Real-time imaging of single DNA molecules with fluorescence microscopy

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ABSTRACT A fluorescence microscopy technique was used to image the dynamics of individual DNA molecules. Lambda, calf thymus, cosmid (circular), and T4 DNA were studied with the fluorescent dye acridine orange. Experiments with DNAase I were conducted, and the results indicate that these observations correspond to DNA molecules. The results of experiments with circular DNA provide strong evidence that these were single DNA molecules. Molecules were observed free in solution or attached to a glass or copper surface at one or several points. The Brownian motion of these molecules was observed, indicating that DNA in solution exists in a partially supercoiled state. Some molecules appeared stretched and were attached to the surface by their termini; the lengths of these molecules were measured. Such molecules also exhibited elastic behavior upon breaking. The power of this technique is demonstrated in images of cosmid DNA molecules, catenanes, and DNA extending from T4 phage particles. These results suggest immediate applications to molecular biology, such as examining the dynamics of protein-DNA interactions. Areas of ongoing research are discussed.

INTRODUCTION

DNA is a polymorphic molecule. Many of its accessible conformations involving secondary structure have been well characterized in solution and in the solid state (1, 2). In contrast, the tertiary and higher-order structures of the molecule are much less known. This is due in part to the lack of methods appropriate to detect and characterize the long-range dimensions of these structures.

Methods of visualization such as electron microscopy have been used to study various degrees of aggregation and coiling of the DNA molecule. However, this method yields only a static picture of the structures, provides no information about the mechanism and the dynamic process by which these structures are attained, and may produce substantial alteration of the structures during sample preparation.

Real-time visualization is the most appropriate way of obtaining direct information about the configurations of single DNA molecules in solution; no other method can provide a better appreciation for the flexibility and dynamics of DNA. Morikawa and Yanagida (3) have developed a technique which uses an optical microscope to visualize individual DNA molecules labeled with fluorescent ligands. Using this method they were able to record movements of single DNA molecules in solution (3–6). We have improved on this technique in various ways by altering the conditions of sample preparation and substrate to obtain a larger proportion of molecules in an extended (uncondensed) state. These improvements, and recent technological advances in ultra low-light level video cameras (7), have enabled us to obtain high quality video images. In this paper we present several lines of evidence using linear and circular DNA, which indicate that our observations correspond to single DNA molecules and not to aggregates or bundles. These molecules were observed to undergo several configurational transitions, and their behavior varied depending on whether they were free in solution or attached to a surface. The power of this technique is demonstrated in our observations of catenanes (circular DNA molecules linked in a chainlike fashion) in solution and loops of DNA emanating from bacteriophage T4.

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MATERIALS AND METHODS

Instrumentation

The microscope-camera system used to acquire these data consists of a Zeiss Universal microscope, with a 100X Neofluar objective, equipped for epifluorescence and coupled to a super-intensified video camera (Opelco, Herndon, VA). This system was developed, and is housed, in the Department of Chemistry at the University of New Mexico. All observations were recorded on video tape at standard video rates (30 frames per second).

Nucleic acids and preparation of samples

Lambda DNA, Hind III fragments of lambda DNA, cosmid 30-5-A DNA (8), calf thymus DNA, and bacteriophage T4 were used in these

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experiments. 1 μm is 2.941 kilobase pairs (kb). Lambda DNA is 50 kb (17 μm) long. The four largest Hind III fragments of lambda DNA are: fragment 1, 23.13 kb (7.86 μm); fragment 2, 9.416 kb (3.20 μm); fragment 3, 6.557 kb (3.23 μm); and fragment 4, 4.361 kb (1.48 μm). These data for Hind III fragments of lambda DNA were provided with the digest purchased from Bethesda Research Laboratories. The cosmid vector alone has a contour length of 7.9 kb (2.7 μm), and the total length with the *Neurospora crassa* insert is ~48 kb (16 μm) (8).

Solutions of 0.5–1.0 μg/ml DNA (in 10 mM Tris, pH 6.8; 1 mM EDTA, pH 8.0) or 10^9 particles/ml T4 phage, 2% β-mercaptoethanol (vol:vol), and 0.5–1.0 μg/ml acridine orange (AO) were prepared in a 1.0 mM Tris (pH 6.8)–0.1 mM EDTA (pH 8.0) buffer. These AO concentrations were at or near saturation for the DNA, corresponding to a r value (r = moles dye bound per moles phosphate) of ~0.25. Preparations of bacteriophage T4 were agitated vigorously to fracture the protein capsid and release the DNA. A small drop (5–7 μl) of the solution was placed on a clean glass slide, or a copper EM grid resting on a slide. A clean glass coverslip (24 × 30 mm) was gently placed over the drop, and the edges of the coverslip were sealed with fingernail polish. These samples were then examined under the fluorescence microscope. Test solutions were prepared as above but (*a*) without DNA and (*b*) with 0.5 μg/ml of DNAase I (in 50 mM Tris with 0.5 mM $CaCl_2$, pH 7.4) and incubated for 20 min at 37°C. DNAase I was added to solutions that had previously been examined and found to contain numerous fluorescent particles.

Observation of individual DNA molecules was confirmed by using a modification of the cytochrome *c* spreading procedure (Kleinschmidt technique) (9). This technique is used extensively in electron microscopy to surface spread individual DNA molecules on a protein monolayer. We modified this procedure by preparing the spreading solution (DNA, ammonium acetate, and cytochrome *c*, in ddH_2O) to include 0.5 μg/ml AO and 2% β-mercaptoethanol. A small drop (≈10 μl) of this solution was placed on a piece of parafilm, and an uncoated copper EM grid was then touched to the surface film of the drop so that a small volume (≈7 μl) of solution adhered to the copper. The grid was then transferred to a clean glass slide, covered with a coverslip, and sealed with fingernail polish.

