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Structures and Dynamics of a Supercoiled DNA†

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ABSTRACT: Secondary structures of supercoiled (RF) M13mp7 DNA are investigated by time-resolved fluorescence polarization anisotropy, which monitors the magnitude and uniformity of the torsional rigidity. Tertiary structures are monitored by gel electrophoresis. Seven distinct long-lived structural conformers of this supercoiled DNA are identified: four result directly from different replicates of the same standard preparation procedure; one results from an alternate preparation; and two result from irreversible conversions of such forms to daughter products. These seven conformers all exhibit either of two different, but apparently uniform, torsional rigidities, depending upon the buffer type. These and other data imply that two different secondary structures can prevail in this supercoiled DNA and that neither is ordinary B helix. Each conformer also exhibits one of three basic gel mobilities. The observed dual secondary structures, metastability, and hysteresis of this DNA are shown to follow naturally, if the primary function of supercoiling is actually to facilitate remote control of gene activity by site-specific regulatory proteins. A specific model is proposed for gene regulation by protein control of remote junctions between secondary structure domains. The previously inexplicable stimulatory effect of the prmup-1 mutation in the right operator region of the λ repressor is rationalized by certain aspects of this model.

Supercoiling of DNA is required for transcription, replication, recombination, and site-specific binding of certain proteins (Kornberg, 1980; Bauer, 1978; Cozzarelli, 1980; Gellert, 1981). It is widely suspected that the main function of supercoiling is to induce alterations in DNA secondary structure that critically affect its interactions with proteins involved in gene expression (Kornberg, 1980; Bauer, 1978; Cozzarelli, 1980; Gellert, 1981; Wells et al., 1977). Such structural variants may include locally denatured regions (Wells et al., 1977; Dasgupta et al., 1977), cruciform hairpins at inverted repeats (Wells et al., 1977; Lilley, 1980; Panayotatos & Wells, 1981; Mizuuchi et al., 1982; Courey & Wang, 1983), and stretches of Z helix (Klysik et al., 1981; Peck et al., 1982; Nordheim & Rich, 1983a,b). Enhanced susceptibility to S1 nuclease (Wells et al., 1977; Dasgupta et al., 1977; Lilley, 1980; Panayotatos & Wells, 1981), reduced susceptibility to

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*Eco*RI nuclease (Mizuuchi et al., 1982; Courey & Wang, 1983; Lilley et al., 1983), or a change in sequence specificity for cleavage by antibiotics (Mirabelli et al., 1983) presently comprises the bulk of the evidence for such deviant structures, except in the case of Z helix.

The torsional rigidity of a DNA should depend on its local secondary structure. Thus, the dynamics of its Brownian torsional deformations, as manifested in the time-resolved fluorescence polarization anisotropy (FPA) of intercalated ethidium dye (Barkley & Zimm, 1979; Allison & Schurr, 1979; Wahl et al., 1970; Thomas et al., 1980; Millar et al., 1982), should provide an informative secondary structure probe.

Three unstated assumptions underlie most work on supercoiled DNAs. (1) Equilibration among the accessible tertiary, and also secondary, structures is sufficiently rapid that the DNA is practically always at structural equilibrium. (2) Mobilities in gel electrophoresis faithfully reflect any differences or changes in secondary structure, which are immediately transmitted to the tertiary structure sensed by the gel. Implicit here is the assumption that DNA secondary structure is unaffected by any buffer changes incurred as the DNA is introduced into the gel. (3) Alternate structures induced by supercoiling are always highly sequence dependent and therefore local in extent. We present evidence here for the existence of seven distinct long-lived structural states of a common supercoiled DNA, each of which exhibits one of *two* different, but apparently uniform, torsional rigidities and one of only three basic gel mobilities. Our findings provide substantial evidence that either of *two* distinct global secondary structures can prevail in this DNA and in one way or another vitiate all three of the above assumptions. A discussion is given of how our findings might pertain to the function of supercoiling.

Theoretical Background

Theories relating the decay of the FPA to the torsional rigidity of cylindrically symmetric filaments with immobile symmetry axes have been developed (Barkley & Zimm, 1979; Allison & Schurr, 1979). The problem of how to *correctly* incorporate tumbling of the local symmetry axis into the FPA has also witnessed substantial progress (Robinson et al., 1980; Allison et al., 1982; Schurr, 1982), the most recent of which is described in a forthcoming paper.

