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Helical repeat of DNA in solution. The V curve method

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ABSTRACT

The V-like dependence of the electrophoretic mobility of small DNA rings on their topological constraint, which has been documented in a recent paper [Zivanovic *et al.* (1986), J. Mol. Biol., *192*, 645-660], has been explored as a tool to measure the helical twist of the torsionally unstressed duplex. The method was applied to single mixed sequence fragments approximately 350 to 1400 base pairs in length, providing estimates of their average helical periodicity. It was also used to compare two DNA fragments, and to evaluate the helical repeat of poly(dA.dT).poly(dA.dT) and poly(dA).poly(dT) inserts, and the helix unwindings associated with dA and dC methylations by dam and HhaI methylases, respectively. Data were found to be highly reproducible and helical repeat estimates were in good agreement with those obtained from previous techniques.

INTRODUCTION

Helical twist of DNA in solution has so far been measured by two methods. In one of them, the DNA was adsorbed on a solid surface, and its helical repeat was obtained from the periodicity of the enzymatic or chemical cleavage of the exposed side of the double helix (1-3). In the other, the DNA was inserted into a plasmid and its repeat deduced from the correlative change in the plasmid superhelicity (4). Those methods have produced estimates for mixed sequence DNAs which vary between 10.5 and 10.6 base pairs (bp)/turn (2, 5-7).

A third way to the same purpose was opened more recently by the observation that the probability of a short DNA fragment to circularize under incubation with ligase varies periodically with its length (8). As a consequence, the ratio between adjacent topoisomers in the distribution of the closed circular products varies between 0 (a single topoisomer present) when the mean twist number of the fragment is integral, and 1 (two topoisomers, obtained from equal unwinding and overwinding of the double helix, respectively, present in the same amount) when that number is an integer plus 0.5. The measurement of the exact periodicity of that variation then provided accurate estimates of the helical repeat of the DNA in the ligation buffer. 10.45 and 10.54 bp/turn were independently obtained by two laboratories for 210 to 250 bp rings (9, 10). Once normalized to 25 °C in gel electrophoresis buffer (see its composition in Methods) using the known dependence of the helical twist on temperature (11, 12) and counterion concentration (13), helical repeats become 10.55 and 10.57 bp/turn, respectively, in excellent agreement with above estimates. Unfortunately however, such procedure, which requires extensive constructions of

fragments of different lengths along with accurate quantitations of DNA in gels, can hardly become a practical method suitable for routine measurements.

We have investigated here the use of the V-like dependence of the electrophoretic mobility of small DNA rings on their topological constraint, which has been documented in a recent article from this laboratory (14), as a method for the measurement of the DNA helical twist. The method has been applied to mixed sequence DNAs, poly(dA.dT).poly(dA.dT) and poly(dA).poly(dT), as well as to the measurement of duplex unwindings resulting from methylations of dA and dC by dam and Hha I methylases, respectively. The method was shown to be highly reproducible and to lead to helical repeat figures in good agreement with those previously estimated by above procedures.

MATERIALS AND METHODS

a) Enzymes and reagents.

Netropsin was a gift of Dr. C. Zimmer. Ethidium bromide was from Sigma. Acrylamide and other electrophoresis reagents were from Biorad Laboratories. Restriction endonucleases were from Appligene (Strasbourg), Boehringer, Promega Biotec and New England Biolabs. Alkaline phosphatase and phage T4 DNA ligase were from Boehringer, while phage T4 polynucleotide kinase was from Promega Biotec. [γ^{32}P] ATP (3000 Ci/mmol) was from Amersham.

b) DNA fragments.

The 358, 665 and 506 bp fragments, respectively, were purified from Sau3A and Hinf I digests of pBR322 (15). The 641 bp ori C fragment originates from a HhaI digest of plasmid pOC42 (16). This plasmid was obtained from pBR322 by insertion at the Pst I site of a 1934 bp fragment of the *E. coli* chromosome containing the replication origin (ori C). The pOC42 plasmid with unmethylated dA residues in Mbo I d(GATC) sites was obtained by transformation of dam 3 *E. coli* cells with the methylated plasmid. The effective presence or absence of methylated sites in pOC42 DNAs was checked by comparative digestions with Mbo I and Sau 3A restriction endonucleases. Other fragments derive from the 665 bp fragment and contain inserts of poly(dA.dT).poly(dA.dT) (709 bp fragment), or poly(dA).poly(dT) (721 bp fragment). These fragments were purified from Sau3A digests of plasmids pAT44 and pB10, respectively. Plasmid pAT44 had previously been obtained by insertion of 40 bp of poly(dA.dT).poly(dA.dT) at the filled-in Hind III site of pBR322 (6). Plasmid pB10 was recently constructed by tailing of Hind III-cleaved pBR322 DNA using terminal deoxynucleotidyl transferase, followed by annealing and transformation of *E. coli* HB101, as previously described for plasmids pAA24 and pAA82 (6). Insert and surrounding regions were sequenced as described (17) using the two separated strands of the EcoRI-HhaI fragment. Fig. 1 reveals that the four middle bp of the Hind III site were deleted on both sides and that 65 bp of poly(dA).poly(dT) were inserted, resulting in a 61 bp length difference with the parent plasmid. Such deletions are an expected consequence of the fact that termini were not filled-in prior to tailing.

c) Topoisomers.

Preparation procedures have previously been described in detail (14). Briefly, fragments were dephosphorylated with alkaline phosphatase, labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase,

and circularized with T4 DNA ligase. Netropsin and various concentrations of ethidium bromide were used to prepare positively and negatively supercoiled topoisomers, respectively. 665 bp topoisomers +3 and +4 (see below for topoisomer identification) were made using reverse gyrase from the archaebacterium *Sulfolobus acidocaldarius* (a kind gift from Dr. A. Kikuchi) (18) on ligase-prepared negatively supercoiled topoisomers. Individual topoisomers were purified by gel electrophoresis in the presence or absence of ethidium bromide. Topoisomers were identified by their linking number difference, ΔLk, with the topoisomer (topoisomer 0) which migrates the least in the gel, at 25°C, and which is therefore the closest to relaxation. ΔLk is also referred to as the topoisomer topological constraint.

d) *In vitro* methylation of the 358 bp fragment by Hha I DNA methylase.

20 ng of the ^{32}P-labeled fragment were incubated at 37 °C for 90 min in 20 µl of 50 mM Tris-HCl, 10 mM EDTA, pH 7.5, 5 mM ß-mercaptoethanol and 80 µM S-adenosyl-L-methionine (Boehringer), with 2 units of Hha I DNA methylase (New England Biolabs). [This enzyme methylates the internal dC residue in d(GCGC) into 5mdC (19).] The DNA was then purified and circularized as indicated above. The fragment was also essayed for digestion with a 10-fold excess of HhaI restriction endonuclease and was found to be resistant, while unlabeled pBR322 DNA added as a marker displayed the expected band pattern. Such resistance does not however prove that all sites had received two methyl groups, as shown by the following experiment. Briefly, a 1035 bp Eco RI-Nru I fragment containing the 358 bp fragment near one end and the insert (fig. 1) near the other was prepared from plasmid pB10. The fragment was methylated under above conditions and the two strands of both methylated and unmethylated fragments were separated by gel electrophoresis. The strands were then associated crosswise to give two hybrid duplexes with only one strand methylated, and Hha I-susceptible sites were mapped. Approximately 70% of each duplex was found to be completely resistant, confirming that hemimethylation suffices to prevent the sites from being digested. The susceptible fraction, on the other hand, showed the occasional occurrence of methyl-free sites at all expected positions (ref. 15; 5 of those were in the 358 bp fragment). These results (I.G.; unpublished) therefore demonstrate that sites in the original 1035 bp fragment were mostly double-methylated, but sometimes hemimethylated. Such conclusion must apply to the above 358 bp fragment as well.

e) Gel electrophoreses.