Length measurements

The lengths of DNA molecules in samples prepared by the modified Kleinschmidt procedure were measured. Only the lengths of extended molecules can be measured unambiguously and with an adequate degree of precision in this technique. Molecules whose dimensions appear substantially smaller than their theoretical length can either be highly coiled, be fragments of molecules, or contain local coiling and undulations beyond the level of resolution of the optical microscope. The uncertainty in the determination of the lengths of these molecules under the optical microscope is too large to use them in any statistical analysis. To avoid this problem, we concentrated on the most elongate and extended molecules in the field of view to obtain a size distribution of the longest molecules instead of a complete size distribution for the entire population. We use this approach only to confirm that the dimensions of the longest molecules observed were, in all cases, smaller than or equal to the maximum extended length of the molecule. The lengths of molecules were measured directly from the video screen, and compared with the known lengths of these DNAs. Images on the video monitor were magnified 7,700 times. This was determined by measuring the images of polystyrene spheres of known dimensions. The video camera and monitor did not introduce any noticeable horizontal or vertical distortion of the image.

Imaging

The signal-to-noise ratio was optimized by reducing the background fluorescence in two ways. First, highly polished metal surfaces such as copper were often used because, unlike glass, they display little or no autofluorescence. Second, the amount of fluorescence contributed by the free dye in solution could be reduced by decreasing the sample volume between the surface and coverslip. This combination of factors drastically improved the quality of the video images, which required little or no image processing.

The relative dimensions of the molecules observed under these conditions can be substantially altered in this type of "emission imaging." The spatial resolution of the optical microscope is diffraction limited. This corresponds to a pixel size in the image of ~0.25 μm. The dimensions of any luminous object will, therefore, appear larger than the original object by about this value. In the case of a DNA molecule, which can be quite long (several microns) but is very thin (~2.0 nm or 20×10^{-4} μm), this effect will lead to a substantial magnification anisotropy. Thus, for images produced by the microscope, the length is increased by the product of the microscope's optics (125 times in this case); but the width, being smaller than the size of a pixel (0.25 μm), is magnified by a factor of $(0.25 \times 10^4/20) \times 125$, i.e., >15,000 times. Therefore, the images of the DNA molecules experience a length/width deformation of ~125-fold. This "apparent magnification" of the width makes it possible to visualize individual molecules of DNA with this technique. Images on the video monitor were magnified an additional 61.6 times.

RESULTS AND DISCUSSION

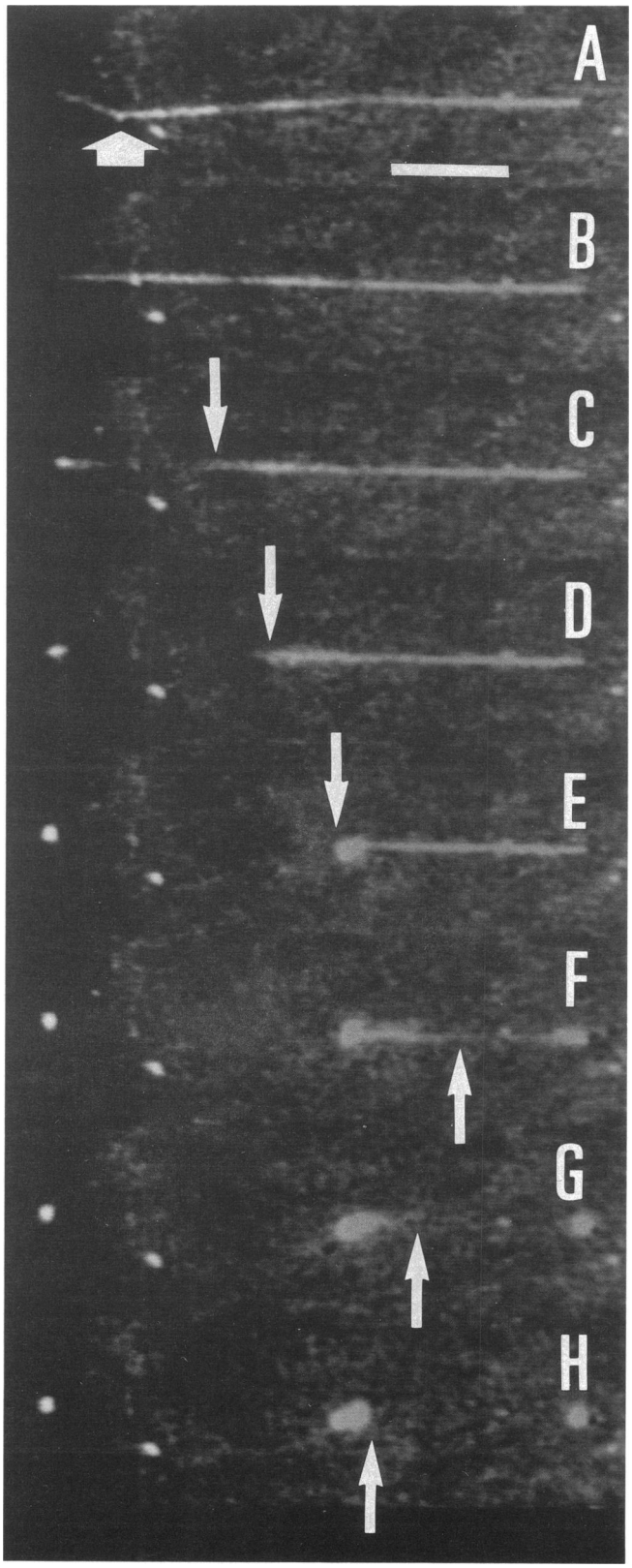
Evidence for DNA

Few fluorescent particles were present in solutions prepared without DNA or in solutions of DNA that had been incubated for 20 min with DNAase I in the presence of $CaCl_2$, and no moving particles were seen in these solutions. Furthermore, the density of fluorescent particles was positively correlated with the concentration of DNA in the sample preparation, as would be expected if the observations corresponded to DNA molecules.

