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Chromatin Reconstitution on Small DNA Rings

V. DNA Thermal Flexibility of Single Nucleosomes

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The thermal flexibility of DNA minicircles reconstituted with single nucleosomes was measured relative to the naked minicircles. The measurement used a new method based on the electrophoretic properties of these molecules, whose mobility strongly depended on the DNA writhe, either of the whole minicircle, when naked, or of the extranucleosomal loop, when reconstituted. The experiment was as follows. The DNA length was first increased by one base-pair (bp), and the correlative shift in mobility resulting from the altered DNA writhe was recorded. Second, the gel temperature was increased so that the former mobility was restored. Under these conditions, the untwisting of the thermally flexible DNA due to the temperature shift exactly compensates for the increase in the DNA mean twist number resulting from the one bp addition. The relative thermal flexibility was then calculated as the ratio between the increases in temperature measured for the naked and the reconstituted DNAs, respectively. The figure, 0·69 (±0·07), was used to derive the length of DNA in interaction with the histones, 109 (±25) bp. Such length was in good agreement with the mean value of 115 bp we have previously obtained from the distribution of the angles between DNAs at the entrance and exit of similar nucleosomes measured from high resolution electron microscopy. This consistence further reinforces our previous conclusion that minicircle-reconstituted nucleosomes, with 1·3(109/83) to 1·4(115/83) turns of superhelical DNA, show no crossing of entering and exiting DNAs when the loop is in its most probable configuration, and therefore, that these nucleosomes behave topologically as "single-turn" particles. The present data are also within the range of values, 50 to 100 bp of thermally rigid DNA per nucleosome, obtained by others for yeast plasmid chromatin, suggesting that the "single-turn" particle notion may be extended to this particular case of naturally-occurring H1-free chromatin. However, these data are quite different from the 230 bp figure derived from thermal measurements of reconstituted H1-free minichromosomes. It is proposed that nucleosome interactions occurring in this chromatin, but not in yeast chromatin, may be partly responsible for the discrepancy.

Keywords: DNA twist; DNA topology; nucleosome interactions; yeast chromatin

The twist of DNA free in solution is temperature-dependent. This can be observed when relaxing with topoisomerase I (or closing with DNA ligase) a closed (or nicked) circular DNA molecule at two temperatures, and measuring the mean linking number of the resulting equilibrium distributions of the topoisomers. Increasing the temperature actually untwists each base-pair (bp†) of the double helix linearly at a rate of 0·012 to 0·014° per deg.C (Depew & Wang, 1975; Pulleyblank *et al.*, 1975). However it is expected that this thermal untwisting can be at least partially inhibited if DNA is complexed with a protein. The overall "thermal flexibility" of a DNA molecule partly involved in such a complex may then be measured relative to the naked DNA, and from this, an estimate of the length of DNA resisting thermal untwisting and therefore interacting with the protein could be derived.

When applied to chromatin in circular mini-chromosomes, this method has produced estimates for the length of DNA in interaction with the histones in a nucleosome. These values have not always been in agreement with figures derived from micrococcal nuclease digestions, about 145 and

† Abbreviations used: bp, base-pair(s); SV40, simian virus 40.

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165 bp for H1-free and H1-containing nucleosomes, respectively (McGhee & Felsenfeld, 1980). In early experiments, DNA in native simian virus 40 (SV40) minichromosomes showed a complete inability to untwist upon a temperature increase (Keller *et al.*, 1978). Later on, a flexibility corresponding to 160 bp of DNA immobilized per H1-containing nucleosome was found, close to the expected figure (Ambrose *et al.*, 1987; Lutter, 1989). Reconstituted chromatin, however, has shown very low DNA thermal flexibilities, corresponding to as much as 230 bp of immobilized DNA per nucleosome (Morse & Cantor, 1985), regardless of the absence or presence of H5 or of the trimming of histone tails with trypsin (Morse & Cantor, 1986). So far, two explanations have been given for this result. The first one involved theoretical considerations which showed that some peculiar thermal distortion in the shape of the histone core surface could be responsible (White *et al.*, 1989). The second one is based on spectroscopic measurements which revealed a non-linear dependence of the amplitude of the circular dichroism of such chromatin with temperatures above 20 °C (Smirnov *et al.*, 1991). This effect was interpreted as reflecting a nucleosome loss above this temperature, although it could also be due to a thermally induced conformational transition of the nucleosomes. The merit of the nucleosome-loss explanation was that, if taken into account, the 230 bp value could be lowered to 175 to 180 bp. Moreover, this 175 to 180 bp value could itself be further lowered to the expected value of 145 bp by a decrease in the salt concentration to 10 mM (Smirnov *et al.*, 1991).

On the other hand, DNA in native yeast plasmid chromatin, present either in cells, spheroplasts, nuclei or nuclear extracts, has shown very large thermal flexibilities (Saavedra & Huberman, 1986) with only 50 to 100 bp of DNA immobilized per nucleosome (Morse *et al.*, 1987). The reason for this large flexibility has been assumed to lie in the differences in primary amino acid sequences between yeast histones and those of higher eucaryotes (Morse *et al.*, 1987), which could also explain the high transcriptional activity of yeast chromatin (Grunstein, 1990). The lack of H1 in this chromatin may indeed not be responsible, as suggested by the above data with reconstituted chromatin and by the observation that the small percentage of SV40 minichromosomes active in transcription, which are supposed to be also devoid of H1, are not more flexible than bulk H1-containing SV40 chromatin (Lutter, 1989). It is interesting, along this line, that purified yeast nucleosome core particles were indeed found to be more susceptible to salt dissociation than their chicken erythrocyte counterparts, but not to be a better template for *in vitro* transcription with *Escherichia coli* RNA polymerase (Pineiro *et al.*, 1991).

