Effect of Pulsed and Reversing Electric Fields on the Orientation of Linear and Supercoiled DNA Molecules in Agarose Gels†

Nancy C. Stellwagen

Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242

Received January 27, 1988; Revised Manuscript Received May 10, 1988

ABSTRACT: When linear or supercoiled DNA molecules are imbedded in agarose gels and subjected to electric fields, they become oriented in the gel matrix and give rise to an electric birefringence signal. The sign of the birefringence is negative, indicating that the DNA molecules are oriented parallel to the electric field lines. If the DNA molecules are larger than about 1.5 kilobase pairs, a delay is observed before the birefringence signal appears. This time lag, which is roughly independent of DNA molecular weight, decreases with increasing electric field strength. The field-free decay of the birefringence is much slower for the DNA molecules imbedded in agarose gels than observed in free solution, indicating that orientation in the gel is accompanied by stretching. Both linear and supercoiled molecules become stretched, although the apparent change in conformation is much less pronounced for supercoiled molecules. When the electric field is rapidly reversed in polarity, very little change in the birefringence signal is observed for linear or supercoiled DNAs if the equilibrium orientation (i.e., birefringence) had been reached before field reversal. Apparently, completely stretched, oriented DNA molecules are able to reverse their direction of migration with little or no loss of orientation. If the steady-state birefringence had not been reached before the field reversal, complicated orientation patterns are observed after field reversal. Very large, partially stretched DNA molecules exhibit a rapid decrease in orientation at field reversal. The rate of decrease of the birefringence signal in the reversing field is faster than the field-free decay of the birefringence and is approximately equal to the rate of orientation in the field (after the lag period). The rates of orientation and field-assisted disorientation decrease linearly with increasing electric field strength and increase slowly with increasing DNA molecular weight. The agarose gel matrix is also oriented by the electric field and exhibits a time-dependent transient in reversing fields. However, the time constants observed for orientation and disorientation of the gel matrix are much faster than those observed for the DNA samples under the conditions studied here.

When DNA molecules are imbedded in agarose gels and subjected to pulsed electric fields, the DNA becomes oriented in the gel, giving rise to a strong electric birefringence signal that can be used to monitor the orientation (Stellwagen, 1985). When the average pore size of the gel is larger than the mean end-to-end length of the DNA molecule, the DNA orients and disorients upon application of the field as though the gel matrix were not present. However, if the median pore size of the gel is smaller than the mean end-to-end length of the DNA fragment in free solution, the DNA molecule becomes stretched during orientation, and the longest or terminal relaxation time observed after removal of the electric field becomes that value characteristic of the fully stretched molecule (Stellwagen, 1985). In terms of reptation theory, this terminal relaxation time represents the time required for the DNA to diffuse from one "tube" into another; in this case, from a tube oriented in the direction of the electric field into a randomly oriented tube. The terminal relaxation time of linear DNA restriction fragments was found to increase according to the 2.8 power of the molecular weight (Stellwagen, 1985), close to the third power dependence predicted theoretically for the renewal of chain conformation (deGennes, 1971).

The orientation of large DNA molecules in agarose gels during electrophoresis leads to nonlinear effects that reduce the molecular weight dependence of the mobility (Southern, 1979; Bearden, 1979; Fangman, 1978; McDonnell et al., 1977; Stellwagen, 1985). Experimentally, the orientation of the DNA molecules during electrophoresis can be disrupted by using pulsed linear (Bean, 1985; Hervet & Bean, 1986; Fesjian et al., 1986), orthogonal (Schwarz & Cantor, 1982, 1984; Carle & Olson, 1984), or reversing (Carle et al., 1986) electric fields. In these time-dependent fields the DNA molecules continually need to orient, disorient, and reorient in the gel during electrophoresis. The finite time required for reorientation leads to improved molecular weight separation of large DNA molecules, if the field-on and field-off time intervals are chosen to coincide with the orientation times of some of the DNA molecules in the sample. Theories describing the center-of-mass velocity and the dynamics of entangled polymers in external electric fields have been proposed by Lerman and Frisch (1982), Lumpkin et al. (1982, 1985), Slater and Noolandi (1986), Olvera de la Cruz et al. (1986), Adolf (1987), and Viovy (1987).

In the present study, the technique of transient electric birefringence is used to compare the orientation of linear and supercoiled DNA molecules in agarose gels in pulsed and reversing electric fields. The measurements are also extended to λ DNA, a molecule of much higher molecular weight than studied previously. In all cases the sign of the birefringence is negative, indicating that the DNA molecules in the gel are oriented parallel to the electric field lines. At low field strengths orientation is preceded by a lag period, when no orientation occurs. The lag period decreases with increasing field strength but is relatively independent of molecular weight, suggesting that it may reflect the finite time required for the

† This research was supported by Grant GM29690 from the National Institute of General Medical Sciences.

0006-2960/88/0427-6417$01.50/0

DNA molecule to find a pore in the gel oriented in the direction of the electric field. When the electric field is rapidly reversed in polarity, very little change in orientation is observed if the signal had reached its equilibrium value before field reversal. However, if the equilibrium orientation is not reached before field reversal, the birefringence signal decreases markedly before increasing again in the new field direction, and complicated orientation patterns can be observed. The agarose gel matrix can also be oriented by the electric field, which adds another degree of complexity to the interpretation of electrophoresis experiments.

Materials and Methods

DNA Samples. Three restriction fragments, 622, 1426, and 2936 bp, were prepared from an *Eco*RI digest of plasmid PB20, as previously described (Stellwagen, 1984). Agarose gel electrophoresis of the fragments confirmed that they were monodisperse. Plasmids pBR322 and PUC19 and the linear 1 kb ladder were obtained from Bethesda Research Laboratories. The pBR322 was linearized by digesting with *Pvu*II (New England Biochemicals) or *Eco*RI (Bethesda Research Laboratories) under standard enzyme buffer conditions. λ DNA was a gift from D. Quick of the DNA Recombinant Core Laboratory here.

Preparation of DNA Solutions and Gels. The DNA samples were stored in T0.1E buffer (10 mM Tris-HCl buffer, pH 8.0, plus 0.1 mM EDTA) at −20 °C. For the measurement of electric birefringence in free solution, the solvent was 0.2 mM Tris buffer (1:50 T0.1E buffer/deionized water). The DNA concentration varied from 7 to 22 μg/mL; the birefringence results were independent of concentration in this range. The deionized water was 18-MΩ water freshly drawn before each experiment from a Barnstad NANOpure II deionizer.