Observations of linear DNA

Most of the molecules in samples prepared following the modified Kleinschmidt procedure appeared fully extended and were attached to the surface in several places. This extension and adhesion is due largely to solution flow in the sample and surface spreading with cytochrome *c*. However, extended molecules were often seen aligned in various directions within a given field, unlike that expected from a simple flow motion. Furthermore, the number of extended molecules increased greatly over a period of 24–48 h after preparation of the slide, suggesting a slow process of elongation for some molecules. The mechanism of this slow process of extension likely involves a specific interaction of the molecule with the surface (10) to overcome the unfavorable reduction in entropy associated with the extended state.

The lengths of the longest and most extended molecules of whole lambda DNA (Fig. 1) and Hind III fragments of lambda DNA, prepared following the modified Kleinschmidt procedure, were measured. These values ranged



from 20.13 to 27.01 μm (mean, 22.46; SD, 1.99; *n*, 11) for whole lambda DNA, and are consistent with the actual length of lambda DNA after allowing for a length increase due to intercalation (of AO) of 25–30% (11, 12). A wide range of sizes was present in the preparation of Hind III fragments. The lengths of 90 of the longest molecules were measured. The longest of these molecules was 9.7 μm, consistent with the length of the largest fragment (fragment 1) after allowing for AO intercalation. The other molecules varied in length from 1.5–7.4 μm; this population probably includes Hind III fragments 1, 2, 3, 4, and broken pieces of the larger fragments. Many shorter fragments were also present, probably due to breakage induced during purification of the DNA or preparation of the samples.

The molecules of DNA (lambda, cosmid, T4, and calf thymus) we observed free in solution were very active. The Brownian motion of a molecule of lambda DNA is represented in Fig. 2. Many tertiary configurations, from an apparently extended filament (Fig. 2, *A* and *B*) to a compact, random coil (Fig. 2, *E* and *H*), were adopted during a 1-s time period. The apparent length of this molecule varied ~200% during this time period; the maximum length observed for this molecule was ~6 μm (Fig. 2 *A*), whereas its apparent length in Fig. 2 *H* is ~2 μm. If this is a whole molecule of lambda DNA it is <30% (when allowing for AO intercalation) of the fully extended length of lambda DNA, suggesting that DNA exists in a partially supercoiled state in solution.

Some of the apparent variation in length may be due to portions of the molecule drifting out of the plane of focus. However, this contribution is probably insignificant, because in most observations the apparent changes in length were accompanied by corresponding changes in fluorescence intensity. This variation in fluorescence provides a reliable means of identifying partially supercoiled or compact regions of the molecules. Due to the limitations of light microscopy, the DNA filament cannot be resolved when in a compact, random coil configuration (Fig. 2 *H*), but rather appears as a bright blob. The intensity of the fluorescence of the molecule in this configuration (Fig. 2 *H*) appears greater than that in the more extended configuration (Fig. 2 *A*). This effect is due to the apparent increase in the local concentration of AO. All of the AO molecules in the focal plane contribute to

FIGURE 1 An extended molecule of lambda DNA ~22 μm long and attached to a copper surface in four places—the two termini and two internal points. One of these internal attachments is indicated by the wide arrow in *A*. Subsequently the molecule disattaches from this point (*B*). (*B–D*) A series of consecutive video frames (33 ms apart) ~1 s after *A*. (*E–H*) Another series of consecutive video frames ~1 s after *D*. Arrows above or below the molecule indicate the ends of the collapsing region of the left or right portions of the molecule, respectively. See text for details. These video images were unprocessed. Scale bar, 5 μm.

