Partially Condensed DNA Conformations Observed by Single Molecule Fluorescence Microscopy

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ABSTRACT To detect partially condensed conformations of a double-stranded DNA molecule, single molecule fluorescence microscopy is performed here. The single DNA molecules are ethidium stained, 670 kilobase pair bacteriophage G genomes that are observed both during and after expulsion from capsids. Expulsion occurs in an agarose gel. Just after expulsion, the entire G DNA molecule typically has a partially condensed conformation not previously described (called a balloon). A balloon subsequently extrudes a filamentous segment of DNA. The filamentous segment becomes gently elongated via diffusion into the network that forms the agarose gel. The elongated DNA molecule usually has bright spots that undergo both appearance/disappearance and apparent motion. These spots are called dynamic spots. A dynamic spot is assumed to be the image of a zone of partially condensed DNA segments (globule). The positions of globules along an elongated DNA molecule 1) are restricted primarily to time-stable regions with comparatively high thermal motion-induced, micrometer-scale bending of the DNA molecule and 2) move within a given region on a time scale smaller than the time scale of recording. Less mobile globules are observed when either magnesium cation or ethanol is added before gel-embedding DNA molecules. These observations are explained by globules induced at equilibrium by a bending-dependent, inter-DNA segment force. Theory has previously predicted that globules are induced by electrostatic forces along an electrically charged polymer at equilibrium. The hypothesis is proposed that intracellular DNA globules assist action-at-a-distance during DNA metabolism.

INTRODUCTION

The averaged conformation of DNA molecules in aqueous solution is (macroscopically) found to be indistinguishable from a random coil. This conclusion is based on both light scattering and analytical ultracentrifugation (for review, see Bloomfield et al., 1974; Cantor and Schimmel, 1980). The same conclusion is drawn from the results of both fluorescence microscopy (Serwer et al., 1995; Haber et al., 2000) and force-extension measurements (Bouchiat et al., 1999; Stigter and Bustamante, 1998) of single DNA molecules. However, these data do not exclude the possibility that more condensed conformations exist either 1) in conditions that resemble biological conditions or (2) at higher resolution in the conditions of past studies. Double-stranded DNA molecules are known to have more condensed conformations in some conditions, including the presence of ethanol (for review, see Bloomfield, 1996; Rouzina and Bloomfield, 1998; Gelbart et al., 2000). Also, all DNA segments have a completely condensed conformation when a double-stranded DNA molecule is packaged in a cavity within the protein capsid of a virus. The cavity has a volume approximately twice the volume of the packaged DNA molecule, in the case of several double-stranded DNA bacteriophages (Earnshaw and Casjens, 1980). Electron microscopy reveals that DNA expelled from particles of bacteriophage G is sometimes partially condensed (Sun and Serwer, 1997).

In theory, multiple partially condensed segments (globules) along a molecule of linear polymer reduce the free energy of the molecule if the molecule is electrically charged. A globule-containing molecule of polymer is sometimes called a necklace (Kantor and Kardar, 1994, 1995; Dobrynin and Rubenstein, 1996; Solis and Olvera de la Cruz, 1998). In principle, necklace-associated globules should be observable by fluorescence microscopy of single DNA molecules at equilibrium if the globules exist. The double-stranded DNA genomes of viruses are uniform-length DNA molecules. However, neither fluorescence microscopy nor any other procedure has yet been a rigorous test for globules in a viral DNA molecule at equilibrium. One reason is DNA length. For example, the size of a double-stranded DNA random coil approaches the resolution of a fluorescence microscope in the case of the 48.5 kilobase pair bacteriophage λ genome (Smith and Bendich, 1990). Some unique-length DNA molecules are long enough to display globules during fluorescence microscopy even when the conformation approximates a random coil. However, overlap of DNA segments causes ambiguity in detecting fine structure when a DNA molecule's conformation approximates a random coil (Serwer et al., 1995).

In contrast, globules might be observable for a DNA molecule that is partially elongated. An elongated DNA molecule, however, is not in an equilibrium state. To approximate equilibrium conditions during a test for globules, DNA molecules should be elongated as gently as possible. Several techniques have previously been used to produce elongated DNA molecules for single molecule microscopy; none begins with a condensed DNA molecule. These techniques include 1) adsorption to a glass surface (Bensimon et al., 1994; Serwer et al., 1995), 2) preparation of a specimen

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in the presence of molten agarose that subsequently gels (Schwartz et al., 1993; Schwartz and Samad, 1997), 3) either magnetically or optically pulling on a bead attached to the end of a DNA molecule (Bustamante et al., 1994; Wang et al., 1997), and 4) exposure of a DNA molecule to a fluid velocity gradient (Perkins et al., 1995; Serwer et al., 1995; LeDuc et al., 1999; Smith et al., 1999). Preliminary evidence of globules is found for DNA molecules that are stretched by either a fluid velocity gradient (Smith et al., 1999) or electrophoretic migration (Ueda et al., 1998). Similar evidence for globules is found for DNA molecules that are retracting after having been stretched in an agarose gel (Schwartz et al., 1993). However, apparently no test for fine structure has been made for DNA molecules that are close to equilibrium. Technique 1 is probably too extreme to permit the survival of globules. The bead used in technique 3 obscures the DNA molecule. Elongation of a DNA molecule should be done as gently as possible without attachment of a foreign object.

The following is a possible strategy for achieving more gentle, partial elongation of a condensed DNA molecule: 1) remove the constraint that condenses the DNA molecule and 2) then allow the DNA molecule to diffuse in the presence of obstacles. The purpose of the obstacles is to cause gentle elongation during decondensation diffusion. This diffusion-based strategy is used in the present study. The 670 kilobase pair (244,000 nm) linear double-stranded DNA genome of bacteriophage G (Hutson et al., 1995) is the specimen. Elongation of a G DNA molecule is achieved by expelling a (condensed) DNA molecule from the G capsid while a particle of bacteriophage G is embedded in an agarose gel. Evidence is found for both DNA globules and a new, higher-level, partially condensed G DNA conformation.

MATERIALS AND METHODS

Preparation of bacteriophage G

Bacteriophage G was preparatively grown in a 0.4% agar overlay. Bacteriophage particles were purified by use of rate zonal centrifugation in a sucrose gradient that contained 6% polyethylene glycol with a molecular weight of 3350. Details have been previously described (Serwer et al., 1995). Bacteriophage G particles were stored without dialysis after centrifugation. The buffer for storage was 0.01 M Tris-Cl, pH 7.4, 0.01 M $MgSO_4$, 6% polyethyene glycol with a molecular weight of 3350 (G buffer). Approximately 15% sucrose was also present. Particles of bacteriophage G were stable for a period of at least 3 years.

