Video Light Microscopy of 670-kb DNA in a Hanging Drop: Shape of the Envelope of DNA

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ABSTRACT Although its conformation has not been observed directly, double-stranded DNA in solution is usually assumed to be randomly coiled at the level of the DNA double helix. By video light microscopy of ethidium-stained DNA at equilibrium in a nonturbulent hanging drop, in the present study, the 670 kb linear bacteriophage G DNA is found to form a flexible filament that has on average 17 double helical segments across its width. This flexible filament 1) has both asymmetry and dimensions expected of a random coil and 2) has ends that move according to the statistics expected of a random walk. After unraveling the flexible filament-associated DNA double helix near the surface of a hanging drop, recompaction occurs without perceptible rotation of the DNA. Both conformational change and intermolecular tangling of the DNA are observed when G DNA undergoes nondiffusive motion in a hanging drop. The characteristics of the G DNA flexible filament are explained by the assumption that the flexible filament is a random coil of double helical segments that are unperturbed by motion of the suspending medium.

INTRODUCTION

For the purposes of both determining hydrodynamic properties and predicting behavior during gel electrophoresis, double-stranded DNA is assumed to be a random coil when no electrical field is present (reviewed in Bloomfield et al., 1974; Cantor and Schimmel, 1980; Zimm and Levene, 1992). Although the time-averaged distribution of the segments of a random coil is spherically symmetric, at any given time, in theory the average random coil is asymmetric. Theoretical analysis was performed by both analytical procedures and simulation (reviews: Šolc, 1977; Weiss and Rubin, 1983; Rudnick and Gaspari, 1987; Saxton, 1993). If the DNA is longer than 50–100 kb, then the resolution of light microscopy is sufficient to observe some aspects of the conformation of single DNA molecules. For observation of conformation, single DNA molecules have been observed by fluorescence light microscopy, after staining with a fluorescent dye (reviewed in Bustamante, 1991). During observation, DNA molecules observed have been adhered to a glass surface (Matsumoto et al., 1981; Bensimon et al., 1994) in buffered solution (Yanagida et al., 1983; Houseal et al., 1989), migrating through an agarose gel (Smith et al., 1989; Schwartz and Koval, 1989; Gurrieri et al., 1990), or in buffered solution that had, in addition, a neutral polymer in an amount sufficient to detectably exclude volume (Minagawa et al., 1994). However, when in buffered solution, the DNA studied by fluorescence microscopy was contained between two glass surfaces, separated by 4–20 μm (Matsumoto et al., 1981; Yanagida et al., 1983; Houseal et al., 1989). The glass surfaces have an unknown effect on the conformation of the DNA. Even for the Brownian motion of spheres, significant effects of glass surfaces were found when the separation between a cover glass and microscope slide was less than two times the radius of the spheres (Schaertl and Sillescu, 1993). In addition, the detail observable within a coil of DNA was limited to 0.2–0.5 μm by the resolution of light microscopes (Slayter and Slayter, 1992). Although evidence for an asymmetric, filament-like organization of 40–50 kb DNA has been obtained by light microscopy of single DNA molecules, both the above limitations and the short lifetime of the observed filament (typically less than 1 s) made uncertain the significance of the filament (Yanagida et al., 1983; Houseal et al., 1989).

For overcoming the limits imposed by both the resolution of light microscopy and the lifetime of DNA conformations, one approach is to increase the length of the DNA observed. However, this approach potentially increases the perturbing effect of containment. In the present study, the effect of containment has been reduced by performing fluorescence light microscopy of DNA suspended in a hanging drop. Microscopy of a comparatively long DNA, the 670-kb linear double-stranded DNA of bacteriophage G (G DNA is most recently described in Hutson et al., 1995), has revealed new aspects of the conformation of DNA in solution.

MATERIALS AND METHODS

Bacteriophage DNAs

By use of pulsed-field agarose gel electrophoresis (PFGE) (reviewed in Birren and Lai, 1993), the double-stranded DNA of bacteriophage G has been found to be 670 kb long (Hutson et al., 1995). By use of the procedure of PFGE described below, the length was not significantly different from that previously determined. Bacteriophage G, originally obtained from Carolina Biological Supply Co. (Burlington, NC), was grown in solid phase (plate stock) by modifying the procedure originally used (Ageno et al., 1973), according to recommendations made by Carolina Biological Supply Co. Both agar layers (Adams, 1959) for the plate stock contained 10 g Bacto tryptone, 5 g NaCl in 1000 ml H_2O. The upper layer of agar was

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cast from the following solution poured at 45–48°C onto the bottom (1%) agar layer at room temperature: 2.5 ml of 0.4% agar to which had been added 4–5 drops of log phase *B. megaterium* (1×10^8 ml; received from Carolina Biological Supply Co.) and 3×10^5 infective particles of bacteriophage G. The plates were incubated at 30°C overnight. Bacteriophage G was extracted from the cleared upper agar layer by, first, adding to each plate 2 ml of the following: 10 g Bacto tryptone, 5 g KCl, 0.002 M $CaCl_2$, 1000 ml H_2O (the $CaCl_2$ was added after autoclaving). Next, the plates were incubated at 30°C for 1 h. After removal from the top of plates, liquid was chloroformed to complete lysis of cells. After twice repeating this extraction, pooled lysate was clarified by centrifugation at 1000 rpm for 45 min at 4°C in a Beckman J21 centrifuge. To pellet bacteriophage from the supernatant, centrifugation was performed at 8000 rpm for 2 h at 4°C. The bacteriophages were resuspended in ice-cold buffer that contained 0.01 M Tris-Cl, pH 7.4, 0.01 M $MgSO_4$, 6% polyethylene glcyol with a molecular weight of 3350. To further fractionate particles of bacteriophage G, rate zonal centrifugation was conducted by use of procedures previously described (Fangman, 1978), but modified as follows: 1) Six percent polyethylene glycol with a molecular weight of 3350 was included in the density gradient used during centrifugation; the polyethylene glycol was not removed before storage. This inclusion of polyethylene glycol stabilized the bacteriophage, preventing the release of DNA for a period of at least 3 years. (2) Bovine serum albumen was removed from the buffer of Fangman (1978).

Double-stranded DNA bacteriophages T4 (DNA length = 170 kb) (Kutter and Rüger, 1983) and T7 (DNA length = 39.936 kb) (Dunn and Studier, 1983) were both grown and purified by procedures previously described (Griess et al., 1991). After purification, a bacteriophage preparation was dialyzed against 0.2 M NaCl, 0.01 M Tris-Cl, pH 7.4, 0.001 M $MgCl_2$. The concentration of both bacteriophage T4- and bacteriophage T7-associated DNA was determined by use of absorbance at 260 nm (Griess et al., 1991). Approximate concentrations (±25%) of bacteriophage G DNA were determined by gel electrophoresis (below), followed by visual comparison of the band intensity (next section) to the band intensity of either T4 or T7 DNA of known amount. To expel DNA from bacteriophages, 1 μl of a bacteriophage preparation was diluted into 9.4 μl of 0.1 M NaCl, 0.01 M sodium phosphate (pH 7.4), 0.001 M EDTA (NPE buffer); this mixture was placed in a water bath for 30 min at the indicated temperature. Expulsion of DNA was the last operation before preparing DNA for light microscopy.

