Imaging of Kinked Configurations of DNA Molecules Undergoing Orthogonal Field Alternating Gel Electrophoresis by Fluorescence Microscopy†

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ABSTRACT: The dynamics of individual DNA molecules undergoing orthogonal field alternating gel electrophoresis (OFAGE) have been studied by use of T2 DNA molecules labeled with a dye and visualized with a fluorescence microscope. The mechanism of reorientation used by a molecule to align itself in the direction of the new orthogonal field depends on the degree of extension of the chain immediately before the application of this field. The formation of kinks is promoted when time is allowed between the application of the two orthogonal fields so that the molecule attains a partially relaxed configuration. In this case, the chain appears bunched up in domains moving along the contour of the molecule. These regions are found to be the locations where the kinks are formed upon application of the second field perpendicular to the chain. The formation of kinks provides a significative retardation of the reorientation of the molecules, relative to molecules that do not form kinks, and appears to play an important role in the fractionation attained with OFAGE. A classification of various reorientation mechanisms observed in molecules that form kinks is presented.

Conventional electrophoresis in agarose gels is an effective tool for size fractionation of DNA molecules up to about 20 kilobase (kbp). However, under commonly employed electrophoretic conditions, there is a loss of the size dependence of mobility for DNA chains longer than about 6 μm (20 kbp) (McDonnell et al., 1977; Fangman, 1978). The size at which the electrophoretic mobility becomes independent of molecular weight depends strongly on the electric field strength and the agarose concentration (Hervet & Bean, 1987).

To overcome this drawback, a series of pulsed-field gel electrophoresis (PFGE) techniques have been developed (Schwartz & Cantor, 1984; Carle & Olson, 1984, 1985; Carle et al., 1986; Southern et al., 1987), in which the electric field is switched between two different directions with pulse times ranging from 0.1 to 1000 s or more (depending on the particular technique and the molecular size). In this way a separation of DNA molecules up to about 2000 kbp is possible. Fractionation is thought to be achieved because the time required by the molecule to change direction and adjust to the changing field depends on the size of the DNA molecule, shorter molecules being able to respond and move faster than longer ones. To provide a physical explanation of this behavior, several reorientation mechanisms (based on the angle between the two electric fields) have been proposed, as recently reviewed by Cantor et al. (1988).

Deutsch (1987) has examined theoretically the case when the chain is elongated immediately before the field is switched 90° (OFAGE: orthogonal field alternating gel electrophoresis). He proposed kinking as the primary reorientation mechanism that aligns the molecules in the direction of the new applied field. According to this view, when the orthogonal field is applied, there is a force acting on all the segments of the chain inducing the molecule to double up on itself forming kinks. Once a certain number of kinks is formed, the longer will grow even longer at the expense of the shorter ones, and the total number of kinks should diminish with time, while their average length increases. Eventually, this growth will terminate with one large kink storing most of the length of the chain; this kink will then completely unfold, moving in the direction of the electric field. Furthermore, Deutsch's theory predicts that a high-density gel (high agarose concentration) suppresses kink formation.

A fluorescence microscopy technique that allows observation of single DNA molecules labeled with a fluorescent ligand in an optical microscope has been described recently (Morikawa & Yanagida, 1981; Matsumoto et al., 1981; Yanagida et al., 1983). Houseal et al. (1989) have used this technique to visualize the dynamics of DNA in solution, and Smith et al. (1989) and Schwartz and Koval (1989) have studied DNA molecules undergoing gel electrophoresis. This technique is applied here to study the dynamics of single DNA molecules during OFAGE and regular electrophoresis. Conditions have been found that favor the observation of DNA molecules in kinked configurations. Specifically, we examined the effect of electric field strength and the effect of the degree of extension of the molecules before the application of the orthogonal field on kink formation in DNA molecules during OFAGE.

