortion occurred, the leading edge was used for measurement of band position. This type of sample concentration dependence has previously been observed for rod-shaped bacteriophages during CFGE (Griess et al., 1990) and is observed by inspection of Figure 4 (below).

Dependence on Electrical Potential Gradient of Arrest during CFGE. Although in Figure 1b the capsid–DNA complex arrested at the origin during CFGE (3 V/cm, 1.5% agarose), the capsid–DNA complex did not arrest at the origin when the electrical potential gradient was lowered to 0.5 V/cm during CFGE of expelled T7 DNA in the 0.2–2.0% agarose gels of a multigel (Figure 4a; C–DNA bands are indicated by a horizontal bar; A is indicated at the top of a lane). As the electrical potential gradient was increased to 1 V/cm (Figure 4b) and 2 V/cm (Figure 4c), the capsid–DNA complexes arrested at the origin of progressively more dilute agarose gels. For 2 V/cm (Figure 4c), the C–DNA band was lost for all gels; most capsid–bound DNA was detected only at the origin. However, a continuous DNA distribution extended from the origin of the more dilute gels in Figure 4c. This continuously-distributed capsid-bound DNA progressively moved toward the origin as the electrical potential gradient was further increased to 3, 4, and 6 V/cm (shown for 6 V/cm in Figure 4d). Further analysis of this apparent

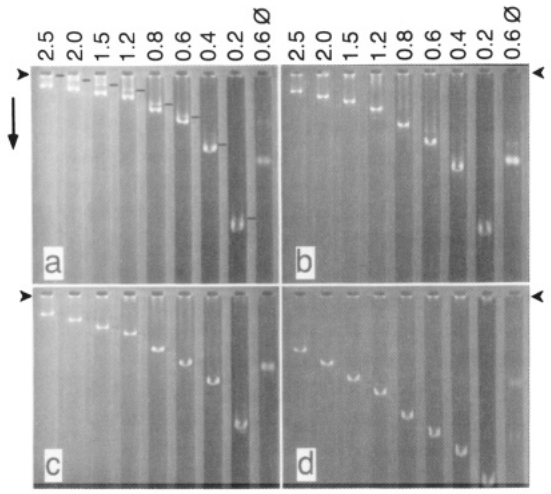

FIGURE 4: Dependence of arrest on electrical potential gradient. After DNA was expelled from bacteriophage T7, 25 μL of the products of expulsion (6.4 μg/mL) was subjected to CFGE in a multigel, by use of the following conditions (electrical potential gradient (volts per centimeter), followed by time of electrophoresis (hours)): (a) 0.5, 40; (b) 1.0, 19.5; (c) 2.0, 9.75; (d) 6.0, 3.3. The origins of electrophoresis are indicated by the arrowheads; the direction of electrophoresis is indicated by the vertical arrow. C–DNA bands are indicated by horizontal bars. The *A* of a running gel is indicated at its top.

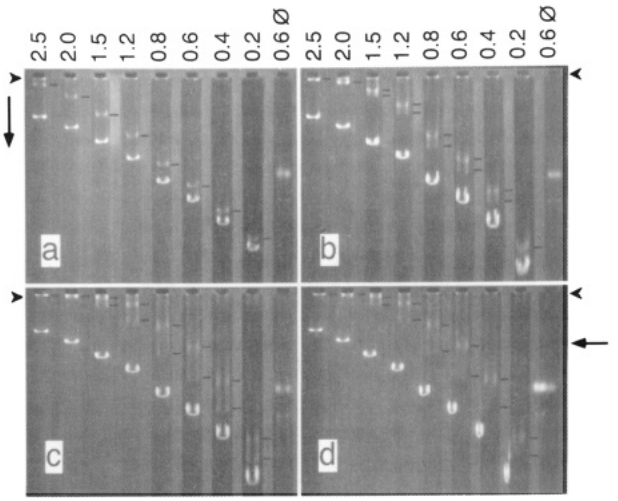

FIGURE 5: Effects of pulse time and gel concentration. After DNA was expelled from bacteriophage T7, 25 μL of the products of expulsion (6.4 μg/mL) was subjected to PFGE, in a multigel at 3 V/cm, by use of the following forward pulse times (seconds): (a) 6, (b) 24, (c) 48, and (d) 48. After PFGE, the gel in (d) was subjected to orthogonally-oriented CFGE (indicated by the horizontal arrow) at 3 V/cm for 30 min. The markings at the top are described in the legend to Figure 4. Additional markings: horizontal lines in the frame gel to indicate both C–DNA_1 (uppermost line) and C–DNA_2 (lowermost line), and a horizontal arrow (next to (d)) that indicates the direction of the second-dimensional electrophoresis in (d).

heterogeneity of capsid–DNA complexes is presented in a subsequent section.