Fluorescence light microscopy and image processing

To stain DNA for fluorescence light microscopy, the following was added to the expelled DNA: 16 μl H_2O, 2 μl 10× NPE buffer, 0.6 μl β-mercaptoethanol, and 1 μl of a fluorescent dye. The fluorescent dye was either ethidium or 4′,6-diamidino-2-phenylindole (DAPI) (Bustamante, 1991). The final concentrations were as follows: DNA, 0.1 μg/ml; NaCl, 0.10 M; β-mercaptoethanol, 2%; dye, indicated in either the text or figure legend. To prepare DNA for fluorescence light microscopy, first, 10 μl of a solution of stained DNA was placed on a cover glass. Subsequently, the cover glass was inverted on a glass microscope slide. Either a flat glass slide or a hanging drop slide was used, as described in the Results. To prevent the solution from undergoing capillary creep, unless otherwise indicated, a piece of parafilm was placed on both the cover glass and the hanging drop slide; a circular hole was cut in both pieces of parafilm to make room for the drop of sample. To improve the parafilm-parafilm seal, the slide was warmed until the parafilm partially cleared, by rapidly passing through a flame, just before use. To improve the glass-parafilm seal, both the microscope slide and the cover glass were warmed gently in a flame until the parafilm melted. The cover glass was cooled to room temperature before use.

To observe DNA by fluorescence microscopy, the microscope slide with DNA was placed on the specimen stage of an Olympus BH2 fluorescence microscope. The microscope ocular had been replaced with a Philips XX1410/SP image intensifier. The image of the intensifier was recorded by a Philips 56770 series video camera connected to a television monitor. Images were videotaped. For the formation of a still image, a videotaped image was digitized by use of procedures previously described (Griess et al., 1992). Images were both marked and measured while digitized. Magnification was determined from the image of a diffraction grating (5276 lines/cm). Subsequently, the images were photographically reproduced, by use of procedures previously described (Griess et al., 1992). In a figure, the time of the digitization of an image is indicated in seconds, on the figure, by use of black numbers on a whitened background. All magnification bars are 10 μm long.

To determine the length of a flexible DNA filament observed by light microscopy, the flexible DNA filament was observed until, by chance, it developed a conformation in which it did not detectably cross itself at any point. The total projected length of this filament was determined by hand-tracing the contour of the filament in a digitized image, by use of the program National Institutes of Health IMAGE (Rasband, W.; see Griess et al., 1992). When a section of the filament became unfocused in a single image, an additional, refocused image was used to obtain the originally unfocused part of the contour. To correct the measured length for projection on the plane of the image, the measured length was multiplied by a factor of $4/\pi$; the corrected length will be called L_F. This factor is derived by averaging the projected length over all directions.

To quantify the asymmetry of a filament observed by light microscopy, three procedures were used: 1) The size of an enclosing rectangular box was decreased until all four sides touched, but were not traversed by, a randomly oriented flexible filament. The length of the larger side of the box is called the large span of the image (S_2); the length of the smaller side is called the small span (S_1; the concept of span is reviewed in Weiss et al., 1983). The ratio of the mean value of S_2 ($\bar{S}_2$) to the mean value of S_1 ($\bar{S}_1$) is a measure of the asymmetry of the random coil; $(\bar{S}_1 + \bar{S}_2)/2$ is a measure of the size of the random coil (Rubin and Mazur, 1975; Rubin et al., 1976). 2) A second measure of asymmetry is the ratio of maximum span across the random coil to the orthogonally oriented span (Rubin and Mazur, 1975); this ratio will be called S_m. 3) The final measure of asymmetry is the standard deviation (σ) of measurements of one of the spans. The ratio of σ to the mean span (for example, $\sigma(S_2)/\bar{S}_2$) is reported. For obtaining the spans, the image used was a composite of images recorded during a through-focal series. This series included 3–16 frames, each separated from nearest-neighboring frames by 0.05 s. The composite image was used to ensure that the spans included a projection of the entire filament. To help determine the outer limits of a flexible filament, a composite image was binarized (Griess et al., 1993) while including all signal that appeared significantly above background. By use of a binarized image, S_1 and S_2 were determined by use of software (written by G. A. Griess) that was combined with National Institutes of Health IMAGE. After obtaining S_1 and S_2, the maximum span was similarly determined by using National Institutes of Health IMAGE to rotate the rectangular box used to obtain S_1 and S_2.

Gel electrophoresis

To determine the length distribution of the DNA double helix released from bacteriophage G, this DNA was subjected to PFGE in a horizontal, submerged agarose gel. To increase the density of samples for loading in this gel, Nycodenz (Accurate Chemical Co.; final concentration = 10%) was added to the solution of DNA; to provide a tracking dye, 100–200 μg/ml bromophenol blue was also added. When 3–10% sucrose was used instead of 10% Nycodenz (the Nycodenz solution is more dense) to increase the density of samples used for PFGE, the performance of PFGE was compromised by the spontaneous expulsion of bacteriophage G DNA from sample wells. This effect, possibly caused by viscoelastic relaxation of the DNA (Chapter 12 in Cantor and Schimmel, 1980), was not observed for either T4 or shorter DNAs. After loading of samples, PFGE was performed for 25 h at 2 V/cm, 20°C, through a 1.0% Seakem LE agarose gel (FMC Bioproducts, Rockland, ME); the electrophoresis buffer was 0.01 M sodium phosphate, pH 7.4, 0.001 M EDTA. Pulsing of the electrical field was achieved by rotating the gel according to the following program

executed by procedures previously described (Arshad et al., 1993): no rotation for 150 s at an angle of 0.3π radians relative to the direction of net electrophoresis, followed by rotation through 1.4π radians at a speed of 0.7π radians/s, to an angle of -0.3π radians relative to the direction of net electrophoresis, followed by no rotation for 150 s, followed by reversal of the direction of rotation and rotation through 1.4π radians at 0.7π radians/s (see Louie and Serwer, 1991). After PFGE, all gels were stained with 1 μg/ml ethidium bromide; the gels were destained for at least 2 days. Images were recorded by photography, followed by digitization of the photographic negative (Griess et al., 1992). Standards for the length of DNA included the DNAs of bacteriophages T7 and T4; shorter DNA standards were restriction endonuclease *Hin*dIII fragments of λ DNA (obtained from New England Biolabs, Beverly, MA); additional DNA standards were *Saccharomyces cerevisiae* chromosomal DNAs (obtained from FMC Bioproducts).

RESULTS

Bacteriophage G DNA

After release of DNA from bacteriophage G (but before preparation for light microscopy), PFGE revealed a major band (Fig. 1 *a, lane 2*; the band is indicated by a horizontal arrow), accompanied by a comparatively weak and broad zone possibly formed by broken DNA of variable length (indicated by brackets; the positions of length standards are indicated by the length [kb] at the right). To determine whether preparing G DNA for light microscopy caused breakage of the DNA, two identical samples of G DNA were prepared for light microscopy. After removing (by pipetting) the DNA solution from the cover glass of both preparations, the DNA solutions were first pooled and then analyzed by PFGE. The PFGE profile after preparation for microscopy (Fig. 1 *a, lane 1*) was similar to that before microscopy (Fig. 1 *a, lane 2*). Thus, if the G DNA does not break during observation, most of the G DNA mass observed will be in intact DNA molecules.

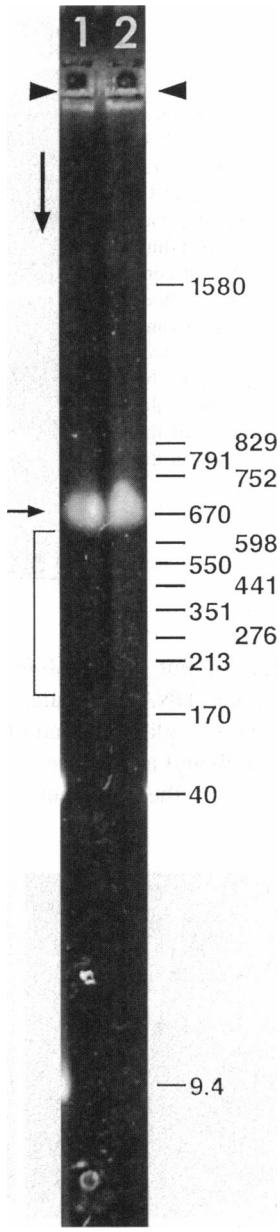

FIGURE 1 PFGE of bacteriophage G DNA. Bacteriophage G DNA was fractionated by use of PFGE. (*1*) DNA after preparation for light microscopy; (*2*) DNA before preparation for light microscopy. For DNA length standards fractionated in separate lanes of the same gel (not shown), the position is indicated in kilobases. The arrowheads indicate the origins of electrophoresis; the vertical arrow indicates the direction of electrophoresis.