When rapid librations of the fluorophore relative to the filament are ignored, the FPA of an elastic filament of arbitrary length is given by

$$r(t) \equiv \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} = r_0[[(3/2)\cos^2\epsilon - (1/2)]^2 F_0(t) + 3\sin^2\epsilon\cos^2\epsilon\, C_1(t)F_1(t) + (3/4)\sin^4\epsilon\, C_2(t)F_2(t)] \quad (1)$$

where $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the emission intensities with polarizations parallel and perpendicular, respectively, to that of the instantaneous excitation pulse, r_0 is the initial anisotropy, and $\epsilon = 70.5°$ is the polar angle of the fluorophore transition dipole with respect to the local symmetry axis. The twisting correlation functions, $C_n(t) \equiv \langle \exp[-n^2\langle\Delta_z(t)^2\rangle/2]\rangle_R$, $n = 1$ or 2, and the tumbling correlation functions, $F_n(t) \equiv \langle \exp[-(6 - n^2)\langle\Delta_x(t)^2\rangle/2]\rangle_R$, $n = 0$, 1, or 2, are defined in terms of mean-squared angular displacements $\langle\Delta_j(t)^2\rangle$ about the local *body-fixed* symmetry ($j = z$) and transverse ($j = x$) axes, respectively, and the subscript R denotes an average over all subunits, or rod elements, to which the fluorophore could bind. The rather different anisotropy expression of Barkley & Zimm (1979) is equivalent to eq 1 only when axial tumbling makes no contribution to the depolarization, i.e., when $F_n(t) = 1.0$, and is otherwise incorrect.

A sequence of simple formulas representing the complete time course of $C_n(t)$ for filaments containing arbitrary numbers $N + 1$ of base pairs has been obtained and is presented elsewhere. For DNAs with $N + 1 \geq 3000$ base pairs and t in the range 10^{-11} to 1.3×10^{-7} s, the well-known intermediate zone formula (Barkley & Zimm, 1979; Allison & Schurr, 1979; Allison et al., 1982; Schurr, 1982) applies:

$$C_n(t) = \exp\left[\frac{-n^2 k_B T t^{1/2}}{(\pi\alpha\gamma)^{1/2}}\right] \quad (2)$$

Here α is the torsion elastic constant between base pairs, and $\gamma = 4\eta\pi a^2 h$ is the friction factor for axial rotation of a rod of radius a and height h in a fluid of viscosity η. Taking $h = 0.34$ nm and $a = 1.2$ nm for one base pair, one has $\gamma = 6.12 \times 10^{-23}$ dyn·cm·s (6.12×10^{-30} J·s) in water at 20 °C.

In this work, we ignore any contribution of helix axis reorientation in the first 120 ns and set $F_0(t) = 1.0 = F_1(t) = F_2(t)$. Barkley and Zimm present an approximate treatment of the bending of very long filaments and propose a formula for $\Delta(t) = 2\langle\Delta_x(t)^2\rangle$, which in turn yields $F_0(t)$, $F_1(t)$, and $F_2(t)$, but its validity is open to question. Our initial attempts to include the longitudinal tensile/compressive forces missing from their treatment strongly suggest that their theory significantly overestimates the tumbling contribution (unpublished calculations). Firm evidence for such overestimation comes from a Brownian dynamics simulation of a short wormlike coil by S. A. Allison and J. A. McCammon (unpublished results). We also have empirical evidence (to be published elsewhere) that the Barkley–Zimm result for $F_0(t)$ does not work as well for 600-base-pair fragments as does the experimental birefringence decay curve of Elias and Eden (obtained from D. Eden), which makes only a negligible contribution during the first 120 ns. If, instead of 1.0, the Barkley–Zimm formula for $F_n(t)$ (with a persistence length of 500 Å) is used in eq 1 to fit either real or simulated data for long DNAs, the best fit value of α is increased by a factor of 1.62 ± 0.03 on all time scales, but no other conclusions are affected. When the correct formulas for $F_n(t)$ are ultimately identified, the present values of α can be scaled up to their true values by a similar fitting exercise.

Validity of eq 1 and 2 with $F_n(t) = 1.0$ was first demonstrated by Thomas et al. (1980), who showed for linear $\phi 29$ DNA that the same value $\alpha = 3.8 \times 10^{-12}$ dyn·cm (3.8×10^{-19} N·m) could be obtained by deconvoluting data collected over different time spans (0–18, 0–32, 0–64, and 0–120 ns). This constitutes an exceedingly stringent test of eq 2, as well as other formulas considered for $C_n(t)$ (Thomas et al., 1980). This also indicates that the torsional rigidity is uniform, free of any major weaknesses in the range from about $^1/_{20}$ to $^1/_{1000} - ^1/_{2000}$ base pairs (Thomas et al., 1980). Millar et al. (1982, 1981, 1980) subsequently obtained the same value of α (corrected to our analysis) from long time span (5–100 ns) data for calf thymus DNA but did not investigate the question of uniformity. They also examined a single sample of supercoiled PBR322 plasmid DNA and obtained a somewhat larger α than observed for calf thymus DNA (Millar et al., 1982) but pursued the problem no further.