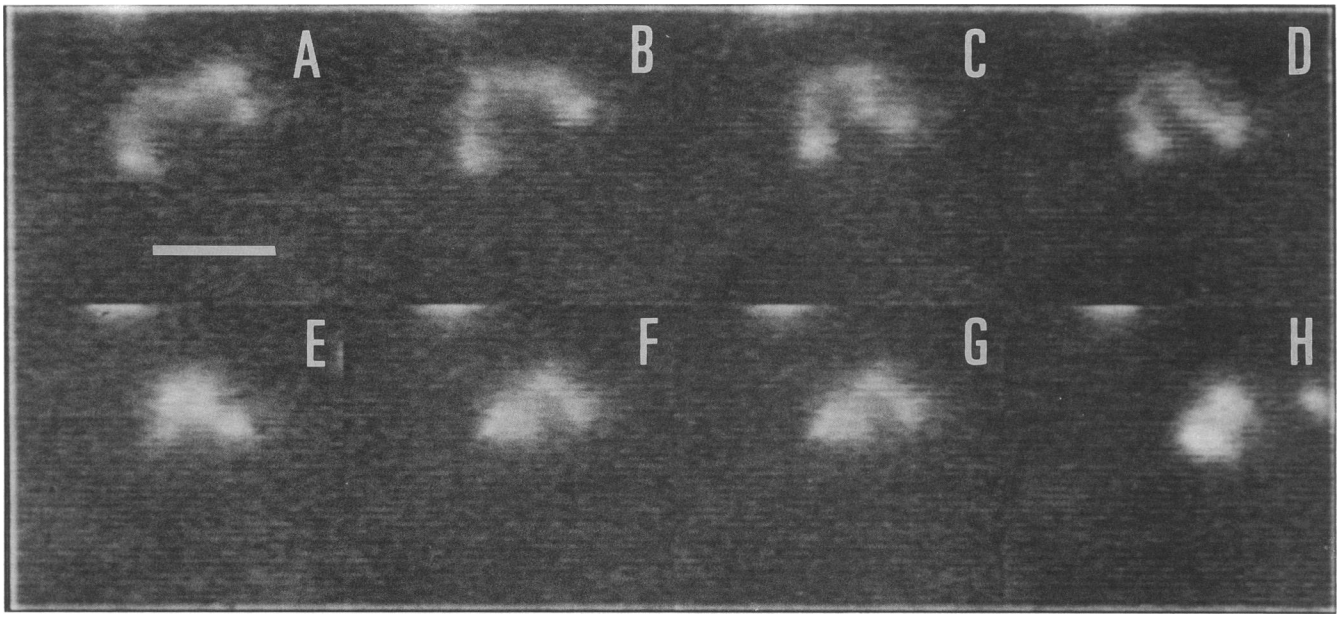


FIGURE 2 A molecule of lambda DNA free in solution. Elapsed time is 1.0 s. Frames are not at a constant time interval but were chosen to demonstrate various tertiary configurations adopted by the molecule in a short time. Note the extended appearance in *A–D*, and the more compact, random coil configuration in *E* and *H*. See text for additional discussion. These video images were treated with a sharpening algorithm. Scale bar, 3 μm.

the detected fluorescence, and the two-dimensional area of the molecule in Fig. 2 *H* is less than that in Fig. 2 *A*. This indicates an increase in the concentration of AO contributing to the detected fluorescence, assuming that the number of AO molecules along this DNA molecule has not changed during the 1 s elapsed from Fig. 2, *A–H*. Due to the analogue nature of the equipment and several variables (such as lamp intensity and camera adjustments), this analysis is valid only in a relative sense, i.e., comparing portions of any given molecule, or molecules within any given field. This effect is best illustrated by the appearance of the supercoils in the molecule in Fig. 3.

Breaking of DNA

Some of the molecules broke during observation. Upon breaking, the fully extended DNA molecules displayed a distinct elastic behavior with the free end collapsing rapidly onto the points of attachment (Figs. 1 and 4). The rates of collapse of the left and right portions of the molecule in Fig. 1 were ~124 and 144 μm per second, respectively. This collapsed portion attached to the surface and appeared irreversibly condensed; it was never observed to fluctuate among the tertiary configurations described above. Similar behavior was observed in all other extended molecules that broke, including T4, calf thymus, and cosmid DNA (Fig. 4). A similar behavior was reported by other investigators, who used DAPI (4′, 6-diamidino-2-phenylindole) as the fluorochrome (6), although these authors report much slower rates of collapse (11–18 μm/s). The breaking and collapsing we observed is consistent with the assumption that these were single molecules. If these fibers were several molecules thick, this observation would imply that all the molecules in the fiber broke simultaneously, and in the same place. Although this scenario is not impossible, it seems highly improbable.

Some molecules attached at their termini were not completely extended, but swayed loosely between the points of attachment. Upon breaking, these molecules exhibited no elasticity, but rather fluctuated between several tertiary configurations similar to molecules free in solution (Fig. 2).

The breaking of the molecules appears to be induced during observation. Photosensitization of the DNA-AO complex with blue light will nick DNA (13), although β-mercaptoethanol drastically reduces the frequency of this reaction (unpublished results). During observation, the molecule in Fig. 1 releases from one of the internal attachment sites (wide arrow in Fig. 1 *A*) due, perhaps, to an increase in tension along the molecule or Brownian motion of the solution. Shortly thereafter a break occurs (Fig. 1 *C*). The break is most likely initiated by a nick in the molecule, induced by photosensitization. The weakened DNA molecule breaks in the region of the nick, probably due to the increased tension experienced by the