To determine whether the capsid-bound DNA arrested at the origin had irreversibly adhered to the gel, this DNA was arrested by CFGE at 3.0 V/cm. Subsequently, the electrical potential gradient was lowered to 0.5 V/cm. This lowering resulted in both the release of the arrest of capsid–DNA complexes and the formation of a C–DNA band (not shown).

Effects of Pulse Time and Gel Concentration during PFGE. During PFGE at 3 V/cm, by use of a 6-s forward pulse time, a C–DNA band formed for all *A* values between 0.2 and 2.5% (Figure 5a; the C–DNA bands are indicated by horizontal lines). Qualitatively, as *A* increased, μ_n decreased for the particles that formed the C–DNA band. The μ_n(C–DNA)/μ_n(DNA) ratio also decreased as *A* increased. In addition, the background of continuously-distributed DNA appeared lower during PFGE (Figure 5a) than it was during CFGE (Figure 4a).

When the forward pulse time was increased to 24 s (Figure 5b) and 48 s (Figure 5c), the C–DNA band split into two C–DNA bands that were further apart for 48 s than they were for 24-s forward pulse time (C–DNA_1 is the more origin-proximal, C–DNA_2 is the more origin-distal band; indicated by horizontal lines in Figure 5b,c). Additional, continuously-distributed DNA was found between the C–DNA_2 band and the origin. Analysis by use of two-dimensional PFGE (forward pulse time = 48 s for both dimensions) revealed that identity was maintained during PFGE by the particles that formed both the C–DNA_1 and C–DNA_2 band (Figure 3b). Furthermore, when the forward pulse time of the first dimension was 48 s and that for the second dimension was 6 s, particles that formed the single band for 6 s were observed to include particles that formed both bands observed for 48 s (Figure 3c).

To further characterize the particles that form the C–DNA_1 and C–DNA_2 bands, after PFGE the gel of Figure 5c was subjected to orthogonally-oriented CFGE at 3 V/cm. During this second-dimensional CFGE, the DNA band moved for all *A* values and the C–DNA_1 band did not; however, the C–DNA_2 band did perceptibly move for $0.2 \leq A \leq 0.8$ (Figure 5d). When the second-dimensional electrophoresis was performed at 6 V/cm, the movement of the C–DNA_2 band was reduced (not shown). Thus, the capsid–DNA complexes that formed the C–DNA_2 band were the capsid–DNA complexes that were also more difficult to arrest in the experiment of Figure 4.

Restriction Endonuclease Analysis of C–DNA_1 and C–DNA_2. Because the position of the capsid on DNA varies for T7 capsid–DNA complexes, difference in this position is a possible explanation for the splitting of the C–DNA band to form the C–DNA_1 and C–DNA_2 bands. To test this hypothesis, after preparative isolation of capsid–DNA complexes, the capsid–DNA complexes were digested with restriction endonuclease *Xba*I. After capsid-free DNA restriction fragments were separated from capsid–DNA restriction fragment complexes by buoyant density centrifugation, analysis by CFGE of density gradient fractions revealed a gradient profile with the following properties: (1) The capsid–DNA restriction fragment complexes (to be identified by the notation, C–DNA, followed by the name of the fragment) were separated from capsid-free DNA fragments according to position in the density gradient (C–DNA_1, Figure 6a; C–DNA_2, Fig. 6b); the C–DNA and the DNA regions of the gradient are both indicated at the top of Figure 6; these two regions are separated by a dashed line [see also Serwer et al. (1992)]. (2) A DNA fragment with bound capsid (to be called a C–DNA fragment) migrated more slowly during CFGE than the same fragment that was capsid-free; in Figure 6, a solid black line connects the band formed by a C–DNA fragment and the band formed by the same DNA fragment that was capsid-free. In the case of C–DNA_2, only the C–DNA–C fragment was observed in the C–DNA region of the density gradient (Figure 6b). The *Xba*I C fragment covers the central region (positions 32–57) of mature T7 DNA (a map of *Xba*I sites is in Figure 6c). Thus, in C–DNA_2, the capsid is bound near the middle of the DNA. However, in the case of C–DNA_1, all four *Xba*I fragments were observed in the C–DNA region of the density gradient (Figure 6a); the

