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Periodicity of DNA folding in higher order chromatin structures

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Each level of DNA folding in cells corresponds to a distinct chromatin structure. The basic chromatin units, nucleosomes, are arranged into solenoids which form chromatin loops. To characterize better the loop organization of chromatin we have assumed that the accessibility of DNA inside these structures is lower than on the outside and examined the size distribution of high mol. wt DNA fragments obtained from cells and isolated nuclei after digestion with endogenous nuclease or topoisomerase II. The largest discrete fragments obtained contain 300 kbp of DNA. Their further degradation proceeds through another discrete size step of 50 kbp. This suggests that chromatin loops contain ~50 kbp of DNA and that they are grouped into hexameric rosettes at the next higher level of chromatin structure. Based upon these observations a model by which the 30 nm chromatin fibre can be folded up into compact metaphase chromosomes is also described.

Key words: chromosome/loops/model/pulsed-field/topoisomerase II

Introduction

Electron microscopy (Paulson and Laemmli, 1977) as well as the results of experiments on the sedimentation of nucleoids (nuclei from which the majority of proteins were dissociated, Cook and Brazell, 1975) and the fluorescence of ethidium bromide treated nuclei (Vogelstein *et al.*, 1980; Pienta and Coffey, 1984) suggested that the 30 nm chromatin fibre, in both interphase nuclei and metaphase chromosomes, is organized into structures called loops containing 34-220 kbp long DNA stretches (for recent reviews, see Paulson, 1988; Van Holde, 1989). Igo-Kemenes and Zachau (1978) were the first to use nucleases to study the chromatin organization of DNA domains of up to 100 kbp. In their classic study, they used conventional agarose gel electrophoresis to fractionate DNA fragments resulting from nuclease treatment of isolated nuclei. Assuming that the observed DNA fragment sizes resulted from regular spacings between nuclease-sensitive sites which in turn result from the higher level of folding of DNA in chromatin, they estimated that the average 'chromatin domain' (synonymous with the chromatin loop) was at least 34 kbp. Using mild digestion conditions they did, however, observe fragments calculated to be as long as 75 kbp. The loops are probably individually constrained and anchored into either the chromosomal 'scaffold' or nuclear 'matrix' by a protein component of chromatin (Comings, 1977; Pienta and Coffey, 1984). Topological considerations (DiNardo *et al.*, 1984) and the results of studies on the localization of immunofluorescent anti-topo II antibodies in metaphase chromosomes (Earnshaw and Hech, 1985; Boy de la Tour and Laemmli, 1988) and in nuclei (Berrios *et al.*, 1985) suggested that this protein component has a topoisomerase II activity.

Studies of the organization of DNA at this and even higher levels of folding in chromatin and chromosomes have now become possible by the recent development of pulsed field gel electrophoresis (Smith and Cantor, 1987). This technique extends the resolving power of DNA agarose electrophoresis and has been used here to determine more precisely the parameters of the periodicity of DNA folding, at the high mol. wt level, by studying the DNA products of fragmentation of the chromatin in cells and in isolated nuclei. The nuclei of eucaryotic cells contain a well characterized enzyme, the Ca/Mg-dependent endonuclease (Hewish and Burgoyne, 1973). This nuclease, which shows little sequence specificity (Stratling *et al.*, 1984) but whose action is easily slowed down by proteins complexed with the DNA, has been used extensively to elucidate the structure of the 30 nm chromatin fibre (Walker and Sikorska, 1986; Walker *et al.*, 1986). We expected that this enzyme, would have a restricted access to the bulk of the DNA in the 'loops' while either the interloop 'linkers' or the external, most exposed, parts of the loops would be more sensitive to digestion. In addition, we have also analysed the size distributions of DNA fragments resulting from the treatment of cells in culture with the antibiotic calcium ionophore A23187 and with the inhibitors of topoisomerase II. These treatments are known to activate DNA breakage in cells by unrelated enzymatic mechanisms as shown by McConkey *et al.* (1989) and Ross *et al.* (1979), respectively. We have found, in all three cases, two periodicities of 50 and 300 kbp of DNA in the distribution of sites in chromatin which are sensitive to enzymes hydrolysing DNA phosphodiester bonds. The 50 kbp periodicity almost certainly reflects the loop organization of the chromatin whereas the 300 kbp periodicity could reflect an aspect of the arrangement of these loops into a higher order chromatin structure. These results are discussed in terms of the ability of chromatin to be folded into highly compacted metaphase chromosomes.