In this work, gel electrophoresis was used to measure the thermal flexibility of DNA minicircles reconstituted with single nucleosomes. This chromatin system has previously been described in detail (Goulet *et al.*, 1988) and has enabled so far the measurement of DNA supercoiling in the H5-free (Zivanovic *et al.*, 1988) and H5-containing (Zivanovic *et al.*, 1990) nucleosomes, and the investigation of the dependence of nucleosome sequence preference on DNA supercoiling (Duband-Goulet *et al.*, 1992). DNA thermal flexibility in this system could, in principle, also be obtained through relaxation with topoisomerase I at two temperatures. However, given the short size of the DNA and consequently the small change in twist induced by the temperature shift, this measurement would be rather inaccurate. An alternative procedure, schematized in Figure 1, made use of the property of such particles to show electrophoretic mobilities strongly dependent on the writhe of their extra-nucleosomal loop, which itself depends on the loop linking number difference, ΔLk_1 (see a definition of ΔLk_1 in Table 1 legend). More specifically, the observation of interest was that nucleosomes on $Lk = 33$ and 32 topoisomers of a 354 bp minicircle

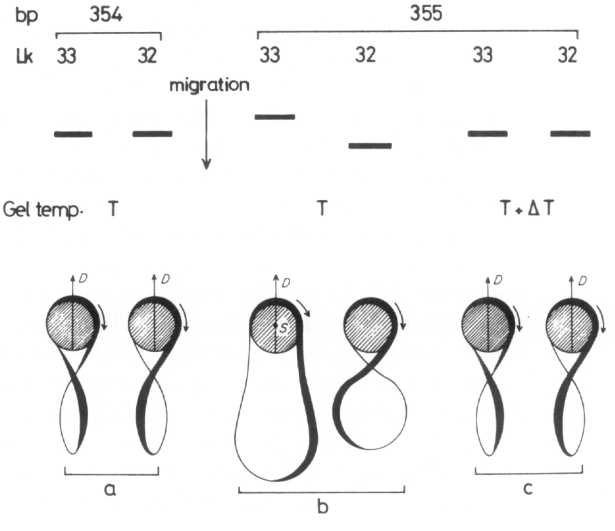

Figure 1. Thermal flexibility of DNA minicircles reconstituted with single nucleosomes. The principle. a, Nucleosomes on $Lk = 33$ and 32 topoisomers of a 354 bp minicircle have the same electrophoretic mobility at temperature t. This comigration is expected to result from the opposite and equal linking number differences applied to their extranucleosomal loop (ΔLk_1 is close to +0·5 and −0·5, respectively; see Table 1). As a consequence, the loops have rotated by equal and opposite angles around the particle dyad axis, D, so that their planes are parallel to the superhelix axis, S. Note that the upper exiting DNA remains above in the loop in the right-hand configuration, while it goes below in the left-hand configuration. b, Upon increase in the DNA size to 355 bp, ΔLk_1 decreases by 0·1 (see Table 1), and the loop partially relaxes in the $Lk = 33$ particle which is retarded, and supercoils more in the $Lk = 32$ particle which is accelerated. These effects are shown exaggerated in the Fig. c, Nucleosome comigration can be restored upon increase in the gel temperature by Δt ($\Delta t > 0$) if the correlative DNA thermal untwisting in the loop exactly compensates for the increase in the DNA mean twist number resulting from the 1 bp addition. Δt, as measured here, is close to 11 deg.C. (T represents t.)

Table 1

Topological parameters of naked and reconstituted topoisomers calculated in TE buffer at 27·5 °C

N (size; bp)	Lk_0†	Lk	ΔLk‡	Lk	ΔLk‡	ΔLk_1§	Lk	ΔLk‡	ΔLk_1§
351	33·14	34	+0·86	33	−0·14	+0·86	32	−1·14	−0·14
353	33·33	34	+0·67	33	−0·33	+0·67	32	−1·33	−0·33
354	33·43	34	+0·57	33	−0·43	+0·57	32	−1·43	−0·43
355	33·52	34	+0·48	33	−0·52	+0·48	32	−1·52	−0·52

† Lk_0 is the linking number of the minicircle most probable configuration and was calculated from the classical equation;

$$Lk = Tw + Wr, \tag{1}$$

(White, 1969; Fuller, 1971; Crick, 1976), in which Lk, Tw and Wr are the topoisomer linking number, twist and writhe, respectively. Making $Wr = 0$ in eqn (1), we find $Lk_0 = Tw_0 = N/h_0$ (Horowitz & Wang, 1984). h_0 = 10·59 (±0·03) bp/turn is the helical periodicity of *torsionally unconstrained* mixed-sequence DNA at 27·5 °C in TE buffer (10 mM-Tris·HCl and 1 mM·EDTA, pH 7·5), as measured in Zivanovic *et al.* (1988) using the V-curve method (Zivanovic *et al.*, 1986; Goulet *et al.*, 1987).

‡ ΔLk is the topoisomer linking number difference relative to the minicircle most probable configuration, and is given by:

$$\Delta Lk = Lk - Lk_0 \tag{2}$$

(Wang *et al.*, 1982). ΔLk depends on the temperature, t, as does Lk_0, according to the equation:

$$\Delta Lk\ (t) = \Delta Lk\ (27{\cdot}5) + 0{\cdot}0125 \times N\ (t - 27{\cdot}5)/360, \tag{3}$$

in which 0·0125 (in °/deg.C per bp) is the rate of DNA thermal untwisting measured here (see text). ΔLk partitions into twist and writhe, according to the differential of eqn (1):

$$\Delta Lk = \Delta Tw + Wr. \tag{4}$$

Wr in this equation governs the topoisomer gel electrophoretic mobility.

§ ΔLk_1 is the linking number difference applied to the loop in the minicircle-reconstituted nucleosome. In this definition, the loop is considered as an independent topological domain delimited by the clamping of DNA to the histones, and its size is assumed to be constant, that is, independent of ΔLk_1 and of the gel temperature. ΔLk_1 was calculated from the equation:

$$\Delta Lk = \Delta Lk_n + \Delta Lk_1 \tag{5}$$

in which ΔLk is given by eqn (2) and ΔLk_n is the linking number increment associated with single nucleosome formation (see Zivanovic *et al.* (1988, 1990) for a more complete definition of ΔLk_n). ΔLk_n was equal to −1·1 (±0·1) at 37 °C under conditions of relaxation with topoisomerase I (50 mM-Tris·HCl (pH 7·5), 0·1 mM-EDTA, 50 mM-KCl, 5 mM-$MgCl_2$ and 0·5 mM-dithiothreitol), and was calculated to increase to −1·0 (±0·1) in TE buffer at room temperature (Zivanovic *et al.*, 1988). ΔLk_1 also depends on temperature according to an equation similar to eqn (3) above:

$$\Delta Lk_1\ (t) = \Delta Lk_1\ (27{\cdot}5) + 0{\cdot}0125 \times N \times 0{\cdot}69\ (t - 27{\cdot}5)/360, \tag{6}$$

in which $N \times 0{\cdot}69$ is the length of the thermally flexible loop DNA measured here (see text). As above for naked DNA (see eqn (4)), ΔLk_1 provides the driving force which induces the loop to writhe, and this writhe in turn governs the electrophoretic mobility of the particle, assuming that a change in ΔLk_1 does not otherwise alter the structure of the nucleosome.