Experimental Procedures

Filamentous M13mp7 virus is a commonly used cloning vector that contains single-strand DNA (Messing et al., 1981). We work with the double-strand replicative form (RF) of M13mp7 DNA, which contains 7238 base pairs, including a

single inverted repeat of 48 base pairs (Denhardt et al., 1981). This DNA exists normally as covalently closed circles containing approximately 50 negative superhelical turns (Bauer, 1977).

Incubation and harvesting of the cells and isolation of the RF DNA are carried out by the alkaline extraction method of Birnboim & Doly (1979). The resulting ethanol-precipitated RF DNA is redissolved in TES buffer [0.05 M tris(hydroxymethyl)aminomethane (Tris), 0.005 M ethylenediaminetetraacetic acid (EDTA), 0.05 M NaCl, pH 8], extracted with BRL ultrapure phenol, which is preequilibrated with 3% NaCl and titrated to pH 6.7, and then dialyzed vs. TES for ≥5 h at 4 °C. CsCl is added to 0.98 g/mL and ethidium bromide (EB) to 0.7 mg/mL, and the sample is then centrifuged at 14 000 rpm in a Beckman 65 rotor to remove debris. The supernatant is collected and centrifuged at 45 000 rpm in the same rotor for 24 h to band the RF DNA. The band is visualized by UV illumination, collected, and rebanded by centrifugation for another 24 h in the same solvent. Ethidium is removed by repeated extractions with 2-propanol saturated with 20× standard saline citrate (SSC) (0.15 M NaCl, 0.015 M sodium citrate). The DNA solution is then dialyzed against one of the following buffers: (1) 2 mM Tris, 0.05% $CHCl_3$, pH 7.0–8.6; (2) 0.1× SSC, 2 mM Na_2EDTA, 0.05% $CHCl_3$, pH 7.0; (3) 0.01× SSC, 0.2 mM Na_2EDTA, 0.05% $CHCl_3$, pH 7.0. In one case, half of the sample was dialyzed vs. buffer 1 and half vs. distilled water, which had no effect on the results.

After dialysis, sodium dodecyl sulfate (SDS) and proteinase K (Boehringer Mannheim) were added to 0.1% and 75 μg/mL, respectively, and incubated at 37 °C for ≥1 h. Phenol extractions were repeated until no precipitate formed at the interface, and the solution was dialyzed into its final buffer, usually 1 or 3 above. Aliquots were assayed for contaminating ammonium ions with fluorescamine (after dialyzing out any Tris) (Udenfriend et al., 1972). The assay level of contamination was less than or equal to that produced by 0.005 g of bovine serum albumin/g of DNA, which is practically at the sensitivity limit of our fluorescamine test. Typically, 90% or more of the DNA was present in the supercoiled form, the remainder in relaxed circles. The conformer designated 2a in the sequel was prepared by an alternate procedure, namely, column chromatography using the NACS-37 resin and procedures described in the BRL manual, followed by dialysis into 10 mM Tris, 0.01 M NaCl, and 1 mM Na_2EDTA, pH 7.0.

Horizontal slab gels containing 0.3% agarose, 40 mM Tris, 5 mM H_3BO_3, and 1 mM Na_2EDTA at pH 9 were prepared immediately prior to use. Typically, 0.25 μg of DNA was run in each track for 24 h at 30 V, with the first 0.5 h at 60 V. After being stained, the bands were visualized with a UV transilluminator and migration distances measured to the nearest millimeter directly, and also occasionally in photographs.

Fluorescence polarization anisotropy measurements were performed with the apparatus and procedures described by Thomas et al. (1980). The Terak/LSI11 microprocessor is now linked to a VAX 11/780, which carries out the deconvolutions in less than 1 min and graphically displays the experimental data, best fit curve, and their difference on a terminal at the site. Correction of a minor error in the weights used in the fitting program for the anisotropy has decreased all our reduced χ^2 values to 1.0–1.5 but left the previous best fit values of α virtually unchanged.

Sample solutions contained 0.02–0.05 mg/mL DNA, and the base pair to EB ratio was greater than 150:1. Assuming an unwinding angle of 26° for intercalated EB (Pulleyblank & Morgan, 1975; Shure et al., 1977; Wang, 1974), this amount of dye causes a loss of 3.5 supercoils out of 50. Gels were run both before and immediately after the FPA measurements to ensure that no persistent changes had occurred. All FPA measurements were made near neutral pH at 20 °C. Circular dichroism (CD) spectra were measured on a Dichrograph Mark III spectrometer.