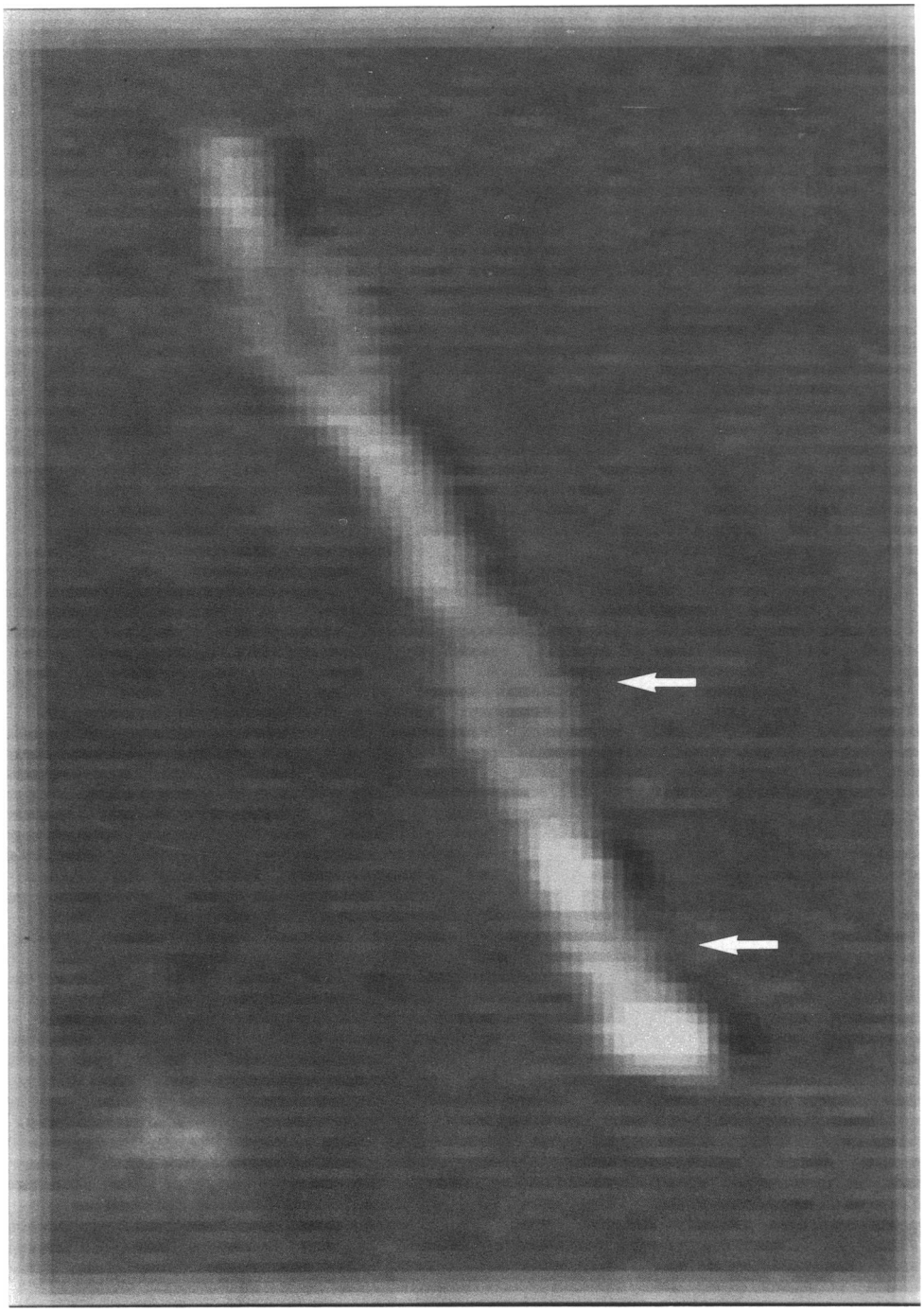


FIGURE 3 A partially supercoiled molecule of cosmid DNA. The contour length is ~18 μm. Note the two open regions, and the twisted regions at the top, center, and bottom of the molecule. There is increased fluorescence associated with the supercoiled regions. The regions of the molecule indicated by an arrow flickered rapidly between open and closed states. This video image was improved by background subtraction.

single DNA strand in the nicked region. The rates of collapse we measured were ~10 times faster than the rates (11–18 μm/s) reported by Yanagida et al. (6). The contraction of the molecules could involve different mechanisms of collapse and may depend strongly on the nature of binding of the fluorescent dye (DAPI in the case of reference 6), thus accounting for this discrepancy.

The contraction of the molecule upon breaking resembles the case of a stretched spring immersed in a viscous medium. The spring is released from one end and allowed