nearly comigrated at room temperature, while nucleosomes on either Lk = 34 and 33 or 33 and 32 topoisomers of a 359 bp minicircle, in contrast, migrated quite differently (Zivanovic *et al.*, 1988). Such comigration was thought to result from the opposite and almost equal values of ΔLk_1 in these nucleosomes (Table 1). We reasoned that a one bp addition to the DNA length (to 355 bp), in increasing the DNA mean twist number ($Tw_0 = Lk_0$ in Table 1) by approximately 0·1, would decrease ΔLk and therefore ΔLk_1 by the same value. As a result, the mobility of the first particle (Lk = 33; $\Delta Lk_1 > 0$) would decrease, while the mobility of the second (Lk = 32; $\Delta Lk_1 < 0$) would increase (see Fig. 1 and Table 1 for corresponding values of ΔLk_1). If now the gel temperature was increased by the proper Δt, then the thermally flexible DNA in the loop could be untwisted by the same angle (about 34°) and the comigration restored (Fig. 1).

A similar experiment could be done with naked Lk = 34 and 33 topoisomers of the same 354 bp minicircle, since these topoisomers are expected to comigrate near room temperature (their linking number differences, ΔLk, are again opposite and almost equal at 27·5 °C; see Table 1). Let now Δt (DNA) be the temperature increase necessary to restore the comigration after the one bp addition. Δt (DNA) is expected to be smaller than Δt (chromatin) since now all, rather than part, of the DNA is thermally flexible. The ratio Δt (DNA)/Δt (chromatin) then gives the relative length of the thermally flexible DNA. It is noteworthy that this procedure does not require the exact overtwisting resulting from the one bp addition nor the rate of DNA thermal untwisting to be known.

In the actual experiment, two additional minicircles of 351 and 353 bp were used. Topological characteristics of the topoisomers, both before and after reconstitution, are listed in Table 1. Figure 2(a) shows the results obtained upon gel electrophoresis at two temperatures of naked Lk = 33 and 34 topoisomers of the four different minicircles. As shown in the 20 °C gel, the increase in DNA size results in an acceleration of Lk = 33 topoisomers, while a retardation of a similar amplitude is observed for Lk = 34 topoisomers. These features are in agreement with the increase in the absolute value of ΔLk with DNA size for Lk = 33 topoisomers, while the reverse occurs for Lk = 34 topoisomers (Table 1). Upon increase in the gel

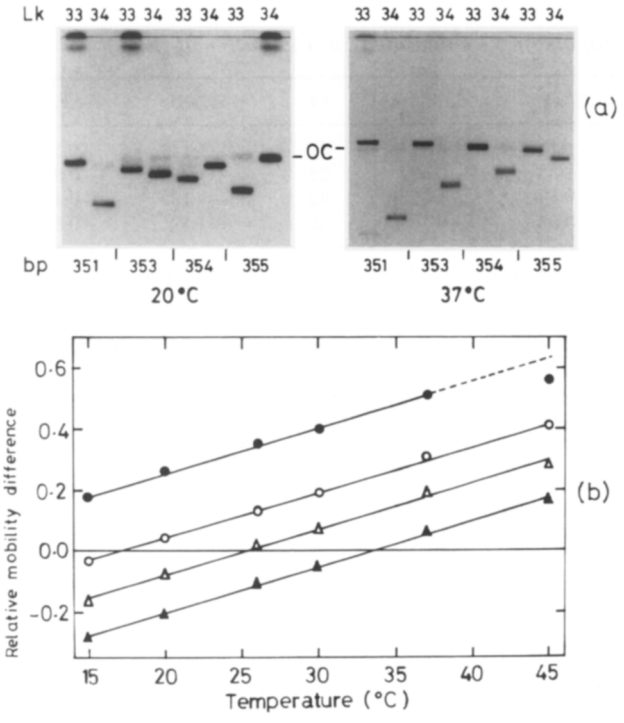

Figure 2. Thermal flexibility of naked topoisomers. (a) Topoisomers of the different minicircles were electrophoresed in 4% polyacrylamide (acrylamide/bisacrylamide = 20 : 1; w/w) slab (0·12 cm × 17 cm × 20 cm) gels either in TAE buffer (6·7 mM-Tris·HCl, 3·3 mM-sodium acetate and 1 mM-EDTA, pH 7·5) or in TE buffer (10 mM-Tris·HCl and 1 mM-EDTA, pH 7·5) buffers at the temperatures indicated. A Pharmacia GE 2/4LS apparatus was used, which had been modified to achieve a temperature regulation of ±0·05 deg.C. The gels were pre-electrophoresed at about 250 V for 1 h and electrophoresed for about 4 h at the same voltage under extensive recirculation of the buffer between the 2 reservoirs, until the xylene cyanol dye reached the bottom of the gel. The exact buffer used is not specified since identical results were obtained with both. Upper bands mark the gel start and were obtained by loading linearized pBR322 plasmid DNA 10 s before terminating the run. The weak band immediately below is at the position of the xylene cyanol dye and presumably results from a carriage of free [γ-^{32}P]ATP remaining after the ethanol precipitation of the end-labeled DNA. OC: open circular DNAs. Autoradiograms of the dried gels are shown. (b) Relative mobility differences between $Lk = 34$ and 33 topoisomers in the gels shown in (a) and in 4 additional gels run at other temperatures were plotted for each minicircle as a function of temperatures (t). If d_{34} and d_{33} are the distances migrated by the topoisomers from the start, the relative mobility difference is $y = 2(d_{34}-d_{33})/(d_{33}+d_{34})$. These distances were measured in densitometric profiles of the film obtained with a Joyce–Loebl microdensitometer. Straight lines were obtained using a least squares procedure. Their equations were y (351 bp, ●—●) = 0·0150 t − 0·0405; y (353 bp, ○—○) = 0·0150 t − 0·2641; y (354 bp, △—△) = 0·0152 t − 0·3877; and y (355 bp, ▲—▲) = 0·0152 t − 0·5091. The 45 °C data point for the 351 bp minicircle was not taken into account in the calculation of the upper straight line since its deviation is due to a reversal in the sign of ΔLk of the $Lk = 33$ topoisomer from negative at lower temperatures to positive at this temperature ($\Delta Lk = +0{\cdot}07$ at 45 °C, as calculated using eqn (3) in Table 1 legend). Topoisomers were prepared by circularization of the ^{32}P end-labeled fragments and purified by gel electrophoresis as previously described (Zivanovic *et al.*, 1986). The fragments originated from *Taq*I cleavage of plasmids pB351, pB353, pB354 and pB355. These plasmids were constructed through deletions of 17, 15, 14 and 13 bp, respectively, at the unique *Ssp*I site of pBR322 at position 4168 in the Sutcliffe (1978) sequence, as corrected in Watson (1988). The plasmids were named by reference to the length of their *Taq*I fragment of interest (the undeleted *Taq*I fragment extends between positions 4017 and 24 of pBR322 and is 368 bp long). After linearization, the DNA was digested from the ends with S_1 (Sigma) and *Bal*31 (Boehringer) nucleases, and recircularized using DNA ligase. The DNAs were then propagated through transformation of *E. coli* HB 101 cells. Deletions were checked by sequencing 1 strand of the supercoiled plasmids using a synthetic primer and the sequencing technique of Sanger *et al.* (1977) with the Sequenase (version 2.0) kit of United States Biochemical.