The agarose used for all measurements was Marine Colloids Seakem LE agarose, purchased from Bethesda Research Laboratories as "electrophoresis grade agarose", lot no. 20105A. A typical analysis taken from the catalog was as follows: gelling temperature of a 1% solution, 36–42 °C; sulfate, <0.35%; ash, <0.75%; electroendosmosis, 0.10–0.15. To prepare the DNA–agarose gels, about 10 mg of agarose was dissolved in enough deionized water to make a 1.0% gel by boiling in a microwave oven. The solution was weighed before and after heating; evaporation was negligible. The gel solution was then equilibrated in a water bath at 55–60 °C, a 300-μL aliquot placed in an Eppendorf tube at room temperature, a calculated volume of thawed DNA stock solution (ca. 1 μg/μL) added, and the mixture vortexed. The gel mixture was then quickly pipetted into the space between a pair of birefringence electrodes, which had been sealed on one side with a piece of Teflon. The DNA–agarose mixture solidified within 1 min. After a time interval ranging from 15 min to 2 h, the Teflon plate was removed and the electrode assembly inserted into a birefringence cell containing deionized water. The DNA concentration in the gel ranged from 10 to 16 μg/mL, approximately the same as control experiments in free solution. The resistance of the cell was also approximately the same, whether the DNA was dissolved in 0.2 mM Tris buffer or imbedded in the agarose gel. Hence, the electrical environment of the DNA molecules was approximately the same in the two cases. All birefringence results were independent of the DNA concentration and the time of aging of the DNA–agarose gel. Agarose gels containing no DNA were also studied as controls.

Electric Birefringence Measurements. The electric birefringence apparatus used in the present studies has been described briefly (Stellwagen, 1985). The Kerr cell was a 1.00-cm path length quartz spectrophotometer cell, chosen for its negligible strain birefringence. The electrodes were parallel platinum plates with a 2.0-mm electrode separation, mounted on a lexan support of standard design (Pytkowicz & O'Konski, 1959). The electronic time constant of the detecting system was about 0.2 μs. The stray light constant (Fredericq & Houssier, 1973) ranged from 0.5×10^{-5} to 2×10^{-5}, depending on the DNA molecular weight and solution concentration. With DNA–agarose gels in the cell, the stray light constant was about 5–10 times larger, because of light scattered by the gel. The scattered light had no effect on the birefringence measurements. All measurements were made at 20.0 °C; the calculated temperature rise during any pulse was always less than 0.1 °C.

The electric field pulses were generated by Cober high-power pulse amplifiers, Model 605P. Single or reversing square wave pulses, varying from 7 μs to 3 ms in duration, and 0.5–10 kV/cm in amplitude could be generated by these pulsers. The birefringence results in free solution or in the gels were independent of pulsing history; repeated pulsing, or returning to lower electric fields after higher voltage pulses, caused no significant change in the birefringence amplitudes or relaxation times. The amplitude of the birefringence and the various birefringence relaxation times were usually reproducible within ±10% when duplicate solutions or duplicate gels were measured. The specific birefringence (i.e., the observed birefringence divided by DNA concentration) was independent of DNA concentration under all conditions. Electrophoresis in alkaline 1.0% agarose gels after extensive birefringence measurements of the DNA fragments in free solution showed no evidence of nicking.

The electric birefringence experiment is described in several reviews [O'Konski, 1976; Fredericq & Houssier, 1973; Krause, 1980; see also Stellwagen (1981)]. When an electric field is applied to polar macromolecules in free solution or imbedded in a gel, the macromolecules will tend to orient in the field. If the macromolecules are asymmetric in shape and have a refractive index different from that of the solvent, the refractive index of the solution becomes different in directions parallel and perpendicular to the electric field. This difference in refractive index, called the electric birefringence or electric double refraction, can be measured for DNA molecules in free solution or imbedded in gels [e.g., Stellwagen (1985) and Wijmenga and Maxwell (1986)] and is a direct measure of the orientation of the macromolecules upon application of an electric field.

At low field strengths the amplitude of the birefringence is proportional to the square of the applied electric field strength (Fredericq & Houssier, 1973) as shown:

$$\Delta n = KnE^2 \qquad (1)$$

Here, Δn is the birefringence, n is the mean refractive index, E is the electric field strength, and the proportionality constant K, called the Kerr constant after the discoverer of the effect [see, e.g., O'Konski (1976)], is related to the optical and electrical anisotropy of the molecules being oriented in the electric field. Equation 1 is known as the Kerr law. If very high electric fields are applied to solutions of macromolecules, the Kerr law breaks down and the amplitude of the birefringence approaches a saturation value, characteristic of complete orientation of the macromolecules in the solution (O'Konski et al., 1959).

The field-free decay of the birefringence after removal of the electric field is due to the randomization of the orientation of the birefringent particle because of Brownian motion. In