Materials and Methods

The microscope–camera system used in this study is that described by Houseal et al. (1989). A suspension of 1.25% (w/v) agarose (Sigma) in 0.5 × Tris–borate–EDTA (TBE) buffer (45 mM Tris, 45 mM borate, 1 mM EDTA) was heated to about 90 °C. The molten gel was cooled to 50 °C, and 2-mercaptoethanol (2-ME) was added to a final concentration

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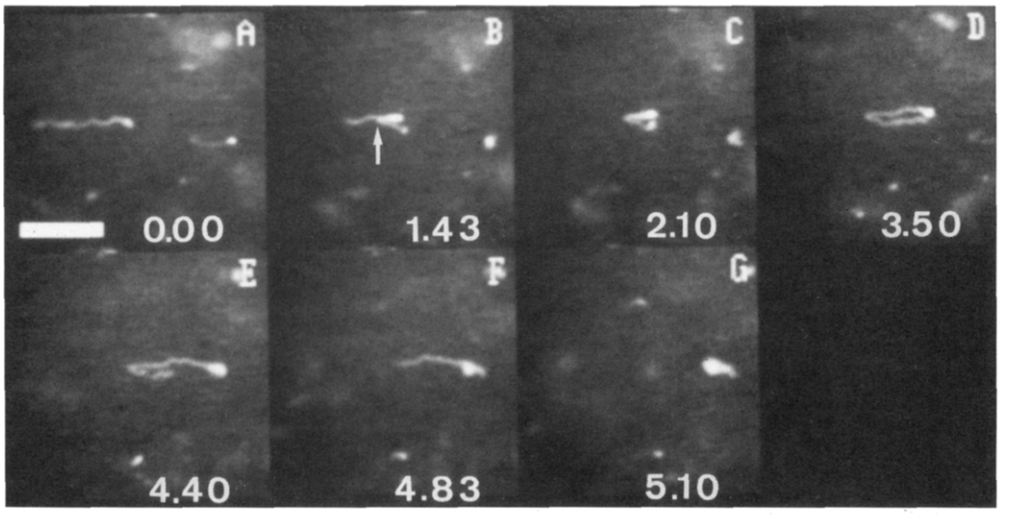

FIGURE 1: Sequence showing a molecule of T2 DNA undergoing electrophoresis in a thin agarose gel and demonstrating one of the most common mechanisms of retardation in regular gel electrophoresis. In this and all other figures, the numbers on the lower right corner depict the time elapsed (in seconds) since the begining of the sequence. The molecule moves from left to right. In (A), the molecule is partially extended as it travels through the gel but bunches up in the leading end. In (B), the molecule has encountered an obstacle (arrow) and folds on itself, as the trailing end of the molecule overshoots the leading end (B and C), to create a characteristic *narrow* U-shape. In (D), the trailing end becomes the leading part of the molecule, and it appears bunched up. Eventually, this end slips the molecule around the obstacle (E and F), and the molecule finally collapses into a blob (G). Field = 10 V/cm. Scale bar = 8 μm.

of 4% (v/v); 40 μL of the 2-ME–agarose solution was mixed with 10 μL of a solution containing 0.5 μg/mL T2 DNA (M_r = 110 × 10^6; 164 kbp), 1 μg/mL acridine orange (AO), and 4% (v/v) 2-ME in 0.5 × TBE. The final concentrations were 1% (w/v) agarose, 0.1 μg/mL T2 DNA, 0.2 μg/mL AO, and 4% (v/v) 2-Me. A 20-μL drop of this molten mixture was placed between two microscope coverslips (24 × 24 mm) and allowed to cool for 10 min. This sandwich arrangement was placed on top of a regular microscope slide and sealed at the four corners with fingernail polish. The slide had been previously prepared with four copper electrodes fixed on it with epoxy adhesive. This apparatus was then refrigerated at about 5 °C for 30 min. Molten agarose was dropped over the electrodes to complete the electrical connection, and TBE buffer (0.5 ×) was occasionally added to keep it wet.

The procedure described above allowed us to optimize the signal-to-noise ratio of the images, to obtain a reduced and homogeneous background fluorescence, and to improve drastically the quality of the video images. Moreover, it was possible to obtain a homogeneous gel (about 15–20 μm thick) and to minimize the liquid layer that often formed between the gel and the coverslip. A switchbox connected to the four electrodes and to two different power supplies was used to switch the field between two orthogonal directions. The sample was irradiated with blue light (490 nm) from a xenon source, and green wavelengths, around 520 nm, were selected for visualization. The length of the kinks was measured directly from the screen, previously calibrated with fluorescent beads of known dimensions. The photographs presented in this paper are illustrations of the typical behavior encountered in the course of a large number of observations.