temperature to 37 °C (Fig. 2(a)), $Lk = 34$ topoisomers increase their mobility relative to $Lk = 33$ topoisomers, again as expected from the positive and negative values of ΔLk, respectively (Table 1). Surprisingly, however, the mobility dependence on DNA size appears now much smaller for $Lk = 33$ topoisomers than for $Lk = 34$ topoisomers. The reason for this dissymmetry is twofold. First, the onset of writhing of DNA minicircles has been proposed on theoretical grounds not to occur before ΔLk is large enough (Le Bret, 1984). This prediction has subsequently been confirmed by the U-shaped dependence of the electrophoretic mobility of ~350 bp minicircles on ΔLk (Zivanovic *et al.*, 1986), which is also observed here with the present minicircles (not shown). (In contrast, 500 to 700 bp minicircles showed a V-like migration, due to their larger interactions with the gel matrix (Zivanovic *et al.*, 1986).) Second, $Lk = 33$ and 34 topoisomers are nearly symmetrically positioned on the U at 20 °C but not at 37 °C. (ΔLk, as calculated using equation (3) in Table 1 legend, is found to vary with the DNA length between −0·23 and −0·61 and +0·39 and +0·77, respectively, at 20 °C; at 37 °C, in contrast, ΔLk varies between −0·02 and −0·40 and +0·60 and +0·98, which brings $Lk = 33$ topoisomers closer to the bottom of the U.)

The relative difference in mobility between $Lk = 34$ and 33 topoisomers was measured for each minicircle in the gels of Figure 2(a) and other similar gels run at four additional temperatures ranging from 15 to 45 °C (not shown). These differences were plotted in Figure 2(b) as a function of gel temperatures, resulting in virtually parallel straight lines (see their equations in the Fig. legend). Their intersections with the abscissa then give the topoisomer comigration temperatures $t = 2{\cdot}7$, 17·6, 25·5 and 33·5 °C for 351, 353, 354 and 355 bp minicircles, which in turn lead to temperature differences $\Delta t = 14{\cdot}9$ deg.C (for a 2 bp difference in length, i.e. $14{\cdot}9/2 = 7{\cdot}4_5$ deg.C for 1 bp), 7·9 deg.C (for 1 bp) and 8·0 deg.C (for 1 bp), respectively. Assuming

these measurements have the same precision as the gel temperature readings, ±0·05 deg.C, we find Δt (DNA) = 7·8 (±0·3) deg.C per bp.

As a control of the whole procedure, it is interesting to calculate the rate of thermal untwisting of the minicircle. This can be done using the above Δt measurement and the twist angle of 1 bp, 360/10·59 (±0·03) = 34·0 (±0·1)°, in which 10·59 (±0·03) bp per turn is the DNA helical periodicity previously measured under present gel electrophoresis conditions at 27·5 °C (see legend to Table 1). When using the minicircle with the intermediate size, 34 (±0·1)/(353 × 7·8 (±0·3)) = 0·012 to 0·013°/deg.C per bp is obtained, a figure essentially identical to the rates previously obtained by others (see above), and by ourselves (0·012 to 0·014°/deg.C per bp) through the V-curve method (Goulet *et al.*, 1987; Zivanovic *et al.*, 1986).

An experiment equivalent to that shown in Figure 2 was performed with chromatin reconstituted on Lk = 33 and 32 topoisomers of the same minicircles. Figure 3(a) displays gels obtained with this material at two temperatures. While unreacted naked Lk = 33 topoisomers accelerate upon increase in the DNA size, as expected (see Fig. 2(a)), Lk = 32 topoisomers behave anomalously since their mobility remains approximately constant at 37 °C, and even decreases at 20 °C, instead of increasing. This presumably reflects departures from *B* form DNA induced by their large negative ΔLk values (see Table 1), which lead to their partial relaxation. Among the chromatin bands, M is prominent and D is minor and multiple (Fig. 3(a)). On the basis of sedimentation velocity, micrococcal nuclease digestion, histone content and electron microscopy, M and D bands have previously been ascribed to mono- and dinucleosomes, respectively (Goulet *et al.*, 1988). Moreover, the splitting of the D band appeared to result from a variable histone complement in the second nucleosome due to an impaired co-operativity in octamer assembly resulting from the small ring size (Goulet *et al.*, 1988). Interestingly, a splitting is also observed for the M band, especially for Lk = 33 topoisomers and at the lower temperature (Fig. 3(a)). The different origin of the M band splitting, as compared to the D band splitting, is emphasized by its dependence on the temperature but not on the histone/DNA ratio used in the reconstitution, while the appearance of the D band does depend on this ratio (not shown), but not on the temperature (compare the two gels in Fig. 3(a)). M band splitting has previously been reported and was shown to reflect a partial fractionation of molecules with the nucleosome at different locations (Duband-Goulet *et al.*, 1992). The fractionation was due in this particular case to a strongly curved region in the minicircle made of synthetic DNA, which, depending on its position on the nucleosome or in the loop, could modulate the particle mobility. The present minicircles also contain a curved region, actually one of the most highly curved region in pBR322 around position 4200 in the Sutcliffe (1978) sequence (Muzard *et al.*,