They were performed in a Pharmacia GE2/4LS apparatus in which the plastic coil had been replaced with a longer glass coil of larger inside diameter so as to regulate the gel temperature within ±0.1°C. The buffer (20 mM sodium acetate, 2 mM EDTA and 40 mM Tris-acetate, pH 7.8) was recirculated between the two reservoirs. Gels (0.12 x 17 x 20 cm) were pre-electrophoresed for 1 to 2 h and electrophoresed at 250 V (about 50 W) until the xylene cyanol dye reached the vicinity of the bottom.

PRINCIPLE OF THE MEASUREMENT

a) At the temperature (T_o) of relaxation of topoisomer 0.

When topoisomer 0 occupies the very apex of the V, its mobility is minimal and its configuration must therefore be as expanded as possible. This was the basis for the assumption, made in ref. 14, that

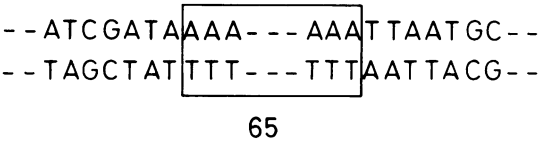

Figure 1 : Nucleotide sequence of insert and surrounding regions of recombinant plasmid pB10. The number about the box indicates the length (in bp) of the inserted poly(dA).poly(dT) stretch. Sequences of surrounding regions were identical to those reported in ref. 34.

such positioning actually signaled its relaxation. Making Wr=0 in equation

$$Lk = Tw + Wr \qquad (1)$$

which relates topoisomer linking, twisting and writhing numbers (in turns) (20-22), gives for topoisomer 0

$$Tw_0 = Lk_0 \qquad (2)$$

The helical repeat (in bp/turn) of the relaxed, i.e. *torsionally unconstrained*, DNA ring in the gel can in turn be calculated from the equation

$$h = N/Tw_0 = N/Lk_0 \qquad (3)$$

where N is the ring size (in bp). h can subsequently be normalized to the reference temperature of 25 °C by replacing Tw_0 in equation (3) by $Tw_0 + \Delta Tw$, with

$$\Delta Tw = -N \times \Delta T \times Th/360 \qquad (4)$$

ΔTw (in turn) is the twist increment resulting from a temperature shift of ΔT (in °C)= 25-T_0, and Th (in °/°C per bp) the DNA thermal untwisting coefficient (Th>0).

b) At other temperatures.

Fig. 2 displays a schematic V obtained at temperature T_0, with topoisomer 0 at the apex and

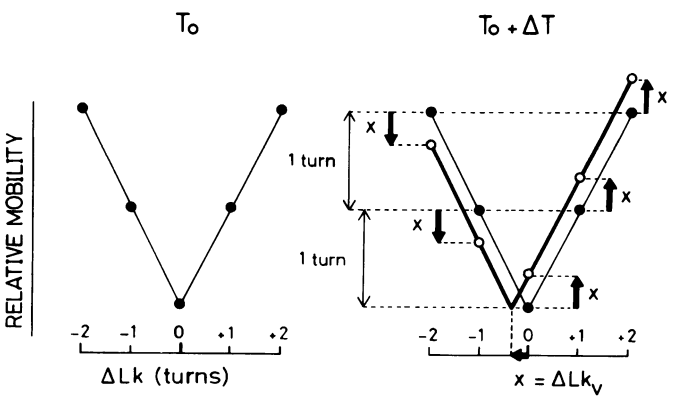

Figure 2 : Schematic representation of the V and of its temperature dependence. The V corresponds to a plot of the mobility of the topoisomers as a function of their topological constraint, ΔLk (see Methods for a definition of that parameter). The mobility is here measured relative to that of the linear DNA, as performed in fig. 3B, below. See Principle of the Measurement for an explanation of x.

topoisomers -2, -1, +1 and +2 symmetrically positioned on the two branches. Upon increase in the gel temperature by ΔT ($\Delta T>0$), the twist increments by $\Delta Tw=x$ (in fraction of a turn; $\Delta Tw<0$) and the writhing by $\Delta Wr=-\Delta Tw=-x$, as shown by differentiation of equation (1) ($\Delta Wr>0$). Topoisomer 0, from relaxed, becomes positively supercoiled, while "negative" and "positive" topoisomers, respectively, become less negatively and more positively supercoiled. If all topoisomers undergo the same ΔTw and therefore ΔWr increments, their mobility is expected to decrease or increase by the same fraction, x, of the distance separating two of them in the gel, as shown in fig. 2. [This is what is actually observed in Results with 665 bp topoisomers, in which case a 25 to 45 °C increase in temperature leads to $x \sim 0.5$; see fig. 3B.] As a result, it is clear that the V is displaced parallel to the ΔLk axis by the same fraction of a turn, x (fig. 2). The position of the apex on this axis, termed ΔLk_V in ref. 14, then is $\Delta Lk_V=x=\Delta Tw$.

Let us consider now an hypothetical nicked ring in which the strands can rotate freely about the nick. At temperature T_0, the strands are aligned at the nick, the mean twist number of the nicked ring, Tw_n, is integral, and equal to Tw_0 in equation (2). At temperature $T_0+\Delta T$, the strands are expected to rotate about one another by the same ΔTw as the topoisomers, which gives $Tw_n = Tw_0 + \Delta Tw$, and after replacement of Tw_0 and ΔTw

$$Tw_n=Lk_0 + \Delta Lk_V \qquad (5)$$

This equation, like equation (2), permits to obtain the helical repeat of the torsionally unconstrained duplex by replacing Tw_0 by Tw_n in equation (3). [The above virtual nicked ring would remain unconstrained and therefore located at the V apex at all temperatures. The real ring, in contrast, has its strand rotation impeded by stacking interactions between base pairs across the nick when the constraint is small enough, and behaves very much like topoisomer 0 (results not shown; this point is also discussed in ref. 8).]

RESULTS

a) Helical repeat of single DNA fragments.