Results and Discussion

The experiments started with the observation of single DNA molecules during conventional agarose gel electrophoresis. It was possible to reproduce most of the results recently published by Smith et al. (1989). The molecules move showing elastic behavior, stretching in the direction of the applied electric field and then contracting into rather dense balls. Moreover, the leading end of a chain appears bunched up, looking brighter than the other parts of the molecule as it has to find a new way through the gel pores, while the rest of the chain just follows it along the same path. Increasing the electric field strength, the molecules become, on average, better aligned in the direction of the field and more elongated, but seldom do they become totally elongated. They often remain hooked around obstacles, extending both of the arms down field forming a *narrow* U-shape (Figure 1) and then slipping off, with the longer arm driving the chain.

The main goal was then to find out in which conditions the formation of kinks, as predicted by Deutsch's (1987) theory, takes place. It was found that in DNA molecules undergoing *conventional* OFAGE the configuration of the chain, immediately before the field is switched 90°, affects strongly the mechanism of realignment of the molecules along the direction of the new applied electric field. Although, as said before, the molecules usually do not stretch to their full length after the application of the first field, the partial elongation is enough to hamper the formation of kinks in a conventional OFAGE experiment. Instead, the head of the chain (that is bunched) remains the leading end of the molecule and turns the corners in the direction of the new field, entering new gel pores. In some cases the tail end can also turn in the direction of the field and even succeed in driving the chain. These observations were recorded on a group of 50 DNA molecules with an electric field of 20 V/cm. One example of this behavior is shown in Figure 2, under a smaller field strength. However, molecules undergoing *conventional* OFAGE can sometimes form kinks when they are aligned but not too stretched, but this behavior was observed only in a small fraction of the molecules.

To promote the formation of kinks, a *modified* OFAGE experiment was designed. In this case, the first field was applied for about 10 s to align molecules in a given direction; this field was then turned off for an interval ranging from 2 to 10 s to allow the molecules to relax from their partially elongated state; then, the orthogonal field was applied for a time ranging between 5 and 10 s. For statistical purposes, the behavior of 66 molecules under an electric field of 14 V/cm and 40 molecules under an electric field of 20 V/cm was studied. In both cases the average apparent length of the molecules, right before the orthogonal field was turned on, was

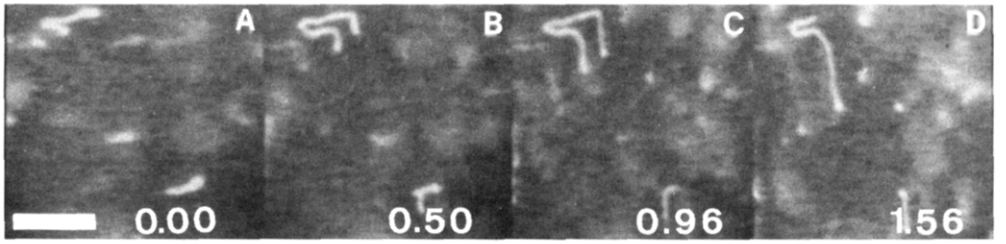

FIGURE 2: Molecule of T2 DNA (upper left corner) undergoing *conventional* OFAGE. The molecule was in a narrow U-shaped configuration before the application of the orthogonal field (A), in the downward direction. In (B), the molecule is seen to adapt to the new field by turning both ends in this direction. The two ends then begin to compete to lead the chain (C) until one of them prevails (D). Field = 10 V/cm. Scale bar = 6 μm.