Adjusting the procedure of observation

In initial studies, bacteriophage G DNA was observed by use of a procedure that differed in three respects from the procedure ultimately used: 1) DAPI (0.5 μg/ml) was used, instead of ethidium, for staining the DNA; the exciting radiation was ultraviolet rather than visible (Bustamante, 1991). 2) DNA was released from the bacteriophage at 85°C instead of 60°C. 3) The double layer of parafilm was omitted between the cover glass and the hanging drop slide. Omission of the parafilm freed the DNA-containing sample to creep between the cover glass and the slide. Possibly because of creeping, together with other effects, the following was observed by microscopy. When fields were chosen at random, most fields had no observable DNA. However, occasionally, multiple loosely entangled molecules of DNA moved through the field of view. Although some force was clearly holding these DNA molecules together, the pattern of entanglements could not be determined. Based on the movements of the DNA, the solution was in a state of turbulent, multidirectional motion. Some of the DNA molecules had no visible attachment to the cover glass. Usually, two regions were observed in each of these molecules: an unstructured, comparatively bright, roughly spherical region (Fig. 2 *a, arrows 1*) and an extended region, possibly a single double helix (Fig. 2 *a, arrows 2*). The bright region presumably had DNA sufficiently condensed so that separate DNA segments were not seen. The image of the extended region is significantly blurred by turbulent motion. Other DNA molecules were apparently caught (trapped) by protrusions from the cover glass. These molecules were

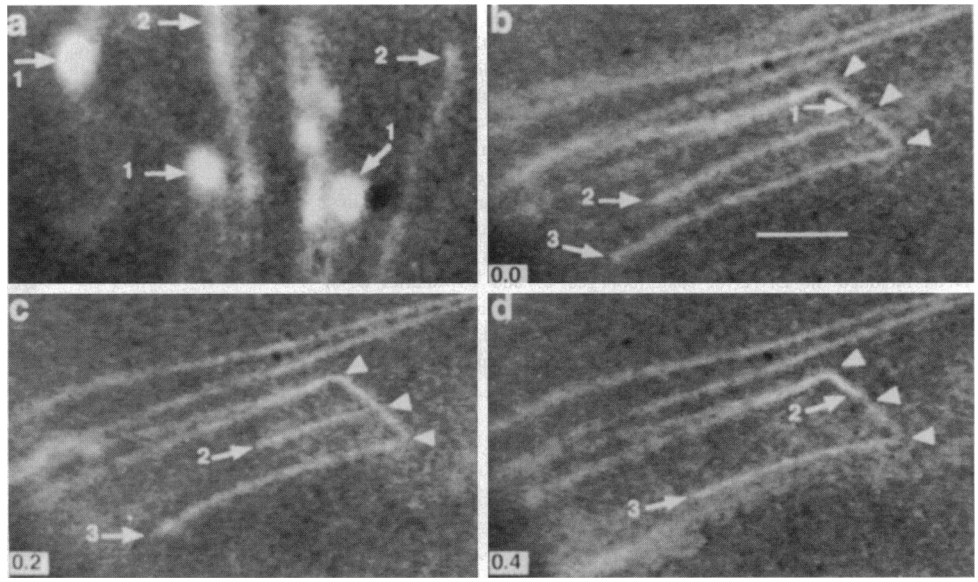

FIGURE 2 DNA in a turbulent hanging drop. Bacteriophage G DNA, stained by 0.5 μg/ml DAPI after release from the bacteriophage at 85°C, was observed in a hanging drop that was not sealed by use of parafilm. DNA is shown in two types of condition: in turbulent motion without attachment to the cover glass (*a*) and in motion in the plane of the cover glass (*b–d*), apparently caught by projections (*arrowheads*) from the cover glass. The significance of the numbered arrows is described in the text.

sometimes aggregated, but still mobile. In Fig. 2, *b–d*, several such aggregated DNA molecules are sliding around protusions from the cover glass (an end of a DNA molecule is indicated by a numbered arrow; the protrusions are indicated by arrowheads). Other molecules were sufficiently trapped so that they did not slide; occasionally such molecules appeared to be broken by a surge of turbulence. In Fig. 3 *a* is such a DNA molecule, trapped by the cover glass at a position of DNA condensation (*arrow 1*); the trapping was sufficient to prevent any translational motion of this part of

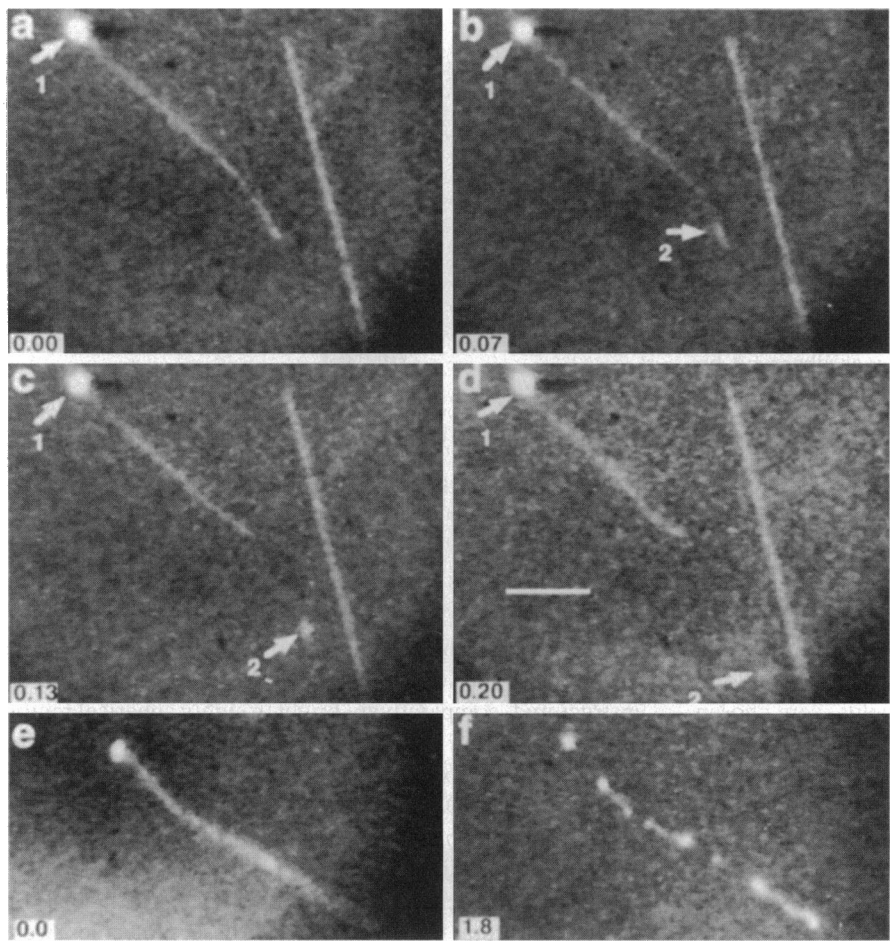

FIGURE 3 Breakage of G DNA during microscopy. Bacteriophage G DNA, stained by 0.5 μg/ml DAPI after release from the bacteriophage at 85°C, was observed in a hanging drop that was not sealed by use of parafilm. (*a–d*) Hydrodynamic shear-induced breakage of the free end (*arrow 2*) of a DNA molecule that was trapped at the point indicated by arrow 1; (*e*) a DNA molecule adhered to the cover glass in a comparatively nonturbulent region; (*f*) the DNA molecule in *e* after exposure to the exciting illumination for 1.8 s.

the DNA molecule. In Fig. 3, *b–d*, the same molecule is shown during a surge of turbulence that is associated with breakage of the unattached end of the DNA molecule (the broken end is indicated by arrow 2 in Fig. 3, *b–d*). In areas of turbulence, breakage was both rare and associated with a surge in turbulence. This breakage was probably caused by hydrodynamic shear (Zimm and Reese, 1990).