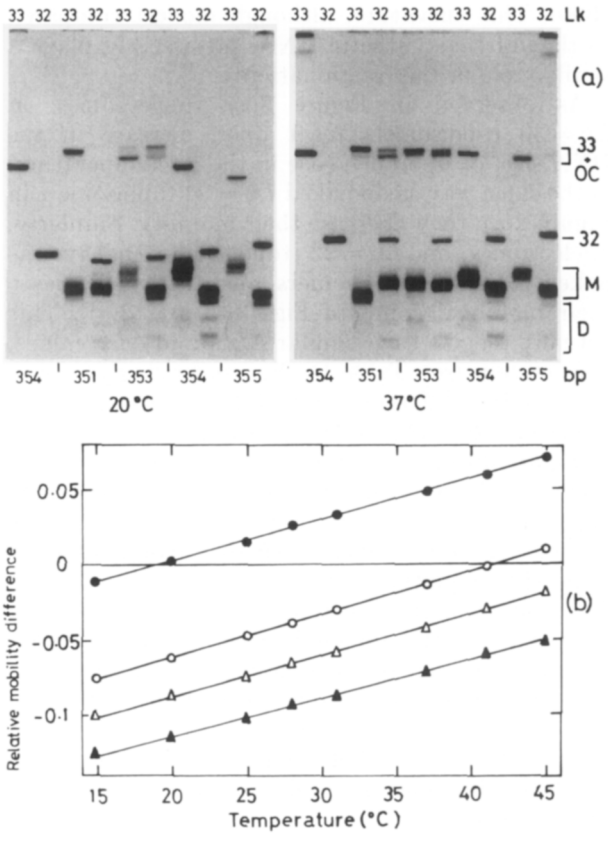

Figure 3. Thermal flexibility of reconstituted topoisomers. Chromatins were reconstituted using the "salt-jump" procedure of Stein (1979) as described (Zivanovic *et al.*, 1990). Briefly, the procedure involved the addition of labeled minicircle DNAs to unlabeled plasmid pBR322 (form I), followed by the suitable amount of core histones from duck erythrocytes (the histone/DNA weight ratios used varied between 0·1 and 0·4, depending on the topoisomers) to final DNA and NaCl concentrations of 200 μg/ml and 2 M, respectively. The mixture was first incubated at 37 °C, subsequently diluted to 50 μg DNA per ml and 0·5 M-NaCl, further incubated at the same temperature, and finally dialyzed at 4 °C against 10 mM-Tris·HCl and 0·25 mM-EDTA (pH 7·5). (a) Chromatins were electrophoresed as nucleoproteins, together with naked 354 bp topoisomers used as controls (first two lanes to the left), in the gels and under conditions described in legend to Fig. 2. Both TAE and TE buffers were also used with indistinguishable results. Unreacted naked topoisomers, mono- (M) and dinucleosomes (D) are indicated. OC, open circular DNAs. Autoradiograms of the dried gels are shown. (b) Relative mobility differences (y) between nucleosomes on Lk = 33 and 32 topoisomers were measured in gels shown in (a) and in 6 additional gels run at other temperatures as described in legend to Fig. 2 and were plotted as a function of gel temperatures (t) for each minicircle (see symbols for the different minicircles in legend to Fig. 2). The equations of the straight lines were: y (351 bp) = 0·0027 t − 0·0520; y (353 bp) = 0·0029 t − 0·1185; y (354 bp) = 0·0027 t − 0·1428; and y (355 bp) = 0·0026 t − 0·1663. Some peaks in the densitometer profiles were asymmetrical, and in that case, their center of gravity, rather than their maximum, was considered.

1990). This curvature, although modest compared to that of the synthetic DNA, presumably plays a similar role in the fractionation.

As observed in Figure 3(a), nucleosomes on $Lk = 33$ topoisomers react upon increase in the DNA size, or upon decrease in the gel temperature, in the same way as do naked $Lk = 34$ topoisomers in Figure 2(a): they decrease their mobility. Similarly, nucleosomes on $Lk = 32$ topoisomers behave as naked $Lk = 33$ topoisomers and instead increase their mobility (compare Figs 3(a) and 2(a)). This actually reflects their similar ΔLk_1 and ΔLk values, respectively (see Table 1). Not surprisingly, therefore, relative differences in mobility between nucleosomes on $Lk = 33$ and 32 topoisomers, when plotted in Figure 3(b) as a function of gel temperatures for each minicircle, result in straight lines of the same appearance as those in Figure 2(b). Their slope, however, is about fivefold smaller (compare their respective equations in the Fig. legends). This discrepancy originates from two factors. First, the compaction of the minicircle upon wrapping around the histones increases the mobility, and therefore, the denominator of the relative mobility difference fraction. Second, the reduced size of the loop compared to the whole minicircle decreases the absolute difference in mobility between the two topoisomers.

