Supercoiling-Regulated Liquid-Crystalline Packaging of Topologically-Constrained, Nucleosome-Free DNA Molecules†

Ziv Reich,‡ Smadar Levin-Zaidman,‡ Susan B. Gutman,§ Talmon Arad,§ and Abraham Minsky*,‡

Department of Organic Chemistry and Center of Electron Microscopy, The Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT: Electron microscopy and circular dichroism studies of cholesteric aggregates derived from topologically-constrained DNA molecules indicate that the overall morphology and structural properties of these aggregates are fundamentally different from those characterizing condensed structures of nonconstrained DNA species. Specifically, the cholesteric pitch and twist of all hitherto characterized lyotropic mesophases of biopolymers—including those obtained from linear DNA—depend predominantly upon environmental parameters such as the dielectric constant of the solvent. In contrast, the properties of aggregates derived from closed circular supercoiled DNA are found to be *solely and directly* dictated by the superhelical density and handedness. On the basis of these results, as well as on the demonstrated ubiquity of liquid-crystalline DNA organizations *in vivo*, we suggest that supercoiling-regulated liquid crystallinity represents an effective packaging mode of nucleosome-free, topologically-constrained DNA molecules in living systems.

Several families of bacterial plasmids may reach, under certain growth conditions, a copy number larger than 1000 per cell, corresponding to a total amount of DNA that may exceed the amount of DNA within the bacterial chromosome. Such a large copy number necessarily imposes severe storage and spatial organizational demands upon the host. The issue is underscored by the observation that the axis length of a relatively small, 3 kb, supercoiled plasmid [4000 Å; ~40% of the DNA contour length (Boles et al., 1990)] is about half the diameter of the bacterial cytoplasmic compartment. The storage problem transcends the specific topic of plasmid packaging: most extrachromosomal DNA, including many viruses, plasmids, and mitochondrial and chloroplast DNA, as well as substantial portions of the prokaryotic chromosomal DNA adopts a nucleosome-free, plectonemic conformation (Bauer et al., 1980; Yagil, 1991). Notably, whereas the solenoidal DNA organization encountered in nucleosomal complexes allows efficient packaging, DNA compaction associated with the plectonemic conformation is negligible (Boles et al., 1990). How does the cellular system cope with the requirement to accommodate a large number of DNA molecules which do not present a packed nucleosomal organization, and whose interwound topology *intrinsically* precludes intramolecular compaction processes?

We have recently been able to show that isolated supercoiled plasmids spontaneously form a cholesteric liquid-crystalline phase at physiological ionic strength and physiological DNA concentrations, thus raising the possibility that intracellular plasmid molecules may assume a liquid-crystalline organization as well (Reich et al., 1994). Indeed, X-ray scattering experiments conducted on intact bacteria suggested that within the bacteria supercoiled plasmids aggregate into condensed clusters which are characterized by a lateral order (Reich et al., 1994). The results presented in this study indicate that the structural features of the packed aggregates derived from interwound DNA topological states are directly and uniquely determined by the supercoiling parameters which provide, as such, a means for the regulation of the macroscopic properties of the packed DNA organization. These observations, combined with the demonstrated propensity of superhelical DNA species to form condensed liquid-crystalline phases in bacteria, as well as in viruses and mitochondria, support the notion that supercoiling-regulated liquid crystallinity represents an effective packaging mode for plectonemic, nucleosome-free DNA molecules in living systems.

THEORETICAL BACKGROUND

Superhelical Handedness. The topology of a circular, covalently closed DNA molecule is described by the linking number Lk which corresponds to the total number of times the two DNA strands are interwound about each other when the molecule is confined to a plane. The linking number is provided by the relationship Lk = Tw + Wr, where Tw stands for the twist of the DNA strands and Wr, or writhe, is the number of times the double helix winds around the superhelix axis, thus providing both the extent and the handedness of the superhelicity (White, 1969; Cozzarelli et al., 1990). Native closed circular DNA molecules are characterized by a negative writhe, corresponding to a right-handed supercoiling. Since Lk is a topological invariant, conditions that promote DNA unwinding—and hence reduction of Tw—relax and finally reverse the supercoiling, rendering it positive or left-handed (Vinograd et al., 1968). A well-documented example of supercoiling relaxation and subsequent reversal of the superhelical handedness is provided by intercalating drugs which unwind the DNA molecules upon binding (Bauer & Vinograd, 1968; Waring, 1970).

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* To whom correspondence should be addressed. Incumbent of the Victor L. Ehrlich Career Development Chair.

‡ Department of Organic Chemistry.

§ Center of Electron Microscopy.

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Limited melting of a closed-circular DNA molecule can be accommodated as a change in the twist and writhe; thus, the formation of melted regions within a supercoiled plasmid is equivalent to an unwinding process, and hence expected to affect the topological parameters in a manner similar to intercalation, Z-DNA or cruciforms (Frank-Kamenetskii, 1990; Bowater et al., 1991). Indeed, relaxed conformations of plasmids were found to form left-handed, positive supercoils upon increasing the temperature (Anderson & Bauer, 1978). Reversal of the superhelical sense induced by DNA-denaturing agents such as hydroxyl ions or chaotropic species has been proposed as a topological outcome of the linking number invariance (Vinograd et al., 1968), but heretofore not experimentally demonstrated. Notably, for a 3000 base pair plasmid chracterized by a supercoiling density of 0.05, only 150 base pairs—corresponding to 5% of the total length—need undergo a helix−coil transition in order to effect complete relaxation; additional melting of a few base pairs would result in small positive superheling. Such an extent of supercoiling-promoted DNA melting is readily attainable: large-scale melting of susceptible DNA sequences, e.g., AT-rich regions, has been shown to occur in supercoiled molecules at ambient temperatures (Kowalski et al., 1988; Bowater et al., 1991).