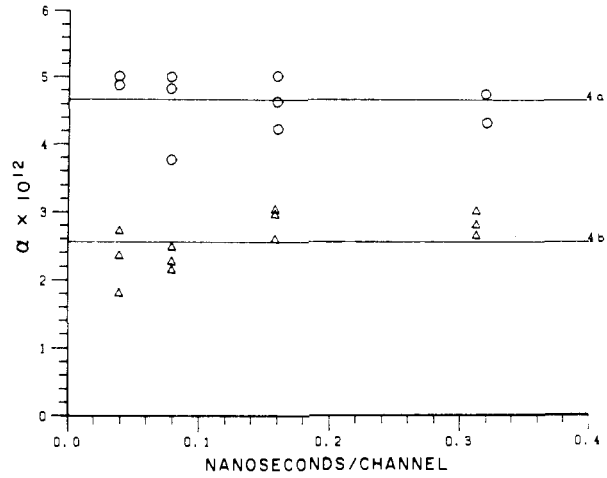


FIGURE 1: Best fit values of the torsion constant α vs. time span of the experiment for conformers 4a (circles) in Tris buffer and 4b (triangles) in citrate buffer. The time spans corresponding to the indicated channel delays are very nearly 0–18, 0–35, 0–70, and 0–120 ns. The α values are obtained by least-squares deconvolution of the difference data $[I_\parallel(t) - I_\perp(t)]$ with eq 1 and 2 in conjunction with the excitation profiles and best fit fluorescence decay curves, exactly as described by Thomas et al. (1980).

Results

The fluorescence lifetime of the dye is 21.0 ± 0.7 ns in all cases, which indicates a normal intercalation site. Figure 1 presents the torsion constants α obtained by least-squares deconvolution of the difference $[I_\parallel(t) - I_\perp(t)]$ data as a function of experimental time span for two different samples with the same gel mobility. Within the experimental uncertainty, the best fit values of α for all of our supercoiled and linearized DNAs are independent of the time spans employed in these experiments, which are 0–18, 0–35, 0–70, and 0–120 ns. This implies that the torsional rigidities of these DNAs are uniform, free of major weaknesses, over domains of about 1000–2000 base pairs. Our gels normally exhibit the predominant supercoiled species migrating as a narrow band, trailed by a faint narrow band 2–3 cm behind representing relaxed circles, and no other bands. (These gels are not capable of resolving the natural population of topoisomers of the supercoiled form.) By these criteria the M13mp7 DNAs in every sample are uniform and essentially homogeneous. Nevertheless, the DNAs in different samples are by no means always identical, as is apparent from Figure 1.

We have identified seven distinct structural forms of supercoiled M13mp7 DNA that result either directly from our preparations or from apparently irreversible conversions of such forms to daughter products. Moreover, each of these forms was observed at least twice, in the sense that it resulted from two or more different preparations or preparation–conversion sequences. Table I presents the gel mobilities and torsion constants α measured for these seven forms. The α values apply for 0.01 M Na^+ ion, except for conformers 4a, 1b, and 4′b, which were measured in, respectively, 0.002, 0.02, and 0.02 M Na^+ ion.

The torsion elastic constants of these seven conformers fall into *two* classes, one labeled a with $\alpha = (4.8 \pm 0.6) \times 10^{-12}$

Table I: Torsional Rigidities and Gel Mobilities of Different Conformers of Supercoiled (RF) M13mp7 DNA[a]

conformer	1a	1a′	2a	4a	1b	2′b	4′b
α ($\times 10^{-12}$ dyn·cm)	5.0 ± 0.5	4.2 ± 0.6	5.4 ± 0.4	4.7 ± 0.2	3.0 ± 0.3	2.6 ± 0.4	2.6 ± 0.3
gel mobility (cm/day)	12.5–13	12.5–13	8.5–9	6	12.5–13	9.5–9.7	5

[a] α values for conformers labeled a were measured in buffer containing 2 mM Tris and 10 mM NaCl, except for 4a, which had only the Tris. α values for conformers labeled b were measured in buffer containing 1.5 mM citrate, 0.2 mM EDTA, and 20 mM NaCl, except for 2b where the NaCl was 10 mM.