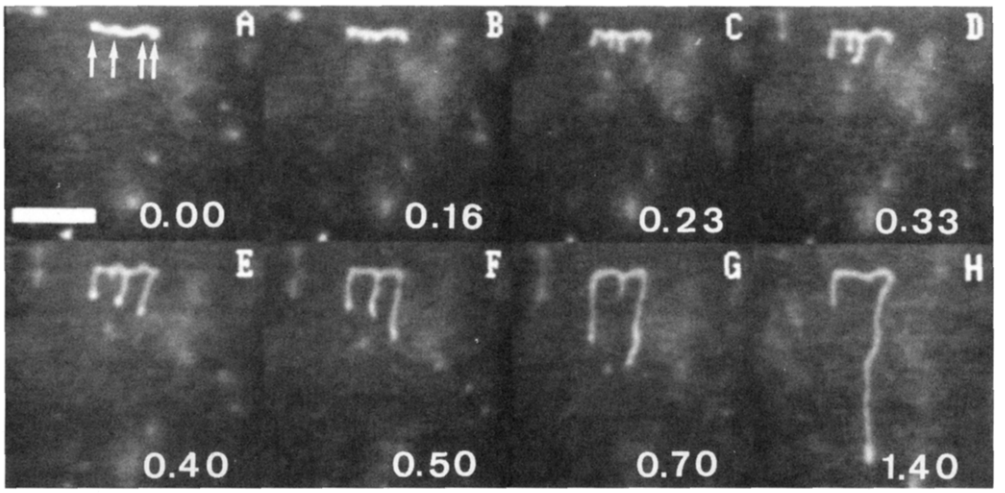

FIGURE 3: Sequence illustrating *reorientation mechanism 1*, in a T2 molecule undergoing a *modified* OFAGE experiment. (A) A molecule appears aligned along the horizontal direction a few seconds after the aligning field was turned off and just before the orthogonal field in the vertical direction was turned on. The regions where the molecule bunches up forming beads are indicated by arrows. These regions are the places where the kinks begin to grow as seen in frames B and C. The three central kinks experience substantial competition from the two ends of the molecule that have turned in reaction to the new field (D and E). Eventually, only one kink, the central one, is able to continue growing in the direction of the field until reaching a maximum size (F), but finally, it also disappears feeding the right-end of the molecule. Field = 20 V/cm. Scale bar = 8 μm. Notice that the growth of the right end of the molecule, which ultimately becomes the leading end, is rather slow relative to the growth rate of the other parts in the molecule (see C and D).

6.9 μm with standard deviation of 1.8 μm.

Figure 3 shows a complete time sequence of a DNA molecule displaying a typical behavior, termed here *reorientation mechanism 1*. It was observed that when the molecule is aligned, but partially relaxed, the length of the chains is inhomogeneously distributed between the two ends. The regions where the molecule bunches up store a substantial fraction of its length and appear, under the fluorescence microscope, as bright domains or "beads" moving randomly along the contour of the chain (arrows in Figure 3A). These beads resemble, on a larger scale, the "defects" described by de Gennes (1971) in the reptation theory of polymers. For T2 DNA their number usually range between 1 and 5, and it is precisely at these locations in the chain that the kinks form (Figure 3B). The distribution of the kinks along the molecule is therefore controlled by the location of these beads at the moment when the orthogonal field is applied. Since the molecule is usually compact at the ends, these tend to grow also in the direction of the orthogonal field, giving the molecule the appearance shown later on in Figure 3G. The number and size of the kinks grow with the time elapsed since the orthogonal field was turned on until a maximum number of them is formed (Figure 3C). From that moment on, usually, the longer kink grows at the expense of the shorter ones (Figure 3D,E), but in a few cases the opposite behavior was also observed. The remaining kink will reach a maximum length (Figure 3F); then, typically, one of the ends "wins" over the other end and over this kink (Figure 3G) and succeeds in driving the chain (Figure 3H).

In Deutsch's theory, the molecules are assumed to be infinitely large so that end effects can be neglected. For real, finite chains, we find that frequently the leading and trailing ends of the molecule turn in the direction of the new orthogonal field. As a result (see below), most of the time the kinks formed along the chain dissapear, being pulled back by the turning ends. As will be seen later, this "end effect" has important consequences in the dynamic behavior of kinks. Notice that the tip of the growing kinks appears always brighter than the elongated parts of the molecule, suggesting that the chain is bunched there also. Whether or not a kink continues to grow depends on the ability of this bunched end to find additional pores to extend the "reptating tube" defined around the kink. Because a kink has less freedom of lateral motion than a simple reptating molecule, often kinks cease to grow. This also explains why sometimes shorter kinks grow at the expense of the longer ones. As will be seen below, the length of the molecule stored at the end of the kink can provide alternative mechanisms of extension, such as "branching".