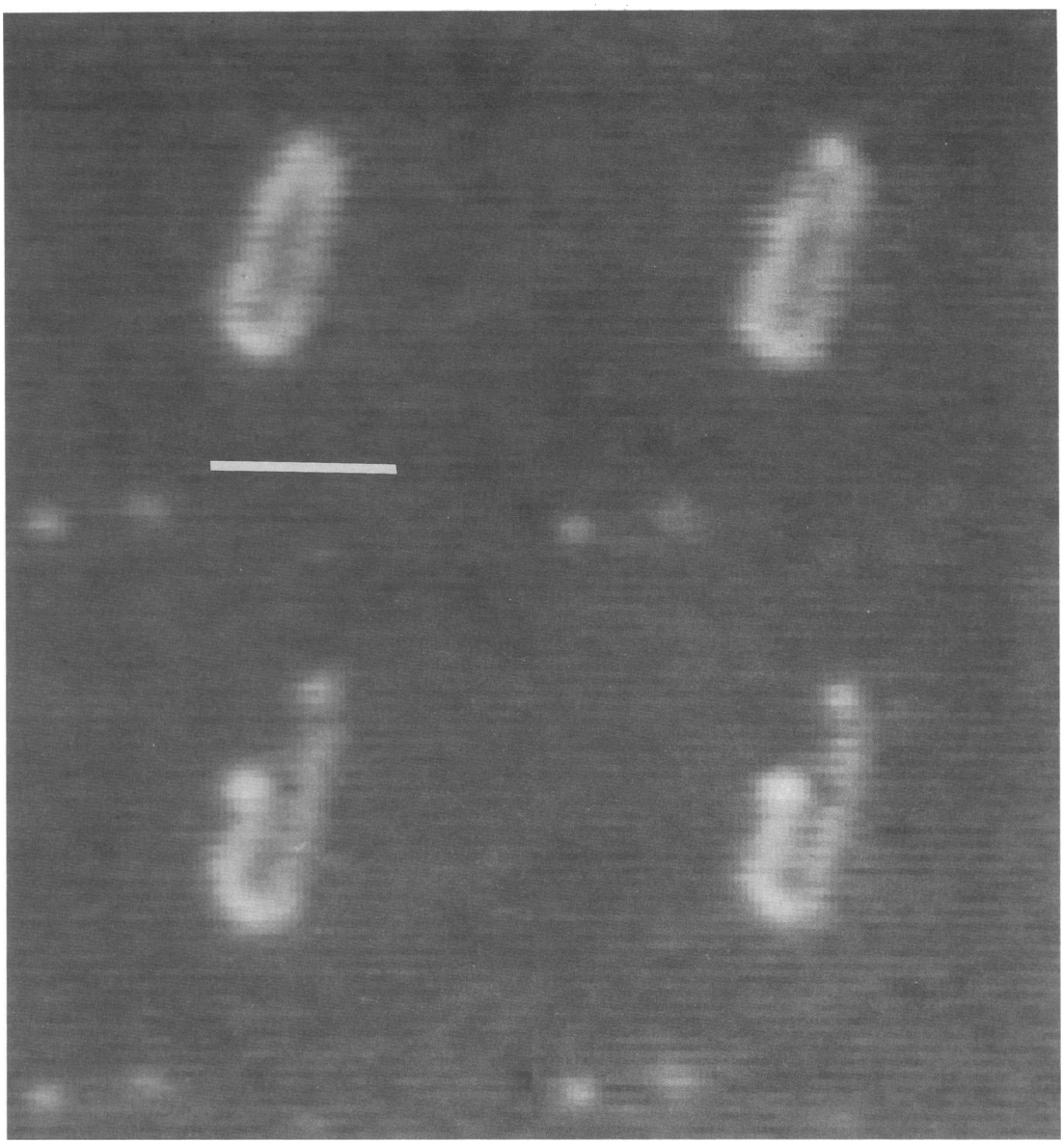


FIGURE 4 An apparently relaxed molecule of cosmid DNA. The approximate contour length is 5.5 μm. The first frame (*upper left*) is the appearance of the molecule when observations began. The second and third frames are consecutive video frames ~0.5 s after the first frame. The fourth frame is ~0.5 s after the third frame. See text for details. These video images were unprocessed. Scale bar, 2 μm.

to contract. The dynamics of the contraction is determined by the viscoelastic properties of the system (14). The Voigt-Kelvin model (15) is appropriate for the case of a single molecule surrounded by the solvent, as in the observations presented here. This model is comprised of an elastic spring coupled in parallel with a dashpot and can satisfactorily predict the dynamic behavior of the molecular relaxation upon breaking. An exponential time dependence of the molecular elongation then results if a constant frictional coefficient is assumed:

$$y = y_0 e^{-t/\tau_r},$$

where τ_r is the relaxation time of the molecule and is given by

$$\tau_r = f/E,$$

where f and E are the frictional coefficient and Young's Modulus of the molecule, respectively. When the data corresponding to Fig. 1 are treated according to this model, a value of $\tau_r = 0.080$ s is obtained. If the value of f is known or can be independently estimated, a value for the Young's Modulus of the DNA molecule can be obtained.

Observations of circular DNA

Many circular cosmid molecules were observed. The partially supercoiled cosmid in Fig. 3 has a contour length of ~18 μm, consistent with the actual length of this DNA molecule. The supercoils are easily identified as regions of increased fluorescence. The regions of this molecule indicated by an arrow fluctuated between an open and closed state. Other cosmid molecules had shorter contour lengths. The cosmid DNA molecule in Fig. 4 has a contour length of ~5.5 μm. This size discrepancy is probably due to a large deletion within the 40 kb insert, a common occurrence among cosmid preparations. The smooth contour of the molecule in Fig. 4 and the lack of variation in fluorescence intensity (indicative of folded or supercoiled regions) strongly suggests that these are not composed of molecules wrapped around each other in bundles, but made of a single double-stranded DNA molecule. Some of these cosmid molecules with shorter contour lengths displayed greater fluorescence intensity (Fig. 5) and as explained before it is not clear whether they were smaller molecules or larger, supercoiled circles.

On two occasions catenanes were observed; one of these is shown in Fig. 6. At various times during the motions of this catenane the circular appearance of one or the other molecule was easily identified. Upon breaking, a short, linear segment (representing the broken molecule) was