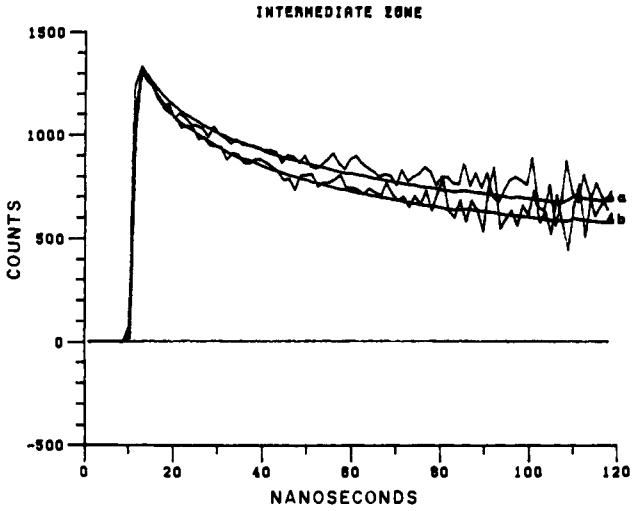


FIGURE 2: Quotient $[I_{\parallel}(t) - I_{\perp}(t)]/S(t)$ of the difference data and the best fit fluorescence decay curve $S(t)$ vs. time for conformers 4a (upper) and 4b (lower) on the 0–120-ns time scale. The pertinent data sets for each conformer have been preaveraged together prior to least-squares fitting for the purpose of the present display. The values of α obtained in this manner are (4.57 ● 0.13) × 10^{-12} dyn·cm (4a) and (2.83 ± 0.09) × 10^{-12} dyn·cm (4b), which are in the ranges of those that appear in Figure 1.

dyn·cm and the other labeled b with $\alpha = (2.8 \pm 0.3) \times 10^{-12}$ dyn·cm. A qualitative indication of the degree of discrimination between the a and b conformers can be gleaned from the data presented in Figures 1 and 2. The gel mobilities are grouped into three classes labeled 1, 2, and 4, which migrate, respectively, 12.5–13, 8.5–9.7, and 5 or 6 cm/day. The conformer label simply combines the designation for gel mobility with that for the torsional rigidity. The primes indicate a presumably minor, but real, distinction. For example, 4′b really does not migrate quite as rapidly as 4a. Likewise, 1a′ is distinguished from 1a by its (probably) significantly smaller α, as well as by other measurements not shown. Such additional measurements include circular dichroism (CD), dynamic light scattering, scattered intensity, and susceptibility to *Bgl*I, all of which will be described elsewhere.

Our standard DNA preparation produces usually class 1 conformers, occasionally class 4 conformers, and never (to date) a class 2 conformer. No obvious correlation between buffer type and mobility class has been observed (to date). In contrast, our supercoiled DNAs *invariably* exhibit a class a torsional rigidity when the buffer is Tris and a class b rigidity when it is citrate or cacodylate, regardless of the mobility class.

Conformer 1a converts spontaneously to a species with class 2 mobility, whose α is class a in Tris and class b in cacodylate. This 1a → 2 conversion is facilitated by high NaCl concentration, is catalyzed by contact with dialysis tubing, and proceeds in a completely homogeneous fashion over a period of about 2 weeks in 1.0 M NaCl. Both supercoiled and relaxed bands migrate with progressively slower mobilities, when analyzed every third day, until the supercoiled band stabilizes at 8.5–9.0 cm/day with the relaxed band about 2.5 cm/day behind. We have been unable to reverse this transformation by any means, including exhaustive dialysis into very low ionic strengths. We also observed the conversions of conformers 4a and 4′b to a species with class 1 mobility after 1.5–2.5 months in 0.01 M NaCl but lacked sufficient material for examination of its FPA. On the basis of these somewhat limited observations, we tentatively nominate class 2 as the equilibrium (tertiary) form of supercoiled M13mp7 DNA. The other mobility classes, which are indeed very long lived, are evidently metastable.

Immediately after linearization of conformer 2′b with *Bgl*I, its mobility increased to 10.2 cm/day, and its α decreased to $(1.33 \pm 0.16) \times 10^{-12}$ dyn·cm. Immediately after linearization of conformer 1a′ with *Bgl*I, its mobility decreased to 11 cm/day, and its α decreased to $(2.78 \pm 0.12) \times 10^{-12}$ dyn·cm. However, over a period of several weeks, the torsion constant of this latter DNA increased to the value characteristic of normal linear DNAs (3.8×10^{-12} dyn·cm), but its mobility remained constant at 11 cm/day, which agrees well with molecular weight markers obtained from a *Hin*dIII digest of λ DNA.

Conclusions and Interpretation

There clearly exist multiple metastable states of supercoiled M13mp7 DNA in addition to its equilibrium structures in Tris and citrate (or cacodylate) buffers. Evidently, equilibration among the different conformers is extraordinarily slow, especially at low NaCl concentration (≤0.01 M). Moreover, long-lived memory of some of these states extends even to their relaxed and linear forms. Two very different conformers of cop608 supercoiled DNA have been observed recently by Brady et al. (1983). We are unaware of any firm evidence for such different metastable conformers in other systems, although anecdotal evidence abounds.

