Fluctuations in the velocity of individual DNA Molecules during agarose gel electrophoresis

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ABSTRACT The velocity of the center of mass of individual T4 DNA molecules during agarose gel electrophoresis, computed from digitized video-microscopic images, fluctuated between 0 and 4.5 μm/s after a field E = 5 V/cm was applied; the amplitude of the velocity peaks was twice the averaged steady-state velocity. The velocity fluctuations correlated with changes in molecular configuration. The mean velocity (10 molecules) showed a sharp rise in less than 0.2 s, followed by a shallow minimum and a broad peak, before reaching a plateau. The much smaller amplitude of these oscillatory features demonstrated that the velocity fluctuations of individual molecules were largely, but not entirely, uncorrelated with the onset of the field. The components of the shape tensor **S** of individual chains, which are a measure of the extension of the chains, were also determined for each image sequence. Only the principal component in the direction of E, $\mathbf{S}_{xx}$, increased.

INTRODUCTION

A most remarkable aspect of the movement of DNA through the pores of agarose gels during electrophoresis is that individual DNA chains oscillate between compact and highly extended conformations. These conformational fluctuations are a prominent feature of several computer simulations of gel electrophoresis (1–8); they have also been directly observed by video microscopy (9–13). The simulations and microscopy observations also suggest that, because of these conformational fluctuations, individual chains advance not with a steady velocity but instead by a series of starts and stops. One reason for such starts and stops is that both ends of a chain often advance simultaneously upon application of the electric field, in contrast to early theoretical models. As a consequence, the chains can become arrested in "U" conformations due to competition between electrical forces on the two arms of the U and the restraining force of the gel fiber(s) inside the base of the U.

Here, we build upon this earlier work by digitizing and analyzing image sequences quantitatively. A study by Rampino, which appeared since the completion of this work, also uses this approach (13). Our long term goal is to make quantitative comparisons to predictions of the computer models and also to make connections between the motions of individual chains and the average behavior of a large number of chains, such as make up a band during electrophoresis. Two measures of this average behavior, instantaneous velocity and alignment, show oscillations when a field is first applied or immediately after field inversion (14–17). The existence of such oscillations shows that the conformational fluctuations of individual molecules can have macroscopic effects if the pulse protocol synchronizes the motions of individual molecules to some extent. Computer simulations have recently had considerable success in duplicating these features (6, 7, 18, 19).

In this study, the velocity and changes in shape of individual T4 DNA molecules were measured in the first 14 s after a field was first applied. T4 DNA, which contains 170 kb, was selected because it had a unique length and was long enough to show features characteristic of pulsed field gel electrophoresis, such as a deep mobility minimum at specific pulse durations (20, 21). Larger chains would be difficult to bring into focus throughout their length and would have to be observed over greater distances to characterize their fluctuations. They would also be degraded more easily by shear during sample preparation.

MATERIALS AND METHODS

Our procedures for tagging and visualizing single DNA molecules in the microscope are based largely on the work of Yanagida (22, 23) and of Smith (9).

Buffer

The buffer contained 45 mM Tris base, 45 mM boric acid, and 1.25 mM EDTA, pH 8.2–8.3 (0.5 × TBE).

T4 DNA preparation

Intact T4 phage (Carolina Biological Supply, Burlington, NC) was diluted with buffer to a DNA concentration of 0.5 μg/ml, heated to 60°C for 15 min to release the DNA, then quench-cooled to 0°C. An equal volume of phenol:chloroform (1:1, vol:vol) was added, stirred overnight at 1 rpm, then centrifuged for 3 min. The upper layer, containing the DNA, was removed. T4 DNA is normally glucosylated and contains 170 kb.

DNA in agarose

Gels were prepared from agarose with endosmosis = 0.10–0.15, gel point 36°C (Sea-Kem LE grade; FMC, Rockland, ME). A solution of agarose, ethidium bromide (EB), β-mercaptoethanol (β-ME), and buffer was heated to boiling, filtered through a 0.6 μm Nuclepore filter (Pleasanton, CA), then cooled to 50°C. 20 μl of T4 DNA solution was added to 180 μl of the liquid EB/agarose/β-ME mixture to give a solution containing 1.0% agarose, 1.0 μg/ml EB, 2.0% β-ME, and ~0.05 μg/ml DNA.