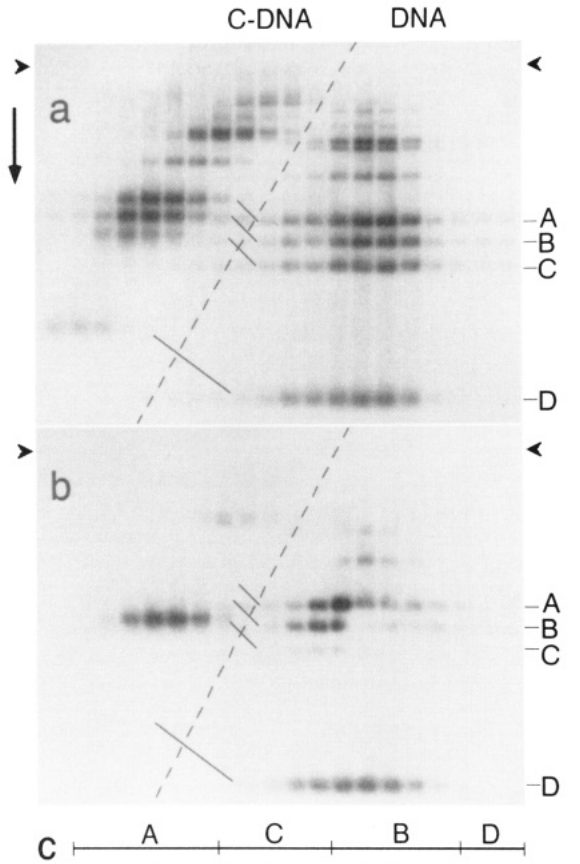

FIGURE 6: Restriction endonuclease analysis. After preparative PFGE by use of the conditions of Figure 5c, capsid–DNA complexes from both the C–DNA_1 and the C–DNA_2 regions of a preparative 1.5% SeaPlaque agarose gel were isolated in an agarose plug. The plugs were digested; the DNA released was digested with restriction endonuclease *Xba*I and, then, subjected to buoyant density centrifugation in a 1.2-mL Metrizamide density gradient (initial density = 1.160 g/mL) at 29 000 rpm for 17 h. After centrifugation, a 10-μL portion of each fraction was subjected to CFGE at 0.5 V/cm for 28.8 h in a 0.7% agarose gel at room temperature (22 ± 3 °C). The electrophoresis buffer was 0.05 M sodium phosphate, pH 7.4, 0.001 M EDTA. After electrophoresis, the gel was probed with ^{32}P-labeled T7 DNA. Panels: (a) C–DNA_1, (b) C–DNA_2. The origins of electrophoresis are indicated by the arrowheads; the direction of electrophoresis is indicated by the arrow. The C–DNA and DNA regions of the density gradient are indicated at the top. The restriction endonuclease fragments are indicated by letters. The unmarked, weaker fragments at the top are the products of partial digestion by *Xba*I. An *Xba*I map of mature T7 DNA is in (c).

C–DNA–C fragment was present in a submolar amount. For C–DNA_1, quantification (Materials and Methods) reveals that the molar amount of C–DNA–C was lower than the molar amount of the C–DNA–B fragment by a factor of 0.57, even though DNA-C and DNA-B were roughly equimolar in Figure 6a. Thus, the data of Fig. 6 indicate that the C–DNA_1 band was formed primarily by capsid–DNA complexes that have capsids more than 18% of the mature T7 DNA length from the center of T7 DNA; higher resolution analysis has not yet been performed. However, C–DNA_1 also appears to be contaminated with a background of C–DNA_2. Presumably, this background includes the interband background in PFGE patterns and is caused, in part, by binding of a second capsid to capsid–DNA complexes.