When the temperature of DNA release from bacteriophage G was reduced from 85°C (used for Figs. 2 and 3 *a–d*) to 60°C, the DNA-DNA tangling was reduced in samples observed by the procedure of Figs. 2 and 3 *a–d*. However, motion of the suspending fluid still occurred. When, in addition, parafilm was inserted between the cover glass and hanging drop slide (see Materials and Methods), turbulent motion was reduced to a level below that perceptible to the eye.

However, when motions of both DAPI-stained G DNA and the suspending fluid were suppressed, breakage of the DNA dramatically increased during observation. This breakage was associated with exposure to the exciting illumination of the microscope. For example, Fig. 3 *e* shows a stretched DNA molecule adhering to the cover glass at several points, to the extent that no internal motion was occurring. This molecule had been in the presence of DAPI for 10–20 min. However, after illumination for less than 2 s, the DNA molecule fragmented to form six pieces, each of which had at least one point of adherence to the cover glass; the unadhered regions of each piece retracted (Fig. 3 *f*). Qualitatively, the rate of illumination-dependent breakage was greater for stretched DNA than it was for unstretched DNA. To avoid this illumination-dependent fragmentation of DNA, DAPI staining was replaced with ethidium staining; green illumination was used. The result was the reduction of illumination-dependent DNA breakage to the point that it was no longer significant. Quantitatively, ten randomly chosen G DNA molecules were observed for 5 min each. The number of breakage events was 0. For longer observation times, significant fading of the image occurred. For other types of preparation, Bustamante (1991) has previously reported that DAPI staining produces more illumination-dependent DNA breaks than does ethidium staining.

To optimize the visibility of DNA, the effect was determined of the ethidium concentration on the visibility of DNA. The following observations were made: 1) For either 0.01 μg/ml ethidium or less, the signal-to-noise ratio was too low to observe DNA. 2) For 0.025 μg/ml ethidium, the DNA was visible, but substructure (described in the next section) observed at higher concentrations of ethidium was not. 3) As the concentration of ethidium bromide increased, the visibility of the DNA improved until an optimum was reached at 0.25–0.6 μg/ml. 4) As the concentration of ethidium bromide increased above 0.6 μg/ml, the visibility of the DNA progressively decreased until substructure was lost at 1–2 μg/ml; the DNA became invisible at 5 μg/ml. For both the range of ethidium bromide concentrations in which the DNA was visible and the range of observation times used (1–300 s), no easily visible change in the dimensions of the DNA was observed. Quantification of dependence on ethidium bromide concentration is described in the next section.

The Conformation of G DNA

When ethidium-stained bacteriophage G DNA was observed by video fluorescence microscopy of a nonturbulent hanging drop, the most massive DNA molecules observed appeared to be flexible particles (Fig. 4; in-focus molecules are indicated by arrowheads). As predicted for linear, but not circular, random coils (reviewed in Weiss and Rubin, 1983; Rudnick and Gaspari, 1987), at any given time, most (>90%) of these flexible particles were prolate, i.e., filamentous. Because of both the predominance of unbroken G DNA after preparation for light microscopy and the absence of breakage during microscopy, these flexible filaments are assumed to be unbroken G DNA. Qualitatively, these unbroken flexible filaments had the following characteristics: 1) The total length (L_F) was less than that of the DNA double helix (L_D). The comparatively low value of L_F is explained by the hypothesis that the observed flexible filament is formed by a finer filament, the DNA double helix, that has bent to form an unresolved array of segments; the conformation of the double helix is possibly a random coil. 2) The flexible filament had comparatively bright zones (beads) that sometimes produced a beads-on-a-string appearance. The beads changed position along a filament,

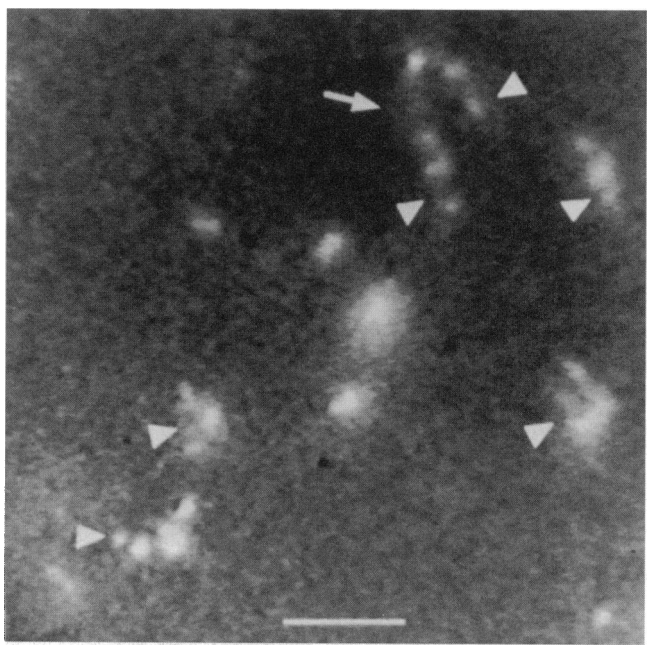

FIGURE 4 Several G DNA molecules in a hanging drop. Bacteriophage G DNA, stained by 0.4 μg/ml ethidium bromide after release at 60°C, was observed in a hanging drop that was sealed by use of parafilm. When most of a flexible filament is in focus, it is indicated by an arrowhead. Two flexible filaments too close to resolve separately are indicated by an arrow. The latter two flexible filaments separated from each other in subsequent video frames.

apparently at random. 3) At all positions along the flexible filament, the extent of bending changed continuously with time; the changes in bending appeared random. For all in-focus DNA molecules in Fig. 4, the presence of the beads is apparent. Images of four DNA flexible filaments (Fig. 5 *a–d*) are shown at the times indicated in each panel of Fig. 5. Both flexing of the filament and movement of the beads are observed by comparing images of any one molecule. By the criterion of rate zonal centrifugation, the buffer constituents of Fig. 4 do not cause any departure from the state of DNA usually assumed to be a random coil (Lerman, 1973; Serwer et al., 1978).

Quantitatively, when L_F was both determined and averaged for 18 randomly chosen flexible filaments, the average L_F ($\bar{L}_F$) was 0.057 times the L_D determined by assuming 0.34 nm per base pair and correcting for intercalation of ethidium (Chapter 23 in Cantor and Schimmel, 1980) (Table 1, 0.6 μg/ml ethidium). The $\bar{L}_F$ did not significantly change when the concentration of ethidium changed (Table 1). The finding of $\bar{L}_F$ less than L_D confirms the hypothesis that the observed flexible filament has across its width more than one segment (i.e., 17 segments on average) of the DNA double helix.