We ascribe the two classes of torsional rigidity, a and b, to different secondary structures in supercoiled M13mp7 DNA. This structural interpretation is supported by CD measurements shown in Figure 3. The fact that the linearized progeny of 1a′ and 2′b still exhibit distinct, though significantly smaller, torsional rigidities immediately after linearization also argues convincingly for a difference in secondary structures of their parent conformers. Neither a nor b secondary structure appears to be Z helix, because their CD spectra are not of that type and, in any case, (linear) Z DNA does not bind ethidium (Pohl & Jovin, 1972). It is also unlikely that either a or b is ordinary B helix, because the structures resulting initially from linearization are metastable and exhibit torsional rigidities too small for B DNA. A more definitive structural assignment cannot be made at present. The uniformity of the torsional rigidities over substantial domains (1000–2000 base pairs), as implied by our results, indicates that the differences in secondary structure between a and b are not primarily sequence dependent and local but are effectively global.

Certain alternating purine–pyrimidine sequences in supertwisted DNAs, undergo a transition to Z helix under physiological conditions (Nordheim & Rich, 1983a,b; Azorin et al., 1983). Quite surprisingly, this equilibrium shifts *away* from Z helix with *increasing salt in superhelical* DNAs but *toward* Z helix *in linear* DNAs (Azorin et al., 1983). We suggest that the equilibrium *in supercoiled DNAs* is a (or b)

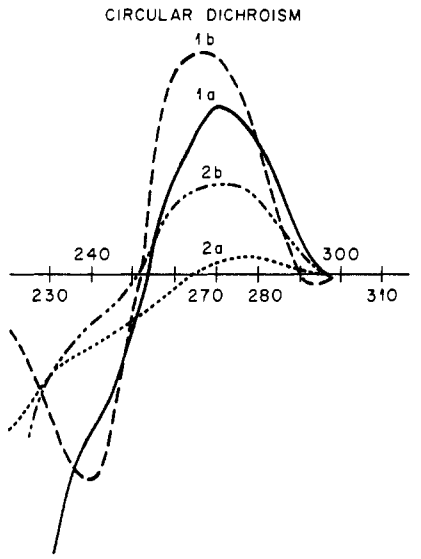


FIGURE 3: Circular dichroism spectra for conformers 1a, 1b, 2a, and 2b. The DNA concentration is 0.05 mg/mL for 1a (in 2 mM Tris-HCl), 0.03 mg/mL for 1b (in 0.1× SSC, 2 mM Na_2EDTA), 0.05 mg/mL for 2a (in 0.2× TES), and 0.04 mg/mL for 2b (in 0.01 M NaCl, 2 mM cacodylate). The spectra are normalized to 0.05 mg/mL DNA concentration.

$\rightleftharpoons$ Z instead of B $\rightleftharpoons$ Z, in which case the salt dependence of the latter may well not apply.

We tentatively ascribe the three classes of gel mobility, 1, 2, and 4, to different tertiary structures. Such an assignment is supported by light scattering measurements (not shown). If these assignments are correct, then the coupling between secondary and tertiary structures is evidently sufficiently weak that either can change without causing an immediate change in the other.

Cruciform extrusion (or absorption) at the 48-base-pair inverted repeat in M13mp7 cannot *by itself* account for most of our observations. In principle, our measurements could be affected either directly by the *local cruciform structure* itself or indirectly by some *global manifestation* of the reduced twisting stress or change in linking number, 4.6 turns in the present case. A direct effect is unlikely, because one small region of anomalous structure in 7238 base pairs could not significantly affect our FPA measurements, unless the dye were strongly clustered at that site. Dye clustering on the cruciform hairpins would yield a highly nonuniform apparent torsional rigidity, as well as a substantial decrease in initial anisotropy due to excitation transfer (Genest et al., 1982; J. C. Thomas, unpublished results), neither of which was observed. Depending upon the nature of the potential surface, the global effect of a reduction in twisting stress could be either a simple decrease in the equilibrium twisting strain of the B helix or a perturbation of the prevailing equilibrium between two or more distinct secondary and/or tertiary structures. If the twisting potential of B helix were somewhat anharmonic, a change in its equilibrium twisting strain could alter its torsional rigidity. Were this the main cause of the change in rigidity from a to b, both conformers should exhibit the rigidity of unstrained B helix immediately upon linearization, contrary to observation. A simple reduction in linking number by 4.6 turns cannot account for the enormous differences in gel mobilities between these supercoiled forms, which exceed the respective differences between these forms and their fully relaxed counterparts. Differences in tertiary structure between these conformers are evidently much more profound. Some coupling of cruciform extrusion to the a $\rightleftharpoons$ b and/or 1 $\rightleftharpoons$ 2 $\rightleftharpoons$ 4 conformational equilibria is not ruled out by our data. Future experiments on DNAs devoid of such palindromes will hopefully illuminate the extent to which cruciform extrusion is coupled to the equilibria between distinct secondary or tertiary structures.