Patterns of Sieving. During PFGE at 3 V/cm (forward pulse time = 6 s), a μ_n vs A plot for capsid-free DNA had a slope that decreased in magnitude as A increased (Figure 7a, DNA, 6). This curvature, to be called concave, has previously been observed during CFGE [reviewed in Stellwagen (1987) and Serwer (1990)]. The concave curvature for DNA was in contrast to convex curvature observed for the capsid–DNA complex (Figure 7a, C–DNA, 6). For A values as low as 0.075, extrapolation to $A = 0$ yielded μ_n(C–DNA)/μ_n(DNA) that approached 0.95 (not shown). Thus, at the higher A values, the separation of DNA from the capsid–DNA complex is caused primarily by sieving of the gel, not by a difference in average electrical surface charge density (Shaw, 1969).

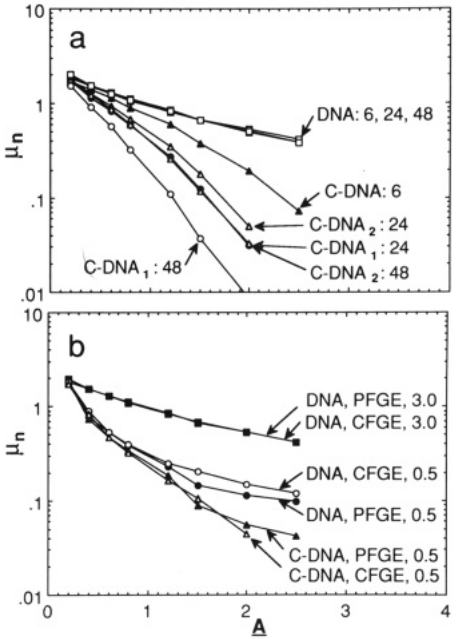

FIGURE 7: Quantification of μ_n. The following are plotted as a function of A for the DNA and C–DNA bands (indicated at the right): μ_n during PFGE at 3 V/cm (forward pulse time (seconds) indicated at the right) and (b) μ_n during either PFGE (forward pulse time = 6 s) or CFGE at the electrical potential gradient (volts per centimeter) indicated at the right.

When the forward pulse time was raised to 24 s at 3 V/cm, (1) the μ_n vs A plot for capsid-free DNA was not significantly altered (Figure 7a, DNA, 24); (2) C–DNA_2 had a μ_n vs A plot that was more convex than the μ_n vs A plot for the single C–DNA band observed for a forward pulse time = 6 s (Figure 7a, C–DNA_2, 24); and (3) the plot for C–DNA_1 was even more convex (Figure 7a, C–DNA_1, 24). Raising the forward pulse time to 48 s did not alter the μ_n vs A plot for DNA (Figure 7a, DNA, 48), but it did increase the convexity of the μ_n vs A plots for C–DNA_1 and C–DNA_2 (Figure 7a, C–DNA_1 and C–DNA_2, 48).

During CFGE at 0.5 V/cm, (1) the μ_n vs A plot for the DNA band (Figure 7b, DNA, CFGE, 0.5) was both steeper and more concave than it was at 3.0 V/cm during either CFGE (Figure 7b, DNA, CFGE, 3.0) or PFGE (Figure 7b, DNA, PFGE, 3.0), and (2) the plot for the C–DNA band was, at the lower A values, more similar to the plot for the DNA band than it was during PFGE at 3.0 V/cm; concave curvature was observed at the lower A values (Figure 7b, C–DNA, CFGE, 0.5). During PFGE at 0.5 V/cm, the results for both the DNA band (Figure 7b, DNA, PFGE, 0.5) and the C–DNA band (Figure 7b, C–DNA, PFGE, 0.5) resembled those for CFGE at 0.5 V/cm. Thus, the arrest of capsid–DNA complexes at 3.0 V/cm during CFGE is associated with an increase during PFGE in both the convexity of μ_n vs A plots and the differential in the sieving of DNA and capsid–DNA complex. The simplest assumption is that transient arrest during PFGE is the cause of these differences.