Results

Influence of the conditions of the isolation of liver nuclei on the autodigestion of chromatin

The composition of the buffer used in the isolation of chromosomes or nuclei (the 'isolation buffer') influences both the intactness of the DNA in the chromatin and its further behavior during incubation at 37°C in the 'digestion buffer' (Blumenthal *et al.*, 1979; Walker and Sikorska, 1986). Our aim was to find the conditions under which the degradation

of DNA during the nuclear isolation procedure would be negligible and the endogenous nuclease could still be activated by adding Mg^{2+} and Ca^{2+} to the digestion buffer. The isolation buffers examined contained in addition to Tris−HCl, sucrose and phenylmethylsulfonyl fluoride (PMSF) also (i) 20, 70, or 150 mM KCl, (ii) either 5 mM $MgCl_2$ or polyamines and EDTA, (iii) either no, or 1 mM EGTA. The nuclei were isolated in these buffers, washed, resuspended in digestion buffer, autodigested at 37°C for the desired period of time in the presence or absence of 0.1 mM $CaCl_2$ and embedded in agarose. The agarose blocks were processed as described in Materials and methods. After electrophoresis and ethidium bromide staining, the gels were examined for the presence of diffused bands of partial DNA digestion products (see for example Figure 1A). Table I summarizes the results of these experiments. In the nuclei isolated using 20 and 70 mM KCl in the absence of EGTA and EDTA, partial digestion of DNA takes place even in the absence of exogenous Ca^{2+} in the digestion buffer. The presence of 2 mM EDTA in

Table I. Determination of the optimal composition of the nuclear isolation buffer

Isolation buffer	+ EGTA						− EGTA					
		20 mM KCl		70 mM KCl		150 mM KCl	20 mM KCl		70 mM KCl		150 mM KCl	
		Mg	Sper + EDTA	Mg	Sper + EDTA	Mg	Sper + EDTA	Mg	Sper + EDTA	Mg	Sper + EDTA	Mg	Sper + EDTA
Digestion buffer	− $CaCl_2$	+	−	−	−	−	−	+	+	+	−	−	−
	+ $CaCl_2$	+	+	+	−	−	−	+	+	+	+	−	−

The rat liver nuclei were isolated in a buffer containing 0.25 M sucrose, 50 mM Tris−HCl pH 7.4, 0.2 mM PMSF and (i) 0 or 1 mM EGTA, (ii) 20, 70 or 150 mM KCl, (iii) either 5 mM $MgCl_2$ or 0.25 mM spermine−0.5 mM spermidine−2 mM EDTA. They were then suspended in the digestion buffer (10 mM Tris−HCl pH 7.4, 70 mM KCl, 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$). This last component was omitted in the control samples. After incubation for 15 min at 37°C the 'plugs' were prepared and electrophoresed as described. The gels after staining were examined for the presence (+) or absence (−) of partially digested DNA.