Fig. 3A shows the electrophoresis of topoisomers of a pBR322 665 bp fragment at three temperatures, and fig. 3B the Vs obtained by plotting the mobility of the topoisomers relative to that of the linear fragment as a function of their topological constraint (see the figure legend). The deviation of topoisomer -4 toward the outside of the Vs is typical of a structural departure from the regular B form double helix induced by negative torsional stress, such as the B-Z transition (23, 24) or cruciform extrusion (25, 26). These transitions lead to an expansion of the molecule and therefore to a relative decrease in its mobility. The origin of the smaller deviations observed for topoisomers +3 and +4 is less clear. The most salient features of those Vs are: 1) an essentially identical mobility of the apex at all temperatures, consistent with the notion that this mobility is that of the relaxed ring, either real (at approximately 25°C) or virtual (at other temperatures; reference is made here to the hypothetical nicked ring described in the principle section); and 2) approximately equal and opposite shifts in the mobility of "negative" topoisomers and "positive" topoisomers plus topoisomer 0, respectively, upon increase in the

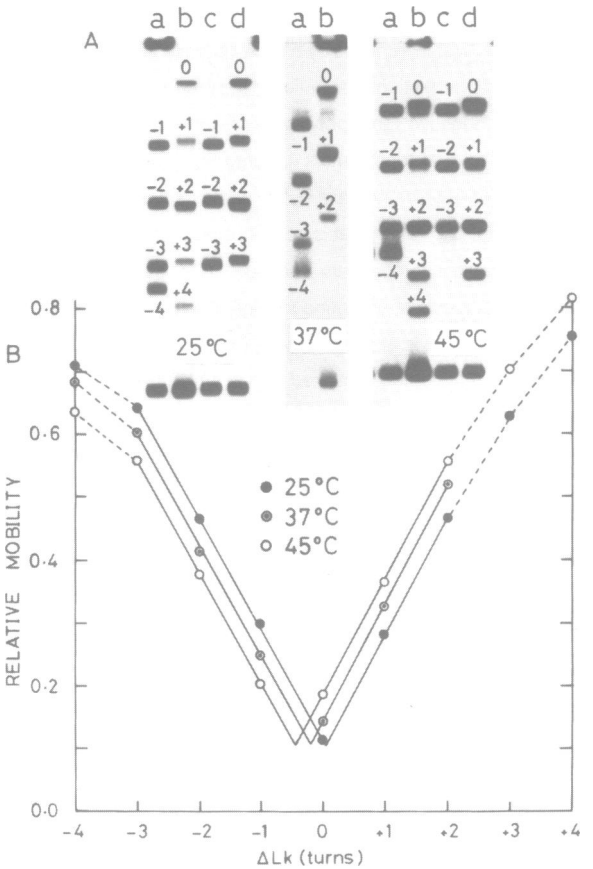

Figure 3 : V-like dependence of the electrophoretic mobility of topoisomers of a pBR322 665 bp fragment on topological constraint. (A) Topoisomers (numbers) were individually purified, appropriately mixed and electrophoresed in 4% polyacrylamide (acrylamide/bisacrylamide=30/1; w/w) gels at the temperatures indicated, along with the linear fragment (bottom bands). Autoradiograms are shown. The radioactivity at the top of the gels was added after the completion of the electrophoreses to show the position of the start. (B) Ratios between distances migrated from the start by the topoisomers and by the linear DNA in lanes a and b were plotted as functions of ΔLk. Vs were constructed by joining through continuous straight lines (the actual branches of the Vs) lower negatively and positively supercoiled topoisomers, respectively. Higher topoisomers which were not used in the constructions because of their significant deviations are shown by broken lines. Recognizing the supercoiling polarity is straightforward for most topoisomers, except sometimes for topoisomer 0 when it is close to relaxation, for example at 25 and 37°C. In those cases, a smaller (25°C) or a larger (37°C) mobility of topoisomer +1 relative to topoisomer -1 was taken as a reflection of a negative or positive supercoiling of topoisomer 0, respectively (see ref. 14 for a justification of this procedure and for more details on the construction of the V). Apexes of the Vs were obtained by extrapolating the branches until they intersect. If their equation, derived by a least squares procedure, is Mob=a. ΔLk + b, with Mob being the topoisomer mobility, then ΔLk at the apex is given by $\Delta Lk_V=(b_- - b_+)/(a_+ - a_-)$, where the + and - subscripts refer to the "positive" and "negative" branches, respectively. It comes ΔLk_V=+0.054, -0.200 and -0.442 turn at 25, 37 and 45 °C, respectively. 25 and 45 °C gels and corresponding Vs have previously been shown in fig. 2 in ref. 14.

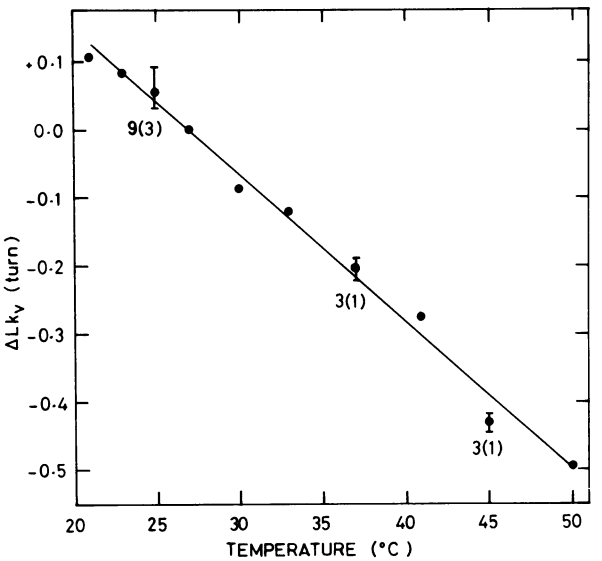

Figure 4 : Temperature dependence of the apex location for the pBR322 665 bp fragment. ΔLk values at the apex, ΔLk_V, derived from Vs shown in fig. 3B and others at the same or different temperatures (see for example figs. 5B and 7 below) were plotted as a function of gel temperatures. 25, 37 and 45 °C points correspond to mean values calculated from data obtained on several gels, whose number is indicated. In some of these gels, samples were electrophoresed in duplicate. The number of such gels is shown between parentheses. The bars span the extreme values obtained. Means and extreme values were +0.056, +0.032 and +0.092 turn at 25 °C; -0.203, -0.190 and -0.219 turn at 37 °C; and -0.430, -0.417 and -0.442 turn at 45 °C. Data points were fitted with a straight line using a least squares procedure.

gel temperature. This results in a net displacement of the V towards smaller ΔLk values parallel to the ΔLk axis, which reflects the thermal untwisting of the duplex.

Apex abscissae, ΔLk_V, were measured on those Vs and others obtained at the same or different temperatures as described in legend to fig. 3B, and were plotted in fig. 4 as a function of gel temperatures. Absolute differences between ΔLk_V values derived from duplicate migrations within the same gel (see fig. 4 and lanes c and d in gels in fig. 3A) were found to vary between 0.001 and 0.011 turn, with a mean (for five measurements) of 0.005 turn = 1.8 ° in helix rotation. The reproducibility in ΔLk_V from one gel to another is not as good, however (see error bars in fig. 4 and the figure legend). As shown in the figure, data points appear to fall on a straight line of slope, measured by least squares fitting, equal to -0.012 °/°C per bp. Potential helical repeat values of the torsionally unconstrained duplex at 25°C were calculated from equations (5) and (3) in the principle section using $\Delta Lk_V = +0.06 \pm 0.03$ turn (see legend to fig. 4). One obtains, with Lk_0 = 62, 63 or 64 turns, h =10.715, 10.546 or 10.381 ± 0.005 bp/turn, respectively (means ± maximal deviations for 12 measurements). The underlined, median, value will be shown in Discussion to be the most probable one.