Intersections of the straight lines with the abscissa now gave comigration temperatures $t = 19{\cdot}3$, $40{\cdot}8$, $52{\cdot}9$ and $64{\cdot}1$ °C for 351, 353, 354 and 355 bp minicircles. Interestingly, these temperatures appear to be quite high compared to the comigration temperatures of the corresponding naked topoisomers (see above). If the comigration of naked $Lk = 34$ and 33 topoisomers of the 354 bp minicircle, for example, occurs at 25·5 °C for similar ΔLk values close to $+0{\cdot}5$ and $-0{\cdot}5$ (see Table 1), as expected, the comigration of corresponding nucleosomes on 354 bp $Lk = 33$ and 32 topoisomers occurs at 52·9 °C for widely different ΔLk_1 values (ΔLk_1, as calculated at 52·9 °C from equation (6) in Table 1 legend, is equal to $+0{\cdot}79$ and $-0{\cdot}21$, respectively). This contradicts the assumption in Figure 1, made for the sake of simplicity, that nucleosome comigration required similar ΔLk_1 values. In fact, an asymmetry of the same polarity has previously been reported and was ascribed to the left-handedness of the DNA superhelix around the histone octamer (Zivanovic *et al.*, 1988). A negative writhe of the loop, induced by a negative ΔLk_1 value, indeed respects the geometry of the superhelix with the DNA above at the exit remaining above (see loop configuration of $Lk = 32$ topoisomer in Fig. 1(a)). In contrast, the upper DNA has to go below when ΔLk_1 and therefore the writhe is positive (see loop configuration of $Lk = 33$ topoisomer; Fig. 1(a)), which requires more DNA bending and therefore more supercoiling energy.

From the above comigration temperatures, one obtains $\Delta t = 21{\cdot}5$ deg.C (for 2 bp, i.e. $21{\cdot}5/2 = 10{\cdot}7_5$ deg.C for 1 bp), 12·1 deg.C (for 1 bp) and 11·2 deg.C (for 1 bp), respectively. Assuming again a $\pm 0{\cdot}05$ deg.C reproducibility in these temperatures, one obtains Δt (chromatin) = $11{\cdot}3_5$ ($\pm 0{\cdot}7$) deg.C per bp. The relative thermal flexibility of the reconstituted minicircle then is $7{\cdot}8$ ($\pm 0{\cdot}3$)/$11{\cdot}3_5$ ($\pm 0{\cdot}7$) = 69 (± 7)%. Considering again the intermediate size minicircle, the lengths of the thermally flexible and rigid DNAs, respectively, are 353 ($0{\cdot}69$ ($\pm 0{\cdot}07$)) = 244 (± 25) bp and $353 - 244$ (± 25) = 109 (± 25) bp. (This figure increases slightly with the size of the minicircle used in the calculation, from 108·8 bp for the 351 bp minicircle, to 110 bp for the 355-bp minicircle.)

This calculation assumes that the DNA in the loop thermally untwists at the same rate as the naked DNA. It is possible, however, that a transition zone exists at the edges of the nucleosome between the completely free and the completely immobilized DNA. In that case, the rate of thermal untwisting would be smaller on average, and more bp would be necessary to untwist the double helix by the same angle. As a consequence, the size of the thermally flexible DNA (including the transition zone) would be larger, and therefore the size of the completely rigid DNA smaller, than the above 244 and 109 bp figures, respectively. Another assumption is that the two topological domains of the wrapped minicircle, the histone-bound DNA and the loop, respectively, have fixed dimensions and are independent from each other (see legend to Table 1). Arguments may, however, be given to the contrary. For example, the length of the histone-bound DNA may decrease upon increase in the temperature, consistent with the peeling off of 20 or so bp of DNA at each side of the 145 bp core particle at about 40 °C suggested by physico-chemical studies involving thermal fusion, circular dichroism, sedimentation and nuclear magnetic resonance (van Holde, 1989, and references therein). Such release of the DNA at the edges may also be favored by positive ΔLk_1 values (see Table 1), consistent with a destabilization of the nucleosome by positive DNA supercoiling (Pfaffle *et al.*, 1990; Lee & Garrard, 1991; but see Clark & Felsenfeld, 1991). On the contrary, the nucleosome may be stabilized and the length of DNA interacting with the histones may increase at negative ΔLk_1 values (the opposite was suggested by Garner *et al.* (1987), based on experiments with reconstituted minichromosomes supercoiled with DNA gyrase; but see an alternative interpretation of the Garner *et al.* (1987) results in Zivanovic *et al.* (1988)).

If the length of the thermally flexible DNA was actually increasing with temperature, the temperature increment necessary to compensate the increase in the mean twist number resulting from the 1 bp addition would be smaller at higher temperatures than at lower temperatures. If one further assumes that this temperature dependence of the flexible DNA length is linear, so that the curves of Figure 3(b) could remain straight, as observed, one would then expect the straight lines to converge. The first two lines from the top (slopes of 0·0027 and 0·0029; see equations in the Fig. 3 legend) are indeed

convergent, but the last three (slopes of 0·0029, 0·0027 and 0·0026) are instead divergent. Similarly, if the thermally flexible DNA was longer at positive ΔLk_1 values and shorter at negative values, the thermal flexibility would decrease from the 351 bp minicircle (mean $\Delta Lk_1 = +0{\cdot}36$ at the intermediate temperature of 27·5 °C) to the 355 bp minicircle (mean $\Delta Lk_1 = -0{\cdot}02$ at the same temperature). (ΔLk_1 (351) $= +0{\cdot}86$ and $-0{\cdot}14$ for $Lk = 33$ and 32 topoisomers, respectively, at 27·5 °C (see Table 1), that is, ΔLk_1 (351; mean for the two topoisomers) $= (+0{\cdot}86 - 0{\cdot}14)/2 = +0{\cdot}36$; ΔLk_1 (355) $= +0{\cdot}48$ and $-0{\cdot}52$ (see Table 1), i.e. ΔLk_1 (355; mean) $= (+0{\cdot}48 - 0{\cdot}52)/2 = -0{\cdot}02$.) As a consequence, the increment in temperature necessary to compensate for the 1 bp addition would be expected to increase with the size of the minicircle. Inspection of the above Δt values, 10·75, 12·1 and 11·2 deg.C, respectively, shows instead no consistent trend. It may therefore be concluded that the data of Figure 3(b) show no evidence for a significant temperature or ΔLk_1 dependence of the length of the thermally flexible loop DNA, at least within the present variation ranges of these variables.