Observation of any one G DNA molecule revealed, qualitatively, that its conformation remained a flexible filament for periods as long as 5 min. To quantitatively determine whether the motions of the flexible filament were those of a random coil (i.e., a random coil superimposed on the network of double helical segments), the displacement (ΔX) along the x axis (direction arbitrary) of an end of the filament was measured in the time interval Δt. For random movement, a plot of frequency (F) versus ΔX is Gaussian (pp. 986–989 in Cantor and Schimmel, 1980). When F versus ΔX was determined by use of both Δt = 0.2 s and 2508 measurements of ΔX (33 different DNA molecules were used), the result was a Gaussian plot (Fig. 6). Thus, the movements of the ends of the flexible filament were indistinguishable from those expected of a random coil.

TABLE 1 The length of flexible filaments of G DNA*

Concentration of ethidium (μg/ml)	L_D (μm)‡	$\bar{L}_F$ (μm)
0.05	229	11 ± 4
0.6	244	14 ± 4
2.0	267	14 ± 2

*Values were determined by procedures described in Materials and Methods. For $\bar{L}_F$, values obtained for 10–18 molecules were averaged.
‡L_D was determined by multiplying the length in nucleotides by 0.34 nm per nucleotide and correcting for an expected increase caused by intercalation of ethidium (Chapter 23 in Cantor and Schimmel, 1980).

The effect of introducing a second glass boundary during microscopy

Because the length of the flexible filament of G DNA is comparable to the distance between the cover glass and a flat glass slide, the flat slide may cause a visible change in the conformation of G DNA. When G DNA was observed between a cover glass and a flat slide (average separation

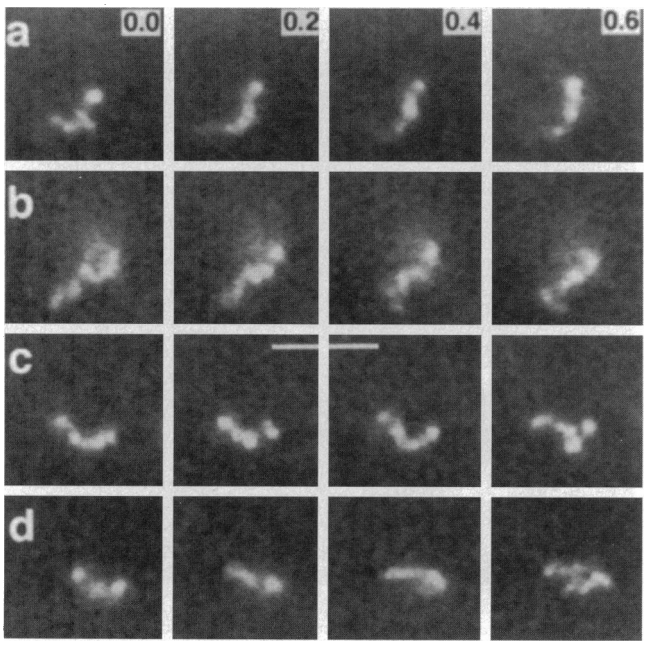

FIGURE 5 Images of single molecules of G DNA in a hanging drop. Bacteriophage G DNA, stained by either 0.4 μg/ml (*a, b*) or 0.6 μg/ml (*c, d*) ethidium bromide after release at 60°C, was observed in a hanging drop that was sealed by use of parafilm. (*a–d*) Four different G DNA molecules visualized at the time indicated (s).

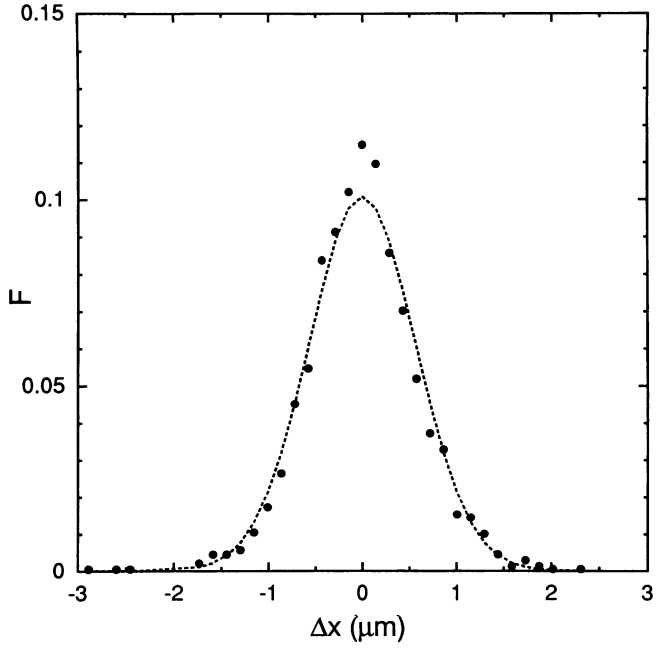

FIGURE 6 F versus ΔX for G DNA. By use of G DNA images obtained by the procedures of Fig. 5 (0.6 μg/ml ethidium bromide), values of F versus ΔX were determined after digitization of the images. The time between measurements was 0.2 s. Data (2508 values of ΔX) were obtained from 33 DNA molecules. The continuous line is the best-fit Gaussian plot.

between glass boundaries = 21 μm), qualitatively, most fields were like that of Fig. 4, except that more molecules had both ends visible. That is, the flexible filaments appeared to have, on the average, flattened in the plane of the cover glass (images not shown). However, basically, the DNA molecules observed in most fields of the flat slide preparation were the flexible filaments observed in the hanging drop preparations. In a few fields, some DNA molecules differed slightly from flexible filaments in the following way. The filament became partially oriented and straightened, as though the DNA was in a region of comparatively small separation between cover glass and glass slide. Images of straightened flexible filaments are shown in the next section.

Unraveling of DNA that forms the flexible filament

The observation that $\bar{L}_F$ is less than L_D is explained by the hypothesis that several double helical segments are arrayed across the flexible filament observed in Figs. 4 and 5. To test this hypothesis, attempts were made to unravel the flexible filament. Unraveling was achieved by either 1) putting pressure on the cover glass of a flat slide preparation or 2) causing a hanging drop to move by either shaking or tapping a hanging drop slide. Immediately after putting pressure on the cover glass of a flat slide preparation, the flexible filaments stretched to many times their length (Fig. 7, *a–c, left panel*). This stretching must have been caused by unraveling of the flexible filament. Thus, the above hypothesis is confirmed. When a stretched filament was allowed to re-equilibrate without further disturbance, it progressively shortened to form a flexible filament (Fig. 7, *a–c, right four panels*). However, the final filament was not that of Figs. 4 and 5. Instead, a more straightened filament was observed. The time to achieve a straightened filament varied from 0.5 to 5 min; this variation in the time to achieve a straightened filament was presumably caused by variation in the distance between the cover glass and flat slide. After formation of a straightened filament, further equilibration for times as long as 5 min did not cause the straightened filament to become a flexible filament. However, the difference between the two filaments appears minor; the straightened filament underwent bending motions almost as large as those of the flexible filament.

Causing movement of a hanging drop was the second procedure used to unravel the DNA double helix of flexible filaments. When either an entire DNA molecule or part of a DNA molecule was deposited in the drop's wake outside of the drop, this DNA molecule was irreversibly both unraveled and straightened. Almost all molecules were entangled with other molecules (Fig. 8). Thus, determination of the length of these unraveled molecules could not be made. However, stretches of DNA were often observed to have lengths between 100 μm and 200 μm (arrow in Fig. 8). Thus, these unraveled, straightened regions of DNA are assumed to be single segments of the DNA double helix.