Shadowing electron microscopy studies of closed-circular DNA molecules provide an indirect indication for a pH-induced reversal of the superhelical handedness. At neutral pH, plasmids are detected as interwound species (Figure 1A); at pH 10.5, a significant population of the plasmids appears as open, relaxed circles, whereas at pH 11.0 a supercoiled conformation predominates (Figure 1D,E, respectively). Although these observations do not provide conclusive evidence for a pH-dependent handedness reversal, the reestablishment of an interwound conformation at high pH supports the notion that such a process is indeed induced.

Optical Properties of Cholesteric DNA Phases. At high nucleic acid densities, linear DNA molecules have been found to form a cholesteric liquid-crystalline phase (Livolant, 1984, 1991; Rill, 1986; Strzelecka et al., 1988; Livolant et al., 1989; Leforestier & Livolant, 1993; Reich et al., 1994) characterized by unusual optical properties (Keller & Bustamante, 1986; Livolant & Maestre, 1988; Yevdokimov et al., 1988; Bustamante et al., 1991). The high solute concentrations, usually required for the formation of lyotropic mesophases, seriously hinder the investigation of these phases by means of circular dichroism. Packaging of DNA molecules into tightly-packed aggregates can, however, be induced under appropriate conditions of salts and dehydrating agents even at very low nucleic acid densities (Huey & Mohr, 1981). The resulting chiral structures, which are amenable to circular dichroism studies, exhibit very large, nonconservative ellipticities (Jordan et al., 1972), attributed to the presence of cholesteric microdomains that are formed once a critical *local* DNA concentration has been affected by the condensing agents (Maestre & Reich, 1980; Keller & Bustamante, 1986; Livolant & Maestre, 1988; Bustamante et al., 1991). The molecular organization—and hence the optical parameters—of cholesteric microdomains is assumed to be identical to that of the liquid-crystalline macrophase.

The sign of the nonconservative ellipticities reflects the sense of the supramolecular twist within the cholesteric microdomains: positive nonconservative circular dichroism signals result from a right-handed cholesteric twist whereas

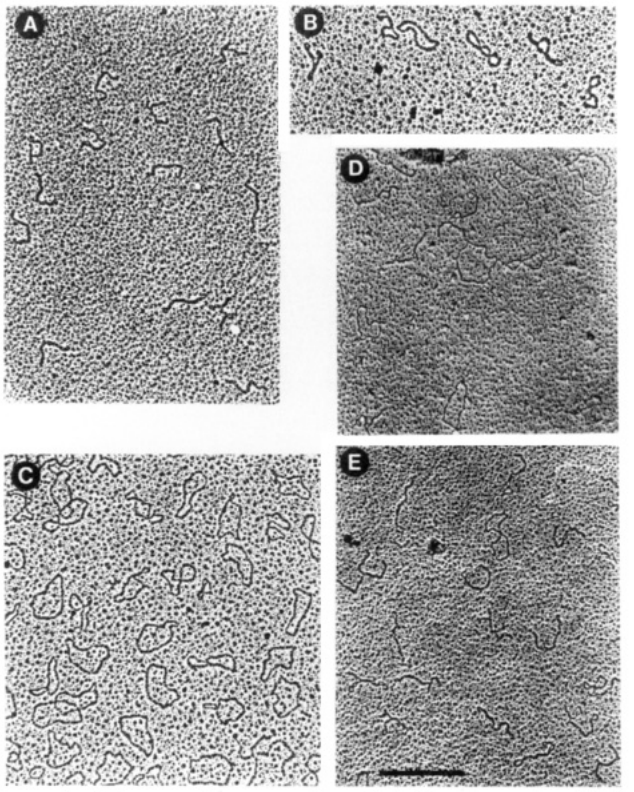

FIGURE 1: Electron microscopy of metal-shadowed samples of (A) fully interwound plasmid DNA molecules, (B) extensively relaxed plasmids, obtained with topoisomerase II, (C) nickel-circular plasmids, obtained with DNase I, (D) supercoiled plasmids, pH 10.5, and (E) supercoiled plasmids, pH 11.0. Note the relatively large population of "open" plasmids of pH 10.5 versus the interwound conformation which predominates at pH 11.0, pointing toward the possibility of a pH-induced superhelical handedness reversal. Spreading and shadowing procedures are detailed under Materials and Methods. Scale bar, 0.5 μm.