Fluorescence microscopy

Specimens with intact bacteriophage G particles were made at room temperature (22 ± 3°C) by dilution in G buffer that had, in addition, 2% β-mercaptoethanol and 0.5 μg/ml ethidium bromide (final concentration). The ethidium cation is the fluorescent stain. Specimens with expelled G DNA were prepared for fluorescence microscopy by dilution of bacteriophage G particles into a buffer that caused expulsion of G DNA from its capsid. This latter buffer was 0.1 M NaCl, 0.01 M Tris-Cl, pH 7.4. 0.02 M EDTA, 0.5 μg/ml ethidium bromide, 2% β-mercaptoethanol (DNA buffer) (see Figs. 1–6). The expected (Griess et al., 1985) molar ratio of bound ethidium cations to nucleotide pairs is roughly 0.05. DNA buffer also had 6% polyethylene glycol when indicated. To embed bacteriophage G particles in an agarose gel, agarose was added to one of these mixtures. Either SeaPlaque agarose or SeaPrep agarose was used at the concentration indicated. Both agarose preparations were low-melting point, low-gelling point (hydroxyethylated) agarose preparations from FMC Bioproducts (Rockland, ME). The agarose had been dissolved by boiling in a microwave oven. The EDTA (a chelator) in DNA buffer destabilized bacteriophage G, thereby causing expulsion of DNA from the G capsid. Specimens were prepared for microscopy by placing a drop (10–20 μl) on a glass microscope slide. A cover glass was placed on the drop. Some specimens were prepared at room temperature (22 ± 3°C) as indicated. Expulsion of DNA occurred more slowly as the temperature became lower. Thus, expulsion of DNA before gelation of the agarose was sometimes slowed by use of the following procedure. Bacteriophage G particles were chilled immediately after dilution in a solution of molten agarose (40°C). The bacteriophage G particles were chilled by placing a specimen on a glass microscope slide that had been equilibrated with a chilled aluminum block. The temperature of the block is indicated in figure legends. An ice-cold cover glass was placed on the specimen. The specimen was kept cold until gelation occurred ~10 min later.

The specimen was subsequently observed on the specimen stage of an Olympus BX60 fluorescence microscope. The specimen stage was at room temperature; chilled slides warmed to room temperature during observation. The fluorescence microscope was used with a U-MWIG filter set (green excitation, orange emission) from Olympus. Images were recorded by a Philips 56770 series video camera and preserved on videotape. Images were recorded beginning at the time of placing the cold glass slide on the specimen stage at room temperature. For the formation of a still image, a videotaped frame was digitized by use of an Avid ABVB board set and Avid Xpress 2.1 for Macintosh software (Tewksbury, MA). The color output was excluded to reduce noise. Most residual noise is from the video camera. To show the dynamics of a DNA molecule, the images of successive frames were digitized. The time between frames was 0.033 s (video frame rate). A time-ordered collection of the images will be called an image train. The time of an image in an image train (seconds) will be indicated in figures. Photo bleaching was variable. However, DNA-fluorescence in DNA buffer never had a half-life less than 3 min in the experiments reported here.

The asymmetry of the image of a single DNA molecule was quantified by determining the value for the following: the ratio of the maximal length to the width of a rectangle that encloses the image of the DNA molecule (maximal span ratio). This strategy for quantifying asymmetry is discussed in Rubin and Mazur (1975). Fluorescence intensity was quantified by use of procedures previously described (Sun et al., 1999).

A change in the above procedure was made for Fig. 7 *b* (see Fig. 7). In this case, DNA was expelled by raising temperature to 60°C for 10 min after diluting bacteriophage particles 10-fold into 0.1 M NaCl, 0.01 M Tris-Cl, pH 7.4. The expelled DNA was further diluted 10-fold into a buffer that contained 0.01 M Tris-Cl, pH 7.4, 0.1 M NaCl, 0.75% SeaPlaque agarose, 2% β-mercaptoethanol, and 0.6 μg/ml ethidium bromide. The agarose was gelled at 0°C. Fluorescence microscopy was performed as described above.

RESULTS

Fluorescence microscopy of ethidium-stained bacteriophage G

Fluorescence microscopy of ethidium-stained single particles of bacteriophage G revealed diffraction-limited point sources of light. Some bacteriophage G particles were dif-

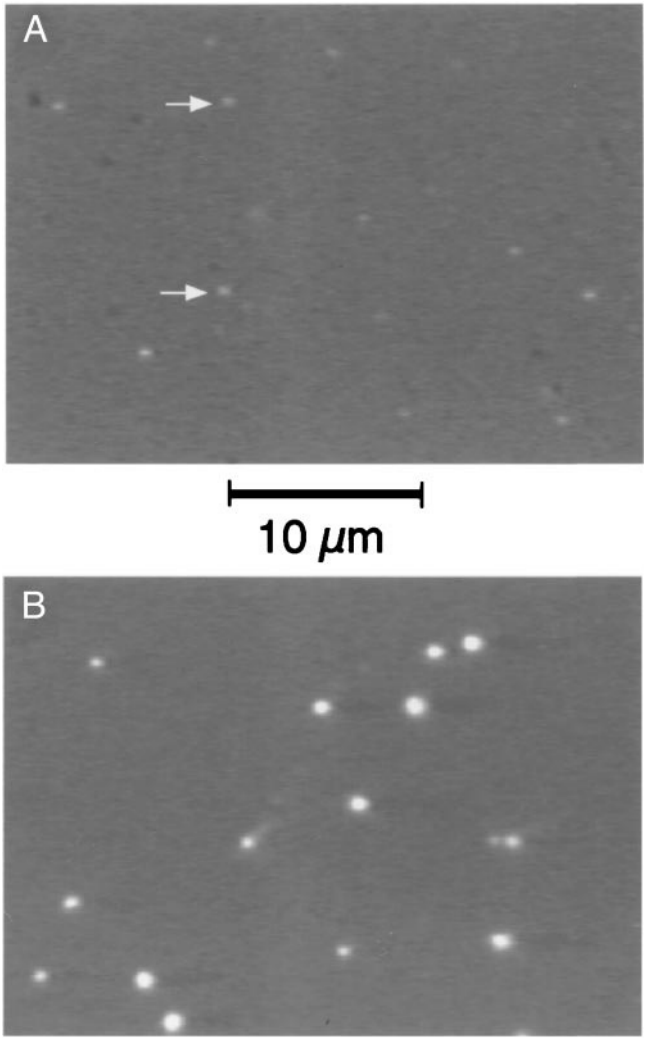

FIGURE 1 Fluorescence microscopy of bacteriophage G adsorbed to a cover glass. Bacteriophage G particles were observed by fluorescence microscopy, after specimen-preparation in (*a*) G buffer, 1.5% SeaPlaque agarose, gelled at 4°C, and (*b*) DNA buffer, 0.75% Seaplaque agarose, gelled at room temperature (7×10^{10} particles/ml).