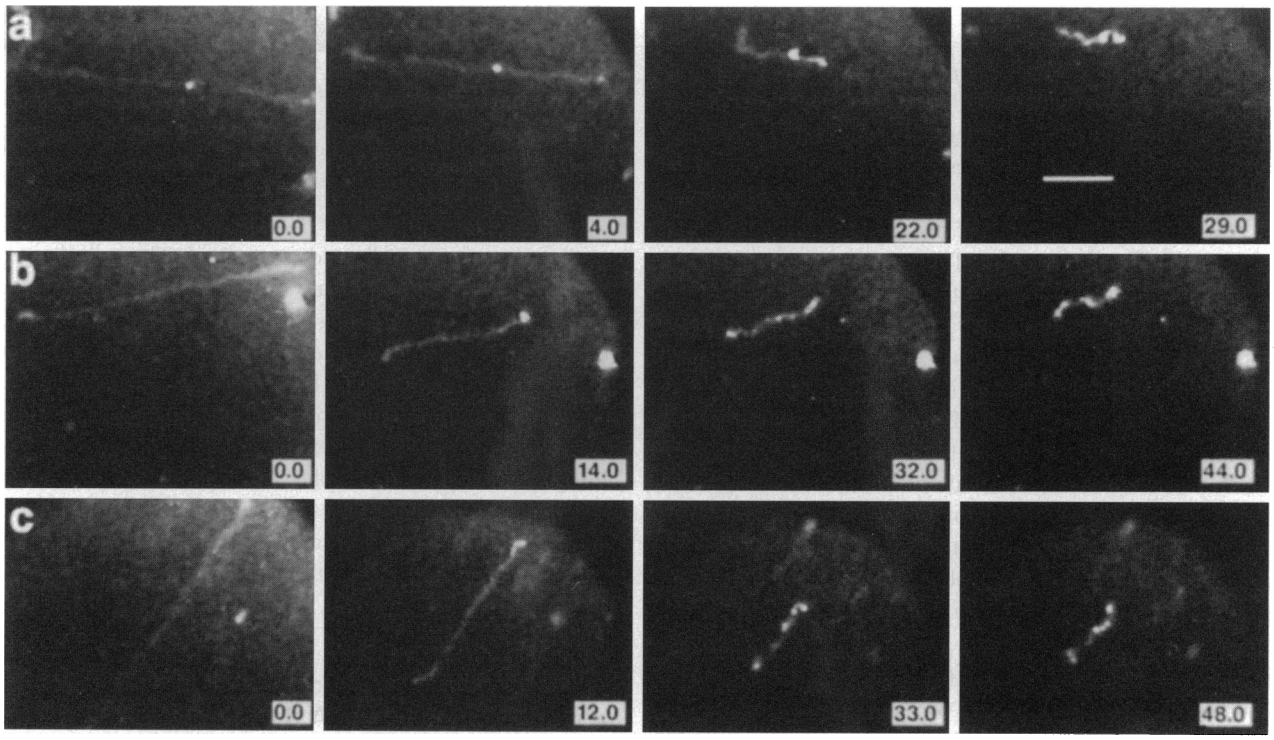

FIGURE 7 Unraveling and compaction of G DNA flexible filaments in a flat slide preparation. Bacteriophage G DNA, stained by 0.6 μg/ml ethidium bromide after release at 60°C, was stretched by lowering the microscope's objective lens to put pressure on the cover glass of a flat slide preparation. (*a–c*) Three molecules imaged as a function of time after stretching.

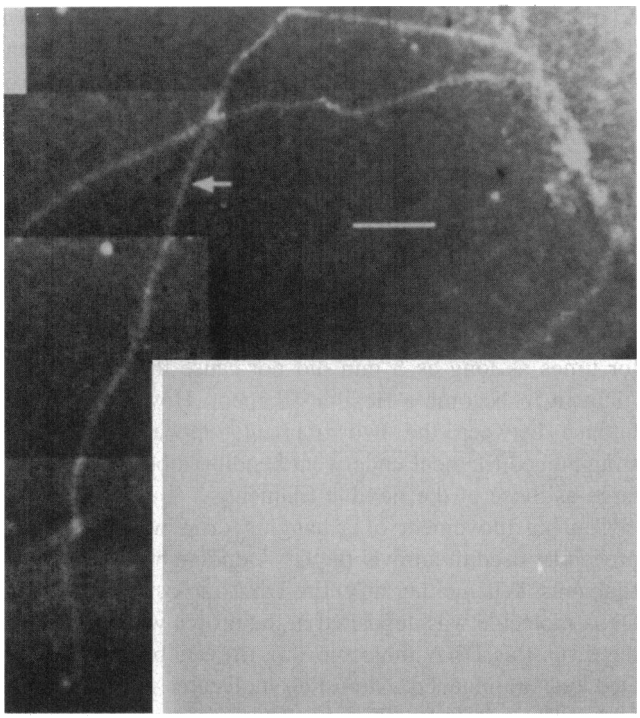

FIGURE 8 Bacteriophage G DNA dried in the wake of a drop. Bacteriophage G DNA, stained by 0.6 μg/ml ethidium bromide after release at 60°C, was observed in the dried wake of a drop that had been made to move in a hanging drop preparation. A composite image of five fields is shown.

The process of forming either a flexible or a straightened filament

To analyze the process of forming a flexible filament, G DNA molecules were observed near the edge of a hanging drop. These DNA molecules were in three classes: 1) Some DNA molecules had an appearance indistinguishable from that of DNA molecules further inside of the hanging drop (Fig. 9 *a, arrows 1*; the edge of the drop is indicated by an arrowhead, which is outside of the drop). 2) Other DNA molecules had aggregated to form amorphous lumps of DNA (the lumps are indicated by arrows 2 in Fig. 9 *a*). When the image intensifier was bypassed, strands of DNA were seen within the lumps; the image intensifier has caused overexposure of the lumps in Fig. 9 *a*. 3) The remaining DNA molecules had a property that is useful for analysis of the formation of a flexible filament: the molecule was unraveled inside the drop. In some cases, part of the molecule was outside of the hanging drop (Fig. 9 *b, arrows*). Inside of the drop, the DNA molecule had presumably been unraveled by the drop's motion. During subsequent observation, the partially unraveled DNA compacted, thereby reforming a flexible filament. To help determine the mechanism of compaction, evidence was sought that an end of the DNA was rotating during compaction. No evidence for rotation (for example, motion that swept outside of the precontraction perimeter of the DNA) was observed. Compaction is shown in Fig. 10 *a–c* for three DNA molecules,

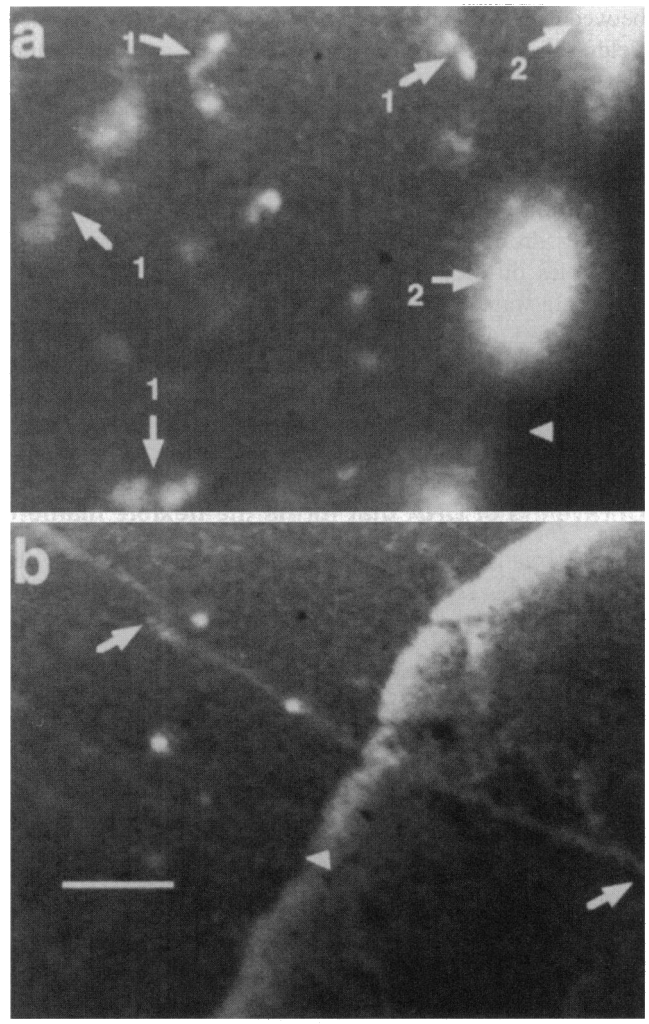

FIGURE 9 Bacteriophage G DNA molecules near the edge of a hanging drop. Bacteriophage G DNA, stained by 0.4 μg/ml ethidium bromide after release at 60°C, was observed near the edge of a hanging drop. *a* and *b* have two different fields. The edge of a drop is indicated by an arrowhead.

at the times (min) indicated on a panel. The arrows next to the DNA molecules indicate the directions of compaction.