Another example of the temperature dependence of the apex location is provided by a pBR322

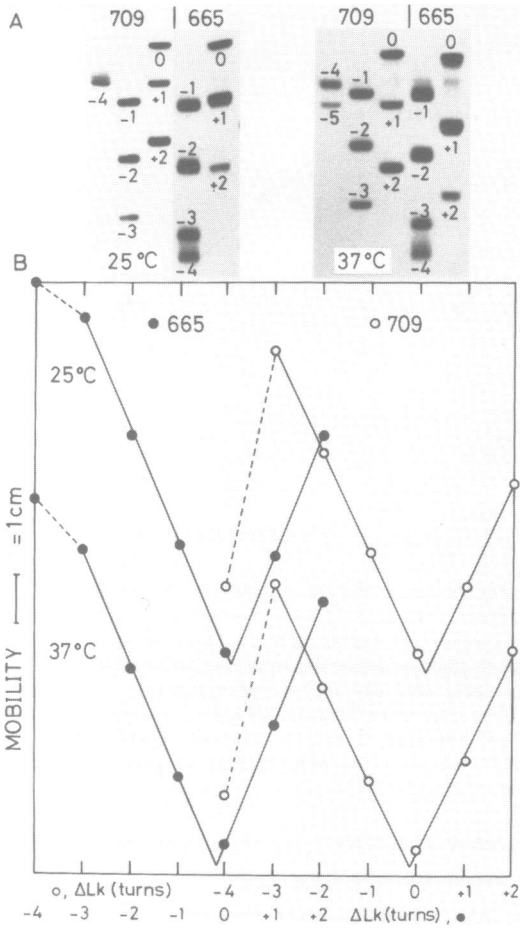

Figure 5 : Comparison between the 665 bp fragment and its poly(dA.dT). poly(dA.dT)-containing 709 bp derivative. (A) Topoisomers were mixed and electrophoresed in 4% polyacrylamide (acrylamide/bisacrylamide =30/1; w/w) gels at the temperatures indicated. Autoradiograms are shown. (B) Distances migrated from the start by the topoisomers were plotted as functions of ΔLk, and Vs were constructed as described in legend to fig. 3B. The V for the larger fragment was shifted by 4 turns up the ΔLk axis, relative to the V of the smaller. Four turns indeed correspond to the most probable difference between the linking numbers of the two topoisomers 0 (ΔLk_0 in equation 6 of Results)(see Discussion). ΔLk_V values were +0.092 and +0.173 turn at 25 °C; and -0.190 and -0.130 turn at 37 °C for 665 and 709 bp fragments, respectively.

506 bp fragment whose Vs at 25 and 37°C have previously been shown in ref. 14. ΔLk_V values measured on those Vs and others at 21, 32 and 41°C (not shown) were -0.23 and -0.46 turn, and -0.14, -0.37 and -0.53 turn, respectively. As observed above for the 665 bp fragment, those points appear to fall on a straight line (not shown) of slope -0.014°/°C per bp. Potential h values were calculated from ΔLk_V using

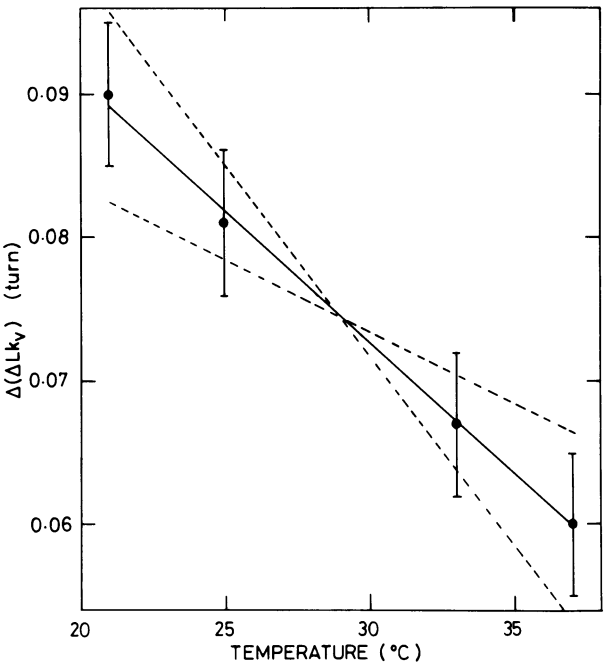

Figure 6 : Temperature dependence of the difference in V apex locations between the 665 bp fragment and its poly(dA.dT).poly(dA.dT)-containing 709 bp derivative. ΔLk_V differences between the larger and the smaller fragments, $\Delta(\Delta Lk_V)$, were measured for the two V couples in fig. 5B and two more at different temperatures (not shown), and were plotted as a function of gel temperatures. Data points were fitted with a straight line using a least squares procedure (continuous line). Error bars indicate the dispersion expected for the data if the measurement was repeated by running four more gels at the same four temperatures. They correspond to twice the average difference observed between ΔLk_V measurements of duplicate samples of the 665 bp fragment (0.005 turn; see Results). Broken lines tentatively show the extreme positions that the continuous line may adopt. Their slopes correspond to untwisting rates of 0.008 and 0.022 °/°C per bp. [Vs obtained for 665 and 709 bp fragments had ΔLk_V values of +0.107 and +0.197 turn at 21°C; and -0.120 and -0.053 turn at 33°C.]

equations (5) and (3), with Lk_0 = 47, 48 or 49 turns. h values were subsequently normalized to 25°C as indicated in the Principle section, using the thermal untwisting coefficient just determined. One obtains h=10.818, 10.592 and 10.374± 0.002 bp/turn (means ± maximal deviations for five measurements)

b) Comparison between two DNA fragments.