A different behavior is observed when the bunched regions are localized mostly in the middle of the chain. In this case, the molecule adapts to the new field forming kinks that pull back both of the ends (*reorientation mechanism 2*). Usually, the longest kink keeps growing, fed by the other kinks and the two adjacent horizontal sections of the chain. This process continues until the molecule reaches a configuration where it is doubled up on itself and all its length is stored in a large kink pointing in the direction of the electric field. Then, the

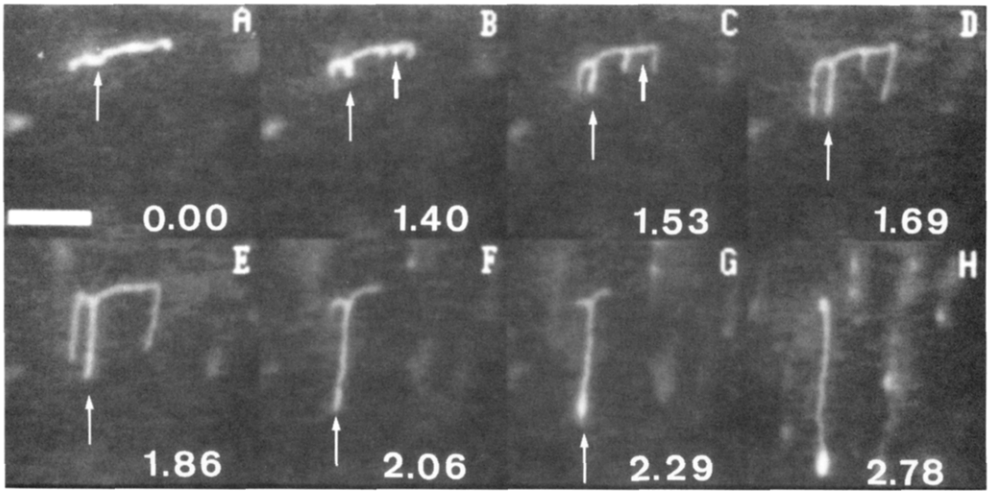

FIGURE 4: Sequence illustrating *reorientation mechanism 2* in a *modified* OFAGE experiment. Same conditions as in Figure 3. Throughout the whole sequence, the kink that ultimately drives the molecule is indicated by the long arrow. Besides this kink two additional kinks can be seen growing in (B). But one of them (short arrow in frames B and C) stops growing and disappears, giving away (D) to the right end of the molecule and the adjacent kink. In the following frames, the leading kink keeps growing uninterruptedly, first at expense of all other internal kinks (E) and then at the expense of the two ends of the molecule, which are pulled until the molecule is completely folded on itself (G); then, the molecule begins to unfold (H). Because at no point are the molecules completely stretched, it is not easy to estimate the actual location of the kink along the molecule and determine whether or not this mechanism is favored when a kink forms at the center of mass of the molecule. Field = 20 V/cm. Scale bar = 6 μm.

chain unfolds and collapses in a rather dense ball. This reorientation mechanism is shown in Figure 4.

Notice that even in the case illustrated in Figure 4, both ends of the molecules turn in the direction of the field and compete with the kinks being formed (see figure caption). Despite this difference, reorientation mechanism 2 is in many ways closer to the behavior described by Deutsch's theory. According to this theory, the length of a kink should grow proportional to time, and since the theory assumes that the total length stored in the form of kinks is constant, the average number of kinks should decrease as $1/t$. We have found that the kinks grow indeed linearly with time, but this linear dependence is better at the early stages of growth and for molecules that follow reorientation mechanism 2. At later times, the effect of the ends is to compete efficiently with the kink growth, introducing deviations from this linear behavior. In this case of the kink that drives the chain in Figure 4, the rate of growth was 8 μm/s at 20 V/cm. On the other hand, the assumption of the theory that the total length stored in the form of kinks is conserved would be valid only if the chains were completely or almost completely elongated. The observations carried out here, and those made by other authors (Smith et al., 1989; Schwartz & Koval, 1989), have shown that DNA molecules are seldom close to this complete elongation under normal electrophoretic field strengths. This fact invalidates the inverse time dependence of the number of kinks.