Electrophoresis

A miniature electrophoresis chamber, with liquid buffer wells at either end and a thin agarose gel of known thickness between a cover slip and

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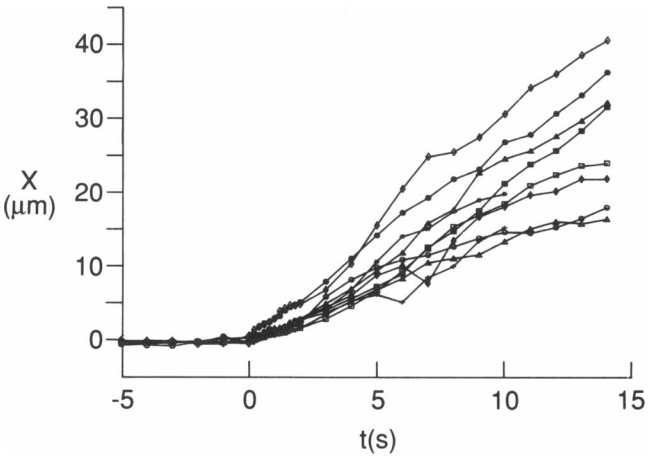

FIGURE 1 Displacement of 10 T4 DNA molecules through a 1% agarose gel during electrophoresis in 0.5 × TBE. An electric field of 5 V/cm was applied at $t = 0$.

a microscope slide, was assembled as follows. Two 12.7 μm Mylar film spacers were set near the edges of a 25 × 75 mm microscope slide preheated to 50°C. After 15 μl of the DNA/agarose solution had been placed on the slide, an 18 × 18 mm coverslip was set on top of the T4/agarose solution and clamped while the agarose cooled and gelled.

The slide was then placed into a plastic chamber with 1 ml buffer wells on opposite sides, each with a platinum electrode and an agarose bridge connecting the buffer with the agarose layer beneath the coverslip. A small electronic circuit was constructed to synchronize (±0.017 s) the beginning of the electrophoretic pulse and the recording of image sequences. The field experienced by the molecules, measured from the voltage drop across the coverslip, was 5.0 ± 0.2 V/cm.

Microscopy and image acquisition

Images were acquired using a Zeiss Axiophot microscope (Thornwood, NY) with a 100×/NA1.4 oil-immersion objective, epifluorescence illumination, Videoscope (Washington, DC) image intensifier, and Hamamatsu video camera (Bridgewater, NJ) with Newvicon tube. The slide was scanned until a suitably isolated molecule of sufficient brightness was found approximately midway between the coverslip and the slide; molecules close to the coverslip or slide behave anomalously (9). Often there were three or four suitable molecules in a given field. Five images taken 1 s apart were recorded (Panasonic TQ-2028F; Secaucus, NJ) in the absence of a field. The field was then turned on. Simultaneously, continuous storage of images at five frames/second was begun and continued for ~15 s. At longer times the molecules were no longer in focus.

Image analysis

The analog image data were converted to 512 × 480 × 8-bit digital arrays by a Matrox PIP board (Dorval, Quebec). The average pixel intensities for the DNA where segment densities were high, for example, at the ends of the molecule, had gray values of ~85 Gray Scale Units (GSU) above background; full scale = 255 GSU. In regions where the segment density was low, for example, where the DNA chain was stretched into a U shape, the brightness was only 5–8 GSU above the background.

Background noise was dealt with in the following manner. First, a "paint" function in the Image 1/AT software package (Universal Imaging, West Chester, PA) was used to define the fraction of the frame which contained the molecule. The background immediately surrounding the molecule was removed digitally by subtracting a precalculated baseline, which allowed a minimum of the molecule to be removed.

The center-of-mass coordinates X_k and Y_k of a DNA molecule at time t_k were calculated from the gray level I_{ij} at each pixel (i, j) in image k:

$$X_k = \Sigma_i \Sigma_j x_i I_{ij}/I$$
$$Y_k = \Sigma_i \Sigma_j y_j I_{ij}/I.$$

Here x_j and y_j are coordinates of pixel (i, j), and I is the sum of all the intensities in image k. A series of values of X_k and Y_k were obtained from the sequence of images for a given DNA chain. The transient velocity components $v_x(t)$ and $v_y(t)$ of individual molecules were calculated by differentiating X and Y with respect to t. Three-dimensional plots of image intensity were prepared with AXUM software.