An alternative interpretation of the FPA data could probably be constructed in which the species a and b differed not by their torsional rigidities but instead by their polar angles ϵ between the ethidium transition dipoles and their helix axes. This interpretation would not alter our conclusion that two distinct secondary structures can prevail in supercoiled M13mp7 DNA, depending upon the buffer type, and would affect none of our subsequent discussion.

The present results demonstrate unequivocally that gel electrophoretic mobilities do not faithfully reflect all differences or changes in secondary structure of supercoiled DNAs. Elaboration of this finding, and similar results pertaining to linear DNAs, is deferred to a subsequent paper.

We address now the possible significance of the two secondary structures, metastability, and hysteresis exhibited by supercoiled M13mp7 DNA. Let us suppose that the primary function of supercoiling is to facilitate *remote control* of gene expression by site-specific regulatory proteins, which bind several tens of base pairs, or more, away from the specific polymerase binding site. Any scheme for remote control of polymerase activity necessarily imposes three basic requirements on the *supercoiled* DNA. (1) It must exhibit at least two distinct secondary structures, e.g., a and b, to provide a molecular basis for transmitting information through the intervening n base pairs between the regulatory protein and the polymerase. (2) The free energies (per base pair) $G°_a$ and $G°_b$ of these two secondary structures must be nearly equal. This crucial feature follows from the requirement that a single bound protein must be able to convert an additional n base pairs beyond its binding site from the state b (say) of lower free energy to the state a (say) of higher free energy. That is, there must be a negative free-energy change for the overall process

$$\text{prot·b} + n\text{b} \rightleftharpoons \text{prot·a} + n\text{a} + J_{a|b} + J'_{a|b}$$

where prot·a and prot·b denote complexes of the site-specific protein (which could be either the regulatory or polymerase species) with DNA in, respectively, the a and b conformations and $J_{a|b}$ and $J'_{a|b}$ denote junctions between a and b conformations. This requirement reduces to

$$G°_a - G°_b < [(G°_{\text{prot·b}} - G°_{\text{prot·a}}) - (G°_J + G°_{J'})]/n \qquad (3)$$

where $G°_X$ denotes the standard-state free energy of the species X. We assume that $G°_a > G°_b$ and $G°_{\text{prot·a}} < G°_{\text{prot·b}}$. The junction free energies are necessarily positive [cf. (3) below]. If n is actually as large as several tens of base pairs, then $G°_a - G°_b$ is necessarily rather small *for any realistic value of* $G°_{\text{prot·b}} - G°_{\text{prot·a}}$. (3) The junction free energies must be positive and sufficiently large to ensure propagation of a given secondary structure over a distance of at least n base pairs beyond the binding site. In other words, the degree of cooperativity must be sufficiently large that the average size of a secondary structure domain must substantially exceed n base pairs. DNA usually exhibits a high degree of cooperativity between different secondary structure types, so this is not an exceptional property.

These three requirements for remote control of gene activity, namely, the existence of two or more distinct structural states with nearly equal free energies per subunit and a high free energy to form an interface between them, suffice to induce metastability and hysteresis in any system. Thus, if the function of supercoiling actually is to facilitate remote control

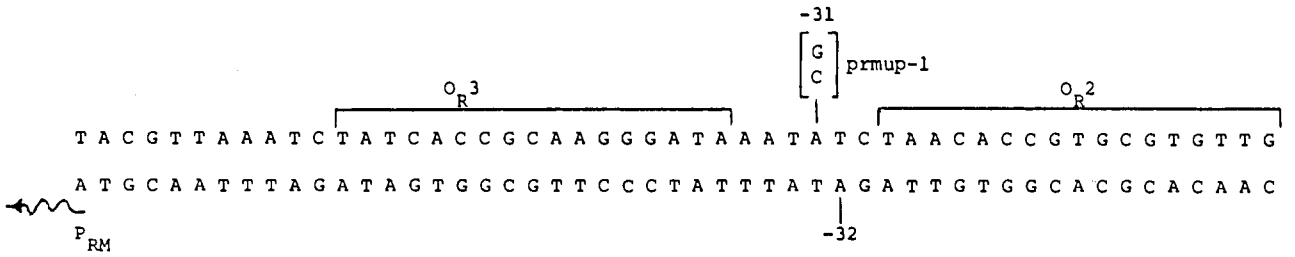


FIGURE 4: Sequence of the right operator region of λ repressor from the start point of transcription of P_{RM} to position −50 at the right end of the O_R2 footprint. The prmup-1 mutation at position −31 is indicated. These data are taken from Meyer et al. (1980).

of gene expression, then metastability and hysteresis follow naturally. The present observations do not establish that remote control of gene activity occurs in supercoiled DNAs but are consistent with that possibility.