negative signals correspond to a left-handed long-range chirality (Maestre & Reich, 1980; Livolant & Maestre, 1988). The sense of the cholesteric twist in lyotropic phases of helical polymers depends, in turn, upon two factors: the handedness of the molecule—right-handed helices stabilize a left-handed cholesteric twist and vice versa (Rudall, 1955). The second factor, found to predominate in most lyotropic phases of helical polymers hitherto investigated, is the dielectric properties of the medium (Robinson, 1961; Samulski & Samulski, 1977). Indeed, the long-range twist in cholesteric phases of a given polypeptide, or in packed chiral forms of linear DNA segments, depends mainly upon the packaging medium, which modifies the dipole interactions within the molecules but does not alter their helical sense (Robinson, 1961; Livolant & Maestre, 1988; Bustamante et al., 1991). The magnitude of the ellipticities exhibited by a cholesteric DNA organization is determined by several factors, including the degree of compaction and the size of the chiral aggregates as well as the pitch and twist of the cholesteric phase which determine the extent of the long-range chirality (Kim et al., 1986). Thus, the sign and magnitude of the nonconservative CD signals provide a sensitive means to investigate the structural parameters of the cholesteric phase and were used in the current study to elucidate the unique properties exhibited by condensed, ordered aggregates obtained from the topologically-constrained DNA molecules.

MATERIALS AND METHODS

Nucleic Acids. The BlueScript plasmid (2960 base pairs) was isolated and purified from *Escherichia coli* JM109 cells according to Sambrook et al. (1989).

(A) Nicked-Circular DNA Molecules. One milligram of the supercoiled plasmid was incubated at room temperature with 25 units od DNase I (Boehringer-Mannheim, grade II, lyophilized, 2000 units/mg) for 30 s in the presence of 60 mM NaCl, 10 mM $MgCl_2$, and 5 mM Tris-HCl (pH 7.5). The reaction was quenched with 1 mL of EDTA (pH 8.0, 0.5 M) and phenol followed by extraction with 2 × 400 μL of chloroform and EtOH precipitation. A virtually pure population of nicked-circular molecules was obtained according to agarose-gel electrophoresis and shadowing electron microscopy.

(B) Relaxed Closed-Circular Plasmids. Three milligrams of the supercoiled plasmid in 400 μL was incubated at 30 °C with 100 units of topoisomerase II (USB, 20 units/μL) in the presence of 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM ATP. After 10 h, 200 μL of the reaction mixture was removed and treated with 200 μL of phenol at 55 °C for 5 min to quench the relaxation process, followed by chloroform extraction and EtOH precipitation. To the remaining 200 μL were added 100 units of the topoisomerase; the reaction was allowed to proceed for an additional 24 h and then treated as above. Two topological plasmid populations, i.e., moderately relaxed and extensively relaxed molecules (approximate average superhelical density of −0.04 and −0.01, respectively), were thus obtained, as estimated from the electrophoretic migration of the resulting conformations.

(C) Linear DNA. One milligram of the supercoiled plasmid was treated with 200 units of *Eco*RI in 50 mM NaCl, 100 mM Tris-HCl (pH 7.5), and 10 mM $MgCl_2$ for 20 h at 37 °C. The enzyme was heat-inactivated (65 °C, 20 min), and DNA was EtOH-precipitated.

DNA Packaging (Huey & Mohr, 1981). A 10 μL solution containing 3.5 mg/mL (for circular dichroism studies) or 100 μg/mL (for electron microscopy samples) DNA was treated with the appropriate amounts of 5 M NaCl or 0.1 M $MgCl_2$ solutions to provide the final salt concentrations indicated in the figure legends. The samples were diluted with H_2O and brought to a volume of 1 mL with either 200 (20%) or 350 (35%) μL of EtOH. Addition of EtOH was always carried out last, 10 min prior to the spectroscopic measurements or the preparation of the grids for electron microscopy.

Circular Dichroism. CD spectra were recorded in a 1-cm light-path cell on a Jasco J-500 spectropolarimeter equipped with a DP-500N data processor; final DNA concentration in all samples: 35 μg/mL (=5 × 10^{-5} M, in base pairs). Maxima of the nonconservative CD spectra (Figures 3−7) are in the range of $\lambda = 268 \pm 10$ nm.

Electron Microscopy. Samples for shadowing electron microscopy were prepared by the Kleinschmidt method (Kleinschmidt et al., 1959). Aliquots of 100 μL of DNA (3.5 μg/mL) in 0.5 M ammonium acetate, 0.1 M Tris (at the pH indicated in the figure legend), and 2.5 mM EDTA were treated with 10 μL of cytochrome *c* (2.5 mg/mL) and spread on the surface of a solution of the above buffer diluted in a ratio of 1:20. The resulting monolayers were lifted onto parlodion-coated grids, washed, and blotted dry. Grids were subsequently rotary-shadowed with platinum−palladium (80:20). Samples of condensed DNA species were prepared by applying 10 μL of the various packaging solutions (final DNA concentration, 1 μg/mL) on a carbon-coated, glow-discharged grid for 30 s, followed by removal of the excess with filter paper. Specimens were stained with 1% (w/v) uranyl acetate dissolved in H_2O−EtOH (25% v/v) and examined on an EM-400T.