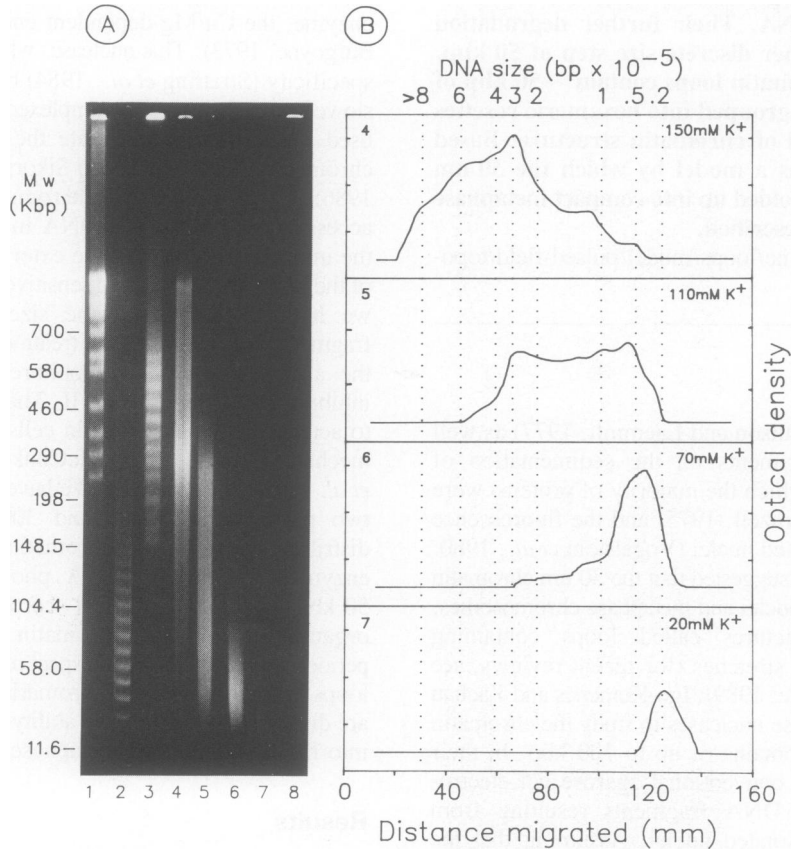

Fig. 1. Influence of the concentration of KCl in the digestion buffer on the size distribution of DNA fragments produced in rat liver nuclei. (**A**) Nuclei isolated in the buffer containing 20 mM KCl, spermine, spermidine, EGTA and EDTA as described in Materials and methods were incubated for 15 min at 37°C in buffer containing 150 mM (**lane 4**) 110 mM (**lane 5**), 70 mM (**lane 6**) and 20 mM (**lane 7**) KCl, 10 mM Tris−HCl pH 7.4, 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. **Lane 8** contained a non-digested control, **lanes 1, 2** and **3** contained mol. wt standards: yeast chromosomal DNA (*Saccharomyces cerevisiae*) 11.6 kbp plasmid ladder and 49.5 kbp lambda DNA ladder, respectively. (**B**) **Lanes 4−7** were scanned to show the size distribution of the DNA fragments. The ordinate is calibrated in arbitrary optical density units. The curves actually represent the optical density of the negative of the photograph of the gel stained by EtBr and photographed in the UV light. This is approximately proportional to the DNA concentration in the gel.

the isolation buffer protected the DNA in the nuclei isolated in 70 mM KCl against digestion but did not protect it if the concentration of KCl was 20 mM. If both EGTA and EDTA were present in the isolation buffer digestion was always negligible regardless of the KCl concentration of the isolation buffer prior to the addition of $CaCl_2$. The DNA in nuclei isolated using the buffer containing 150 mM KCl was practically undegraded but addition of the $CaCl_2$ did not activate the endogenous nuclease.

All further experiments were thus performed using nuclei prepared in the isolation buffer containing 20 mM KCl, polyamines, EDTA and EGTA.

Influence of the parameters of the reaction on the size of DNA fragments produced during autodigestion of chromatin in isolated rat liver nuclei

In order to obtain the release of biologically relevant high mol. wt chromatin fragments, isolated nuclei must be incubated under conditions which minimize changes to the native chromatin structure, but allow active endogenous nuclease access to the most exposed sites. Initially, the nuclei were incubated, at a pH of 7.4, in buffer containing 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and KCl in various concentrations. The resulting mixture of DNA fragments was fractionated by pulsed field gel electrophoresis with resolution in the range of 10–700 kbp. The peak which sometimes appears at 800 kbp is created artificially by the co-migration of fragments of varying sizes >800 kbp which are not resolved by these electrophoresis conditions. In the presence of 150 mM KCl in the digestion buffer large DNA fragments were generated with a pronounced peak at 300 kbp (Figure 1). At an intermediate KCl concentration of 110 mM all the DNA was cut into pieces of 50–300 kbp, whereas at 70 mM KCl virtually all of the DNA had been fragmented into pieces 50 kbp in size. Autodigestion of the nuclei in buffer containing a low concentration of KCl of 20 mM resulted in the degradation of the 50 kbp fragments. A similar size distribution of fragments was produced when digestions were carried out at a fixed KCl concentration of 70 mM, but at various concentrations of $MgCl_2$ (Figure 2). At low concentrations of $MgCl_2$ a pronounced peak at 300 kbp was observed, and this was gradually reduced to a peak of 50 kbp at higher $MgCl_2$ concentrations.