The helical twist difference between two fragments, ΔTw_n, is given by the differential of equation (5) in Principle. It comes

$$\Delta Tw_n = \Delta Lk_0 + \Delta(\Delta Lk_V) \qquad (6)$$

where ΔLk_0 and $\Delta(\Delta Lk_V)$ are the differences between the linking numbers of the two topoisomers 0, and between the apex locations measured on the Vs, respectively. Because the intra-gel reproducibility of the

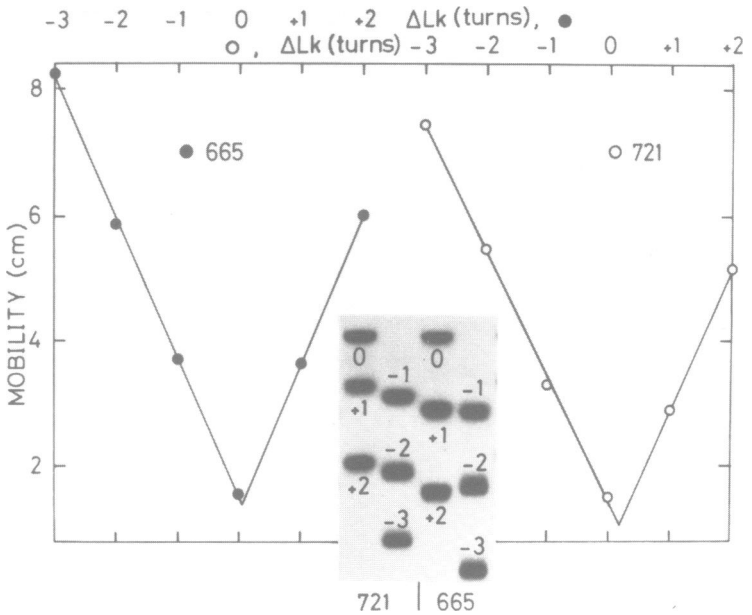

Figure 7 : Comparison between the 665 bp fragment and its poly(dA).poly(dT)-containing 721 bp derivative. Purified topoisomers were mixed and electrophoresed in a 4% polyacrylamide (acrylamide/bisacrylamide=30/1; w/w) gel at 25 °C. An autoradiogram is shown. Vs were represented as described in legend to fig. 5B. The displacement of one V relative to the other was taken equal to 6 turns.

ΔLk_v measurement appears to be significantly better than its inter-gel reproducibility (see above), such comparison will always be performed within the same gel.

i) Helical repeat of poly(dA.dT).poly(dA.dT) : Fig. 5A shows the electrophoretic patterns obtained at two temperatures with topoisomers of the above 665 bp fragment, and of a 709 bp fragment derived from it by insertion of poly(dA.dT). poly(dA.dT). The relative decrease in the mobility of topoisomer -4 as compared to topoisomer -3 appears to be much larger for the 709 bp fragment than for the 665 bp fragment (see above). This is presumably due to the extrusion of a large cruciform out of the self-complementary insert. Corresponding Vs are shown in fig. 5B. ΔLk_v values were measured from those Vs and from two more V couples obtained at other temperatures (not shown), and their difference, $\Delta(\Delta Lk_v)$ in equation (6), was plotted in fig. 6 as a function of gel temperature. The slope of the least squares straight line constructed on the points gives the thermal untwisting rate of the insert, 0.015°/°C per bp. Given the small total twist variation of the insert, however, this measurement may be prone to a significant error. This error was tentatively estimated from the above intra-gel reproducibility of the ΔLk_v measurement to be ± 0.007 °/°C per bp (see the figure legend). The number of helical turns made by the insert, Tw_i ($Tw_i=\Delta Tw_n$ in equation 6) was then calculated from $\Delta(\Delta Lk_v)$ in fig. 6 and $\Delta Lk_o=4$ turns. Tw_i values obtained at the different temperatures were subsequently normalized to 25 °C using equation (4)

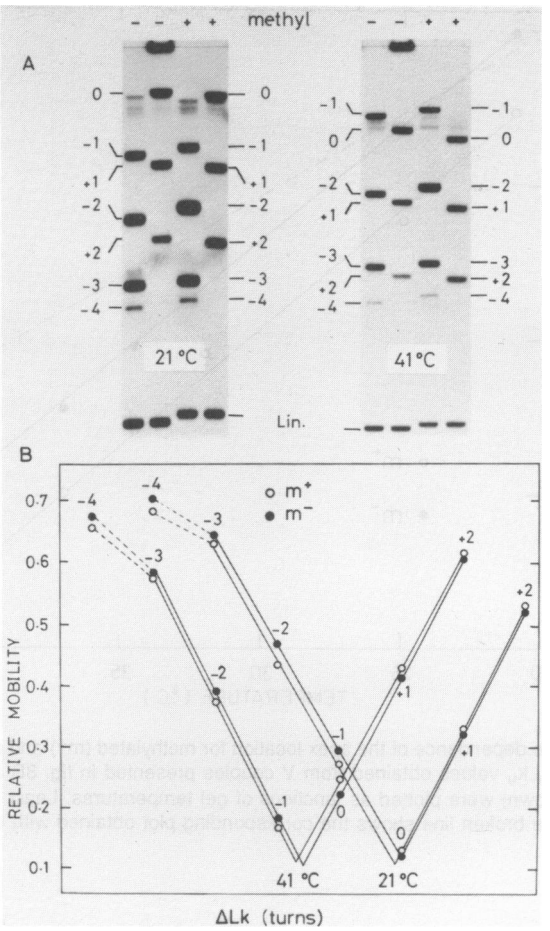

Figure 8 : Comparison between methylated (m^+) and unmethylated (m^-) 641 bp oriC fragments. (A) Topoisomers were mixed and electrophoresed at the temperatures indicated in 4% polyacrylamide (acrylamide/ bisacrylamide = 30/1; w/w) gels, along with corresponding linear DNAs (Lin.). Autoradiograms are shown. The radioactivity at the top of the gels indicates the position of the start. (B) The ratio between the distance migrated by each topoisomer and the averaged distance traveled by methylated and unmethylated linear DNAs was plotted as a function of ΔLk. Vs were constructed by joining positively and negatively supercoiled topoisomers, respectively, through continuous straight lines, as described in legend to fig. 3B. ΔLk_v values were, for m^+ and m^- fragments, respectively, -0.104 and -0.00 turn at 21 °C; and -0.725 and -0.604 turn at 41 °C.

in the Principle section with Th = 0.015 ± 0.007 °/°C per bp. One obtains Tw_i = 4.08 ± 0.01 turns and from equation (3) in Principle, h_i = 44/Tw_i = 10.78 ± 0.03 bp/turn, (mean ± maximal deviations for 4 measurements). ΔLk_0 = 3 or 5 turns would lead to h_i = 14.28 ± 0.04 or 8.66 ± 0.02 bp/turn, respectively.

ii) Helical repeat of poly(dA).poly(dT) : Fig. 7 shows electrophoretic patterns obtained at 25 °C with

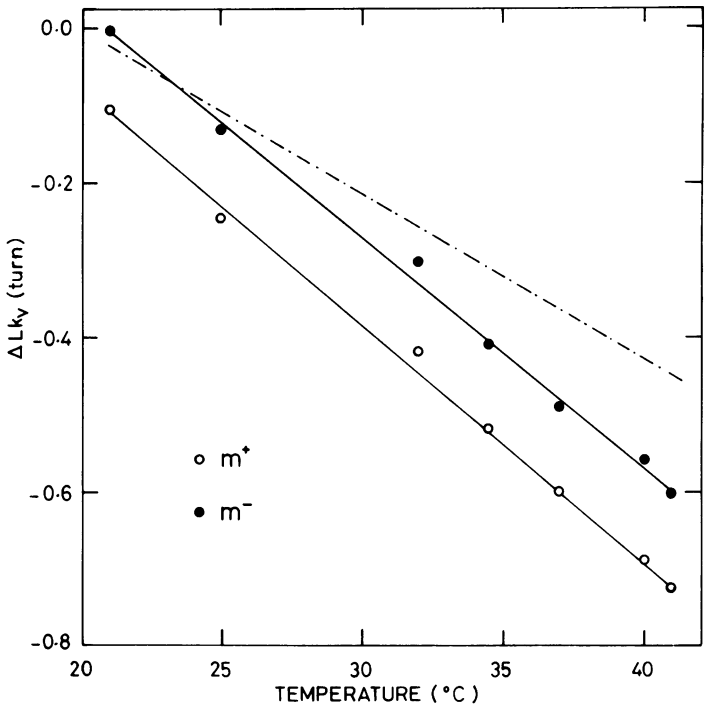

Figure 9 : Temperature dependence of the apex location for methylated (m^+) and unmethylated (m^-) 641 bp ori C fragments. ΔLk_V values obtained from V couples presented in fig. 8B and others at different temperatures (not shown) were plotted as functions of gel temperatures. Least squares straight lines were constructed. The broken line shows the corresponding plot obtained with the 665 bp fragment in fig. 4.