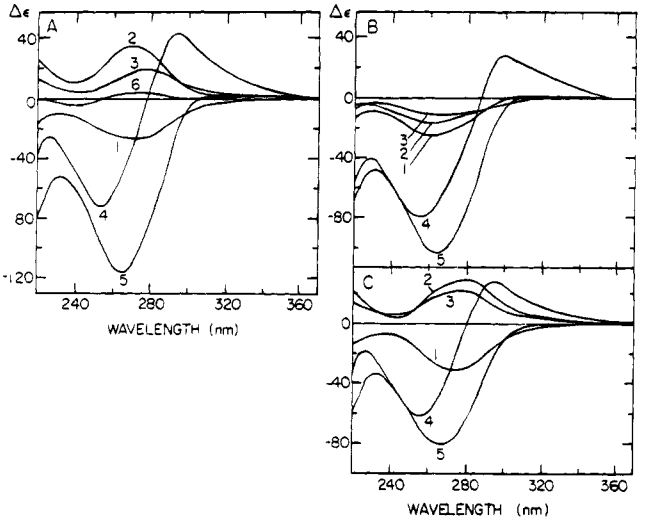

FIGURE 2: Circular dichroism spectra exhibited by aggregates of (1) supercoiled plasmid, (2) linear DNA obtained from the plasmid, (3) nicked-circular plasmid, (4) moderately relaxed plasmid, (5) extensively relaxed plasmid, and (6) supercoiled plasmid. Packaging conditions were (A) 0.8 M NaCl, 35% EtOH for curves 1−5, 0.8 M NaCl for curve 6; (B) 2.2 M NaCl, 35% EtOH; and (C) 10 mM $MgCl_2$, 20% EtOH. Samples were monitored at room temperature and pH 7.0. Note that curve 6 depicts a conservative CD spectrum obtained under nonpackaging conditions (i.e., in the absence of a dehydrating agent), and reflects the chirality associated with the B-DNA conformation. As such, spectrum 6 is characteristic of, and identical for, all the various DNA topological forms in their noncondensed conformation.

RESULTS

CD Studies. The optical properties exhibited by five different DNA topological forms, i.e., closed-circular supercoiled, nicked-circular, and moderately and extensively relaxed, as well as linear conformations, have been determined at those conditions of salt and dehydrating agents previously shown to affect packaging of linear DNA into highly condensed aggregates of long-range chirality (Eickbush & Moudrianakis, 1978; Reich et al., 1980, 1990; Huey & Mohr, 1981; Shin & Eichhorn, 1984; Shin et al., 1986). Whereas all topological states form, under such conditions, chiral aggregates, as evidenced by the characteristic nonconservative CD signals, the properties of the resulting packed conformations markedly differ (Figure 2). Specifically, the nonconservative ellipticities exhibited by aggregates derived from the topologically-constrained molecules (i.e., the fully interwound and the partially relaxed species) are negative under all examined packaging conditions. In contrast, the sign of the packed states obtained from the linear and the nicked-circular species depends upon the medium, being either positive or negative. Also, under all packaging conditions, a clear trend in the relative magnitudes of the nonconservative CD signals is observed: a decrease of the superhelical density of the molecules from which the packed aggregates are composed is accompanied by a substantial increase of the signal size. Thus, the ellipticities exhibited

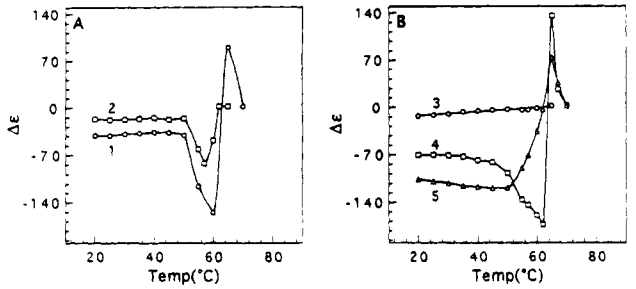

FIGURE 3: Ellipticity maxima ($\lambda = 268 \pm 10$ nm) exhibited by aggregates derived from the various DNA topologies as a function of temperature. Aggregates were obtained at 2.2 M NaCl, 35% EtOH, pH 7.0. Curves 1−5 represent DNA topologies specified in the legend to Figure 2. Notably, aggregates derived from intermediates along the temperature-induced transition of the fully supercoiled molecules to relaxed conformations exhibit very similar nonconservative CD spectra (in terms of sign, magnitude, and position of maxima) to those derived from species of *initially* reduced superhelical density.

by the condensed conformation derived from extensively relaxed plasmids are significantly larger than those revealed by aggregates composed of moderately relaxed molecules which are, in turn, larger than the signals characterizing the compact states of the fully interwound species (Figure 2, curves 5, 4, and 1, respectively). Aggregates derived from the nickel-circular molecules consistently exhibit the smallest nonconservative signals.

The prominent difference between the properties of the packed aggregates obtained from the topologically-constrained DNA molecules and those characterizing the packed conformations derived from the unconstrained species is further manifested when the various aggregates are studied as function of temperature. The nonconservative ellipticities exhibited by the packed phases derived from the fully interwound plasmids are not affected by a temperature increase up to 50 °C, whereupon a very large increase of the magnitude of the negative CD spectrum is observed, followed by a sharp reversal of the signal, leading to large *positive* nonconservative ellipticity (Figure 3A). Similar behavior is revealed by the aggregates composed from the moderately relaxed plasmids (Figure 3B). The packed conformations of the extensively relaxed molecules, characterized by very large negative ellipticities already at room temperature, exhibit only a moderate initial increase of the magnitude of the CD signals which is then followed by a sign reversal. The long-range chiral DNA organization is completely abolished at 70 °C, as indicated by the disappearance of the nonconservative CD signals. Chiral aggregates derived from the linear DNA molecules exhibit a temperature-dependent increase in the intensity of the ellipticity which, significantly, is not followed by a sign reversal; the initially small nonconservative CD bands characterizing packed conformations of nicked-circular molecules do not reveal any temperature dependence.