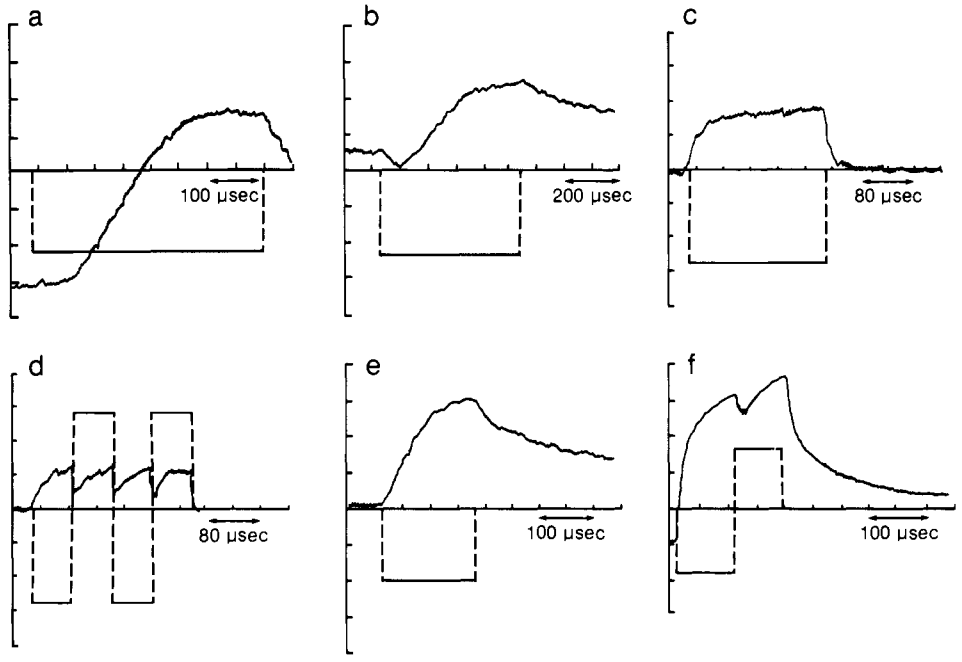

FIGURE 1: Oscilloscope traces of typical birefringence signals observed for DNA samples in 1% agarose gels and in free solution: (a) 2936 bp restriction fragment in 1% agarose, E = 1.1 kV/cm, 410-μs orienting pulse; (b) supercoiled pBR322 in 1% agarose, E = 1.0 kV/cm, 510-μs orienting pulse; (c) 1% agarose gel alone, 200-μs orienting pulse, E = 2.8 kV/cm; (d) 1% agarose gel, 60-μs reversing pulse, E = 2.8 kV/cm; (e) supercoiled pBR322 in 0.2 mM Tris buffer, E = 1.2 kV/cm, 130-μs orienting pulse; (f) 2936 bp restriction fragment in 0.2 mM Tris buffer, E = 2.2 kV/cm, 110-μs reversing pulse. In all cases, the vertical scale is arbitrary; the horizontal scale is indicated. The birefringence signal (upward going in all cases) is arbitrarily offset from the position of zero birefringence to increase clarity. The sign of the steady-state birefringence is negative if the gel or solution contains DNA (a, b, e, f) and positive for agarose alone (c, d). In (a) and (b) note the significant lapse of time before the onset of the birefringence after the pulse (downward-going signal) is applied (d). In (c–f) note that the birefringence signal begins to build up immediately after application of the electric field. In (d) and (f) the birefringence signal begins to decrease immediately after the electric field is reversed in direction.

free solution the decay of the birefringence of an assembly of monodisperse rigid particles is given by

$$\Delta n/\Delta n_0 = e^{-t/\tau} \quad (2)$$

where Δn is the birefringence at any time t, Δn_0 is the birefringence at the moment the field is removed, and τ is the relaxation time, which is related to the rotational diffusion constant θ as shown:

$$\theta = 1/6\tau \quad (3)$$

The rotational diffusion constant can be related to particle length by an equation given by Broersma (1981):

$$\theta = (kT/\pi\eta L^3)[\ln 2p - 1.43 + 7(1/\ln 2p - 0.27)^2] \quad (4)$$

where k is Boltzmann's constant, T is the absolute temperature, η is the solvent viscosity, p is the axial ratio, and L is the apparent hydrodynamic length of the particle. Equations 2–4 indicate that the birefringence relaxation time is approximately dependent on the third power of macromolecular length. If the macromolecules are not rigid, the decay of the birefringence will not be exponential but will contain a series of relaxation times, the longest of which is characteristic of the maximum dimension of the macromolecule rotating in solution (Stellwagen, 1981; Hagerman, 1981; Diekmann et al., 1982).

For DNA fragments imbedded in agarose gels, it may be more appropriate to describe the loss of orientation after removal of the electric field in terms of the disentanglement of the DNA molecules from the gel matrix. The time required for the disentanglement of a macromolecule from a three-dimensional network has been analyzed theoretically by de-Gennes (1971, 1976a,b, 1979). Disentanglement requires the macromolecule, or the tube in which it is contained, to undergo a displacement on the order of its contour length. From a scaling analysis, the terminal relaxation time T_R, the time required for a complete renewal of the chain conformation, is

$$T_R = (6\pi\eta R^3/kT)(c/c^*) \quad (5)$$

where R is the radius of the coiled macromolecule, c is the concentration of macromolecules in the solution, c^* is the overlap concentration where the domains of the macromolecules begin to overlap, and the other terms have been defined above. Equation 5 shows that T_R scales approximately as N^3, where N is the number of monomer units (base pairs) in the chain. Hence both T_R and τ exhibit a similar dependence on macromolecular length.

Results and Discussion

Large DNA Molecules Experience a Delay before Orientation in Agarose Gels. When DNA molecules are imbedded in agarose gels and subjected to electric field pulses, the gels become birefringent. The sign of the birefringence is negative (Stellwagen, 1985), as observed for DNA in free solution (Benoit, 1951; Houssier & Fredericq, 1964; Stellwagen, 1967), indicating that the DNA molecules in the gel are being oriented parallel to the electric field lines. If the DNA molecules are ≥1.5 kb in size, the birefringence signal (and hence the orientation) is observed only after a significant time delay, as shown in Figure 1a,b. The delay before orientation is observed for both linear and supercoiled molecules, although under some conditions supercoiled molecules exhibit a slow positive-going birefringence signal before the onset of the negative steady-state birefringence (Figure 1b). In free solution, no delay is observed before orientation, as shown in Figure 1e for supercoiled pBR322 and in the literature for linear DNA restriction fragments (Elias & Eden, 1981; Diekmann & Porschke, 1982; Stellwagen, 1985). Linear DNA fragments

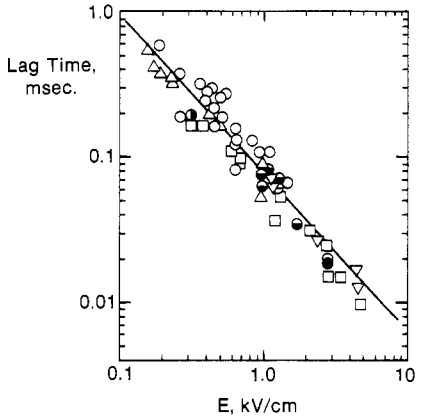

FIGURE 2: Log–log plot of the dependence of the lag period before orientation on the applied electric field strength, E. The DNA samples are (●) a 1426 bp restriction fragment, (○), a 2936 bp restriction fragment, (△) λ DNA, (▽) plasmid 82-6B, (◒) circular plasmid pBR322, and (□) *Pvu*II cut pBR322. The slope of the drawn line is −1.1.