topoisomers of the 665 bp fragment and of its 721 bp long derivative obtained through the insertion of poly(dA).poly(dT), together with the corresponding Vs. An additional couple of Vs was obtained from duplicate patterns within the same gel (not shown), and two more couples from another gel run under identical conditions with duplicate samples. ΔLk_V was measured and Tw_i ($Tw_i = \Delta Tw_n$ in equation 6) subsequently calculated using ΔLk_0=6 turns. It came Tw_i=6.12 ± 0.01 turns and h_i=61/Tw_i=9.97 ± 0.02 bp/turn (mean ± maximal deviations for 4 measurements). ΔLk_0=5 or 7 turns would lead to h_i=11.92 ± 0.03 or 8.57 ± 0.02 bp/turn, respectively.

iii) Unwinding associated with methylation of dA : Fig. 8A shows electrophoretic patterns obtained at two temperatures with topoisomers of a 641 bp ori C fragment before (m^-) and after (m^+) *in vivo* methylation of the dA residue in d(GATC). The fragment contains 19 such sites (27). The unwinding caused by the presence of the methyl groups in fragment 641m^+ is readily seen in the figure. Indeed, "positive" topoisomers migrate more in 641m^+ than in 641m^-, whereas the opposite trend is observed for

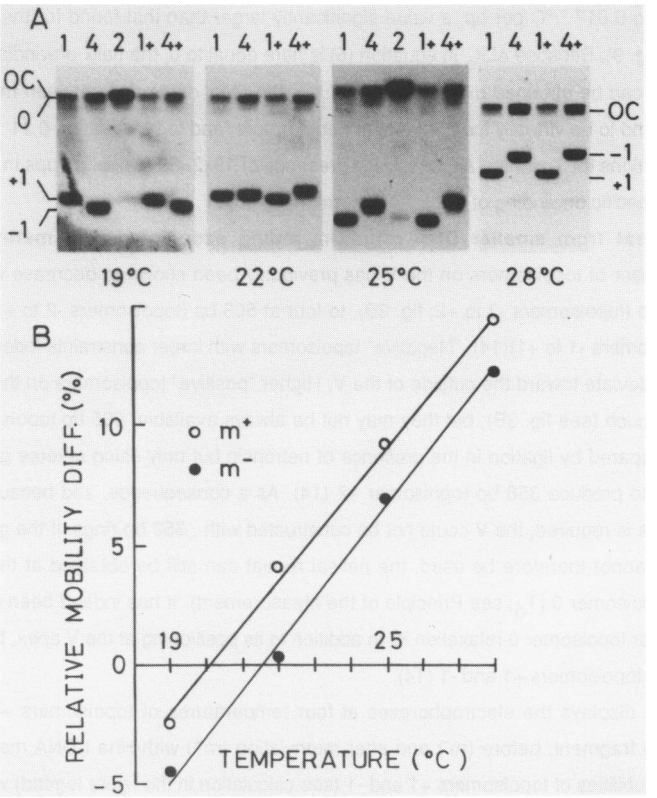

Figure 10 : Comigration temperature of topoisomers +1 and -1 of a pBR322 358 bp fragment, and its dependence on methylation. (A) Electrophoreses of total unmethylated (1, 2 and 4) and methylated fractions (1+ and 4+) (see Methods) were performed in 5% polyacrylamide (acrylamide/bisacrylamide=20/1; w/w) gels at the temperatures indicated. Autoradiograms are shown. OC : open circular DNA. (B) Relative differences in the mobilities of unmethylated (m^-) and methylated (m^+) topoisomers +1 and -1 were plotted as functions of gel temperatures. With d_{+1} and d_{-1} being the distances migrated beyond topoisomer 0 by topoisomers +1 and -1, respectively, the relative mobility difference is given by $(d_{+1}-d_{-1})/2 \times d_{\pm 1}$. Either d_{+1} or d_{-1} was used in the denominator, depending on which distance is larger. The factor 2 takes into account that the displacement of one topoisomer relative to the other is contributed equally by each of them (see fig. 2). Slopes of the least squares straight lines were found to be close to 1.9 %/°C. Such value corresponds to an apparent thermal untwisting coefficient of 1.9 x 360/100/358 = 0.019 °/°C per bp.

"negative" topoisomers. Methyl groups also appear to cause a retardation of the linear DNA. Fig. 8B shows the two corresponding couples of Vs.

ΔLk_V values measured from those Vs and other V couples obtained at different temperatures (not shown) display the same linear dependence on gel temperatures for m^+ and m^- fragments (fig. 9). Corresponding thermal untwisting coefficients calculated from the slopes of the least squares straight

lines are close to 0.017 °/°C per bp, a value significantly larger than that found for the 665 bp fragment (broken line in fig. 9). Because ΔLk_0 in equation (6) is here equal to 0, the helix unwinding associated with the methylation can be obtained directly from $\Delta(\Delta Lk_V)$, the ΔLk_V difference between m^+ and m^- DNAs. $\Delta(\Delta Lk_V)$ was found to be virtually independent on temperature, and to be equal to -0.11 ± 0.01 turn (mean ± maximal deviations for 7 points). Assuming the presence of 19x2=38 methyl groups in the m^+ fragment, this leads to a specific unwinding of 1.1 ± 0.1° per methyl group.

c) Helical repeat from smaller DNA rings.Unwinding associated with methylation of dC.

The number of topoisomers on the V has previously been shown to decrease with the ring size: from 6 at 665 bp (topoisomers -3 to +2; fig. 3B), to four at 506 bp (topoisomers -2 to +1) and to three at ~350 bp (topoisomers -1 to +1)(14). "Negative" topoisomers with larger constraints indeed start structural transitions and deviate toward the outside of the V. Higher "positive" topoisomers on the other hand may not deviate as much (see fig. 3B), but they may not be always available. 665 bp topoisomers +3 and +4 could not be prepared by ligation in the presence of netropsin but only using reverse gyrase, while both methods failed to produce 358 bp topoisomer +2 (14). As a consequence, and because a minimum of four topoisomers is required, the V could not be constructed with ~350 bp rings. If the general method of measurement cannot therefore be used, the helical repeat can still be obtained at the temperature of relaxation of topoisomer 0 (T_0; see Principle of the Measurement). It has indeed been previously shown that a criterion for topoisomer 0 relaxation is, in addition to its positioning at the V apex, the comigration of its two neighbor topoisomers +1 and -1 (14).