The temperature-dependent modifications of the nonconservative CD signals exhibited by the chiral compact phases of the fully interwound plasmids (Figure 3A, curve 1) are detected only under high-salt conditions. When packaging of the supercoiled DNA molecules is induced at lower NaCl concentrations, elevated temperatures merely lead to a negation of the long-range chiral order, as clearly indicated by the disappearance of the nonconservative ellipticities (Figure 4, curve 1). Temperature-dependent modifications

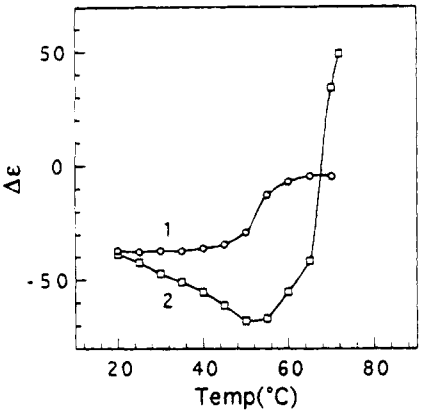

FIGURE 4: Ellipticity maxima of aggregates derived from supercoiled plasmid DNA molecules at (1) 0.8 M NaCl, 35% EtOH, and (2) 0.8 M $NaClO_4$, 35% EtOH, as a function of temperature.

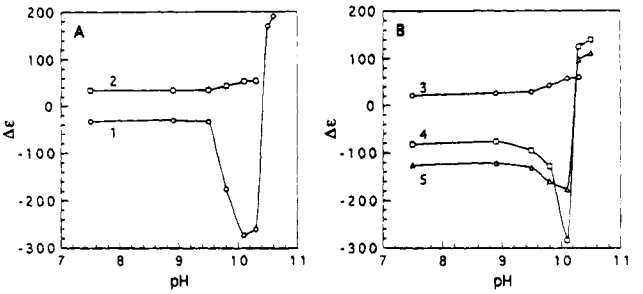

FIGURE 5: Ellipticity maxima exhibited by aggregates derived from the various DNA topologies as a function of the pH. Aggregates were obtained at 10 mM $MgCl_2$, 20% EtOH, and monitored at room temperature. Curves 1−5 represent DNA topologies specified in the legend to Figure 2. Each data point represents a CD maximum obtained from an independently prepared sample.

of the ellipticities resembling those occurring at high NaCl concentrations, i.e., increased intensity followed by a sign reversal, are detected already at a relatively low ionic strength when $NaClO_4$ substitutes for NaCl as a packaging agent (Figure 4, curve 2).

The effects of increasing pH values on the properties of the chiral organizations obtained from the various DNA topological states are very similar to those observed upon increasing the temperature of the corresponding packed structures (Figure 5). Specifically, a large increase followed by a very sharp negative-to-positive reversal of the nonconservative CD signals is exhibited as the packed phases of the constrained DNA conformations are exposed to increasing pH values in the range between 9.5 and 10.5. The huge CD values obtained at this pH range should be noted. No pH-dependent effects are revealed by the chiral organizations obtained from the linear and the nicked-circular molecules (Figure 5, curves 2, 3). Significantly, the pH-induced modifications of the chiral aggregates of the interwound plasmids can be negated upon decreasing the temperature: the large positive ellipticity which characterizes packed aggregates at evaluated pH values decreases as the temperature is gradually reduced, resulting eventually in a positive-to-negative reversal (Figure 6).

The effects of the DNA-intercalating drug actinomycin on the features of the various packed chiral organizations are depicted in Figure 7. Three different modes of modification of the optical properties can be discerned as the drug-to-DNA molar ratio is progressively increased. The initial increase of the magnitude of the negative ellipticity exhibited

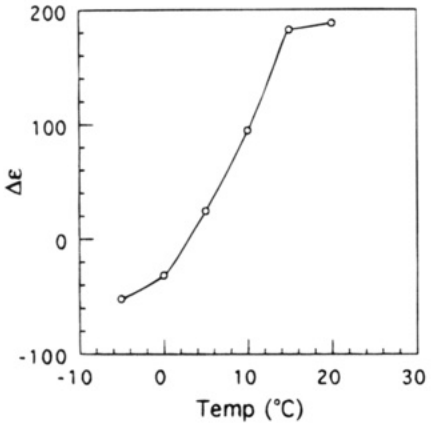

FIGURE 6: Ellipticity maxima of aggregates derived from supercoiled plasmid DNA molecules at 10 mM $MgCl_2$, 20% EtOH, and monitored at pH 10.6, as a function of temperature. Each data point represents a CD maximum obtained from an independently prepared sample.