The number of turns of the nucleosomal DNA superhelix can now be calculated to be 1·3 (109/83), in fair agreement with the mean value of 1·4 (115/83) derived from the distribution of the angles between DNAs at the entrance and exit of similar, relaxed ($\Delta Lk_1 = 0$), minicircle-reconstituted mononucleosomes measured by high resolution electron microscopy (Zivanovic *et al.*, 1988). In these experiments, nucleosomes were examined in TE buffer, one of the two gel electrophoresis buffers used here (see legend to Fig. 2), and also in TE buffer plus 50 or 100 mM-NaCl. The lack of any significant changes in this mean figure of 1·4 under these different salt conditions (Zivanovic *et al.*, 1988, 1990) suggests that the thermal flexibility measurement may similarly not depend significantly on the ionic strength of the buffer. Such consistency between thermal and electron microscopic data further reinforces our previous basic conclusion that, in the most probable (relaxed) conformation of the loop, DNAs at the entrance and exit of a minicircle-reconstituted nucleosome do not cross (Zivanovic *et al.*, 1988), and that such a nucleosome, with only one crossing of the DNA, behaves topologically as a "single-turn" particle (Le Bret, 1988).

The question at this point is whether the "single-turn" particle notion applies only to the minicircle system or has a more general value. This question is especially relevant in view of the consistence between the present result (109 ± 25 bp of thermally immobilized DNA) and that obtained for yeast chromatin (50 to 100 bp; see above), which suggests that this notion can be extended to this particular example of naturally-occurring H1-free chromatin. This problem arises because it may be suspected that the loop is too short, given the bending rigidity of DNA, and the histone–DNA binding interactions at the core positions not strong enough, for the relaxed nucleosome on the minicircle to remain wrapped with 1·8 turns of DNA. In the core particle, 1·8 turns have been observed (Finch *et al.*, 1977; Richmond *et al.*, 1984, 1988; Struck *et al.*, 1992), and are generally believed to be also present in the nucleosomes of long native or reconstituted chromatin regardless of the presence or absence of H1(H5). This wrapping would indeed largely increase the curvature of the DNA in the loop compared to the nucleosome with 1·4 turns (see an illustration of this in Fig. 1, panel b) and possibly require more energy than that provided by histone-DNA binding interactions at the core positions (see a discussion of this issue in Zivanovic *et al.*, 1988).

To obviate this DNA rigidity problem inherent to the minicircle system, linear fragments 250 to 350 bp in length were used for reconstitution of single nucleosomes. In a recently published characterization of this material, such "linear" mononucleosomes were shown to fractionate upon gel electrophoresis according to the position of the nucleosome on the fragment (Duband-Goulet *et al.*, 1992). This fractionation was similar to that observed with CAP protein (Wu & Crothers, 1984), and presumably also resulted from the small angle formed by the DNAs at the entrance and exit of the particle. This angle could not, however, be directly measured in these experiments (Duband-Goulet *et al.*, 1992). In a subsequent work, the linear mononucleosomes were visualized under the scanning transmission electron microscope under different ionic strength conditions, TE buffer, TE buffer plus 50 or 100 mM-NaCl. Particles with about one to no more than 1·5 turns of DNA were observed (A. Hamiche, P. Schultz, P. Oudet & A. Prunell, unpublished results). The close agreement with the results previously obtained with the circular, relaxed, mononucleosomes (Zivanovic *et al.*, 1988), and referred to above, suggests that the rigidity of the DNA in the loop was actually not a problem in these experiments, and that the failure of entering and exiting DNAs to cross may be the rule, rather than the exception, for H1 (H5)-free mononucleosomes. Interestingly, these results with the linear mononucleosomes were more recently confirmed using the more sophisticated techniques of cryo-electron microscopy (Adrian *et al.*, 1984; Dubochet *et al.*, 1988) and 3-dimensional reconstruction (Dustin *et al.*, 1991), which allowed the nucleosomes to be directly observed in their aqueous environment by means of the vitrification of a thin layer of solution (P. Furrer, J. Bednar, J. Dubochet, A. Hamiche & A. Prunell, unpublished results).

On the other hand, cleavage of similar circular and linear mononucleosomes with DNase I and/or hydroxyl radicals under conditions preserving their structures (in general only 1 nick was introduced onto the molecules), has shown large footprints of 145 bp up to 190 bp (Pina *et al.*, 1990; Hayes *et al.*, 1990, 1991). 146 to 160 bp footprints were also obtained in our hands using DNase I on the linear mononucleosomes just described (Duband-Goulet *et al.*, 1992). Although their exact boundaries may sometimes be difficult to specify, such footprints

appear more consistent with 1·8 to 2-turn particles than with 1·4-turn particles. It is also significant that similar footprinting results were obtained with these two probes, since DNase I, due to its large size compared to the chemical probe, might have been suspected to lead to an overestimate of the size of the DNA in close contact with the histones. (Such difference in footprint sizes between DNase I and a chemical probe has previously been observed with a transcriptional activator protein by Sawadogo & Roeder (1985).) Micrococcal nuclease also digests circular and linear mononucleosomes (and yeast chromatin; Thomas & Furber, 1976; Lohr *et al.*, 1977; Pineiro *et al.*, 1991) into standard 145 bp and 1·8-turn core particles (Goulet *et al.*, 1988), while exo III shows the expected strong pauses at the core positions when digesting linear mononucleosomes (Duban-Goulet *et al.*, 1992). It is noteworthy, however, that micrococcal nuclease and exo III may not provide as stringent a test as DNase I or hydroxyl radicals, since, in contrast to these probes, they do not preserve the overall structure of the molecules.

In conclusion, it appears from thermal untwisting and/or electron microscopic data that the 20 or so bp of DNA at each end of both linear and circular mononucleosomes (taking the 145 bp and 1·8-turn core particle as reference) are not in interaction with the histones, while digestion results suggest they are. We propose, to reconcile these apparently contradictory data, that some histone patches protrude from the main histone body and interact with the distal DNA. During the thermal unwinding, these histone patches are expected to follow the DNA in its rotation, possibly inhibiting it to some extent. In this case, this region could be the transition zone between the thermally flexible and immobilized DNA referred to above. In this model, the nature of these protruding histone patches would be unclear, although they seem unlikely to be made of the unstructured tails since trypsin digestion was not found to alter the size of the footprints significantly (Ausio *et al.*, 1989; Hayes *et al.*, 1991). This model would be in keeping with physicochemical data (see above) and also exonuclease III digestion (Prunell, 1983) which have both pointed to the existence of a tripartite nucleosome with two outer ~20 bp regions and a more stable ~105 bp central region. Exonuclease III indeed pauses with two different periodicities in the outer and inner regions (11 and 10 nucleotides, respectively), and revealed an enhanced accessibility of the amino groups of histone basic amino acids to crosslinking with dithiobis (succinimidyl propionate or Lomant's reagent) in the outer regions as compared with the inner region (Prunell, 1983). In the present model, these histone patches, together with the DNA bound to them, would have the ability to fold back on the histone core, possibly in a wing-like manner, as observed under the electron microscope upon H1(H5) binding (Zivanovic *et al.*, 1990) or upon trimming of the DNA to the core positions with micrococcal nuclease. These features would be suggestive of the structural dynamics of the nucleosome under transcription or replication.