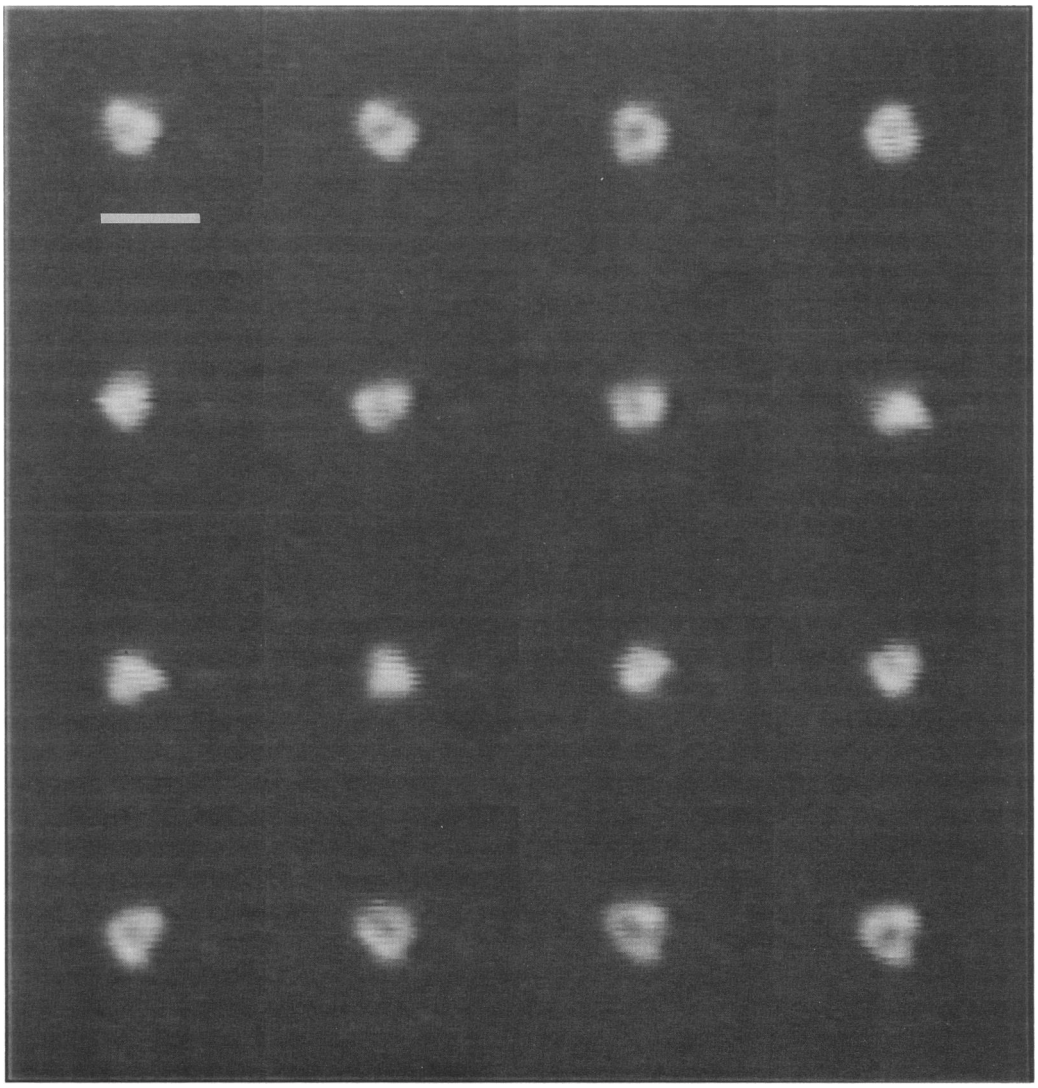


FIGURE 5 16 consecutive video frames (elapsed time, 0.5 s) of a small cosmid DNA molecule partially attached to the surface. Note how rapidly the molecule shifts between the open and closed states. See text for additional discussion. These video images were treated with a sharpening algorithm. Scale bar, 2 μm.

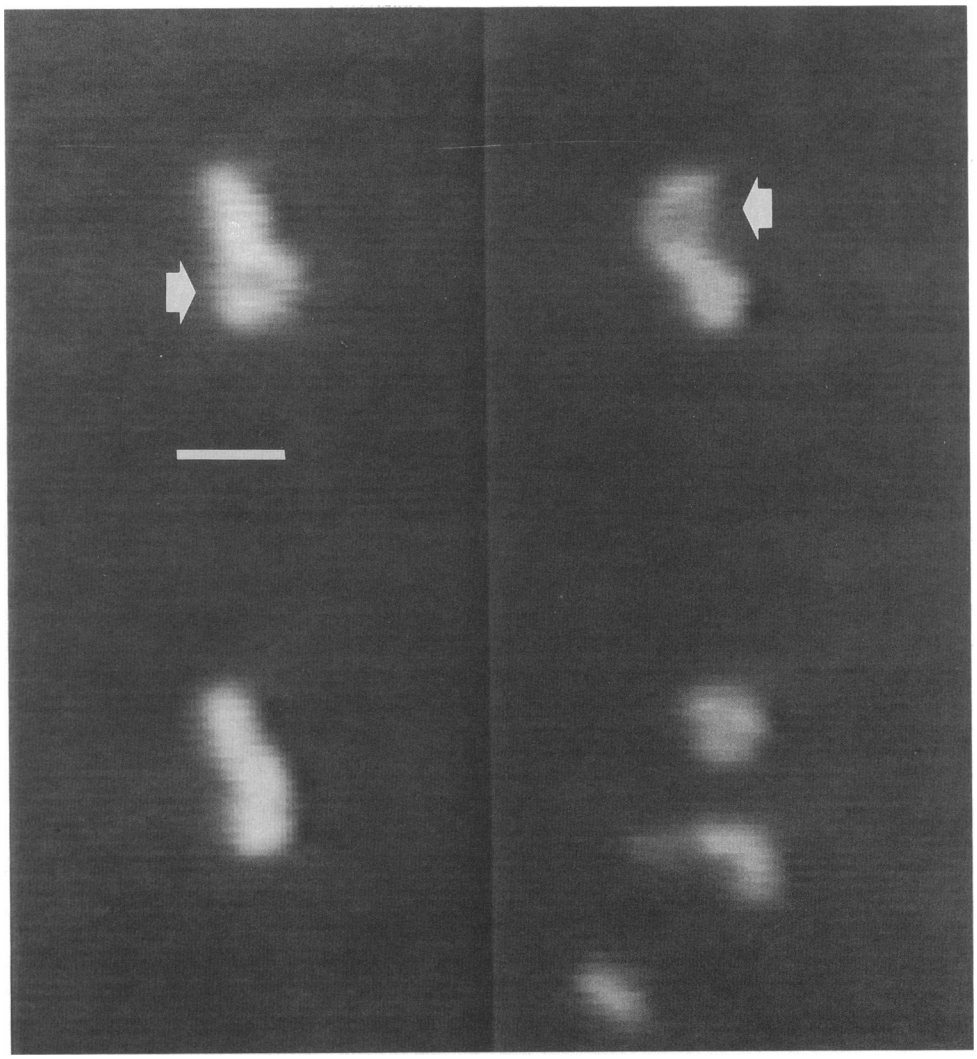


FIGURE 6 A catenane of cosmid DNA. These frames are in chronological order and were chosen merely to demonstrate several configurations adopted by these molecules. The open appearance of each molecule is indicated by the arrow in the first or second frames. A more common configuration is shown in the third frame. The fourth frame is the appearance of the two molecules after one had broken; the circular or linear appearance of these molecules is clearly evident. See text for additional comments. These video images were unprocessed. Scale bar, 1 μm.