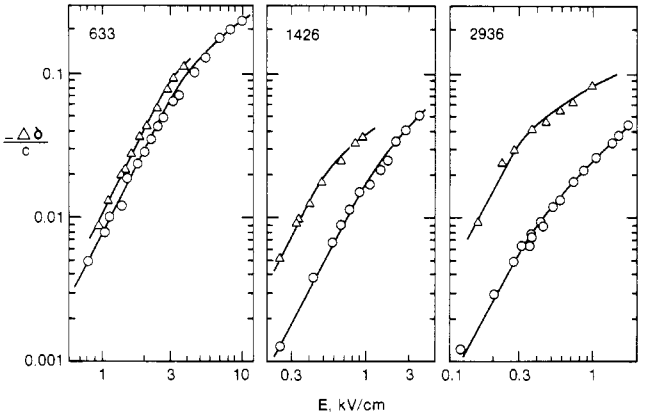

FIGURE 3: Comparison of the saturation curves observed for three DNA restriction fragments in 0.2 mM Tris buffer and in 0.5% agarose gels. This gel concentration was chosen so that Kerr law behavior (eq 1) would be observed over the same range of electric field strength in the gel as in free solution. In all cases, the logarithm of the amplitude of the observed birefringence, corrected for the birefringence of agarose and divided by the DNA concentration, $\Delta\delta/c$, in deg/(μg/mL), is plotted as a function of the logarithm of the electric field strength, E. The drawn lines have slopes of 2.0 at low values of E, indicating that the Kerr law is obeyed. (○) DNA alone; (△) DNA imbedded in 0.5% agarose gel. The molecular weight of the restriction fragment is indicated for each pair of curves.

less than 1 kb in size imbedded in agarose gels also do not exhibit a delay before orientation (Stellwagen, 1985). The observed lag time suggests that large DNA molecules need a finite time to locate a favorably oriented pore in the gel matrix before orientation can occur.

The lag time observed before orientation of the large DNA molecules in agarose gels is inversely dependent on the applied electric field strength, as shown in Figure 2, where the slope of the drawn line is −1.1. The lag time is approximately independent of molecular weight, although at any given electric field strength there is a small but systematic increase of the lag time with increasing DNA molecular weight. These results are consistent with the fact that the driving force for orientation is field strength dependent (Lerman, 1982; Lumpkin et al., 1982, 1985), but the charge density of DNA is independent of molecular weight. Assuming the relationship in Figure 2 to hold at still lower electric field strengths, the delay time before orientation at E = 10 V/cm (a typical voltage used in pulsed field electrophoresis experiments) is estimated to be 10 ms.

Agarose Gels Can Be Oriented by an Applied Electric Field. When orienting pulses of 1 kV/cm or greater are applied to an agarose gel without DNA, a positive birefringence signal is observed with no lag time at the beginning of the pulse (Figure 1c). The appearance of a birefringence signal indicates that the agarose gel matrix can be oriented by the electric field, reaching an apparent steady-state orientation after about 100 μs. However, when much longer pulses are applied to the gel, the birefringence signal begins to increase again after about 1 ms, becoming ~20% more positive than the apparent steady-state value after 2.6 ms (not shown). These effects are more pronounced at higher voltages: at E = 3.6 kV/cm, an apparent steady-state orientation is reached after a 190-μs pulse; however, the signal (i.e., orientation) increases by ~30% if the pulse is lengthened to 2.6 ms (not shown). These results indicate that, after an apparent equilibrium has been reached, still longer electric field pulses increase the orientation of the agarose gel matrix, possibly because of deformation. Deformation of the agarose gel matrix has also been observed at lower electric field strengths by Jonsson et al. (1988). Orientation and/or deformation of the gel matrix in response to an electric field could open tubes or pores which would enable the DNA molecules to orient along the electric field lines.

The orientation of a 1.0% agarose gel in a reversing electric field is shown in Figure 1d for a 60-μs reversing pulse of 2.8 kV/cm. Upon field reversal the birefringence signal decreases to about half its equilibrium value, after which it returns to its equilibrium value before repeating the cycle. This result indicates that both permanent and induced dipole moments (Tinoco & Yamaoka, 1959) contribute to the orientation of the agarose gel matrix and that reorientation of the matrix upon field reversal occurs within ~60 μs under the high-field conditions used here.

Since the sign of the birefringence of the agarose gel is positive and the sign of the birefringence of the DNA is negative, it might be thought that the delay in the appearance of the birefringence signal in Figure 1a,b was due to a fortuitous cancellation of the signals from the DNA and the gel at early orientation times. However, the absolute magnitude of the signal from the agarose gel was more than an order of magnitude less than that exhibited by the DNA samples at these field strengths. In addition, no delay in the appearance of the birefringence signal was observed for small DNA restriction fragments in agarose gels (Stellwagen, 1985) or for larger fragments in higher electric fields (data not shown). Therefore, the delay in observing a birefringence signal at low field strengths for the DNA molecules studied here probably reflects the time required for the large DNA molecules to locate a tube in the gel oriented in the direction of the applied electric field and/or the time required to create such a tube by orientation of the matrix.

The Amplitude of the Electric Birefringence Signal Observed for DNA Molecules Imbedded in Agarose Gels Increases with Increasing Molecular Weight. The amplitudes of the electric birefringence signals observed for three linear DNA molecules in free solution and imbedded in 0.5% agarose gels are compared in Figure 3. The free solution values were measured in 0.2 mM Tris buffer, because the conductivity of DNA solutions in this buffer is comparable to that observed in agarose gels. In all cases the amplitude of the birefringence was corrected for the birefringence of the solvent and the gel matrix. For all three DNA fragments, in free solution or imbedded in agrose gels, the slope of the log–log plot at limiting low fields is 2.0, indicating that the Kerr law (eq 1) is obeyed. Therefore, there is nothing unusual about the mechanism of orientation of DNA in an agarose gel. In all three cases the