In the other cases, two long kinks grow together, "winning" over both of the ends (*reorientation mechanism 3*). In this situation, all the length of the molecule is stored in these kinks and in the segment of chain joining them. These kinks then unfold completely forming a *broad-base* U-shape, and one of the arms becomes the leading end of the molecule. In these broad-base U-shape configurations sometimes the molecules break. It is not clear if this is due to local tension created by the electric field on the chain or if it is due to the photosensitization of the DNA–AO complex with blue light, which is known to nick the DNA (Freifelder et al., 1961), or a combination of both.

It has been mentioned that a DNA molecule undergoing gel electrophoresis often gets hooked around the obstacles of the gel, both of the ends elongating in the direction of the electric field, giving rise to a *narrow* U-shape configuation. If the orthogonal field is turned on when the molecule is trapped in this state, after the two arms have attained partial relaxation, both arms can evolve forming kinks, as shown in Figure 5 (*reorientation mechanism 4*). One of these kinks, or an end, will eventually drive the chain.

Table I

	E = 14 V/cm	E = 20 V/cm
av no. of kinks/molecule in *modified* OFAGE	2.4	2.5
max no. of kinks/molecule in *modified* OFAGE	5	4
av length of kinks in *modified* OFAGE (μm)	1.1	1.4
max length of a kink in *modified* OFAGE (μm)	2.2	3.3
av reorientation time in *conventional* OFAGE (s)	–	1.3
av reorientation time in *modified* OFAGE (s)	4.1	2.5
% of times that a kink drives the chain in modified OFAGE	25.8	27.5

As the kinks grow, the center of their tip can be occasionally stopped by an obstacle in the gel (see above). As it keeps growing, the end of the kink forms a Y-shape (Figure 6). Then, one of the arms usually increases its length at the expense of the other that disappears. The chain then evolves in one of the different ways previously described. This branching effect might become more important as the size of the gel pores become small at higher agarose concentrations.

Since the reorientation mechanism does not seem to depend on the electric field strength, it is possible to report together the results of the 106 molecules observed with the two fields that were used in the *modified* OFAGE experiments. Thus, we found that 72 molecules used *reorientation mechanism 1*, 22 molecules used *reorientation mechanism 2*, 5 molecules used *reorientation mechanism 3*, and 7 molecules used *reorientation mechanism 4*. Table I summarizes the statistical behavior of the molecules studied. The kink parameters (number and length of kinks) were measured at the time when the maximum number of kinks had formed in the molecule. This time depends on the electric field strength. The number of kinks per molecule varies (in our experimental conditions) from one to five, with an average value of about 2.5 that does not seem to depend significantly on the electric field strength.