Fig. 10A displays the electrophoreses at four temperatures of topoisomers +1, 0, and -1 of a pBR322 358 bp fragment, before (m^-) and after methylation (m^+) with Hha I DNA methylase. Relative differences in mobilities of topoisomers +1 and -1 (see calculation in the figure legend) were plotted in fig. 10B as a function of gel temperatures, for both m^- and m^+ DNAs. The points are found to be on approximately parallel straight lines. Intersections of these lines with the abscissa give the topoisomer comigration temperatures and therefore T_0 (see above). 21.5 and 20.1 °C are obtained for m^- and m^+ DNAs, respectively. These results therefore show that the unwinding associated with the methylation can be compensated by the thermal overwinding caused by a 21.5-20.1 = 1.4 °C decrease in the gel temperature.

Assuming a thermal untwisting coefficient of 0.013°/°C per bp (the mean of the values obtained above with 665 and 506 bp fragments), the methylation-induced untwisting can be calculated from equation (4) in Principle to be equal to 0.018 turn. The reproducibility of this measurement, approximately ±0.003 turn, was estimated from the accuracy achieved in the determination of each comigration temperature, about ±0.1°C (see fig. 10B). Knowing that the 5 Hha I sites in each molecule (ref. 15; see also Methods) contain a maximum of 10 methyl groups and a minimum of 5 (see Methods), the unwinding can be calculated to be comprised between (0.018 ± 0.003)x360/10 and (0.018 ± 0.003)x360/5 °, that is, 0.6_5 and 1.3 ± 0.1° per methyl group. Helical periodicities of m^- and m^+ fragments at the respective topoisomer 0 relaxation temperatures were calculated from equation (3) in the principle section. These

helical repeats were subsequently normalized to 25°C using equation (4) and the above thermal untwisting coefficient. It came $h(m^-)$=10.863, 10.543 or 10.242 bp/turn and $h(m^+)$=10.869, 10.549 or 10.247 bp/turn with Lk_0=33, 34 or 35 turns, respectively. The reproducibility of these measurements was found to be smaller than ±0.001 bp/turn.

DISCUSSION

a) Linking numbers.

Lk_0 in equations (2) and (5), or ΔLk_0 in equation (6), which represent the linking number of topoisomer 0, or the difference between these linking numbers in the comparison between two series of rings of different sizes, are generally unknown. For this reason, the method does not provide a unique value for h, but rather a set of discrete potential values upon successive variations of Lk_0 or ΔLk_0 by one unit. The problem is therefore to assign a proper value to Lk_0 or ΔLk_0.

There is little doubt that the correct ΔLk_0 values are those which lead to 10.78 and 9.97 bp/turn

TABLE I: Helical repeats at 25°C

	Fragments					Inserts[4]	
	358[1]	506[2]	641[2]	665[2]	1374[3]	poly(dA.dT). poly(dA.dT)	poly(dA). poly(dT)
			m^+ m^-				
dG+dC(%)[5]	61.7	51.8	45.9	46.5	55.1	–	–
h(bp/turn)	10.54_5	10.59	10.55 10.53	10.54_5	10.58	10.78	9.97
Reproducibility (bp/turn)[6]	<± 0.001	± 0.002	± 0.005	± 0.005	–	± 0.03	± 0.02

1) Only data relative to the unmodified fragment is reported. The helical repeat was first calculated at the temperature at which topoisomers +1 and -1 comigrate, and was subsequently normalized to 25°C (see Results and Discussion).
2) m^+ and m^- refer to the methylated and unmethylated fragments, respectively (see Materials and Methods). Helical periodicities were calculated from the position of the apex of the Vs on the ΔLk axis, as described in Results and Discussion.
3) The 1374 bp fragment originates from a Sau 3A digest of pBR322 (15). Two Vs have previously been obtained for this fragment at 25 and 29 °C. Corresponding ΔLk_V values were -0.13 and -0.33 turn at 25 and 29 °C, respectively, leading to a thermal unwinding of 0.013°/°C per bp (14). The helical repeat was calculated as described in Discussion.
4) Insert helical repeats were derived from the comparison of Vs obtained with 665 and 709 bp fragments (poly(dA.dT).poly(dA.dT)), and 665 and 721 bp fragments (poly(dA).poly(dT)) (see Results).
5) Average dG + dC contents were calculated from sequences reported in refs 27 (641 bp fragment) and 34 (other fragments).
6) Reproducibilities were derived from the accuracy achieved in the determination of the comigration temperatures of topoisomers +1 and -1 (358 bp fragment); from the deviation of the data points around the least squares straight lines in the plots relating apex locations, or differences in apex locations, to gel temperatures (506 and 641 bp fragments; poly(dA.dT).poly(dA.dT) insert); and from the dispersion obtained in several measurements performed at the reference temperature (665 bp fragment; poly(dA).poly(dT) insert) (see Results). Although reproducibilities obtained for the fragments bear on the third decimal point, their helical repeats were rounded off to the second.

(see Results) for the helical repeats of poly(dA.dT).poly(dA.dT) and poly(dA).poly(dT), respectively. These estimates are indeed close to those previously obtained for the same polymers using both the digestion method, 10.5 and 10.0 ± 0.1 bp/turn (5), and the band shift procedure (see Introduction), 10.7 and 10.1 ± 0.1bp/turn (6, 7), while estimates derived from other ΔLk_0 values appear to be aberrant (see Results). In contrast, assigning a value to Lk_0 is more difficult since a one unit variation results in a significantly smaller increment of h, $|\Delta h|$, than observed for ΔLk_0. The reason is the inverse dependence of $|\Delta h|$ on the ring size or insert length, N, as shown by the differential of equation (3) : $|\Delta h|=h^2/N\sim 110/N$. An interesting consequence of this equation, however, is that only the correct estimates of h (derived from the proper values of Lk_0) are expected to remain invariant upon increase in N, while higher and lower estimates should show negative and positive trends, respectively. Inspection of the data obtained for mixed sequence DNAs from pBR322 reveals that only the underlined, median, values of h, 10.543, 10.592 and 10.546 bp/turn for 358, 506 and 665 bp fragments, respectively, meet the invariance criterion satisfactorily, while other values show the expected trends (see Results). [The sum of the squares of the three differences between median h values (4.5×10^{-3}) is found to be almost 10 fold smaller than the equivalent quantity for the higher and lower estimates (34.5×10^{-3} and 36.8×10^{-3}, respectively).]

b) Helical repeats.