Components of the shape tensor S in a given image k were calculated in the usual way (24):

$$\mathbf{S}_{xx} = \Sigma_i \Sigma_j (x_i - X_k)^2 I_{ij}/I$$
$$\mathbf{S}_{yy} = \Sigma_i \Sigma_j (y_i - Y_k)^2 I_{ij}/I$$
$$\mathbf{S}_{xy} = \Sigma_i \Sigma_j (x_i - X_k)(y_j - Y_k) I_{ij}/I.$$

RESULTS

About 80 image sequences were recorded from ~20 gels. Ten image sequences were chosen for further analysis on the basis of their fluorescent intensity and the length of time the DNA remained in focus. Occasional molecules which were shorter than the average, presumably because they had been sheared, were rejected.

The displacement of the center of mass of individual molecules increased promptly and fairly steadily after the field was applied (as shown in Fig. 1) but the various chains did not follow the same curve during the 14 s observation time. Obviously, the slopes of these curves must converge for longer times, since DNA bands have widths of perhaps 0.5 mm after several hours of electrophoresis.

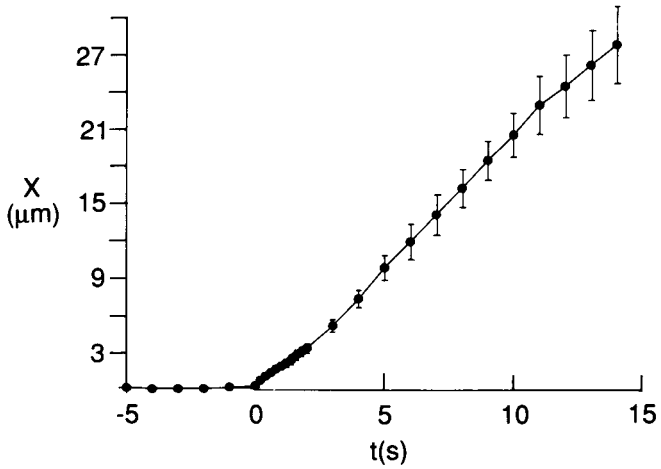

FIGURE 2 Average displacement as a function of time for T4 DNA molecules, computed from Fig. 1. Error bars correspond to ±1 SD calculated for the set of 10 molecules.

The average displacement of the 10 molecules was a much smoother function of t, but was clearly not a straight line, as shown in Fig. 2. The average velocity for the 10 molecules, at various times after E was turned on, was computed by differentiating the data in Fig. 2. Results are shown in Fig. 3. It can be seen that only 0.2 s after the field was turned on, T4 DNA was moving at 2 μm/s. Inspection of the raw images suggested that this rapid increase in velocity was not accompanied by a change in the conformation of the molecule; such changes occurred later. After the initial peak velocity at 0.2 s, there was a slight decrease in velocity between 1 and 2 s, an increase to a plateau between 5 and 10 s, then a gradual decrease, of greater uncertainty, for $t > 10$ s. Although the standard deviation of these results was large, the features of the average velocity are in agreement with precise measurements of the dynamic velocity of a band of T4 DNA, which contains $\sim 10^8$ DNA molecules (Keiner and Holzwarth, unpublished data).

It is useful to compare the fluctuations in the velocity of individual molecules to changes in their configurations; both computer simulations and earlier video microscopic work suggest that velocity minima should correlate with the formation of U-shaped conformations. Our results confirm this, as shown for three T4 molecules in Fig. 4. In Fig. 4 *a* are shown the velocities of the three molecules, with seven specific times marked (*A–G*); Fig. 4 *b* shows the images of the three molecules at the same seven times. Molecule 1 started out with a rather erratic velocity (Fig. 4 *a*) which built to a peak at time *C*, then decreased steadily until reaching zero at *F*. The images for molecule 1 (Fig. 4 *b*) showed that during the increases in v, both ends of the molecule advanced steadily, but part of the molecule remained behind, trapped in the base of the U. This was a common observation just after E was applied. The height of the U, measured from the base to the mean position of the two tips, increased from 7 to 13 μm between images *C* and *E*. At *F* the velocity was zero while the left-hand arm of the U actually moved in the direction opposite to the electric force; the maximum contour length of the U was about 40 μm, or 70% of the contour length of T4 DNA, which is 60 μm. Finally, at *G*, after "resolving" the first U, the

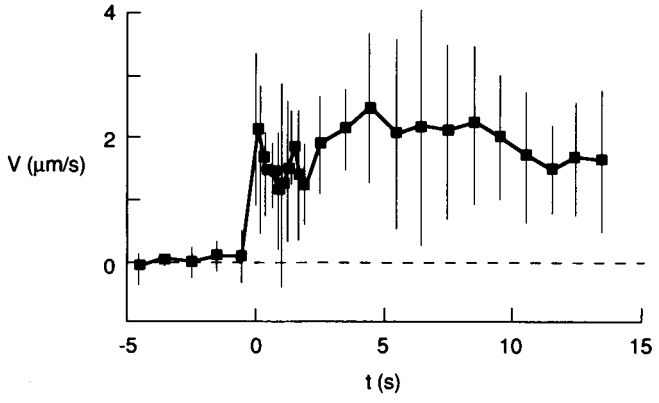

FIGURE 3 Average velocity of T4 DNA after the field was applied. Error bars indicate ±1 SD.