Capsid–DNA Complexes from T7-Infected Cells. To apply PFGE to capsid–DNA complexes from T7-infected cells,

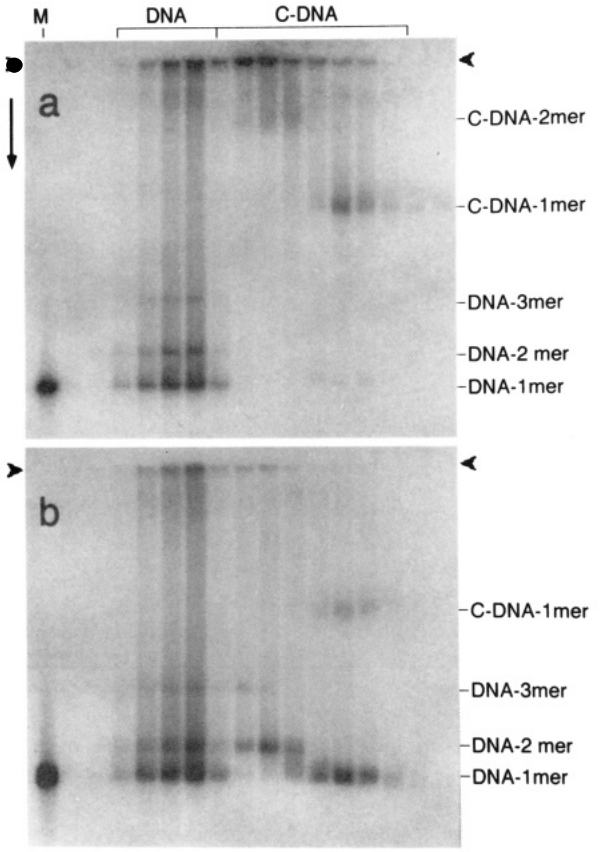

FIGURE 8: Capsid–DNA complexes from T7-infected cells. Capsid–DNA complexes with ^{3}H-labeled DNA were partially purified from a lysate of T7-infected cells, by rate zonal centrifugation. Complexes with a sedimentation coefficient of 40–50 were subjected to buoyant density centrifugation in a 1.2-mL sodium iothalamate density gradient (initial density = 1.163 g/mL) at 29 000 rpm for 16 h at 15 °C. After centrifugation, a 10-μL portion of each fraction was subjected to PFGE at 3 V/cm for 39 h in a 1.5% agarose gel by use of a forward pulse time that was linearly ramped from 9 s to 21 s. The electrophoresis buffer was 0.09 M Tris–acetate, pH 8.4, 0.001 M EDTA. After electrophoresis, the gel was, first, stained with ethidium to locate the following mature bacteriophage DNAs used length standards: T4, T5, and T7. Subsequently, the gel was probed with ^{32}P-labeled T7 DNA and subjected to autoradiography. The PFGE was performed either (a) without any pretreatment of samples or (b) after treatment with 1% Sarkosyl at 37 °C for 30 min. The arrowheads indicate the origins of electrophoresis; the arrow indicates the direction of electrophoresis. The DNA and C–DNA regions of the sodium iothalamate gradient are indicated at the top. The positions of T7 DNA multimers were determined by reference to the length standards. Mature T7 DNA is in the lane marked M.

previously-described (Serwer, 1974; Serwer & Watson, 1981) capsid–DNA complexes were partially purified from a T7-infected cell lysate by rate zonal centrifugation, followed by buoyant density centrifugation. When fractions of the latter gradient were analyzed by PFGE, most DNA at a density of 1.17 g/mL formed a band at the position of capsid–DNA complexes from burst T7 (Figure 8a; C–DNA–1mer). Some capsid-free DNA (density = 1.13 g/mL) formed bands at the positions of mature-length T7 DNA (Figure 8a; DNA–1mer), a dimer of mature-length T7 DNA (Figure 8a; DNA–2mer), and a trimer of mature-length T7 DNA (Figure 8a; DNA–3mer). Some capsid-free DNA was also between the bands. In addition, a comparatively broad band was formed by DNA at a density intermediate to that of capsid-free DNA and the capsid–DNA complex with monomeric DNA. This latter band (Figure 8a; C–DNA–2mer) is potentially a complex of dimeric DNA and a T7 capsid.

To help identify the DNAs bound to capsids in Figure 8a, the PFGE of Figure 8a was repeated after release of capsids from DNA by treatment with ionic detergent (Serwer, 1974). The ionic detergent releases the capsids bound during DNA packaging, but not those bound during expulsion of DNA from mature T7; some expulsion occurs during the process of lysis and pre–fractionation (Serwer, 1974). The results (Figure 8b) were release of (1) monomeric DNA from particles in the C–DNA–1mer region of the gradient, (2) dimeric DNA from particles in the C–DNA–2mer region of the gradient, and (3) trimeric DNA from particles in the region of the gradient between C–DNA–2mer and capsid-free DNA. In other experiments, a much weaker tetrameric DNA was also observed. These results confirm the designation of the C–DNA–2mer band and indicate that complexes of capsids with both trimeric and tetrameric DNA exist. The C–DNA bands for these latter two particles have, however, not yet been identified. Although some evidence for the capsid-dimeric DNA complex was previously obtained (Serwer, 1974; Serwer & Watson, 1981), the data of Figure 8 constitute the first evidence for complexes with trimeric DNA.