readily distinguished from the other molecule, which maintained a circular shape for a short while before it broke too. This observation also suggests that each link in the catenane is comprised of a single DNA molecule.

Observations of T4 DNA

DNA from T4 phage particles attached to a copper surface was observed emanating from the capsid. A large loop (contour length ~6.5 μm) of DNA can be seen stemming from the bacteriophage particle, which appears as a bright spot (Fig. 7). This loop was observed to fold, collapse, rotate, and extend in a few seconds time (Fig. 7). Eventually the loop broke and no noticeable change occurred in the dynamics of the molecule, unlike fully extended molecules which collapsed rapidly. Linear filaments of DNA also were observed extending from phage particles. It is possible that these observations correspond to extrusion of DNA from the capsid, although additional experiments are necessary to test this.

CONCLUSIONS

The use of low-light level video techniques will make possible a number of new studies of DNA structure and dynamics. Several projects have already begun in our laboratory. We are now studying the chemistry of single

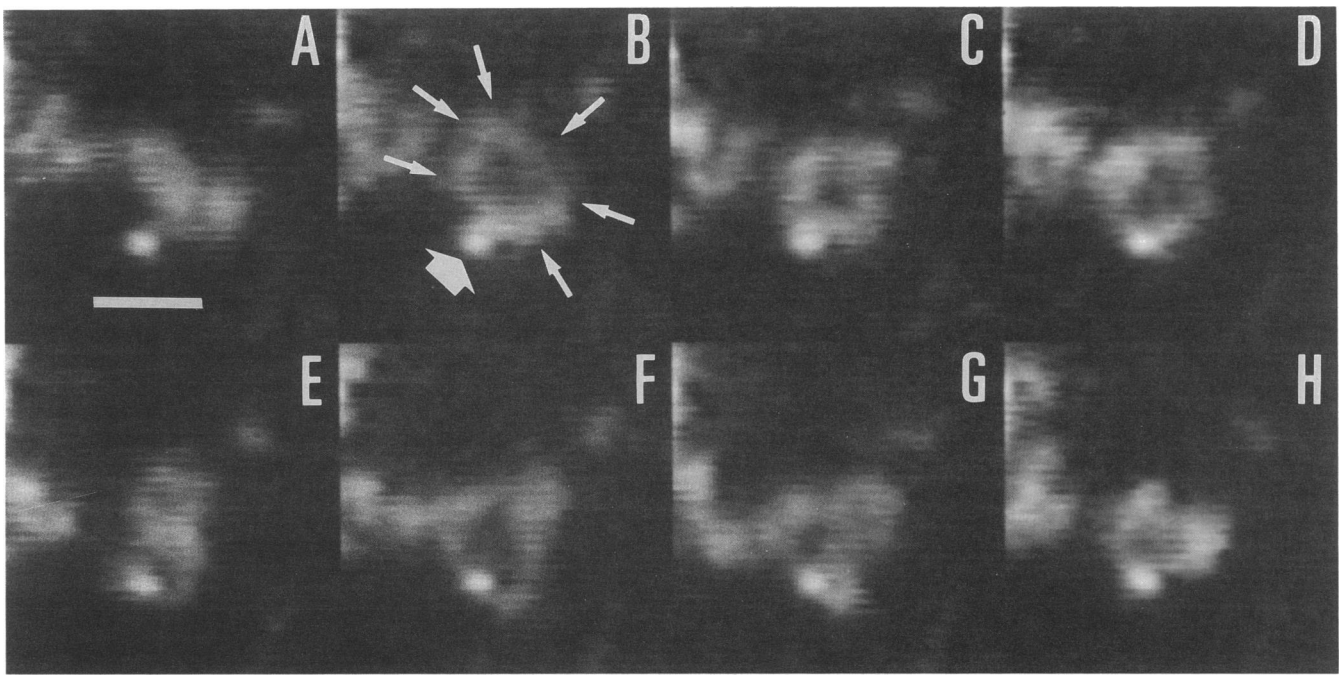


FIGURE 7 A loop of DNA emerging from a T-4 phage particle. Frames are at 0.25-s intervals; elapsed time is 2.0 s. The loop is outlined by the narrow arrows in *B*, and the wide arrow indicates the bright phage particle. Note the compacted (*A*, *H*), relaxed (*B–D*, *F–G*), and twisted (*E*) configurations. These video images were unprocessed. Scale bar, 2 μm.

DNA molecules by using a micro–reaction chamber, allowing us to observe the mode of action of various enzymes which catalyze biologically significant reactions. We can extract information about the primary and secondary structure of DNA, beyond the resolution of fluorescence microscopy, by using DNA probes specific for certain conformations (16, 17). We have already followed the electrophoretic motions of single DNA molecules in agarose microgels and will soon be able to test the various models of the electrophoretic mobility of DNA (18, 19). In addition, this technology provides the framework necessary to initiate spectroscopic studies of single DNA molecules.

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