If the above "wing-like" model of the nucleosome is correct, then the effective number of turns of the nucleosomal DNA superhelix can no longer be directly inferred from the length of the histone-bound DNA. This number of turns is, however, central to the understanding of DNA topology in chromatin. The observed unit linking number reduction ($\Delta Lk_n = -1$; see Table 1 legend) is consistent with the present "single-turn" particle and its single DNA crossing, but not with a 1·8 to 2-turn nucleosome with 2 crossings of the DNA. In fact, this latter particle, as obtained from H5 binding to minicircle-reconstituted mononucleosomes, was found to reduce the DNA linking number by 1·6 rather than 1 (Zivanovic *et al.*, 1990). (It is noteworthy that a linking number reduction of 2 would be expected for a 2-turn particle only in the case of a flat superhelix with a zero pitch.) Long chromatin, in contrast, always showed a unit linking number reduction per nucleosome, whether reconstituted in the absence (Germond *et al.*, 1975; Simpson *et al.*, 1985; Norton *et al.*, 1989) or presence of H5 (Stein, 1980; Morse & Cantor, 1986). This result was also obtained with native H1-containing as well as H1-depleted SV40 minichromosomes (Germond *et al.*, 1975; Keller *et al.*, 1978). If a unit linking number reduction is expected in the absence of H5(H1) for a chain of "single-turn" nucleosomes oriented at random relative to one another (Stein, 1980; Grigoryev & Ioffe, 1981), such a result in contrast is, as mentioned above, inconsistent with the existence of 2-turn nucleosomes in H5(H1)-containing chromatin (Thoma *et al.*, 1979). This so-called linking number paradox (Klug & Lutter, 1981; Morse & Simpson, 1988) is generally thought to reflect an overtwisting of the DNA upon wrapping around the histones (Klug & Travers, 1989; Travers & Klug, 1990), a view supported by numerous measurements through various means of the local helical periodicity of nucleosomal DNA (Drew & Travers, 1985; Gale *et al.*, 1987; Shrader & Crothers, 1990; Costanzo *et al.*, 1990; Hayes *et al.*, 1990). Inasmuch as our results with H5-free and H5-containing minicircle-reconstituted mononucleosomes, which showed no need for an overtwisting of the wrapped DNA to explain the observed linking number differences, could apply to individual nucleosomes in longer chromatin, we propose that the solution to the paradox may rather lie in the linker DNA in H1(H5)-containing chromatin whose actual path remains to be elucidated.

To address the problem of the discrepancy between the different thermal flexibility measurements more directly, it is useful to consider further the observation of Smirnov *et al.* (1991) concerning a dependence of the data on salt concentration (see the introduction section). The length of the thermally rigid DNA, as derived from circular dichroism, 175 to 180 bp, was indeed found by Smirnov *et al.* (1991) to remain constant from physiological salt conditions down to about 50 mM salt, and then to

decrease progressively below 50 mM to reach 145 bp at 10 mM salt. These experiments used native chicken erythrocyte chromatin depleted of H1 by a column procedure which presumably preserved the original nucleosome repeat (208 bp). In their interpretation of these data, Smirnov *et al.* (1991) suggested that the 30 to 35 (175 to 180 − 145) bp of extra DNA bound at both edges of the particle started to be released from the histones below 50 mM salt, this release being completed at 10 mM salt. There are several arguments against this possibility. First, due to their electrostatic nature, histone-DNA binding interactions are rather expected to become stronger upon decrease in the salt concentration. Second, no unfolding was found to occur in this range of salt concentrations using purified large (>145 bp) nucleosomes from calf thymus or chicken erythrocyte chromatin, as investigated by sedimentation velocity or neutron scattering, the only unfolding being observed below 2 mM (Burch & Martinson, 1980; Uberbacher *et al.*, 1983). Third, physiologically spaced nucleosomes in H1-depleted dinucleosomes from chicken erythrocyte chromatin appear to interact strongly with each other at 50 mM salt (and, of course, also above this salt concentration), as revealed by their ability to bend the linker DNA in between and to reach their most compact configuration already at this salt concentration (Yao *et al.*, 1991). Taken together, these data strongly suggest that the true length of the thermally rigid DNA does not depend on salt and that the decrease observed by Smirnov *et al.* (1991) rather reflected a lowering of nucleosome interactions upon decrease in the salt concentration below 50 mM. Interactions of adjacent nucleosomes, in inhibiting their mutual rotation, would indeed sequester the linker DNAs in between. This would prevent the torsional stress induced by the change in temperature to be evacuated through rotation at the ends, and lead to an overestimate of the length of the thermally rigid DNA in this experiment. Other experiments by Smirnov *et al.* (1991) have involved circular dichroism of chromatin reconstituted with a variable number of nucleosomes and the use of an extrapolation procedure, as the experiment of Morse & Cantor (1985). No study of the salt dependence of the thermally rigid DNA length was performed in this case, but the result at physiological ionic strength (175 to 180 bp; Smirnov *et al.*, 1991) was identical to that obtained with the above chicken erythrocyte chromatin at the same salt concentration. This suggests that nucleosomes in such low density chromatin may still interact with each other, at least under these relatively high salt conditions. Such interactions, however, were excluded by Morse & Cantor (1985) as an explanation for their data (230 bp of immobilized DNA; see above) on the basis that nucleosomes in low density chromatin appear to be randomly distributed on the DNA, rather than closely apposed as in native chromatin (Germond *et al.*, 1975; Noll *et al.*, 1980). If such interactions occur, then the large thermal flexibility of yeast chromatin could be explained by the absence of interactions in this chromatin, indeed a possible consequence of the particular primary amino acid sequences of yeast histones. In the absence of nucleosome interactions, data for yeast chromatin could be derived for each nucleosome in isolation, as in the present minicircle system.

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