Table I: Birefringence Relaxation Times and Calculated Lengths of Linear and Supercoiled DNA Molecules Imbedded in 1.0% Agarose Gels

plasmid	free solution (0.2 mM Tris buffer)[a]	1.0% agarose gel[a]	linear DNA	free solution (0.2 mM Tris buffer)[c]	1.0% agarose gel[c]
PUC19	τ = 330 ± 40 μs	τ = 760 ± 60 μs	622 bp[d]	τ = 52 ± 3 μs	τ = 74 ± 5 μs
(2686 bp)	L = 830 bp	L = 1140 bp		L = 460 bp	L = 540 bp
	61% of $^1/_2$ contour length	84% of $^1/_2$ contour length		74% of fully stretched	84% of fully stretched
pBR322	τ = 1.6 ± 0.2 ms	τ = 2.6 ± 0.2 ms	1426 bp[d]	τ = 305 ± 30 μs	τ = 1.12 ± 0.11 ms
(4363 bp)	L = 1490 bp	L = 1800 bp		L = 900 bp	L = 1460 bp
	68% of $^1/_2$ contour length	83% of $^1/_2$ contour length		64% of fully stretched	100% of fully stretched
p82-6B[b]	τ = 12 ± 2 ms	τ = 15.1 ± 0.8 ms	2936 bp[d]	τ = 880 ± 50 μs	τ = 6.9 ± 1.2 ms
(15 kb)	L = 3150 bp	L = 3400 bp		L = 1700 bp	L = 2850 bp
	42% of $^1/_2$ contour length	45% of $^1/_2$ contour length		46% of fully stretched	100% of fully stretched
			λ, 48.5 kb	τ = 8.4 ± 1.1 ms	τ = 60 ± 10 ms
				L = 3.3 kb	L = 6.6 kb
				7% of fully stretched	14% of fully stretched

[a] Lengths calculated from the Broersma (1981) equation, assuming the molecular diameter to be 60 nm. [b] Contains $1^1/_2$ copies of the yeast 2 μ circle (Hartley & Donelson, 1980). [c] Lengths calculated from the Broesma (1981) equation, assuming the molecular diameter to be 26 nm. [d] Adapted from Stellwagen (1985).

amplitude of the birefringence observed for the DNA fragment imbedded in the agarose gel is larger than observed in free solution, the difference increasing approximately linearly with DNA molecular weight. The markedly increased amplitude of the birefringence of the 1426 and 2936 bp fragments probably reflects the stretching of these molecules in the gel, since the rate of disorientation of these fragments after removal of the electric field is characteristic of the fully stretched molecules [Table I and Stellwagen (1985)]. The rate of disorientation of the 633 bp fragment in a 0.5% agarose gel indicates that it retains its free solution conformation in this matrix (Stellwagen, 1985). Stretching of the DNA molecules would be expected to increase the optical anisotropy factor, the measure of the difference in refractive index parallel and perpendicular to the helix axis. Elongation of the DNA molecules in the gel would also be expected to increase the ease of orientation in the electric field (Stellwagen, 1981). Both factors would tend to increase the observed electric birefringence signal of the oriented stretched molecules.

Both Linear and Supercoiled DNA Molecules Become Stretched When Oriented in Agarose Gels. The birefringence relaxation times of supercoiled and linear DNA molecules in free solution and imbedded in 1.0% agarose gels are compared in Table I. Only the longest or terminal relaxation times are given for each sample. The standard deviations of the measurements are also given to indicate the reproducibility of the results. All relaxation times were independent of DNA concentration, pulse length, applied electric field strength, and pulsing history of the solution or gel.

Both linear and supercoiled DNA molecules exhibit increased relaxation times in agarose gels, indicating that both become stretched upon orientation in the gel matrix. The 1426 and 2936 bp linear restriction fragments exhibit relaxation times that are characteristic of the fully stretched molecules (Table I). The 622 bp restriction fragment exhibits a relaxation time in the 1.0% agrose gel that corresponds to the fragment stretched out to 84% of its contour length. This fragment exhibits a relaxation time characteristic of the fully stretched molecule in a 1.5% agarose gel (Stellwagen, 1985). The relaxation times of all three fragments were independent of whether or not steady-state orientation was achieved before removal of the electric field. Different relaxation behavior is observed for λ DNA in a 1.0% agarose gel. Although the relative increase in its relaxation time in the gel was about the same as observed for the 2936 bp fragment, the apparent length calculated for λ DNA was only 14% of the value expected for the fully stretched molecule. Because the longest pulses available from the Cober pulsers used here were about 4 ms in duration, the birefringence signals exhibited by λ DNA in free solution or in the gel were far from the steady-state values (see Figure 5). Under these conditions very large DNA molecules orient by segments in an electric field (Lewis et al., 1987), and the terminal relaxation times reflect the disorientation of these segments (Lewis et al., 1987; see also below). Efforts are currently under way to extend these measurements to longer pulses, to reach the steady-state birefringence of λ DNA.

Supercoiled DNA Molecules Oriented in Agarose Gels Are Less Completely Stretched Than Linear Molecules. The relative change in the terminal relaxation time observed for supercoiled DNA molecules imbedded in agarose gels was much less than observed for linear DNAs. Plasmids pBR322 and PUC19 in free solution exhibit relaxation times that correspond to apparent lengths about 65% of the values calculated for the fully stretched molecules, assuming the fully stretched molecules to be half as long as their contour lengths. When imbedded in 1.0% agarose gels, the relaxation times of plasmids pBR322 and PUC19 correspond to lengths about 80% of the calculated maximum values. Since the PUC plasmids are derived from pBR322 (Yanisch-Perron et al., 1985), it is not surprising that the hydrodynamic behavior of these two plasmids appears to be similar. Plasmid p82-6B appears to be much more highly supercoiled than the other two, with an apparent length equivalent to about 40% of its estimated maximum value in free solution and 45% in 1.0% agarose gels. Electrophoresis of p82-6B in 1% agarose gels showed that p82-6B migrated in two bands, the faster of which comigrated with 10 kb linear DNA. The terminal birefringence relaxation time observed for p82-6B in 1% agarose gels would suggest that its electrophoretic mobility should be equivalent to about a 4 kb linear DNA molecule (Table I). Hence comparison of the mobility of supercoiled DNA molecules with the mobility of linear DNA molecules in gels cannot be used to draw conclusions about the apparent hydrodynamic dimensions of the supercoiled molecules in free solution or in the gel.