fusing in the solution between the cover glass and the glass slide. Other bacteriophage G particles were bound to a cover glass. Glass-bound bacteriophage particles are shown in Fig. 1 *a*; some bacteriophage particles are indicated by arrows in Fig. 1 *a*. Other double-stranded DNA bacteriophages, including Ø29, T4, and T7, had a similar appearance when previously observed by fluorescence microscopy (Kuroiwa, et al., 1981). The image in Fig. 1 *a* was obtained for a specimen prepared at low temperature in G buffer, a buffer that stabilizes bacteriophage G.

A different result was obtained when bacteriophage G was prepared for fluorescence microscopy in DNA buffer at room temperature. First, the average fluorescence intensity of glass-bound particles was higher. A typical field immediately after illumination is in Fig. 1 *b*. Second, the fluorescence intensity sometimes (5–10% of particles randomly selected) increased as a function of time. The presence of polyethylene glycol was used to lower the rate of this increase. The image train of Fig. 2 *a* shows a bacteriophage particle (marked with an arrow) that increased its fluorescence intensity dramatically but comparatively slowly in the presence of DNA buffer that had polyethylene glycol. A second bacteriophage particle in this field (marked with an arrowhead) also increased its fluorescence intensity but to a lesser extent.

New state of partially condensed G DNA

Visually, increase of fluorescence intensity in DNA buffer (without polyethylene glycol) was usually more rapid than it is in Fig. 2 *a*. For example, the image train of Fig. 2 *b* has a particle that underwent a dramatic increase in fluorescence intensity in the 0.00- to 0.25-s frames. That is to say, the bacteriophage particle in Fig. 2 *b* appeared to "inflate" in less than one-half of a second. The product will be called a balloon. Balloons were observed even when agarose was omitted during preparation of a specimen. Some balloons were observed after storage for 24 h at 4°C (data not shown). Like the balloon in Fig. 2 *b*, some balloons were bound to the cover glass, based on absence of thermal motion for at least part of the balloon. Other balloons were unbound, based on presence of thermal motion for the entire balloon.

The following observation indicates that a balloon-associated DNA molecule was at least partially outside of its capsid. A balloon had shape flexibility when bound to a cover glass. A cone-like image is seen after formation of a balloon in the 0.25- to 2.19-s frames of Fig. 2 *b*. The cone is better defined in some frames (1.59-s frame, for example). A more rounded cone-like image is seen in other frames (0.45-, 0.79-, and 1.66-s frames, for example). This change in shape was caused by thermal motion-induced chattering, based on observation at video frame rate. The shape changes were enhanced because the balloon in Fig. 2 *b* remained attached to the cover glass at the point that formed the apex of the cone. Change in shape was not observed for bacteriophage G particles prepared for microscopy in G buffer. Therefore, the apparent flexibility of a balloon indicates that its DNA molecule had been expelled from the protein capsid of a bacteriophage G particle. Before expulsion, the radius of the envelope of the condensed DNA molecule is roughly 80 nm (Sun and Serwer, 1997). The envelope of the DNA molecule must have increased its radius by a factor of at least 2 to 3 to be large enough to observe shape asymmetry. The fluorescence microscope has a resolution of roughly 200 nm. Balloons had an average radius of 280 $\pm$ 60 nm.

However, the following measurement indicates that balloon-associated, expelled DNA molecules were more condensed than a DNA random coil. The mean radius of a G