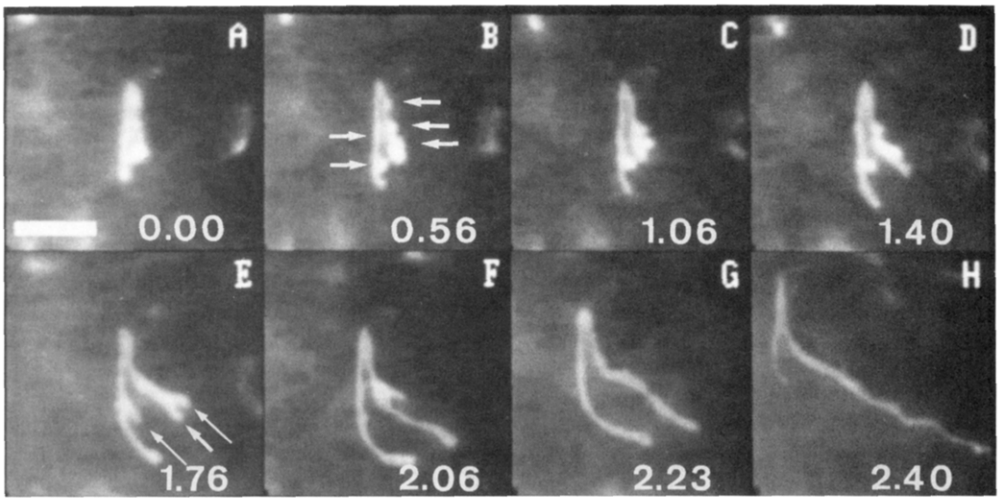

FIGURE 5: Sequence illustrating *reorientation mechanism 4*. The molecule is trapped in a *narrow* U-shape (A), following the application of the initial field (applied downward). The molecule is allowed to relax before the application of the new orthogonal field in the horizontal direction. Upon application of the orthogonal field, kinks begin to form in both arms (B), as indicated by the arrows. These kinks grow, competing with each other to drive the chain (C and D). In (E), only one kink remains in each arm (long arrows), while the ends of the molecule begin to compete efficiently to drive the chain. The right end is indicated by the short arrow. A few moments later, the left-arm kink has disappeared (F), and later, the right-arm kink also succumbs, being pulled back by the right end (G and H). Field = 14 V/cm. Scale bar = 4 μm.

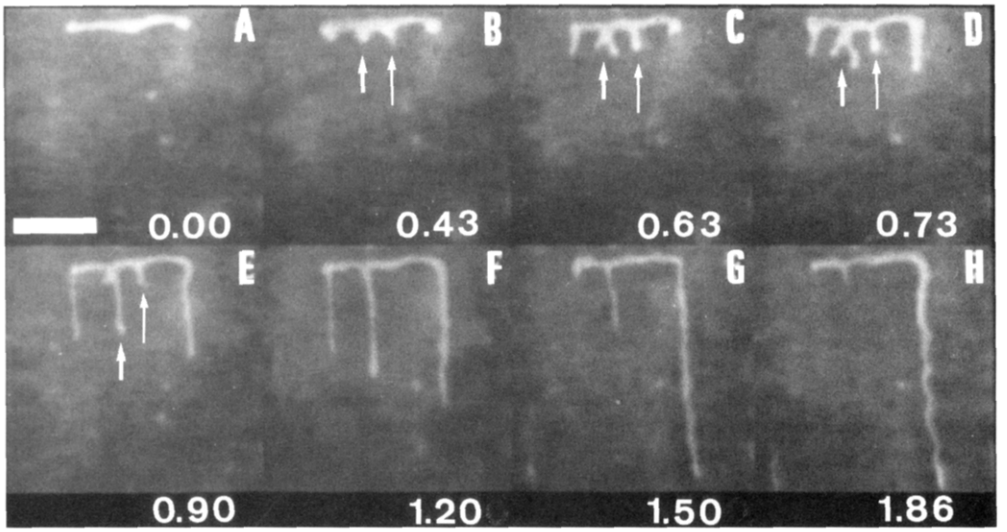

FIGURE 6: Sequence of video frames showing the behavior of kinks when they encounter obstacles in the gel. A molecule of T2 DNA is partially relaxed a few seconds after the initial (horizontal) field is turned off (A). In (B), two kinks are formed and begin to grow few moments later. One of them (short arrow) shortly encounters an obstacle in the gel and splits into two driving heads, forming a characteristic Y-shape, while the other (long arrow) continues to grow (C). Meanwhile, the ends of the molecule have turned in the direction of the field. The right end begins to grow, preventing the growth of the nearest kink (long arrow in frame E), which begin to shorten, while one of the arms of the Y has begun to grow at the expense of the other. This arm of the Y keeps growing as a central kink (F) but is eventually eliminated, pulled back by the right end of the molecule (G and H). Field = 20 V/cm. Scale bar = 4 μm.

The latter affects only the rate at which the kinks grow and the maximum length that the kinks attain. The longest kink observed (after all the others have disappeared) was about 6.5 μm for E = 14 V/cm and 7.5 μm for E = 20 V/cm. Other variables are likely to affect the size of the kinks and their number, such as the length of the molecule, the agarose gel concentration, the time between the application of the two fields, etc., but their effects on kink formation were not tested. It is also possible that kinks will form only above a given electric field strength, but it is not easy to find the value of this threshold since the limit size of a kink discernible under the fluorescence microscope is about 0.5 μm.