A time course of digestion carried out on isolated rat liver nuclei at the optimal mono- and divalent cation concentrations of 70 mM KCl and 5 mM $MgCl_2$ (Figure 3A,B) showed that at the earliest stages of the digestion the 300 kbp fragments were produced and at the later stages 50 kbp fragments were generated. Similar peaks of 300 kbp and 50 kbp were obtained using nuclei isolated from mouse liver (Figure 3C,D). It was apparent, therefore, that under the conditions studied, there were two main spacings between the neigbouring endogenous nuclease-sensitive sites in liver nuclei at 300 kbp and at 50 kbp.

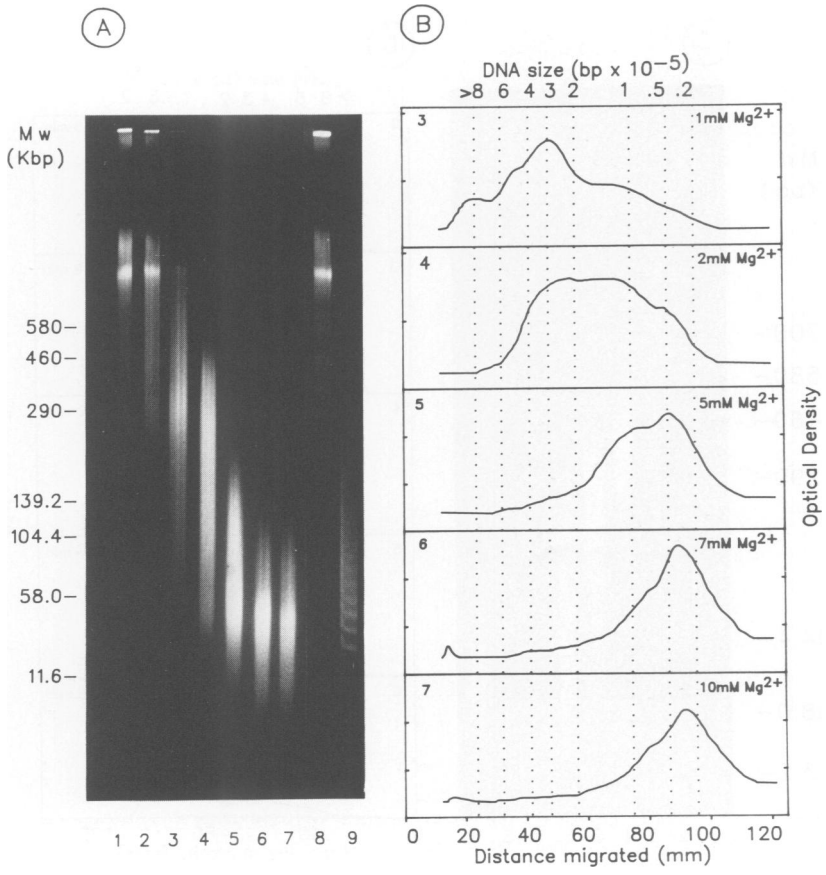

Fig. 2. Influence of the magnesium concentration in the digestion buffer on the size distribution of the DNA fragments generated in rat liver nuclei. **(A)** Isolated nuclei were autodigested in the incubation buffer (see Materials and methods) containing variable concentrations of $MgCl_2$. **Lanes 1–7** correspond to 0, 0.5, 1, 2, 5, 7 and 10 mM $MgCl_2$, respectively. **Lanes 8** and **9** contain mol. wt standards. **(B) Lanes 3–7** were scanned to determine the size distribution of the DNA fragments.