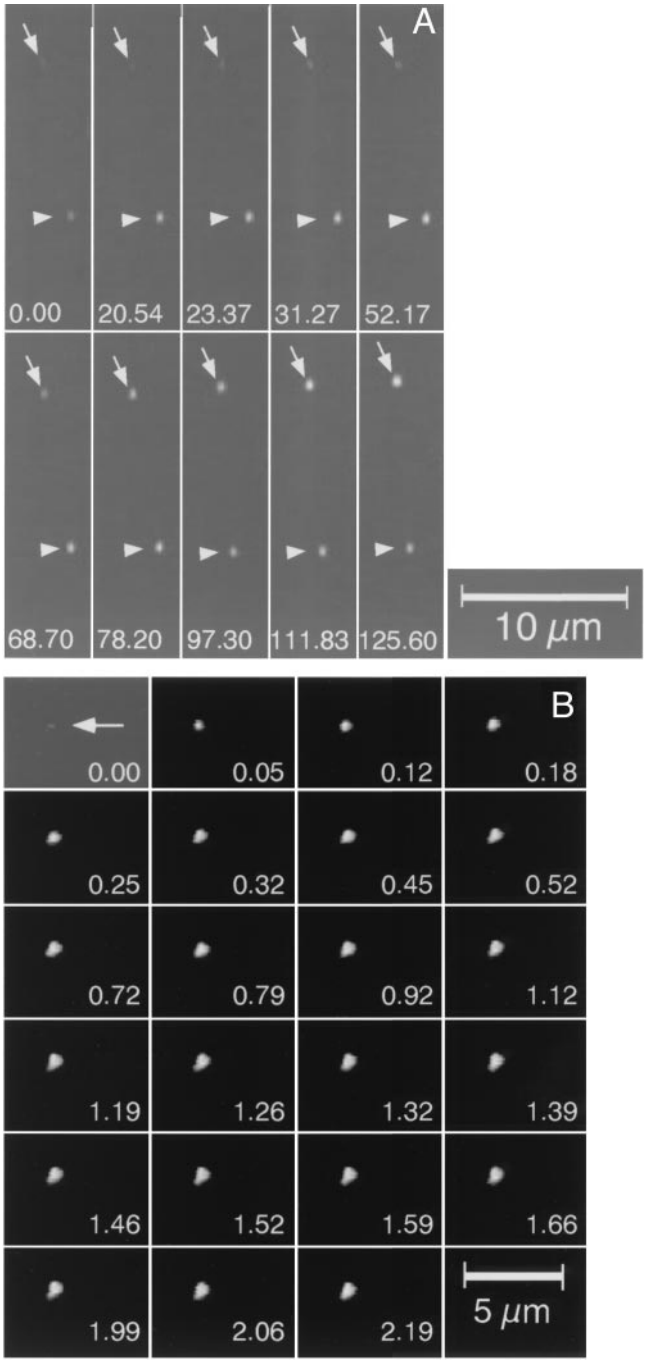

FIGURE 2 Change in fluorescence intensity during microscopy. Specimens (9×10^8 particles/ml) were prepared in DNA buffer with 1.5% SeaPrep agarose, gelled at 4°C. Two independent image trains demonstrate (*a*) gradual increase in fluorescence intensity for the bacteriophage particles indicated with either an arrow or an arrowhead, and (*b*) more sudden increase to form a balloon (0.00- to 0.18-s frames) that subsequently underwent thermal motion-induced change in shape (0.25- to 2.19-s frames). Polyethylene glycol was present in (*a*).

DNA random coil is found to be 2000 ± 650 nm (Serwer et al. 1995), 7× larger than that of a balloon. The following observation suggests that additional partially condensed,

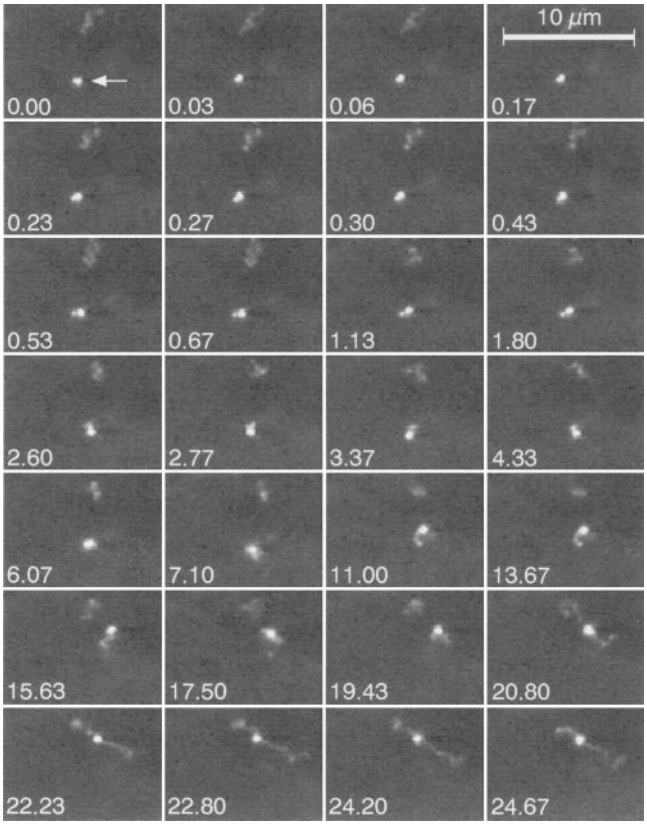

FIGURE 3 Extrusion of a filamentous segment of DNA from a balloon. A specimen (9×10^8 particles/ml) was prepared in DNA buffer with 1.0% SeaPlaque agarose, gelled at room temperature. An image train demonstrates the extrusion of filamentous DNA segments from a balloon.

partially or totally expelled DNA molecules are produced during the slower increase of fluorescence intensity in Fig. 2 *a*: increase in ethidium staining is associated with decondensation of the packaged DNA molecules of bacteriophage T7 (Griess et al., 1985).

Extrusion of a filamentous DNA segment

Balloons are possible precursors for the more elongated DNA molecules needed in a test for globules. Most balloons did change to more elongated DNA molecules when continuously observed. The first stage in forming a more elongated DNA molecule was extrusion of a filamentous DNA segment from a balloon. First evidence of extrusion usually arrived within 10 s after formation of a balloon. The filamentous DNA segment was barely visible at first. A filamentous DNA segment at this early stage is shown in the 0.23- to 1.80-s frames of the image train of Fig. 3. This image train starts with a balloon in the 0.00- to 0.17-s frames. The filamentous DNA segment became longer as time increased in Fig. 3. Eventually, the DNA formed two arms, as though the two ends of a G DNA molecule were separately extruded, as seen in the 20.80- to 24.67-s frames

of Fig. 3. This was typical behavior for a balloon that extruded a filamentous DNA segment. During observation, the extruded filament never became so long that the balloon was gone. The illumination of the microscope appeared to cause eventual stopping of the extrusion. The balloon of Fig. 3 was not attached to the cover glass, based on the observation that the entire balloon underwent thermal motion. The frames of Fig. 3 contain a fixed field of view. Thus, motion of a DNA molecule changed the distances of the DNA molecule from the edges of a frame.

Partial elongation of expelled bacteriophage G DNA

The extrusion of a filamentous DNA segment (illustrated in the 20.80- to 24.67-s frames of the image train of Fig. 3) is potentially the initial stage of a more extensive elongation. Thus, a search was made for DNA molecules at subsequent stages of elongation. This search revealed balloons with filamentous DNA segments that were longer than the filamentous DNA segment in Fig. 3 (data not shown). These latter DNA molecules were presumably at a subsequent stage of elongation. The complete elongation was never observed for any one DNA molecule. The reason is that eventually elongation stopped. In addition, on rare occasion, the DNA molecule cleaved during observation. The apparent cause is the illumination of the fluorescence microscope. Some of the more elongated DNA molecules were not attached to a balloon.

Videotaped image trains revealed that DNA molecules underwent thermal motion at all stages of elongation. The image train of Fig. 4 shows an elongated, balloon-free DNA molecule that was undergoing thermal motion. The molecule was not either 1) undergoing net translational motion or 2) attached to a solid surface. At low resolution, the thermal motion caused bends in the DNA filament, as expected. However, some regions had more visible bends than others for reasons not known. A bend-rich region is indicated by a bracket for the DNA molecule in Fig. 4. A bend-rich region sometimes resembled an irregular standing wave when continuously viewed at video frame rate.

The following observations indicate that the elongation observed here was both more extensive and longer lasting than the elongation produced by a random walk in the absence of a gel. The maximal span ratio was 7 to 20 for elongated DNA molecules like those of Fig. 4 (48 molecules measured). This ratio is 1.6 ± 0.04 for the average randomly coiled G DNA molecule (Serwer et al., 1995). Also, the duration of a state of elongation is a couple of seconds for a typical G DNA random coil in the absence of a gel (Serwer et al., 1995). In contrast, the duration of a state of elongation was greater than 10 min for a typical gel-embedded G DNA molecule. The source of elongation was observed to be decondensation-biased diffusion in the case of balloons that extruded a filamentous segment of DNA during observation.