The speed at which the reorientation of the molecule occurs was much faster when the stronger electric field was applied (starting from molecules with the same average apparent length, as measured directly from the screen). The reorientation time, i.e., the time required for the molecule to become completely aligned in the direction of the new applied electric field, ranged from 1.9 to 7.2 s in the case of E = 14 V/cm (average = 4.1 s) and from 0.9 to 5.1 s in the case of E = 20 V/cm (average = 2.5 s) (see Table I). These mean values are reported here without reference to the standard deviations. This is because all four mechanisms of reorientation contribute to these values and the standard deviations would have been unusually large and statistically meaningless. In fact, the reorientation time depends strongly on the reorientation mechanism used by the molecule to realign itself in the direction of the orthogonal electric field. Very long reorientation times were observed when a broad-base U-shape has formed (reorientation mechanisms 1 and 3) as the two arms "compete" in driving the chain. As mentioned above, the two arms of the broad-base U-shape can be formed by the two ends of the molecule, by two unfolding kinks, or by one kink and one end. Regardless of their origin, however, the arms grow together until they reach almost complete extension. Then one wins over the other and becomes the leading end of the molecule.

The average reorientation time for DNA molecules that do not form kinks during conventional OFAGE (for E = 20 V/cm) is found to be significantly lower than the one measured in the modified OFAGE experiments at the same electric field strength (Table I). In this case the reorientation time ranged from 0.7 to 1.9 s, with an average value of 1.3 s and a standard deviation of 0.3 s. Finally, Table I shows that the percentage of times a kink drives the chain is barely affected by the electric field strength.

Conclusions

The results of this provide the first experimental confirmation of kinked configurations in DNA molecules undergoing OFAGE. The behavior and dynamics of the kinks are in good qualitative agreement with some of the predictions of Deutsch's theory, but important discrepancies have also been observed. These are as follows: (1) Kinks occur in substantial numbers only if the molecules are allowed to relax before application of the orthogonal field. (2) For molecules of finite length, end effects play a very important role. There is a strong tendency of the molecules to realign to the new orthogonal field by turning *both* the leading and trailing ends in the direction of the field. This accounts for why only about one-third of the time a single kink ends up driving the chain. (3) The number of kinks observed per molecule is much smaller than predicted by the theory. This discrepancy can be due to several factors. The most likely one is that the regions where the probability of generating kinks is higher are those in which the chain bunches up to form beads. The early formation of kinks in these locations probably inhibits the formation of kinks in the adjacent, less-folded regions of the chain. These beads or regions of length storage can be considered as preexisting "nuclei" for kink formation. The time interval between the application of the two fields in the *modified* OFAGE experiment favors the relaxation of the molecular stretching and the formation of these nuclei. These results suggest a modification of the theory to incorporate the effect of the uneven distribution of the chain length along the molecule.

The reorientation mechanism and the number of kinks per molecule do not seem to depend on the electric field strength. The latter affects only the rate at which the kinks grow, the maximum length that the kinks attain, and the speed at which the reorientation mechanism occurs.

Kink formation, besides being an important reorientation mechanism, provides a *retardation of the realignment* (relative to molecules that do not form kinks in conventional OFAGE) that can play a crucial role in DNA size fractionation. The longer the molecule, the longer the relaxation time required for the formation of the kink nuclei or beads along the molecule. A careful variation of the time between the application of the two fields in the modified OFAGE experiment might provide further size discrimination. Moreover, the longer the molecule, the longer the average kink dimensions and the longer the time required for the molecule to find its driving configuration under the new applied field. In addition, it is reasonable that the number of beads or bunched up regions along the chain should be greater the longer the molecule. More kinks per molecule could lead to a fiercer competition to drive the chain and to a dilation in the time required by the molecule to realign along the direction of the new field. A further retardation mechanism may be provided by the formation of branches along the leading segments of chain, as was observed in some cases.

A systematic study of the molecular weight dependence of the dynamics of kink formation is currently underway in our laboratory.

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