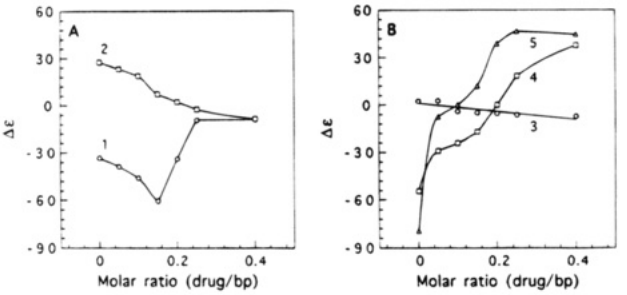

FIGURE 7: Ellipticity maxima exhibited by aggregates of the various DNA topologies obtained in the presence of actinomycin at 10 mM $MgCl_2$, 20% EtOH, as function of the molar drug-to-base pair ratio. Samples were monitored at room temperature. Curves 1−5 represent DNA topologies specified in the legend to Figure 2. Each data point represents a CD maximum obtained from an independently prepared sample. Notably, the indicated drug-to-base pair ratio values represent the ratio of *included* drug to DNA and not of bound drug; the binding ratios could not be measured since under packaging conditions the system is not in equilibrium.

by the packed species of the fully interwound plasmids is followed by a decrease to very low levels, indicating a complete disruption of the long-range chiral organization; no sign reversal is observed in this case (Figure 7, curve 1). Progressive addition of the drug to the packed structures derived from the moderately and extensively relaxed plasmids results in a decrease of the magnitudes of the nonconservative CD signals, leading eventually to a negative-to-positive sign reversal (Figure 7, curves 4, 5). The substantially sharper modifications of the size of the CD signals exhibited by the packed organization of the extensively relaxed molecules relative to those characterizing the packed species derived from the moderately relaxed plasmids, as well as the fact that the sign reversal exhibited by the former aggregates takes place at a lower drug-to-DNA ratio, should be noted. An additional conspicuous point is the observation that in both cases the negative-to-positive inversion as a function of increasing drug-to-DNA ratio occurs in two clearly distinct steps. The presence of actinomycin in the packaging systems of the linear and nicked-circular DNA molecules results in a mere disruption of the long-range organization (Figure 7, curves 2, 3).

Electron Microscopy. The optical properties exhibited by the aggregates derived from the topologically-constrained DNA molecules, and those revealed by the packed conformations of the linear and nicked-circular species, point toward a fundamental difference between the modes of long-range organization of these two structural categories. This dissimilarity is accentuated by electron microscopy studies conducted on the various condensed aggregates (Figure 8). Specifically, both the fully-interwound and the partially relaxed closed-circular DNA molecules form under packaging conditions elongated tightly-packed rodlike conformations, in which a striated texture that is either parallel or perpendicular to the main rod axis can be clearly discerned (Figure 8A,B). In contrast, only fused toroidal conformations, whose overall dimensions are significantly smaller than those characterizing the rodlike forms, are reproducibly observed upon packaging of the linear and nicked-circular DNA species (Figure 8C,D).

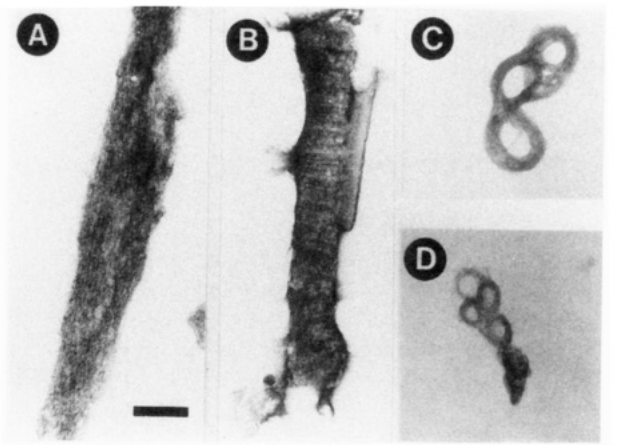

FIGURE 8: Electron microscopy of condensed DNA structures derived from (A) supercoiled plasmids, (B) extensively relaxed plasmids, (C) linear DNA molecules, and (D) nicked-circular plasmids. Samples were prepared by applying 10 μL of DNA (1 μg/mL)−$MgCl_2$ (10 mM)−EtOH (20% v/v) solution on a glow-discharged grid. Scale bar, 100 nm.

DISCUSSION

DNA molecules possess an intrinsic tendency to self-organize into a cholesteric mesophase or cholesteric microdomains (Livolant, 1984, 1991; Rill, 1986; Strzelecka et al., 1988; Livolant et al., 1989; Leforestier & Livolant, 1993; Reich et al., 1994). The observations presented in this study indicate that the topological state of the molecules which form the cholesteric organization crucially affects both the packaging pathway and the fundamental structural properties of the resulting ordered aggregates. Specifically, two distinct families of tightly packed, supramolecular organizations are induced: aggregates obtained from nicked-circular or linear DNA molecules, and those derived from topologically-constrained species. On the basis of the results reported here, a model for the two modes of spontaneous, protein-independent DNA long-range organization is proposed (Figure 9).