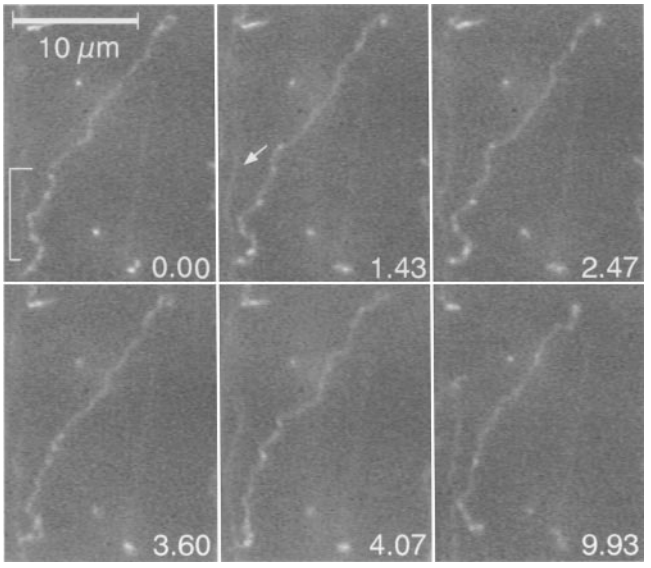

FIGURE 4 Longer DNA segments: dynamic spots. A specimen (9×10^8 particles/ml) was prepared in DNA buffer with 1.0% SeaPlaque agarose gelled at room temperature. An image train is shown for an elongated DNA molecule that has dynamic spots. The bracket indicates a region that has both an increased density of dynamic spots and increased bending.

Substructure of gel-embedded, elongated G DNA molecules

Screening of videotaped image trains revealed that substructure was present in most images of gel-embedded G DNA molecules that had been elongated by decondensation-biased diffusion after being expelled from their capsids. The substructure consisted of bright spots. Bright means fluorescence intensity increased by a factor of at least 2. These spots continually changed both their position and their brightness. They will be called "dynamic spots." To the eye, the changes in position included disappearance/appearance and motion along the axis of the DNA thread. The DNA molecule of Fig. 4 has dynamic spots selectively concentrated in the region indicated by a bracket in the first frame of Fig. 4. The dynamic spot-rich DNA segment of Fig. 4 is also the bend-rich segment discussed in the previous section. Qualitatively, this correlation of bend-rich with dynamic spot-rich DNA segments was observed to be universal. Bend-rich, dynamic spot-rich segments had a lifetime over 30 s.

Quantitatively, the presence of bends was measured by the following ratio (to be called L_N): the total length of a (sometimes bent) DNA segment, divided by the length of a straight line that connects the beginning of the segment to the end of the same segment. The higher L_N is, the more bent the DNA segment is. The value of L_N was measured for

42 globule-rich DNA segments; globule rich means at least one globule per 3 μm. To avoid bias in the measurement, the following procedure was used for selecting frames: 1) frames from an image train were initially selected only for being in-focus throughout the DNA segment. The frames were given numbers. 2) Frames were selected for measurement by a random number generator. Four frames were selected per DNA molecule. The average value of L_N was 1.33 for the globule-rich DNA segments. The average value of L_N was 1.03 for globule-poor DNA segments. The standard deviation of L_N also measures (primarily) the extent of bending; the higher the standard deviation, the more the bending. The standard deviation of L_N does not measure experimental error, because the DNA segments were both moving and changing shape; even a bend-rich DNA segment was sometimes straight. The standard deviation of L_N was 0.34 for the globule-rich DNA segments and 0.02 for the smooth DNA segments; the experimental error was not more than 0.02. These results quantify the association of globules with visible bends.

To the eye, the time-averaged position of the dynamic spots was, with rare exception, uniformly distributed along a bend-rich DNA segment (see the bracketed region in Fig. 4). The dynamic spots resembled spots previously observed at the ends of agarose-stretched DNA molecules that had been cleaved (Schwartz and Samad, 1997). The more uniform distribution of most dynamic spots observed here differentiates these dynamic spots from the spots previously observed only at the ends of DNA molecules. Dynamic spots were observed on most (80–90%, observed at random) extruded, elongated DNA segments, whether or not the DNA segments were attached to a balloon. The assumption is made that a dynamic spot is the image of a region in which DNA segments have partially condensed (globule).

The following is an exception to the above pattern of behavior for dynamic spots. On rare occasion (less than 1% of dynamic spots), a dynamic spot preferentially appeared/disappeared at a single location that was not either at or near the end of a DNA segment (localized dynamic spot). A localized dynamic spot was usually brighter than other dynamic spots of the same DNA segment. The disappearance/reappearance presumably implies that this dynamic spot is not the image of the residual of a balloon. The reason for the existence of localized dynamic spots is not known.

Analysis of G DNA molecules expelled from capsids before preparation of a specimen

Decondensation-biased diffusion of an expelled G DNA molecule is not necessarily the only way that a dynamic spot-containing DNA molecule can be generated. One possible alternative is the process of agarose gel embedding of a G DNA molecule that had been unraveled before mixing with agarose. That is to say, some DNA molecules may have had dynamic spots even though they had been gel embedded in a state that was unrelated to their condensed state in a bacteriophage capsid.