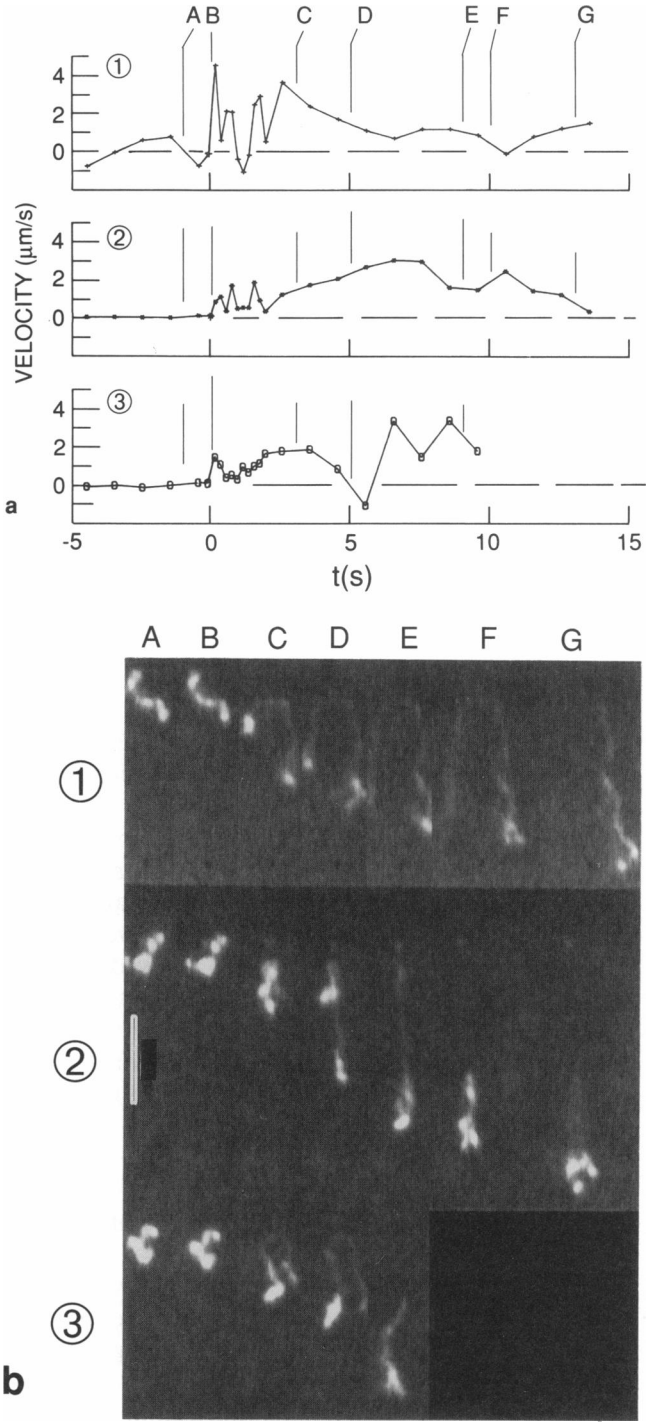

FIGURE 4 Relationship between velocity and configuration of individual T4 DNA chains. (*a*) Velocities of three molecules labeled 1, 2, and 3 after a field is applied. Times designated *A–G* correspond to images in Fig. 4 *b*. (*b*) Video micrographs of molecules 1–3 at the times labeled *A–G* in Fig. 4 *a* above. The field went on at image *B*. The bar is 10 μm long.