Reversing Electric Fields Do Not Cause Disorientation of Completely Oriented DNA Molecules at the Moment of Field Reversal. When the electric field is rapidly reversed in polarity, very little change in the amplitude of the birefringence signal is observed if the DNA molecules had reached steady-state orientation in the gel before field reversal, as shown in Figure 4a for a linear 2936 bp restriction fragment and in Figure 4e for supercoiled pBR322. These results indicate that the orientation of the DNA molecule is virtually unchanged upon field reversal if the DNA had reached its equilibrium orientation before the field was changed in direction. Since orientation of these molecules in the gel is

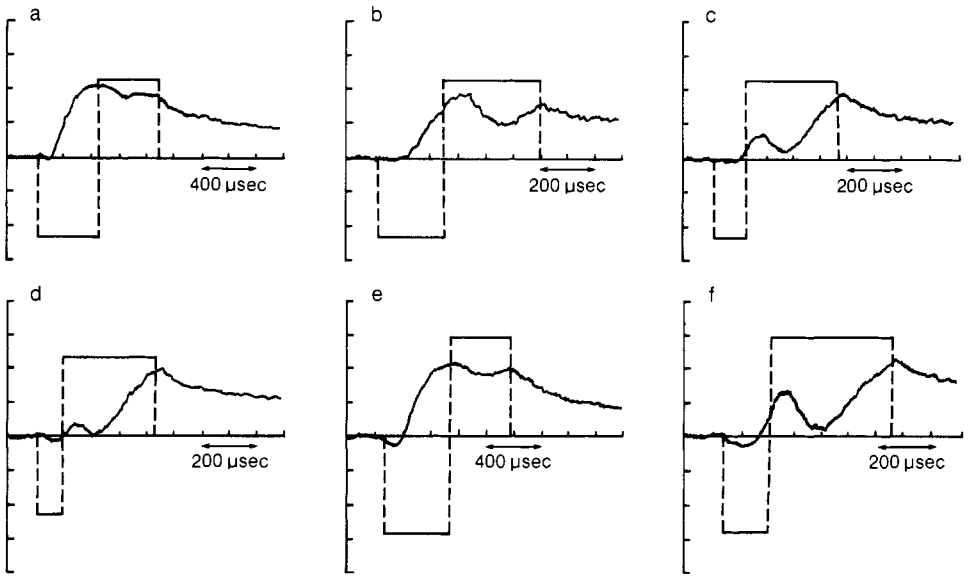

FIGURE 4: Oscilloscope traces of birefringence signals observed for DNA samples in 1% agarose gels when the electric field is rapidly reversed in polarity, as a function of the length of the orienting pulse in the initial field direction: (a) 2936 bp linear DNA, 330/330-μs reversing pulse; (b) 2936 bp linear DNA, 250/330-μs reversing pulse; (c) 2936 linear DNA, 120/330-μs reversing pulse; (d) 2936 bp linear DNA, 70/330-μs reversing pulse; (e) supercoiled pBR322, 450/450-μs reversing pulse; (f) supercoiled pBR322, 170/450-μs reversing pulse. The vertical scale is arbitrary but constant; the direction of negative birefringence is up. The horizontal scale is indicated on each trace. The reversing pulse is indicated by vertical dashed lines between each half of the pulse. In all cases, E = 1.0 kV/cm.

accompanied by stretching (Table I), a completely stretched, oriented DNA molecule can apparently reverse its direction of migration in the gel without having to find a new tube in the new field direction.

Complex Orientation Behavior Can Be Observed if Highly Stretched DNA Molecules Had Not Reached Steady-State Orientation in the Gel before Field Reversal. This is shown in panels b–d and f of Figure 4 for a linear 2936 bp restriction fragment and for supercoiled pBR332, respectively. If the electric field reverses its direction before steady-state orientation is reached, the birefringence signal (and hence the orientation) continues to increase in absolute value during the pulse of opposite polarity until it goes through a maximum, the value of which depends on the length of the initial orienting pulse. The birefringence signal then decreases, passes through a minimum, and increases again, presumably because of the orientation of the DNA molecules in the new field direction. This behavior is observed even if the first pulse is so short that no apparent orientation takes place in the initial field direction (Figure 4c,d,f).

If the pulse in the initial field direction is at least half as long as needed to reach steady-state orientation, the amplitude of the birefringence continues to increase after field reversal until the steady-state value is reached (Figure 4b). For the experiment illustrated in Figure 4b, steady-state birefringence is achieved after a 330-μs pulse (Figure 4a). It is tempting to speculate that, once orientation has reached a certain degree of completion, it continues to equilibrium as long as the electric field is applied, even though the field has changed direction.

The percentage of the birefringence signal remaining at the time of minimum orientation (during the second pulse) is approximately proportional to the length of the first pulse, up to steady-state orientation, because of the increased orientation of the DNA molecules during the first pulse. The time required to reach the minimum orientation during the second pulse increases with DNA molecular weight and also with increasing length of the first pulse, as can be seen from Figure 4. The time at which the minimum orientation is reached decreases linearly with increasing electric field strength, probably because orientation in the new field direction occurs with a shorter lag period at higher electric field strengths (Figure 2).

It is very difficult to rationalize the type of orientation behavior observed if the electric field is reversed in direction before the steady-state birefringence is reached in the initial field direction (Figure 4b,c,d,f). The lack of a transient response at the moment of field reversal is consistent with an "induced dipole" type of orientation mechanism (Tinoco & Yamaoka, 1959). However, this type of orientation mechanism does not explain the subsequent decrease, and then increase, in the amplitude of the birefringence as the pulse is continued in the second field direction. These effects must be attributed to changes in orientation of the DNA molecules in the agarose gel matrix.

A combination of events is probably responsible for the increase, decrease, and subsequent increase in the birefringence signal observed during the second half of the reversing pulse. (1) Partially oriented molecules may continue to orient at field reversal because of the rapid reversal in orientation of the agarose gel matrix (Figure 1d). (2) Completely oriented molecules remain oriented at field reversal, contributing a relatively constant birefringence signal during the second pulse (Figure 4a,e). (3) Other DNA molecules begin to orient in the new field direction, increasing the negative birefringence signal. A lag period would probably be observed before orientation of these molecules, as observed during the first half of the pulse. (4) Field-free and field-assisted decay of the birefringence also occur, decreasing the birefringence signal after field reversal (see below). (5) It is even possible, although energetically less likely, that partially oriented molecules might pass through an orientation roughly perpendicular to the electric field in the process of reorientation, which would give rise to a transiently observed positive birefringence signal. Most of these possibilities imply that the orientation of DNA molecules imbedded in agarose gels has "directional" character. It is possible that individual DNA molecules cannot orient in the electric field unless one end is near a "pore" in the agarose gel matrix; different populations of molecules may have ends near such pores in each of the two electric field directions. A similar "head-first" orientation mechanism was proposed by

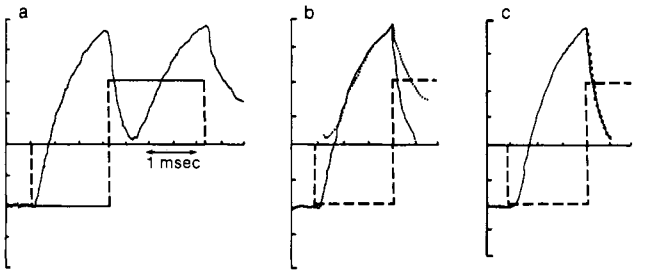

FIGURE 5: Orientation and disorientation of λ DNA imbedded in a 1.0% agarose gel when subjected to a reversing electric field. (a) Oscilloscope trace of the birefringence signal observed during a 1.6/2.0-ms reversing pulse, E = 800 V/cm; (b) superposition of the signal observed during the first half of the reversing pulse (solid line) with the signal observed during the second half of the reversing pulse (dotted line); (c) superposition of the signal observed during the first half of the reversing pulse (solid line) with the inverse of the initial rise curve (dotted line), displaced horizontally to eliminate the lag period. In (b) and (c), the position of the pulse is indicated by a dashed line.