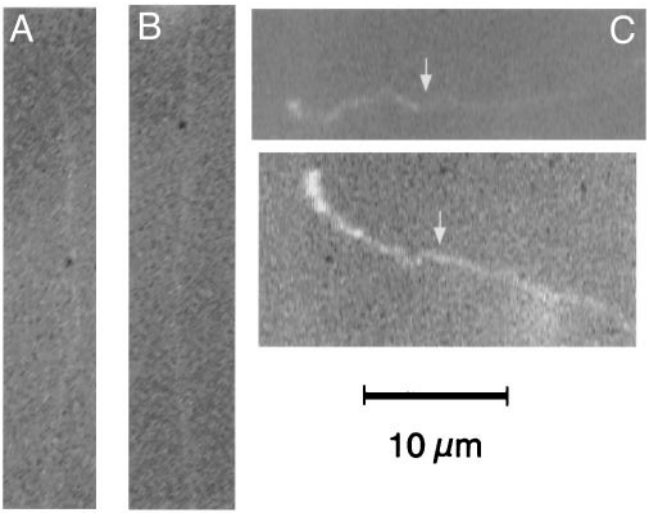

FIGURE 5 Smooth DNA segments. A specimen (9×10^8 particles/ml) was prepared in DNA buffer with polyethylene glycol. (*a* and *b*) Completely smooth DNA molecules are shown. These DNA molecules were in a specimen gelled in 1.5% SeaPrep agarose 4°C. (*c* and *d*) Partially smooth, partially dynamic spot-containing hybrid DNA molecules are shown. The molecule in *c* was prepared by the procedure used for *a* and *b*. The molecule in *d* was in a specimen prepared without polyethylene glycol in 1.0% SeaPlaque agarose at room temperature. An arrow indicates the smooth-dynamic spot boundary in *c* and *d*. The contrast of the DNA molecules in *a* and *b* is close to the limit for detectability.

To determine whether unpackaged G DNA molecules formed dynamic spots when subsequently embedded in an agarose gel, DNA was expelled from bacteriophage G by raising the temperature to 60°C in DNA buffer. Subsequently, a specimen was gel embedded. The specimen was observed by fluorescence microscopy. The result was that most DNA molecules were in aggregates when expelled at the concentration used in Figs. 1 to 4 for agarose gel-embedding bacteriophage particles (data not shown). In contrast, aggregates were not a significant form of DNA in specimens made without previous expulsion of DNA molecules. This observation supports the conclusion that most DNA molecules of Figs. 1 to 4 had been expelled after gel embedding. Nonetheless, a few dynamic spot-containing DNA molecules were observed among the unaggregated DNA molecules produced by expelling DNA molecules before gel embedding. The dynamic spot-containing DNA molecules constituted a much smaller fraction (5–15%) of the isolated single molecules than the dynamic spot-containing DNA molecules observed in the specimens of Figs. 3 and 4 (80–90%). DNA molecules without visible dynamic spots will be called smooth. Examples of smooth DNA molecules are in Fig. 5, *a* and *b*. The DNA molecules in aggregates were greater than 95% smooth. Thus, smooth DNA molecules were the predominant type when G DNA

molecules were gel embedded after expulsion from their capsids.

The data of this section lead to the following conclusion. Dynamic spot-containing, elongated DNA molecules are most efficiently produced during extrusion of DNA segments from a balloon after dilution for specimen preparation. However, dynamic spot-containing DNA molecules can also be produced at low frequency by agarose embedding previously unraveled DNA molecules.

Source of dynamic spots

The following observation suggests that the dynamic spots observed in Fig. 4 were accompanied by smaller dynamic spots that were not resolved. The DNA molecule of Fig. 4 was comparatively thick and unextended in the "smooth" regions that did not have obvious dynamic spots. More extended, thinner, less fluorescent, entirely smooth molecules are shown in Fig. 5, *a* and *b*. The presence of dynamic spots in the thicker smooth DNA segments was confirmed by review of videotapes. Comparatively small, just barely visible dynamic spots were seen in the thicker Fig. 4-like smooth DNA segments (data not shown). These dynamic spots were not seen in smooth molecules like those of Fig. 5, *a* and *b*. The presence of the smaller, but not the larger, dynamic spots in the thicker smooth DNA segments is explained by the following assumption: the larger (easily visible) dynamic spots require inter-DNA segment interaction that is not as great in the thicker smooth DNA segments. That is to say, the larger dynamic spots are not simply the consequence of a random walk confined by the agarose gel. This assumption is supported by the following evidence that the thicker smooth DNA segments were as free to execute a random walk as the dynamic spot-rich DNA segments: the thicker smooth DNA segments underwent lateral thermal motion that was as great as the lateral thermal motion of the dynamic spot-rich DNA segments (data not shown). At least one inter-DNA segment interaction appears to be associated with large-scale, visible bending of the DNA molecule (further discussed below).

The following is additional evidence that the thicker smooth DNA segments were in the same environment as the dynamic spot-rich DNA segments: some DNA molecules were hybrids in which the boundary between a smooth and a dynamic spot-rich DNA segment was sharper than it was for the DNA molecule in Fig. 4. These hybrid DNA molecules sometimes had one segment with no large, easily visible dynamic spots and a second segment with obvious dynamic spots. Both segments underwent thermal motion. Two hybrid molecules are shown in Fig. 5, *c* and *d*. An arrow indicates the smooth-dynamic spot boundary in Fig. 5, *c* and *d*. This boundary was never observed to have a DNA end. Thus, the conclusion is drawn that the hybrid character is not caused by the folding on itself of the DNA molecule. No evidence of such folding was observed for any DNA molecule that had been elongated to the extent of the DNA molecules in Figs. 4 and 5. That is to say, these molecules were never observed to split into more than one segment of DNA. The smooth-dynamic spot-rich boundary was stable for at least 30 s.

The two parts of a hybrid DNA molecule were 1) in the same environment (same ethidium concentration, for example), and 2) undergoing thermal motion. The only visible differences were the degree of bending and the number of dynamic spots per length. The average L_N was 1.17 for the dynamic spot-rich segment and 1.06 for the smooth segment of 42 hybrid DNA molecules. The standard deviations were 0.14 and 0.06. Thus, again, the dynamic spots were correlated with micrometer-scale DNA bending. Other factors (pH, ionic strength, ethidium concentration, for example) might, nonetheless, contribute to the formation of dynamic spots.

Duration of the dynamic spots

The time constant of the motion of the dynamic spots was small in comparison with the time separating video frames. This point is illustrated by the image train of a dynamic spot-containing DNA segment in Fig. 6. This image train has two subtrains of closely spaced images, separated by a thickened vertical bar. The pattern of spots changed dramatically between frames in Fig. 6. The successive images appear to be weakly correlated with respect to the dynamic spots. Quantification by autocorrelation analysis was not performed. Three-dimensional, more rapid, more noise-free recording will be needed for quantification.

The dynamics were more easily observed during running of the videotape at video frame rate. This effect of video frame rate observation was dramatic enough to be surprising. One apparent reason for this effect is that the human eye averages background noise, when images are presented at video frame rate at a fixed point in space. The eye cannot average background noise when successive images are separated in space. A second possible reason is the superposition (unconscious) of interpretation on the train of images. The frames of Fig. 6 were visually selected for being close to focus. That is to say, the dynamics are not being caused by out-of-plane motion.