After moving a hanging drop, assimilation of additional G DNA molecules into lumps (Fig. 9 *a, arrows 2*) was observed. In all cases, the DNA was carried toward a lump by flow of the supporting medium, not by diffusion (images not shown). After observing over 1000 molecules enter lumps, not one DNA molecule was observed to leave a lump. Thus, agitation of a solution of G DNA promotes the formation of lumps by increasing the rate of entry of DNA molecules into lumps, without comparably increasing the rate of exit.

Quantitative comparison of a flexible filament to a randomly coiled DNA double helix

When spans are quantified in three dimensions for a long polymer, the result for an unrestricted random coil (i.e., a coil represented by straight segments that have random

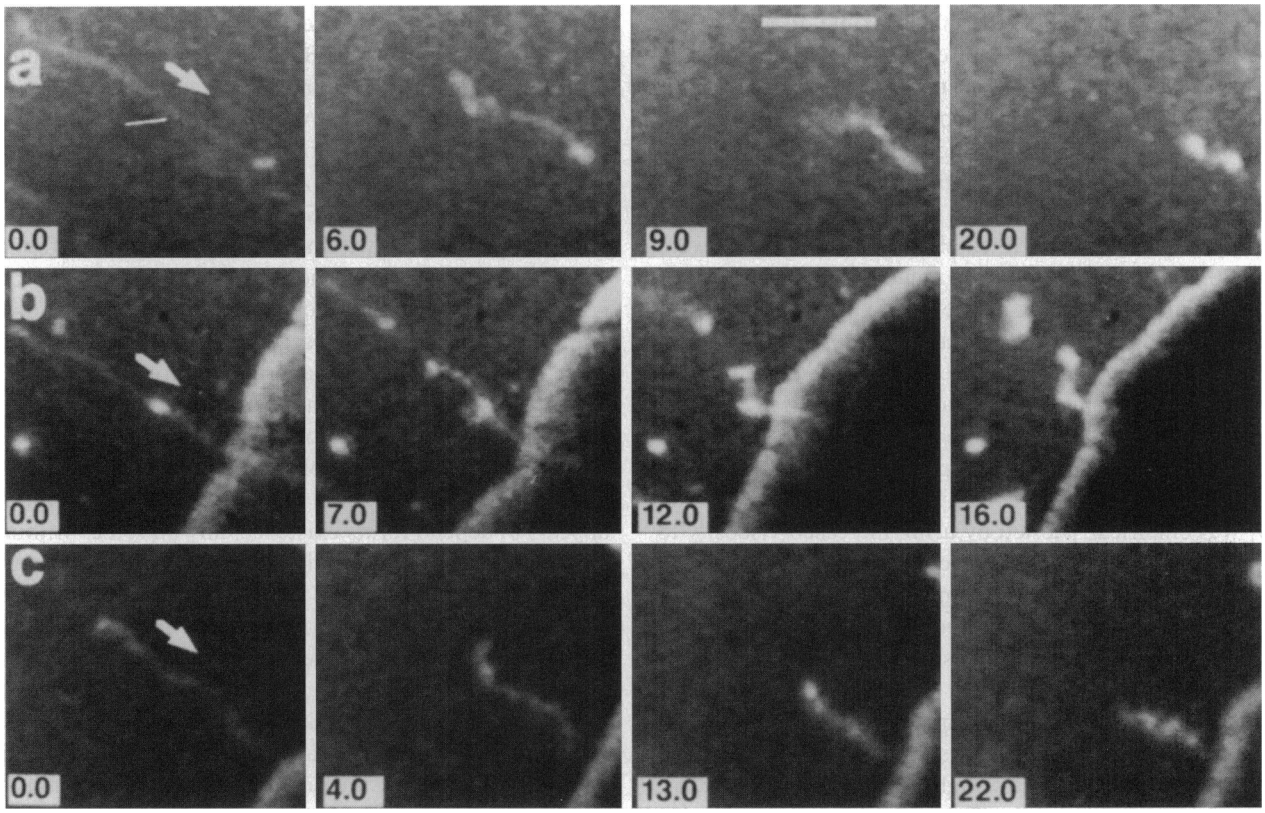

FIGURE 10 Compaction of DNA unraveled at the edge of a hanging drop. Bacteriophage G DNA, stained by 0.4 μg/ml ethidium bromide after release at 60°C, was observed near the edge of a hanging drop that had been made to move. (*a–c*) Single molecules that had previously been extended by the motion of the drop. As a function of time, compaction to form a flexible filament is shown.

orientation unrestricted by steric exclusion) is the finding of mean span ratios that are a) 1.29 for the ratio of the largest to the intermediate span, and b) 1.27 for the ratio of the intermediate to smallest span (Rubin and Mazur, 1975). Both values of S_m were 1.61. In this study, "long polymer" means a polymer that can be represented by at least 150 straight segments, each of length equal to twice the persistence length (*a*). Assuming that the *a* of G DNA is 50 (±5) nm when the ionic strength equals that of the buffer used here (Sobel and Harpst, 1991), then G DNA can be represented by 2440 straight segments. Thus, G DNA is a long polymer by the definition of Rubin and Mazur (1975). Because most of its conformations are sufficiently contracted so that displacement on one axis is independent of displacements on the others, a random coil projected (in two dimensions) to mimic a flexible filament should have a projected $\bar{S}_2/\bar{S}_1$ ratio close to the span ratios of an unprojected random coil. By simulation with 1000 independent computer-generated 2440 segment random coils, the $\bar{S}_2/\bar{S}_1$ ratio was found to be 1.37, slightly higher (Table 2). The exact source of this discrepancy is not known. According to Rubin and Mazur (1975), $\bar{S}_2/\bar{S}_1$ increases by 3–4% after restricting the random coil by preventing any two segments from occupying the same space.

TABLE 2 The asymmetry and mean spans of both flexible filaments and computer-generated random coils

Type of polymer	$\bar{S}_2/\bar{S}_1$	$\sigma(S_2)/\bar{S}_2$	$\sigma(S_1)/\bar{S}_1$	S_m	$(\bar{S}_2 + \bar{S}_1)/2$ (μm)
Flexible filament* in buffer	1.28	0.30	0.30	1.55	3.97 ± 1.30[¶]
Flexible filament[‡] at higher viscosity	1.26	0.29	0.28	1.54	3.93 ± 1.21[¶]
Random coil[§]	1.37 ± 0.03[‖]	0.22 ± 0.02[‖]	0.22 ± 0.02[‖]	1.70 ± 0.04[‖]	3.80 ± 1.03[¶]

* All values in this row were determined by quantification of 100 images of G DNA flexible filaments in buffer with 0.6 μg/ml ethidium bromide, without methyl cellulose.