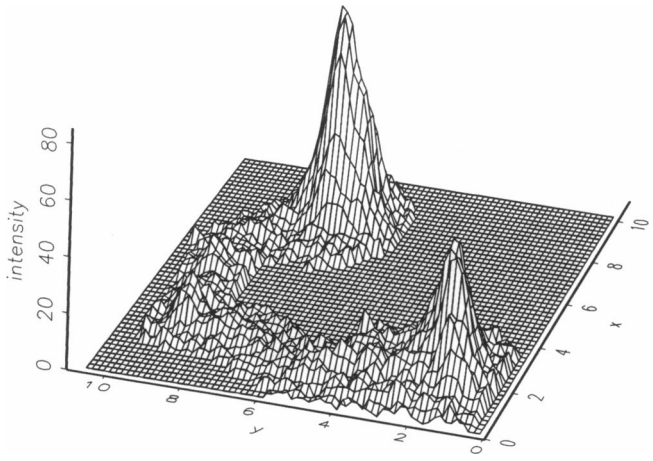

FIGURE 5 Three-dimensional plot of the intensity of a T4 molecule 3 s after the field was turned on. The electric force was in the $-y$ direction. The regions outside the domain of the molecule have been set to zero for clarity. The image used is the same as that in Fig. 4 *b*, molecule 1, time *C*, but with the *x*-axis reversed for clarity. The *x* and *y* scales are in μm; the intensity is in GSU.

molecule again showed a positive velocity as both ends moved off independently, initiating the formation of a new U.

In contrast to molecule 1, molecule 2 showed a high velocity at time *E*; this was consistent with its configuration, with a blob at one end only and no U, an earlier U having been resolved. Later, at time *G*, its velocity was low, and it was in a U. Molecule 3 showed its lowest velocity at *D*; this coincided with a U configuration. Once released from the U at time *E*, the velocity increased. Negative velocities were occasionally computed. These probably represent small errors in our technique for background subtraction.

The data show that individual molecules exhibited large fluctuations in velocity, ranging from ~2 times the average velocity to zero or less. Similar fluctuation amplitudes were reported recently by Rampino (13). Moreover, the motions of the different chains were largely uncorrelated with one another and with the onset of the field. This fact was also noted when viewing the molecules through the microscope.

It was frequently observed that the leading ends of the DNA chain were substantially brighter than the trailing ends of the chain or the base of the U. A quantitative sense of this observation can be obtained from a three-dimensional plot of the intensities, as shown in Fig. 5, for molecule 1 at time C. The two large peaks of DNA at either end of this molecule had peak heights of 82 and 45 GSU, whereas the connector between the two ends had a height of only 5–8 GSU. The integrated intensity of the DNA in this figure was ~15,000 GSU · (pixel)^2, whereas the integrated intensity of the bridge was only 4,500 GSU · (pixel)^2. As the two ends of this molecule continued to advance, the bridge became longer but remained roughly constant in cross-section. Meanwhile, the trailing peak became progressively less intense, until its amplitude equalled that of the connector (molecule 1, time *E*, Fig. 4 *b*).

An additional measure of the shape of the chains was obtained by computing components of the shape tensor S from the digitized images. S_{xx} measures the extension of the molecule in the field direction, S_{yy} measures the extension in the direction perpendicular to *E* but in the plane of the coverslip, and S_{xy} is the cross term. Surprisingly, S_{xx} first decreased for ~1 s, then increased steadily (Fig. 6). Even after 14 s, the molecules were, on the average, still becoming more elongated along the *x* direction. S_{yy} and S_{xy} were almost unchanged by the field; like S_{xx}, both decreased slightly immediately after *E* was turned on. Note that the root-mean-square diameter of the molecules in the *y* direction, $2\sqrt{S_{yy}}$, was less than 5 μm, which was substantially less than the gel thickness, 12.7 μm. Since the diameter in the *z* direction is expected to be the same as that in the *y* direction, on symmetry grounds, the motions of DNA reported here are probably very similar to those which occur in ordinary gels of 4–10 mm thickness.

DISCUSSION

This study showed that the velocity of T4 DNA fluctuated with a period of 5–15 s after a 5 V/cm field was applied. The distance moved by the center of mass during the period of 1 fluctuation was 10–30 μm or 0.2–0.5 contour lengths, which agrees with the displacements at which a band of T4 DNA exhibits oscillatory features after a pulse is applied (15). The amplitude of the velocity fluctuations which we observed, about twice the mean velocity, was quite similar to that reported recently for shear-degraded fragments of *S. pombe* DNA (13).