Lowering the motion of the dynamic spots

Spots observed along a segment of a G DNA molecule could be made both brighter and less mobile. This was done by preparing a specimen that either 1) had 0.5% ethanol or 2) had 0.0001 M $MgCl_2$ in the mixture used to observe an agarose gel-embedded DNA molecule. This concentration of ethanol is more than an order of magnitude lower than the concentration used to either precipitate or condense a complete DNA molecule (for re-

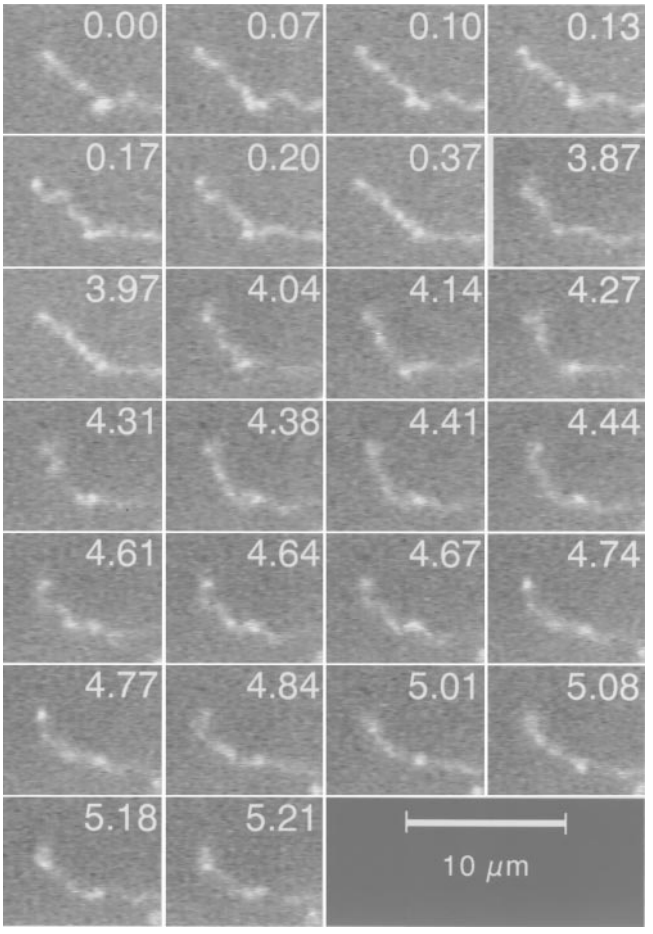

FIGURE 6 Dynamics of dynamic spots. A specimen (9×10^8 particles/ml) was prepared in DNA buffer with 0.5% SeaPlaque agarose gelled at room temperature. An image train illustrates the dynamics of dynamic spots that are typical of most dynamic spot-containing elongated DNA segments.

view, see Bloomfield et al., 1994; Bloomfield, 1996). Magnesium-induced increase in bending has previously been detected via a decrease in DNA persistence length measured in bulk aqueous solution (for review, see Rouzina and Bloomfield, 1998). A typical image is shown in Fig. 7 *a* for an ethanol-containing specimen; a typical image is shown in Fig. 7 *b* for a $MgCl_2$-containing specimen. The spots of both specimens underwent thermal motion. However, the thermal motion was restricted to chattering around a position that appeared fixed, somewhat like localized dynamic spots observed above. The lower threshold for this effect is 0.3% ethanol. The partially frozen spots were observed for ethanol concentrations as high as 1.0%. Higher ethanol concentrations interfered with the imaging of the DNA molecules. Photo bleaching was dramatically increased by the presence of the $MgCl_2$. Photo bleaching was not increased by 0.5% ethanol but was increased by 1.0% ethanol.

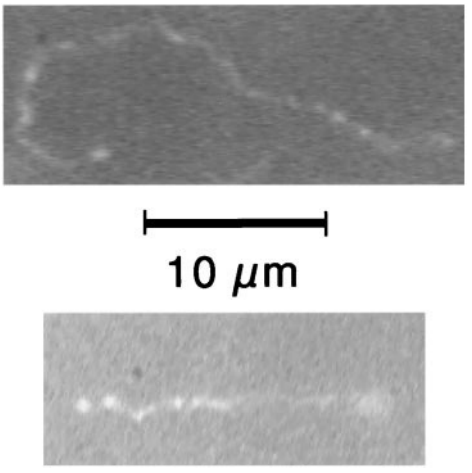

FIGURE 7 Globules with reduced mobility. (*a*) A specimen (9×10^7 particles/ml) was prepared in DNA buffer with both 0.5% ethanol and 0.75% SeaPlaque agarose gelled at 4°C. (*b*) A specimen was prepared by use of the modified procedure described in Materials and Methods. The final concentration of magnesium cation in *b* was 0.0001 M. Molecules with globules of reduced mobility are shown.

DISCUSSION

A G DNA molecule sometimes forms a balloon immediately after expulsion from its capsid. A balloon is a partially condensed G DNA molecule. This type of partially condensed DNA molecule has not previously been reported, to the authors' knowledge. The DNA molecule of a balloon has an average double helical DNA segment density low enough so that nonlocal DNA-DNA interactions cannot stabilize a balloon, if double helical DNA segments are uniformly distributed within a balloon. What, then, causes the (short-term) stability of a balloon? The embedding agarose is not needed. In addition, electron microscopy shows that a G capsid separates from its DNA molecule when the DNA molecule is expelled from the capsid. This latter observation is made for expelled DNA molecules that are partially condensed during electron microscopy (Sun and Serwer, 1997). Thus, presumably, the capsid is also not needed for stability of a balloon. These observations lead to the hypothesis that a balloon is stabilized by interaction of double helical DNA segments that are not uniformly distributed within a balloon. That is to say, these data suggest that condensed domains exist within balloons and, possibly, within a packaged G DNA molecule.

Electron microscopy of bacteriophage G produces data that agree with the presence of condensed domains in a packaged G DNA molecule (Sun and Serwer, 1997). If so, the following question is asked. What is condensing the DNA segments within a balloon-associated domain? The authors do not have an experimentally supported answer to this question. One possibility is a charge-fluctuation force. This type of force can exist either within or between two condensed DNA domains (Podgornik and Parsegian, 1998).

Possibly, interdomain forces stabilize the domains. In any case, the point is emphasized that the existence of balloons is rigorously demonstrated.