Southern et al. (1987) to rationalize the molecular weight separation achieved during pulsed gel electrophoresis. Efforts are currently in progress to extend these studies to lower electric field strengths, which will be more relevant to actual electrophoretic conditions.

Simpler Reversing Field Behavior Is Observed for λ DNA Imbedded in Agarose Gels. The reversing field behavior observed for λ DNA in a 1.0% agarose gel is illustrated in Figure 5a. After a lag period (short on the time scale of the pulses illustrated), the negative birefringence increases during the first pulse, decreases immediately when the field is reversed, and then begins to increase again during the second pulse. None of the complex time-dependent behavior observed for the 2936 bp fragment or for pBR322 during the second half of the reversing pulse is observed for λ DNA.

The relaxation behavior observed for λ DNA in 1% agarose gels was also different from that of the smaller linear DNA molecules (Table I). The relaxation time observed for the linear 2936 bp fragment in 1% agarose gels was characteristic of the fully stretched molecule. However, the relaxation times observed for λ DNA suggest that its apparent end-to-end length increased only from 7% to 14% of its contour length when it was imbedded in the gel. Therefore, the observed birefringence signal must have been due to the orientation of segments of the molecule in the electric field (Lewis et al., 1987), and not to the orientation of the molecule as a whole. The oriented segments apparently lose their orientation relatively quickly in the reversing field, causing the marked decrease in the birefringence signal at field reversal. These results imply that oriented segments cannot simply reverse their direction of migration in the electric field, probably because the rest of the molecule has not yet entered the tube in the gel matrix. Orientation in the new electric field direction apparently must occur from the opposite end of the molecule, or else the previously oriented segment must find a new tube heading in the new field direction. Hence, the type of transient behavior observed at field reversal appears to depend on whether segments of the molecule or essentially the whole molecule has been oriented in the direction of the initial electric field.

The Disorientation of Very Large DNA Molecules during Field Reversal Occurs at a Rate Faster Than the Field-Free Decay of the Orientation. The field-assisted and field-free rates of disorientation can be compared for λ DNA by overlaying the field-free decay on the reversing field-induced decay of the birefringence, as shown in Figure 5b. The rise of the

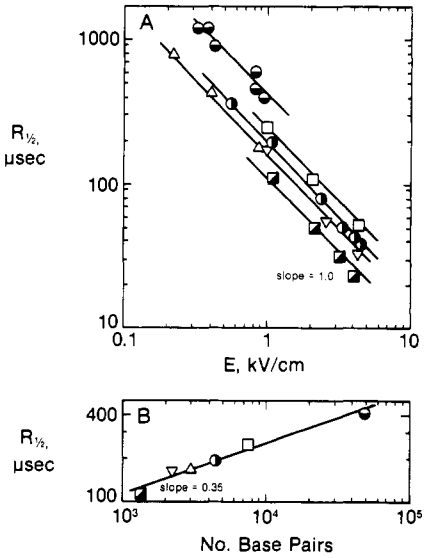

FIGURE 6: (A) Log–log plot of the dependence of the time required for the birefringence to rise to half of its steady-state value, $R_{1/2}$, on the applied electric field strength, E. (B) Log–log plot of the dependence of the rise time ($R_{1/2}$) on DNA molecular weight, in base pairs, at E = 1.0 kV/cm. (◒) λ DNA; (□) plasmid p82-6B; (▽) circular pBR322; (△) 2936 bp fragment; (◑) linearized pBR322; (◪) PUC19. The circular plasmids are plotted as though their molecular weights were half the actual values.

birefringence occurs at the same rate in the two field directions, but the loss of orientation is more rapid in the reversing field, even at the earliest times after the field is removed or reversed. The field-assisted rate of decay of the birefringence is approximately equal to that of the rise of the birefringence (after the lag period), as shown in Figure 5c. Therefore, the rise time can be used as a qualitative measure of the rate of the field-assisted decay.

The rise times of various DNA samples are compared as a function of electric field strength in Figure 6A. Because of the field-dependent lag period before orientation (Figure 2), the rise time is defined here as the time required for the birefringence to rise to half its steady-state value. The rise time defined in this manner is also equal to the decay time at field reversal measured by the intercept of the tangent to the initial decay with the axis of zero birefringence (O'Konski & Haltner, 1956). For all samples, the rise time decreases linearly with increasing electric field strength (Figure 6a). Similar results have been observed by Holzwarth et al., at much lower electric field strengths. The rise time increases with increasing molecular weight, approximately as the 1/3 power (Figure 6b). Hence the rates of orientation and field-assisted disorientation exhibit a markedly lower dependence on molecular weight than the field-free decay of the birefringence, which increases as $N^{2.8}$ (where N is the number of base pairs; Stellwagen, 1985). Figure 6 shows that the rates of orientation and field-assisted disorientation of the birefringence scale as $N^{0.3}E^{-1}$. Slater and Noolandi (1986) have proposed that the stretching of DNA molecules during electrophoresis should scale as $t_{str} \sim N^1E^{-2}$. The difference between the two scaling relations probably reflects the fact that the rise of the birefringence measures orientation as well as stretching.

Very Different Reversing Field Behavior Is Observed for DNA Fragments in Free Solution. The birefringence of DNA in free solution exhibits no delay before orientation, as shown in Figure 1e for supercoiled pBR322. If steady-state orientation is reached before field reversal, a 10–15% decrease in the birefringence signal is observed at the moment of field

reversal, after which the birefringence signal returns to its steady-state value, as observed previously (Stellwagen, 1967; Hanss & Bernengo, 1973; Colson et al., 1974; O'Konski, 1976; Elias & Eden, 1981). If field reversal occurs before the steady-state birefringence is reached, the birefringence signal decreases about 10–15% and then increases to a higher value, approximately equal to the value that would have been reached if the pulse had continued in the original direction without reversing (Figure 1f). No complicated orientation and disorientation patterns are observed in the second half of the reversing pulse in free solution.