Our data showed that the T4 DNA molecules acquired substantial velocity in less than 0.2 s after the field was

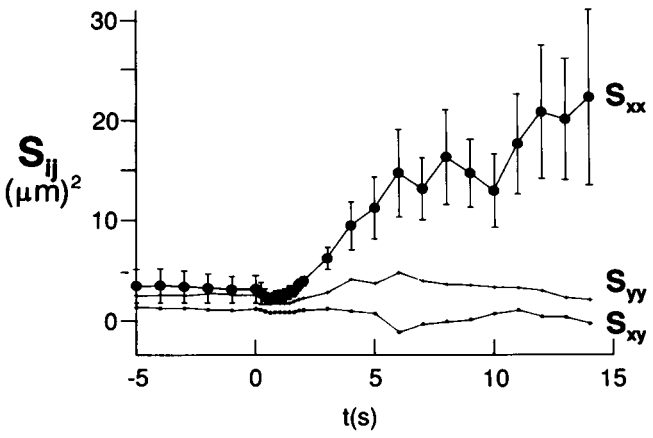

FIGURE 6 Ensemble average of components of the shape tensor S of T4 DNA after a 5 V/cm field was turned on. Error bars correspond to ±1 SD.

turned on. This result is somewhat at variance with our earlier results for a band of T4 DNA, for which v increased more gradually over 5 s after a field of 10 V/cm was applied (15); more recent velocity measurements on DNA bands in our laboratory (Keiner and Holzwarth, unpublished data), which were carried out by an improved method, support these results.

The average velocity of T4 DNA, 2 μm/s, agreed well with measurements for λ DNA in 1% agarose by Smith and his co-workers (12), which were also made with a microscope. They determined the velocity by timing the passage of single DNA chains across a 30-μm span.

Some limitations of the video measurements reported here were the absence of temperature control, the presence of intercalated dye, and the short observation interval, which was a direct consequence of the narrow zone of focus of the microscope; after ~15 s, the molecules are almost invariably out of focus. These limitations have been present in previous video microscopy studies of gel electrophoresis as well. Also, the procedures for selecting the images, and for setting the baseline in those images, were partly subjective. This made it less certain that the measured velocity fluctuations (Fig. 4 *a*) were uncontaminated by instrument noise, and corresponded to a random sample.

As the DNA advanced through the gel, the leading end(s) were usually much brighter than the trailing segments of the chain. For example, 70% of the intensity in Fig. 5 was in the end peaks. This bunching effect near the chain ends has been observed in previous microscopy studies in gels (9–13) but is not observed during DNA electrophoresis in solution (25). It should be noted that the peaks at the ends of the U (Fig. 5) had areas of ~10 $(\mu m)^2$. This meant that each bunched end occupied many pores.

Fluctuations between highly extended, compact, and U-shaped DNA configurations are a critical feature of the more recent computer models of gel electrophoresis (1–8). These models all assume that the constraints imposed by the gel are fixed in space. The agarose is treated as an array of point obstacles, an array of pores of fixed size, or, in the "lakes-straits" theory, as a series of large voids interconnected by narrow passageways. The interconnected set of pores can be represented by a tube; the length of this tube, that is, the number of pores occupied by a given chain, fluctuates as individual pores are filled with different numbers of segments. The DNA moves into new pores at its two ends. In addition, in some of the more recent theories, loops of DNA are allowed to move into adjacent unoccupied pores, so that the tube becomes branched or forked, like a tree. Such a fork is shown in Fig. 4 *b*, molecule 1, images *F* and *G*. The models differ in how they build in chain tension and in whether the individual chain segments are of fixed or variable length. Despite these differences, several models (6, 7, 18, 19) have been successful in reproducing the experimentally observed transient overshoot in the alignment of the DNA upon application of a field (16, 17).

For the lakes-straits model, both the frequency dependence and the amplitude of the velocity fluctuations of the DNA have also been explored. Fourier analysis of the steady-state velocity of a chain with 273 segments (92 kb) shows a broad peak between 5,585 and 6,827 "time units", which at 12 V/cm converts to 4.3–5.3 s (6). This is in excellent accord with our observations of the time dependence of the velocity fluctuations. The amplitude of the velocity fluctuations in this simulation is approximately twice the mean velocity, also in agreement with our observations.

Bunching behavior at the leading end of the chain occurs in several of the computer simulations which include tube-length fluctuations (5–8); the very large bunching effect shown in Fig. 5 seems to be in good accord with the lakes-straits model (6).

We thank Nina S. Allen, Julia Sizemore, and Glenn Crater for getting us started in video microscopy. Richard Whitcomb and Louis Keiner helped us write the ASYST programs for image analysis. We thank Ken Wheeler for his helpful comments on the manuscript.

This research was supported by NSF grants DMB 89-06213 and DIR 87-22684.

Received for publication 6 November 1991 and in final form 8 June 1992.

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