A gel-embedded balloon undergoes elongation to form a DNA molecule that usually has dynamic spots (globules). The data do not determine the role of either the gel or the elongation process in producing the dynamic spots. The dynamic spots are not an expected consequence of an unperturbed random walk for two reasons. First, the dynamic spots are bright enough so that their number per length of observed DNA segment is improbable in dynamic spot-rich DNA regions. This conclusion is based on the following rough calculation. 1) Image elements are not less than 200 nm because of the resolution of the microscope. 2) The fluorescence intensity of the thicker smooth DNA segments indicates the presence of not less than eight statistical segments per 200 nm on average. Each statistical segment has a length of 100 nm (Cantor and Schimmel, 1980). 3) Poisson sampling of these segments would produce a standard deviation of 2.8. Thus, multiplying the brightness by 2 corresponds to a fluctuation of $8.0/2.8 = 2.9$ standard deviations. The frequency of either this level of fluctuation or more is 0.002 from standard tables. In other words, one should observe not more than one twofold brightened dynamic spot per 100 μm, maximum. This number is 30- to 100-fold less than what is observed in dynamic spot-rich regions.

Nonetheless, random walks produce a variety of shapes and textures (Rubin and Mazur, 1975; Serwer et al., 1995; Haber et al., 2000). One might reasonably argue that every image observed here could have been produced by a computer-generated, biased random walk. However, the positions of globules in the globule-rich regions of an image train are uncorrelated among the different images of the train. Assuming that the thermal motions of an entire DNA molecule are uncorrelated on the time scale of recording, the following is a second reason that the data are not a predicted consequence of a random walk. The random walk would have to repeatedly generate a distribution characterized by globules concentrated in the same restricted region(s). In the case of the DNA molecule of Fig. 4, this distribution has almost all globules concentrated near one end of the DNA molecule. The probability of repeatedly generating this distribution through over 300 uncorrelated video frames is orders of magnitude lower than the reciprocal of the total number of DNA molecules observed. Thus, observation of the globule-rich regions is not a predicted consequence of a random walk. A source of potential energy must also be involved in generating the globules.

Empirically, the reasoning of the previous two paragraphs is confirmed by the observation that the thicker smooth DNA segments undergo lateral thermal motion as great as the thermal motion of the dynamic spot-rich DNA segments. The comparatively weak dynamic spots of the thicker, smooth DNA segments are, possibly, the product of a random walk constrained primarily by the presence of agarose gel fibers. In contrast, the dynamic spots of the dynamic spot-rich DNA segments require a physical effect not encompassed in a simple random walk. The existence of this physical effect is further supported by the observation that the globules can be immobilized by adding either ethanol or magnesium cation at very low concentration. The presence of magnesium cation is physiological. The data presented here appear to be the first empirical demonstration of mobile globules within an unstressed DNA molecule that undergoes no motion other than thermal motion.

One current theory predicts that the effect needed to explain globules is produced by the electrical charge of DNA. This theory is based on previous theory that predicts the spontaneous disintegration of an electrically charged water droplet to form smaller droplets. The smaller droplets have, in aggregate, a lower potential energy (Lord Rayleigh, 1882). The current theory is based on minimization of the total free energy. The theory assumes that potential energy is dominated by 1) long-range, charge-charge repulsion among DNA segments, 2) short-range, condensed counterion-induced electrostatic attraction among DNA segments (including charge fluctuation forces), and (3) surface tension. The solution at equilibrium is a string of globules (Kantor and Kardar, 1994, 1995; Solis and Olvera de la Cruz, 1998). A straight string of globules does not explain previous observations of single DNA molecules in the absence of a gel (Serwer et al., 1995; Haber et al., 2000). However, a randomly coiled string of globules is consistent with data obtained in the absence of a gel.

The theory cited above does not encompass all observations. First, the globules observed here are mobile. The theory only describes static globules. The theory does, however, show that the total free energy of a globule is only a weak (in comparison with kT) function of the radius of a globule (Kantor and Kardar, 1995). Thus, qualitatively, thermal motion can change both the size and location of the globules.

Second, the theory does not account for the correlation of globules with micrometer-scale DNA bending. A possible explanation for this correlation is the bending-induced attraction of counterions. The converse has already been observed for nanometer-scale DNA bending. Either asymmetric addition of a condensed counterion or asymmetric elimination of a DNA-phosphate anion causes bending of a double-stranded DNA molecule (for review, see Rouzina and Bloomfield, 1998; Williams and Mayer, 2000). Counterions attracted via DNA bending are presumably mobile and may, therefore, be a catalyst for the dynamic globules observed here. Statistically, micrometer-scale DNA bending causes increased frequency of nanometer-scale DNA bending. Thus, the observed bending will stimulate counterion binding at multiple sites. The initiation of a globule potentially requires nanometer-scale DNA bending at more than one site.

The globules observed here may help to explain some behaviors of double-stranded DNA molecules. For example, studies of the dielectric dispersion of double-stranded DNA solutions have revealed a responsive element much smaller than a complete DNA molecule (for review, see Mandel and Odijk, 1984). This element is possibly the globule observed here. Also, intracellular, metabolizing DNA molecules possibly have globules. The existence of intracellular globules is supported by the observation that intracellular excluded volume causes biochemically significant raising of the activity coefficient of DNA (for review, see Zimmerman and Minton, 1993). This raising of the activity coefficient mimics the raising of the θ temperature in a poor solvent. In theory, rising of the θ temperature has also been found to promote the formation of a (globule-containing) necklace (Dobrynin and Rubenstein, 1996).

The state of intracellular DNA has not yet been determined by direct analysis of intracellular DNA. However, one metabolically active "in vitro" system provides evidence of globules. DNA molecules in this system partition to form both a DNA-rich and a DNA-poor phase. The DNA-rich phase resembles a network of fibers that has globules attached (Sun et al., 1997). Most images of globules in Sun et al. (1997) are larger and less mobile than the images of globules observed here. Details of the relationship of these two types of globule are not yet known. Neither the fine structure nor the function of either globule is known. A possible "in vivo" function of globules is to promote action at a distance. Evidence for cooperative action at a distance is found for the DNA packaging of bacteriophage T7 (Sun et al., 1999). Evidence for cooperative action at a distance is also found for DNA-associated nucleotides that participate in the formation of both prokaryotic and eukaryotic transcription-initiation complexes (for review, see Rippe et al., 1995; Bulger and Groudine, 1999). The data presented here suggest that evolution of the control of gene expression occurred in the context of globule-containing DNA molecules. The simplest evolutionary pathways would, presumably, make use of such globules.

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