In other experiments (not shown), both CFGE and PFGE have been used to detect capsid–monomeric DNA complexes from lysates prefractionated by only rate zonal centrifugation without the buoyant density centrifugation. In theory, capsid–DNA complexes should also be detectable in unprefractionated lysates. However, this goal has not yet been achieved.

DISCUSSION

Sieving of the Capsid–DNA Complex. As the electrical potential gradient was raised from 0.5 to 6.0 V/cm during CFGE, the resultant arrest of capsid–DNA complexes could be caused by either adherence to or steric trapping in the matrix of agarose gel. Because arrest is prevented by either pulsing or lowering of the electrical potential gradient, the conclusion is drawn that steric trapping, not chemical adherence, is the cause of this arrest. In analogy with the results obtained for capsid–DNA complexes, open circular DNA also undergoes elevated electrical potential gradient (3 V/cm) induced arrest that is reversed by pulsing the electrical field [reviewed in Serwer (1990)]. However, during PFGE by use of both 3 V/cm and the other conditions also used here, open circular 48.5-kb DNA has a μ_n that first increases and then decreases as A increases (called atypical sieving; Serwer & Hayes, 1989b). In contrast, the capsid–DNA complexes do not undergo atypical sieving. This difference in sieving for two particles arrested at 3 V/cm is explained by the following two-part hypothesis (to be called the arrest hypothesis; see also Serwer et al., 1990): (1) Open circular DNA is arrested when the DNA is threaded by projections from the gel; the density of projections increases as A decreases. (2) A capsid–DNA complex is arrested when the DNA enters a pore too narrow to allow passage of the capsid; in the absence of the attached DNA, Brownian motion would cause a DNA-free capsid to escape from these pores. However, when bound to DNA, the capsid is held in such a pore by an electrical force that increases as the distance of the capsid from the most distal DNA end increases. In support of part 1 of the arrest hypothesis, electron microscopy of frozen-dried agarose gels has revealed that the density of fibrous projections increases as A decreases (Whytock & Finch, 1991). In support of part 2 of the arrest hypothesis, C–DNA$_2$ (capsid near the middle of the DNA) requires for arrest an electrical potential gradient higher than that required by C–DNA$_1$ (capsid nearer an end). In addition, when the capsid-bound DNA was shortened to

12.8 kb, by digestion with *XbaI*, the electrical potential gradient required for arrest increased from 2–6 V/cm (for 39.9-kb DNA) to 12–18 V/cm (data not shown).

Even when pulsing caused arrest to be transient, the greater susceptibility to arrest of C–DNA_1 was apparent. That is, for the longer, but not the shorter, pulse times C–DNA_1 was more retarded than C–DNA_2. Assuming that pulse times are long enough so that DNA responds to single pulses (i.e., without averaging), then, as the length of a pulse increases, the effect of arrest on sieving should increase (for infinite pulse times, arrest is the only component of sieving). Therefore, the pulse time-dependent increase in the separation of C–DNA_1 and C–DNA_2 is predicted by part 2 of the DNA arrest hypothesis. In any case, the following findings indicate that the electrical force on the capsid has no detectable effect on the behavior of the capsid–DNA complex during either CFGE or PFGE: (1) the absence of effect of the capsid's solid support-free μ on observed sieving and (2) the near identity of the μ of the T7 capsid–DNA complex and the μ of capsid-free T7 DNA when extrapolated to $A = 0$. The latter observation indicates that the net electrophoretic force on the capsid–DNA complex is determined almost exclusively by the electrophoretic force on the DNA component of the capsid–DNA complex (Shaw, 1969).