Linear DNA molecules pack into bent rods (Figure 8C) within which the DNA molecules are suggested to form parallel arrays (Figure 9a; Eickbush & Moudrianakis, 1978). The nonconservative ellipticities revealed by these aggregates result from efficient interhelical chromophore coupling, combined with a salt-dependent twist inbetween the contiguous DNA segments (Figure 9a; Weinberger et al., 1988). Minor salt-induced changes in the local structure of the DNA molecules, conceivably involving modifications in their

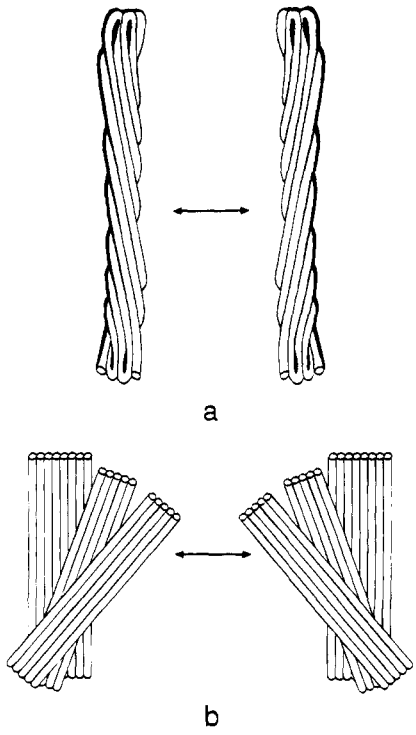

FIGURE 9: Schematic representation of the model suggested for the chiral packaging and handedness inversion of (a) linear DNA molecules and (b) supercoiled plasmid DNA molecules. The bent rodlike segment in (a) represents a single DNA double helix (intramolecular packaging of a single molecule is depicted), whereas each rod in (b) represents a single supercoiled plasmid (i.e., two interwound double helices).

hydration state and surface charge density or distribution (Bustamante et al., 1991), can affect the twist and lead to a reversal of the handedness of the tertiary organization (clearly demonstrated in Figure 2). Thus, ordered chiral aggregates derived from linear DNA molecules represent a "conventional" lyotropic cholesteric-like organization, where the sense of the twist is determined predominantly by the dielectric properties of the medium and not by the molecular handedness (i.e., the DNA right-handed secondary conformation) which, for random DNA sequences, is unaffected by the salt. Elevated temperatures and pH values, as well as the presence of DNA intercalating agents, result in a complete disappearance of the nonconservative optical activities exhibited by the packed aggregates obtained from linear DNA molecules (Figures 3, 5, 7, respectively), indicating a total collapse of their tertiary organization (Reich et al., 1990).

Closed-circular interwound DNA molecules are characterized by a rodlike, relatively rigid conformation (Vologodskii et al., 1992), expected to promote a cholesteric organization (Robinson, 1961). Indeed, plasmid solutions were found to exhibit highly birefringent textures characteristic of a cholesteric mesophase (Reich et al., 1994). The large nonconservative ellipticities exhibited by aggregates obtained from various interwound species remain negative irrespective of the medium (Figure 2), thus indicating that these aggregates assume a cholesteric organization whose left-handed twist is unaffected by the packaging conditions. The predominance of a long-range left-handedness is proposed to result from the right-handed superhelical sense of native plasmids, and to indicate that the cholesteric twist in aggregates derived from supercoiled DNA is directly determined by the molecular superhelicity. The notion that topological constraints play a decisive role in the determination of the properties of the cholesteric arrangement of interwound molecules is buttressed by the pronounced dependence of the magnitudes of the CD signals upon the supercoiling density: a decrease of the superhelical density of the DNA which forms the chiral aggregates leads to a significant increase in the size of the nonconservative ellipticity (Figure 2). This increase is interpreted in terms of a larger cholesteric pitch (Kim et al., 1986) which results, in turn, from an increase of the plasmid diameter that accompanies a reduction of the superhelical density (Boles et al., 1990).

A striking demonstration of the dominant role displayed by the superhelicity upon the properties of the tightly-packed chiral structures of the interwound species is provided by alterations of these properties that are induced by factors which modify the supercoiling parameters (Figures 3–7). Elevated temperatures and pH values, as well as chaotropic anions such as ClO_4^-, promote DNA melting processes. As discussed under Theoretical Background, a limiting melting can be accommodated in closed-circular interwound molecules as a change in the twist, thus resulting in a progressive unwinding. In order to maintain the topological relationship for closed-circular configurations (White, 1969), such an unwinding, which is also affected by DNA intercalating agents, must be compensated by a progressive reduction of the supercoiling density until it is completely relaxed. Indeed, when unwinding is induced either through partial melting or through intercalation, a substantial increase of the magnitudes of the nonconservative ellipticities exhibited by aggregates derived from the various topologically-constrained molecules is detected. The increase is particularly accentuated in ordered structures of fully supercoiled plasmids; the partially relaxed molecules are characterized by an *initial* lower supercoiling density—and accordingly by initial very large negative nonconservative ellipticities.

A further increase of the temperature (Figures 3 and 4), and of the pH values (Figure 5), leads to an elimination of the negative CD signals, followed by the appearance of very large positive nonconservative ellipticities. The large magnitude of the ellipticities is inconsistent with extensive DNA denaturation, and denotes the preservation of a long-range chiral order; the positive sign reflects a right-handed cholesteric twist. Thus, the results depicted in Figures 3–5 indicate a temperature- and pH-dependent left-to-right reversal of the cholesteric twist through a nonchiral phase present at the inversion point. The nonchiral state might reflect a transient disorder or, alternatively, a nematic-like organization where the relaxed plasmids maintain a rodlike conformation due to packaging forces (Torbet & Dicapua, 1989).