Conclusions

The experiments described above have shown that linear and supercoiled DNA molecules can be oriented in electric fields when imbedded in agarose gels. Orientation of DNA molecules larger than 1.5 kb occurs only after a lag period, which decreases with increasing electric field strength and is approximately independent of DNA molecular weight. Reversing electric fields cause an accelerated disorientation of the DNA, compared with the field-free rate of decay, followed by reorientation in the new field direction. If the DNA has reached its equilibrium orientation in the field before the field is reversed in direction, very little disorientation takes place after field reversal, and the DNA can move in the new field direction with little or no hindrance to its motion. However substantial disorientation occurs after field reversal if equilibrium orientation is not achieved in the initial field direction. The times required for disorientation and reorientation of the DNA increase with DNA molecular weight and decrease with increasing electric field strength. The agarose gel matrix is also oriented by the electric field.

References

Adolf, D. (1987) *Macromolecules 20*, 116–121.

Bean, C. P. (1985) *Biophys. J. 47*, 337a.

Bearden, J. C., Jr. (1979) *Gene 6*, 221–234.

Benoit, H. (1951) *J. Chim. Phys. Phys.-Chim. Biol. 48*, 612–614.

Broersma, S. (1981) *J. Chem. Phys. 74*, 6989–6990.

Carle, G. F., & Olson, M. V. (1984) *Nucleic Acids Res. 12*, 5647–5664.

Carle, G. F., Frank, M., & Olson, M. V. (1986) *Science (Washington, D.C.) 232*, 65–68.

Colson, P., Houssier, J. C., & Fredericq, E. (1974) *Biochim. Biophys. Acta 340*, 244–261.

deGennes, P. G. (1971) *J. Chem. Phys. 55*, 572–579.

deGennes, P. G. (1976a) *Macromolecules 9*, 587–593.

deGennes, P. G. (1976b) *Macromolecules 9*, 594–598.

deGennes, P. G. (1979) *Scaling Concepts in Polymer Physics*, Cornell University Press, Ithaca, NY.

Diekmann, S., & Porschke, D. (1982) *Biophys. Chem. 16*, 261–267.

Diekmann, S., Hillen, W., Morgeneyer, B., Wells, R. D., & Porshke, D. (1982) *Biophys. Chem. 15*, 263–270.

Elias, J. G., & Eden, D. (1981) *Macromolecules 14*, 410–419.

Fangman, W. L. (1978) *Nucleic Acids Res. 5*, 653–665.

Fesjian, S., Frisch, H. L., & Jamil, T. (1986) *Biopolymers 25*, 1179–1184.

Fredericq, E., & Houssier, C. (1973) *Electric Dichroism and Electric Birefringence*, Clarendon Press, Oxford.

Hagerman, P. J. (1981) *Biopolymers 20*, 1503–1535.

Hanss, M., & Bernengo, J. C. (1973) *Biopolymers 12*, 2151–2159.

Hartley, J. L., & Donelson, J. E. (1980) *Nature (London) 286*, 860–863.

Hervet, H., & Bean, C. P. (1986) *Biopolymers 26*, 727–742.

Holzwarth, G., McKee, C. B., Steiger, S., & Crater, G. (1987) *Nucleic Acids Res. 15*, 10031–10044.

Houssier, C., & Fredericq, E. (1964) *Biochim. Biophys. Acta. 88*, 450–452.

Jonsson, M., Åkerman, B., & Nordén, B. (1988) *Biopolymers 27*, 381–421.

Krause, S., Ed. (1981) *Molecular Electro-Optics*, Plenum Press, New York.

Lerman, L. S., & Frisch, H. L. (1982) *Biopolymers 21*, 995–997.

Lewis, R. J., Pecora, R., & Eden, D. (1987) *Macromolecules 20*, 2579–2587.

Lumpkin, O. J., & Zimm, B. H. (1982) *Biopolymers 21*, 2315–2316.

Lumpkin, O. J., Dejardin, P., & Zimm, B. H. (1985) *Biopolymers 24*, 1573–1593.

McDonnell, M. W., Simon, M. N., & Studier, F. W. (1977) *J. Mol. Biol. 110*, 119–146.

O'Konski, C. T., Ed. (1976) *Molecular Electro-Optics*, Marcel Dekker, New York.

O'Konski, C. T., & Haltner, A. J. (1956) *J. Am. Chem. Soc. 78*, 3604–3610.

O'Konski, C. T., Yoshioka, K., & Orttung, W. H. (1959) *J. Phys. Chem. 63*, 1558–1565.

Olvera de la Cruz, M., Deutsch, J. M., & Edwards, S. F. (1986) *Phys. Rev. A 33*, 2047–2055.

Pytkowicz, R. M., & O'Konski, C. T. (1959) *Biochim. Biophys. Acta 36*, 466–470.

Schwartz, D. C., & Cantor, C. R. (1984) *Cell (Cambridge, Mass.) 37*, 67–75.

Schwartz, D. C., Saffran, W., Welsh, J., Haas, R., Goldenberg, M., & Cantor, C. R. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 189–195.

Slater, G. W., & Noolandi, J. (1986) *Biopolymers 25*, 431–434.

Southern, E. (1979) *Anal. Biochem. 100*, 319–323.

Southern, E. M., Anand, R., Brown, W. R. A., & Fletcher, D. S. (1987) *Nucleic Acids Res. 15*, 5925–5943.

Stellwagen, N. C. (1967) Ph.D. Thesis, University of California, Berkeley.

Stellwagen, N. C. (1981) *Biopolymers 20*, 399–434.

Stellwagen, N. C. (1984) *Biochemistry 23*, 6311–6319.

Stellwagen, N. C. (1985) *Biomol. Struct. Dyn. 3*, 299–314.

Tinoco, I., Jr., & Yamaoka, K. (1959) *J. Phys. Chem. 63*, 423–427.

Viovy, J. L. (1987) *Biopolymers 26*, 1929–1940.

Wijmenga, S. S., & Maxwell, A. (1986) *Biopolymers 25*, 2173–2186.

Yanisch-Perron, C., Viera, J., & Messing, J. (1985) *Gene 33*, 103–119.