[‡] All values in this row were determined by quantification of 100 images of G DNA flexible filaments in buffer that had 1.0% methyl cellulose of molecular weight 7140. The methyl cellulose increased the viscosity (determined with an Ostwald viscometer at 24°C) from 0.973 centipoise to 3.56 centipoise.

[§] All values in this row were determined by quantification of the projections of 1000 computer-generated unrestricted random coils, each comprising 2440 straight segments that were 100 nm long.

[¶] The error indicated is the σ of $(\bar{S}_2 + \bar{S}_1)/2$.

[‖] The error indicated is the σ of 10 independent measurements, each performed by use of 100 computer-generated random coils.

To help determine whether the flexible filaments observed here have characteristics expected of a random coil, both the $\bar{S}_2/\bar{S}_1$ ratio and the $\sigma/\bar{S}$ ratios were determined for a random collection of 100 apparently unbroken flexible filaments in a nonturbulent hanging drop. The $\bar{S}_2/\bar{S}_1$ ratio was 1.28, slightly smaller than the $\bar{S}_2/\bar{S}_1$ ratio obtained by analysis of computer-generated random coils (Table 2). Possibly because of experimental error, the σ/S ratios were about 20% larger than those of a random coil (Table 2). For flexible filaments, the procedure for determining $\bar{S}_2/\bar{S}_1$ included superimposing several serially focused images of a given flexible filament. Therefore, movement of the flexible filament while changing the plane of focus is a possible source of error in determining $\bar{S}_2/\bar{S}_1$. Although the magnitude of this motion (Fig. 6) appears too small to significantly change $\bar{S}_2/\bar{S}_1$, nonetheless, the effect of movement on $\bar{S}_2/\bar{S}_1$ was empirically tested by using increased viscosity to reduce this movement. After increasing the viscosity 3.7-fold by the addition of 1.0% methyl cellulose, the $\bar{S}_2/\bar{S}_1$ ratio did not significantly change (Table 2). Thus, motion during determination of S values did not have a detectable effect on $\bar{S}_2/\bar{S}_1$.

To further quantify the asymmetry of G DNA flexible filaments, S_m was determined. As found for $\bar{S}_2/\bar{S}_1$, the S_m of the flexible filaments was slightly smaller than the S_m of simulated random coils (Table 2). The diffraction-induced rounding of images of flexible filaments (i.e., the intrinsic limitation of light microscopy) causes a ± 0.5 μm error that is sufficient to explain the difference between the experimental and the modeled values of both $\bar{S}_2/\bar{S}_1$ and S_m.

To compare the size of the flexible filament to the size of a DNA random coil, both $(\bar{S}_1 + \bar{S}_2)/2$ and its σ were both measured for flexible filaments and compared to those expected for a computer-simulated random coil. The values from computer simulation were not significantly different from those observed experimentally for a flexible filament (Table 2).

DISCUSSION

Previous studies (Yanagida et al., 1983; Houseal et al., 1989) of 40–50-kb double-stranded DNA by fluorescence microscopy revealed that the conformation of a single DNA molecule underwent the following transition. A compact form of DNA, indistinguishable from a sphere, reversibly converted to a flexible filament that was 2–6 μm long, at least two times shorter than the DNA double helix stretched to its full length. Thus, across the diameter of the flexible filament must be more than one segment of the double helix. The lifetime of the flexible filament was less than 1 s. The length of the flexible filament for 40–50-kb DNA was 0.2–0.5 times the separation between the glass surfaces (boundaries). Thus, existence of the flexible filament in unbounded solution could not be concluded. In comparison, the 670-kb flexible filament observed here was both longer and more stable than the flexible filament of shorter DNA. Only minor changes in the 670-kb flexible filament occurred when a flat slide preparation was used instead of a hanging drop preparation. Because the additional boundary of a flat slide preparation had so little effect on the conformation of the 670-kb DNA, the assumption is made that removing the secondary boundary (i.e., placing the DNA molecules in unbounded solution) would also not qualitatively change the flexible filament observed here.

Although currents within a hanging drop were found to alter DNA conformation, no evidence was found that currents within the hanging drop were necessary for formation of the flexible filament observed. On the contrary, turbulence converted G DNA to alternative forms shown in Figs. 2 and 3. In agreement, the motions of the ends of the flexible filament were random. Thus, periodic motion of the suspending medium, possibly caused by standing waves, is not the cause of the motion observed. The flexible filament appears to depend for its existence only on forces within the DNA molecules at equilibrium with immobile surrounding buffer and dye. The dye used here to observe flexible filaments was ethidium, an intercalator. However, although DNA breakage was a problem, flexible filaments were also observed when DAPI, a nonintercalator, was used (not shown). Both this observation and the observation that the flexible filament persisted as the concentration of ethidium was lowered support the conclusion that the flexible filament exists in the absence of any dye. However, experiments to further test this conclusion have not yet been performed.

The measured $\bar{L}_F$ was 17 times less than L_D for G DNA. If the assumption is made that none of the DNA was invisible in a flexible filament, then 17 double helices must, on average, be arrayed across the width of the flexible filament. That all DNA was visible is concluded from the visibility of DNA unraveled to form a strand that was a single double helix (Fig. 8).

Two types of array are possible for segments of double helix across the width of a flexible filament: 1) The DNA array is random. 2) The DNA array has some order, possibly either back and forth folding, or unidirectional winding. If the DNA were wound unidirectionally, then either unraveling or compaction of the DNA flexible filament would cause an unraveled end of the double helix to rotate. By observation of compaction of DNA molecules at the interface of the outside and the inside of a hanging drop, evidence for rotation was not found. For the following reasons, rotation too rapid to visualize is inconsistent with the observations made here: 1) No evidence exists for movement of the suspending solution during compaction of the flexible filament. In the absence of such movement, the motions of the DNA must be Brownian. 2) However, the data of Fig. 6 indicate that those Brownian steps already observed are too small to coil the DNA in a time less than that between video frames. Thus, the primary mode of compaction could be either folding or random coiling, not winding. Because both the asymmetry and size of the flexible filament agree with those expected of a randomly coiled G DNA molecule, random coiling appears to be the correct explanation. This

agreement found by using the currently accepted *a* value contrasts with the requirement for a higher *a* value to explain the light microscopically observed size of a DNA more than 10-fold shorter than G DNA (Smith and Bendich, 1990). The limits of the resolution of light microscopy are expected to cause an experimental error that increases as the length of the DNA decreases.

Along a flexible filament, the beads observed here could not have been observed throughout the central region of the previously observed 40–50-kb DNA, because of the limitations of the resolution of light microscopy. Although their existence yields the impression of an ordered structure along the DNA, these beads could be no more than random superpositions of segments of a DNA random coil that forms the flexible filament. This hypothesis is supported by the apparently random motion of the beads.

Two types of DNA-DNA aggregation have been observed here: 1) comparatively loose aggregation observed in hanging drops after release of G DNA at comparatively high temperature (Fig. 2 *a*), and 2) tighter aggregation observed primarily at the edges of hanging drops (i.e., the lumps of Fig. 9 *a*). Although neither of these types of aggregation is understood in detail, both can be explained by nondiffusive motion that causes tangling. In the first case, aggregation would be explained by turbulence-induced tangling during expulsion of DNA from the capsid of bacteriophage G. By this hypothesis, raising the expulsion temperature increases the tangling by increasing turbulence. In the case of aggregation to form lumps, nondiffusive movement of DNA was directly observed to produce the aggregation. The observed irreversibility is best explained by tangling. Thus, although we have observed G DNA flexible filaments that are in an equilibrium state, other G DNA molecules were in a nonequilibrium state. Because both types of molecule were present in the same hanging drop, any procedure used to characterize these molecules would have to be capable of observing limited zones, possibly one molecule, of the solution of DNA.

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