Table I lists all helical repeats obtained in this work, together with the reproducibility of the measurements. Helical repeats of the methylated and unmethylated 641 bp fragments were calculated as described in Results for the 506 bp fragment, using Lk_0=61 turns. These repeats appear to be very similar to those of the other fragments. The helical repeat of the 1374 bp fragment was derived from a V previously obtained at 25 °C in a 2.5% agarose gel (14), using Lk_0=130 turns (see the table legend). The size of this fragment is clearly an upper limit for the method since $|\Delta h|$ (see above) is here equal to only 0.08 bp/turn, which makes adjacent h values difficult to discriminate from one another. The repeat obtained using Lk_0=131 turns, 10.50 bp/turn, was however not considered because it lies outside the range observed for the other fragments. dG +dC contents are indicated.

Such excellent reproducibility in the measurements (table I) does not necessarily mean that actual helical repeats are known with this accuracy. The narrow range of variation of h values for mixed sequence DNA, 10.56 ± 0.03 bp/turn, however, suggests that present estimates and true helical repeats are not far from each other. In fact, if potential biases involved in the method have led to random deviations from the true repeats, which may *a priori* be different for each fragment, then ± 0.03 bp/turn may be considered as an overestimate of those deviations. Such conclusion is further supported by the close agreement of those repeats with the two accurate estimates, 10.55 and 10.57 bp/turn, referred to in the Introduction and derived from small DNA rings.

In contrast, the agreement between repeats obtained for poly(dA.dT).poly(dA.dT) and poly(dA).poly(dT) with the different methods is not as close (see above). Discrepancies between the band shift and the present methods are probably related to the fact that the measurement is made on (slightly) supercoiled DNA molecules in the first case, but on relaxed DNA in the second. The band shift method may therefore be more dependent on the dynamic properties of the double helix than the present one. In

particular, a difference in the torsional and/or bending rigidities of the polymer insert relative to flanking DNA is expected to alter the partition of the topological constraint of the ring into twisting and writhing (see equation (1) in the principle section). This will in turn affect the electrophoretic mobility of the insert-containing topoisomers relative to the noninserted ones, and subsequently the measurement of the insert helical periodicity. It is interesting that a detailed study of cruciform extrusion has suggested that poly(dA.dT).poly(dA.dT) inserts may indeed have a smaller torsional rigidity than surrounding DNA (28, 29).

c) DNA thermal untwisting.

665 and 506 bp fragments have been shown to untwist upon increase in gel temperatures from 21 °C to 50 and 41°C, respectively, at rates of 0.012 and 0.014 °/°C per bp (see Results). Similarly, this rate is found to be equal to 0.013 °/°C per bp for the 1374 bp fragment between 25 and 29°C (see legend to table I). These figures appear undistinguishable from those obtained by others, 0.012 (11) and 0.014 °/°C per bp (12), using the band counting method from closure or topoisomerization of plasmid DNAs at temperatures comprised between 0 and 41°C. Interestingly, the thermal untwisting of the 641 bp fragment, 0.017 °/°C per bp, appears to be larger (fig. 9), although its dG + dC content is similar to that of the 665 bp fragment (table I). This may be due to a large contribution to untwisting of the oriC region of the fragment, where the density of d(GATC) sites, targets of dam methylase, is exceptionally high (27). Such ability to untwist may indeed facilitate the formation of alternative structures in this region which also contains several short direct and inverted repeats, and may be relevant to the function of oriC in the initiation of DNA replication (see ref. 30 for a recent review).

The thermal untwisting of the 358 bp fragment is expected to be reflected in the slope of the straight lines in fig. 10B. The large untwisting which is obtained, 0.019 °/°C per bp (see the figure legend), is however unlikely to be significant since an increase over the true value is expected from the U-like dependence on topological constraint observed for the topoisomers of smaller fragments (14). Indeed the U differs from the V by a larger mobility of topoisomer 0 relative to topoisomers +1 and -1, whose mobilities remain virtually unchanged. The distance between topoisomers +1 (or -1) and 0 (the denominator of the fraction measuring the slope of the straight lines in fig. 10B; see the figure legend) therefore decreases, while the distance between topoisomer +1 and -1 (the numerator of the fraction) is constant. [Such U was shown to occur when compaction forces exerted by the gel matrix on the rings are large enough (14).]

d) Helix unwindings associated with dA and dC methylations.

The helix unwinding associated with *in vivo* methylation of dA at the N^6 position in d(GATC) by dam methylase which has been found here, 1.1 ± 0.1° per methyl group, appears to be twice as large as the 0.50 ± 0.02 ° figure obtained recently by others using the band shift method with plasmid DNAs methylated either *in vitro* or *in vivo* (31). The reason for this discrepancy is unknown, although it is unlikely to lie in the different procedures used since these were found to otherwise lead to very similar data.

A unique value for the helix unwinding induced by *in vitro* methylation of the internal dC residue at

the C ^{5} position in d(GCGC) by HhaI DNA methylase could not be derived due to the lack of a determination of the exact number of methyl groups effectively transferred. Experiments described in Methods have however shown that all potential sites were at least hemimethylated, with a large proportion of them fully methylated. This unwinding may therefore be closer to 0.6 ° per methyl group (DNA methylated to 100%) than to 1.3 ° (in case of a 50% methylation), and appears smaller than that associated with dA methylation (see above).

Both methylation-associated unwindings must reflect permanent alterations of the local structure of the double helix, rather than the effects of a thermal melting. For dC methylation, this results from the observation that the presence of 5 mdC rather increases the DNA melting temperature (32). For dA methylation, this conclusion is based on the lack of temperature dependence of the unwinding between 21 and 41 °C (this work) and between 5 and 37°C (31). This feature is particularly relevant in view of the depression in the DNA melting temperature caused by 6 mdA (33).

e) Concluding remarks.

The property of small DNA rings to migrate in a suitable gel according to a V has been shown here to provide an accurate tool to estimate the helical twist of the torsionally unconstrained double helix under defined conditions. The method may be applied to single fragments, and give their average helical repeat; to avoid ambiguities in the assignment of a value to Lk_0, however, these fragments should not be longer than possibly 1000 bp. The method may also be used to compare the twist of two fragments, in which case the helical repeat of an insert or the alteration of the twist due to a modification of the DNA can be investigated.

The method appears less flexible when applied to fragments smaller than 500 bp, for which the V cannot be constructed. Indeed, in that case, only a helical repeat at the temperature of relaxation of topoisomer 0 (T_0) is directly produced, and normalization to 25 °C must assume a value for the thermal untwisting coefficient. This procedure may be prone to error if T_0 is far from the reference temperature, as would be the case with a 354 bp fragment, for example, of T_0 close to 65 °C. This difficulty may potentially be overcome by using the comigration of two adjacent rather than nonadjacent topoisomers like +1 and -1. Such comigration would indeed occur at approximately 25 °C with the 354 bp fragment referred to above. Lk_0 in equation (3) in the principle section would then have to be replaced by Lk_0 +0.5 (for topoisomers +1 and 0) or Lk_0 -0.5 (topoisomers 0 and -1). The transformation of the V into a U, which is observed with smaller fragments (see above and ref. 14), is however expected to reduce the temperature dependence of the mobility of topoisomers close to relaxation and therefore the sensitivity of such measurement. Another possibility is to make head-to-tail dimers of the smaller fragments to form rings of size consistent with the construction of a V.

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