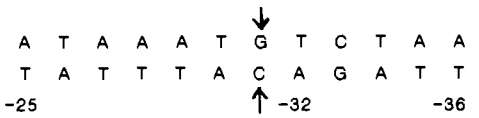
Finally, we note that although the two main secondary structures are to a large extent global and independent of sequence, the free energies of junctions between them very likely depend strongly on the particular local sequence. If so, junctions will occur predominantly only at certain preferred sites along the DNA and will have a very small mobility. This could contribute materially to the very long lifetimes that we observe for these metastable states in the relaxed and linear forms.

Speculations on Remote Control of Gene Activity

It is imagined that the supercoiled DNA exists normally in the low-free-energy conformation (say) b but that the polymerase (in at least some cases) has a strong preference for the alternate high-free-energy conformation a and is obliged to first convert the DNA locally from b to a before it can bind or begin transcription. It is also imagined that the nearest facile a|b junction site is located in a regulatory sequence some distance upstream from the polymerase binding site. The regulatory protein is imagined to bind at or near this specific junction site, and its function is simply to decrease (positive control) or increase (negative control) the free energy to form an a|b junction there. The regulatory protein is not required to convert an extensive domain from one secondary structure to another. Rather, its function is to assist (or inhibit) the polymerase by making an easy (or difficult) junction, when the latter converts a DNA domain from b to a.

One important prediction of this model, namely, that mutations in the remote regulatory sequence (at or near the junction site) can affect gene activity *even in the absence of regulatory proteins*, appears to be fulfilled. Specifically, Ptashne and co-workers (Meyer et al., 1980; Ptashne et al., 1980) report that a particular mutation, prmup-1, in the right operator region of λ repressor, shown in Figure 4, causes 4–5-fold enhancement of repressor gene (P_{RM}) activity *in the absence of regulatory binding proteins*. This enhancement is comparable to the 6-fold stimulation of P_{RM} activity observed when repressor protein binds to the adjacent O_R2 regulatory site in the wild type. The effect of this prmup-1 mutation cannot be rationalized within the model advanced by Ptashne and co-workers, which is based exclusively on interactions between proteins bound to the DNA.

In prmup-1, a single G·C pair is substituted for an A·T pair at position 31 with respect to the start point of transcription of P_{RM}. The prmup-1 sequence from −25 to −36 is



The A·T pair at −32 is sandwiched between two G·C pairs, which are in turn flanked by stretches of A·T pairs. This mutant sequence might well exhibit a favorable junction site at or near position −32, because a single A·T pair sandwiched between two G·C pairs actually forms the most stable internal loop containing a single open base pair. This is predicted from tabulated thermodynamic data (Gralla & Crothers, 1973; Uhlenbeck et al., 1973, 1971; Martin et al., 1971; Bloomfield et al., 1974) and arises because G·C/T·A stacking enthalpies are the lowest measured (Marky & Breslauer, 1982) and also because loop closure by a G·G pair is much more favorable than that by an A·T pair (Gralla & Crothers, 1973; Uhlenbeck et al., 1973, 1971; Martin et al., 1971; Bloomfield et al., 1977; Wilcoxon & Schurr, 1983). NMR melting studies of both tRNAs and synthetic DNAs by D. Hare and B. R. Reid (unpublished data) confirm that the imino proton resonances of A·T (or A·U) base pairs sandwiched between G·C pairs actually do exchange broaden first. The flanking A·T pairs may provide extra flexibility essential to junction formation. We suggest that the prmup-1 mutation creates a more favorable junction site in the DNA, which by itself directly enhances P_{RM} activity without the intervention of regulatory proteins. When repressor is bound to O_R2, the prmup-1 mutation acts synergistically to produce a net $4 \times 6 = 24$-fold enhancement. This is consistent with the possibility that both the mutation and bound repressor contribute independently to reduce the free energy of the *same* junction. Further elaboration of these ideas is deferred to a subsequent paper. This model for gene regulation by protein control of remote junctions between secondary structures is sufficiently explicit and testable in various respects that it may provide useful directions for future work.

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