Transient arrest during PFGE also explains the increased separations of DNA from capsid–DNA complex that occur when the electrical potential gradient is raised from 0.5 to 3.0 V/cm during PFGE. Transient arrest might occur even during the PFGE of protein-free linear DNA, if temporary DNA loops exerted an effect similar to that of a capsid. Additional potential sources (thus far uninvestigated) of transient arrest are cruciform-like secondary structure and branches (Cantor & Schimmel, 1980) that sometimes protrude from a linear DNA. The pores in which T7 capsid–DNA complexes arrest must be smaller than 58 nm, the diameter of a T7 capsid (Stroud et al., 1981). For a 0.2% agarose gel, the average pore has a diameter of 1200 nm (Griess et al., 1989). Thus, in a 0.2% agarose gel, the pore in which a T7 capsid–DNA complex arrests is more than an order of magnitude smaller than the average pore. Potential locations of these smaller pores are zones of agarose fiber junctions [see, for example, Serwer (1990)] that are, in theory, necessary for gelation (Dea et al., 1972; West, 1987).

Electron Microscopy of DNA. To identify capsid–DNA complexes after their fractionation in an agarose gel, in the Results section, electron microscopy was performed on DNA extracted from a liquefied agarose gel. To our knowledge, DNA has not previously been both extracted from an agarose gel and unambiguously differentiated from agarose fibers during subsequent electron microscopy. In Serwer (1990) is shown an image of negatively stained agarose fibers that were not digested. Presumably, the presence of these agarose fibers has, in the past, prevented electron microscopy of DNA extracted from agarose gels. The use of negative staining, in contrast to other techniques for the electron microscopy of DNA [reviewed in Coggins (1987)], was made here to unambiguously identify capsids and, therefore, also to provide a marker for DNA molecules that are part of capsid–DNA complexes. The extent to which other DNA-preparatory techniques can be used to observe DNA from liquified agarose gels has not been determined.

To digest agarose, the agarose was melted before digestion; this step has, thus far, been necessary to obtain complete digestion. To melt agarose at a temperature low enough so that double-stranded DNA is not melted, derivatized, low melt (65 °C) agarose was used [SeaPlaque; see FMC Corp. (1988)]. Although, fortunately, T7 capsid–DNA complexes survived the 65 °C needed to melt SeaPlaque agarose, this temperature will damage some protein–DNA complexes.

Application in either the Study of the Assembly of Viruses or the Mapping of Genomes. Although the elevated electrical potential gradient-induced arrest of capsid–DNA complexes initially impeded the use of gel electrophoresis for the study of viral assembly, once understood, this phenomenon is useful for improving separations made during the study of viral assembly. Capsid–DNA complexes and, presumably, other protein–DNA complexes can be fractionated in a first dimension by use of either a pulsed or low invariant field. Subseqeunt CFGE at an arresting electrical potential gradient (in a second dimension) separates both capsid–DNA complexes and other arrestable DNA (open circular DNA, for example) from uncomplexed linear DNA. In the case of DNA from T7-infected cells, this two-dimensional procedure separates capsid–DNA complexes from over 90% of the remaining cosedimenting DNA. By adjusting pulse time, capsid–DNA complexes can be either grouped in one band or fractionated according to the position of the capsid. Because capsid–DNA complexes do not undergo the atypical sieving of open circular DNA, two-dimensional procedures are readily divised for differentiating mature-length T7 DNA in a capsid–DNA complex from open circular mature-length T7 DNA.

Because of the steric character of the arrest of T7 capsid–DNA complexes, any 58 nm (or larger) sphere should be capable of causing arrest when attached to either T7 length or longer double-stranded DNA. Therefore, nucleotide sequence-specific attachment of a sphere [for potential procedures, see Camerini-Otero and Ferrin (1991) and Ito et al. (1992)], followed by PFGE, is a technique to isolate longer than 40-kb DNA fragments that have targeted nucleotide sequences. In the presence of an excess of other DNA, use can be made of a two-dimensional procedure that consists of PFGE, followed by CFGE (i.e., as in Figure 5d). Streptavidin is a non–capsid particle already shown to cause arrest, when attached (nonspecifically) to single-stranded DNA that is subjected to polyacrylamide gel electrophoresis (Ulanovsky et al., 1990). On the basis of the detection (Results) of a T7 capsid–dimeric DNA complex during PFGE, DNA at least 80 kb long can be used for this purpose. For the causing of arrest, the interdependence of DNA length, sphere diameter, gel concentration, gel type, buffer type, and electrical potential gradient has thus far not been determined.

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