Since the modifications of the optical properties are exhibited only by the ordered structures derived from interwound molecules, we suggest that the inversion of the cholesteric twist reflects a temperature- or pH-induced reversal of the supercoiling sense from the native right-handed into a left-handed conformation (see Figure 1D,E, and considerations outlined under Theoretical Background). Notably, the very large magnitudes of the positive nonconservative CD signals point, according to the arguments presented above, toward a low left-handed superhelical density, for which a titration of only few base pairs is required following relaxation. The hypothesis is strength-

ened by the reversibility of the cholesteric twist inversion: the right-handed cholesteric twist, which is associated with a left-handed supercoiling obtained at high pH values and reflected by the positive CD signals, is eliminated and finally reversed upon progressive cooling of the sample, as indicated by the reversal of the nonconservative ellipticities (Figure 6). Indeed, by promoting DNA annealing and thus negating the pH-induced partial melting, the cooling acts to increase the twist and thus to induce a left-to-right inversion of the supercoiling sense. Furthermore, temperature-dependent sign inversion of the nonconservative ellipticities is observed when DNA packaging is effected at relatively low concentrations of $NaClO_4$ but not of NaCl (Figure 4). Presumably, DNA melting processes which are enhanced by $NaClO_4$ (Hamaguchi & Geiduschek, 1961), and suggested to result in a supercoiling reversal, precede a temperature-induced disruption of the long-range order, whereas in the presence of NaCl such a disruption precedes melting. The initial temperature-dependent increase of the negative CD signals exhibited by the aggregates of the linear DNA species [Figure 3, curve 2; also observed by Widom and Baldwin (1980) and Shin et al. (1986)] might be associated with enhanced DNA bending processes which facilitate, according to the suggested model (Figure 9a), intramolecular packaging.

The drug-induced modifications of the nonconservative optical properties (Figure 7) are particularly informative. The presence of the DNA intercalating agent actinomycin in increasing drug-to-DNA molar ratios results in a simple negation of the nonconservative ellipticities exhibited by the aggregates of linear and nicked-circular molecules, indicating a complete drug-dependent elimination of a long-range order (Reich et al., 1990). Increase of the magnitude and subsequent disappearance of the nonconservative CD signals which is not followed by positive ellipticities are exhibited by clusters of the fully interwound plasmids. We propose that the relatively large amount of drug molecules required to effect a complete relaxation of the fully interwound plasmids, and hence to induce a transition of the corresponding aggregates from a cholesteric to a nonchiral nematic phase, precludes, under packaging conditions (i.e., presence of salts and alcohol), further drug intercalation, which is an energetically unfavorable process for relaxed closed-circular molecules. In contrast, addition of actinomycin to the aggregates obtained from the moderately and extensively relaxed DNA molecules results in a sharp decrease of the, initially large, magnitudes of the negative ellipticities followed by sign inversion. Conceivably, since for these partially relaxed topologies only minute amounts of intercalating drug are required to complete the relaxation, increased drug concentration results in additional binding, leading to a right-to-left-handed inversion of the superhelical sense and hence to the observed left-to-right reversal of the cholesteric twist. The energetic barrier for intercalation of fully relaxed closed-circular molecules is reflected by the observation that the titration curves are composed of two distinct steps (Figure 7, curves 4, 5), which indicate that a substantial increase of the drug concentration is required for a "post-relaxation" intercalation. Indeed, the inflexion point is detected at the range of low-intensity nonconservative ellipticities which, presumably, corresponds to a nematic-like phase associated with a relaxed molecular topology.

A strict and unique correlation is, consequently, indicated between the macroscopic properties of the cholesteric arrangements of the various interwound DNA molecules and the superhelical parameters of these molecules. The long-range twist of the cholesteric mesophase is determined by the handedness of the molecular supercoiling (Rudall, 1955), being left-handed for the native, right-handed closed-circular plasmids and right-handed for left-handed superhelical molecules. The pitch of the packed arrangements is dictated by the diameter of the interwound species which is, in turn, affected by the superhelical density. Fully relaxed plasmids stabilize a nonchiral nematic mesophase.

Plasmid DNA molecules have been shown to form ordered clusters within bacteria and a cholesteric mesophase *in vitro* (Reich et al., 1994). The concentrations of interwound species required for the formation of the cholesteric mesophase were found to be substantially lower than those required for the induction of such an organization in linear DNA molecules, thus indicating that the plectonemic topology promotes and stabilizes a cholesteric arrangement. Here we show that, in contrast to hitherto characterized lyotropic cholesteric phases of biopolymers (including those obtained from linear DNA molecules) which show a complex and indirect dependence of the pitch and twist upon the microscopic properties of the constituent molecules, the features of the mesophases derived from interwound DNA are directly determined by the molecular superhelical parameters. Since the superhelical density responds to environmental and genetic factors (Dorman et al., 1990; McClellan et al., 1990; Drlica, 1992), and following the observations presented above, DNA supercoiling is suggested to effectively promote DNA condensation and to serve as a particularly efficient regulatory link between cellular determinants and DNA packaging pathways. We propose, consequently, that supercoiling-dependent and supercoiling-regulated liquid-crystalline organizations, which have been observed in viruses (Lepault et al., 1987; Booy et al., 1991), bacteria (Reich et al., 1994), and mitochondria (Brugerolle & Mignot, 1979), offer a packaging mode of nucleosome-free DNA molecules *in vivo*, and present, as such, an important alternative mechanism to protein-induced DNA condensation pathways.

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