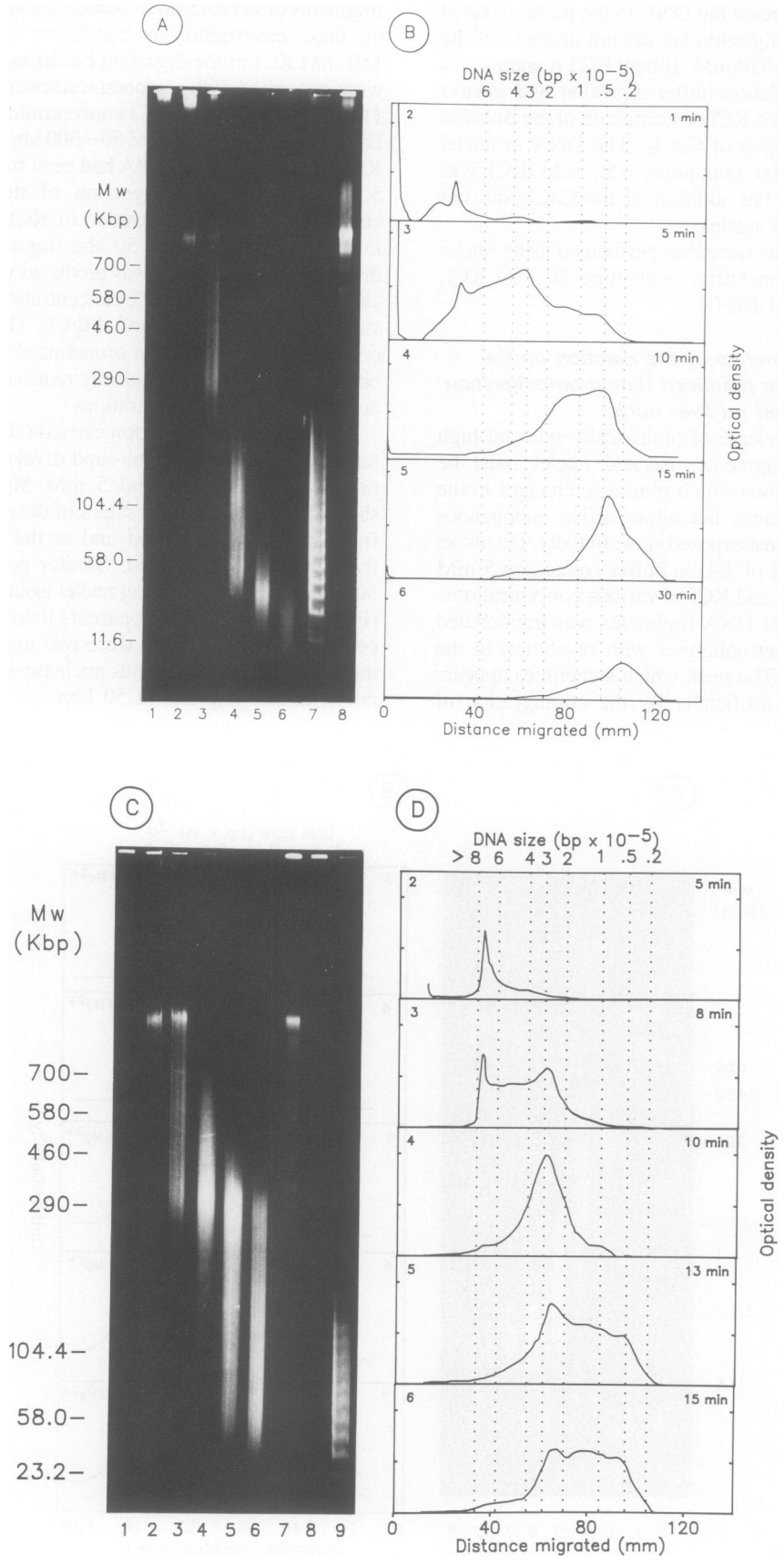

Fig. 3. Time course of the fragmentation of the DNA in isolated rat and mouse liver nuclei. In **A lanes 1–6** correspond to the nuclei from rat liver digested at 37°C for 0, 1, 5, 10, 15 and 30 min in the digestion buffer as described in Materials and methods. **Lanes 7** and **8** contain mol. wt standards. Scans of **lanes 2–6** are shown in **B**. In **C lanes 1–6** correspond to similar digestions of mouse liver nuclei for 0, 5, 8, 10, 13 and 15 min respectively. Mol. wt markers are also shown (**lanes 7–9**) and **lanes 2–6** are scanned in **D**.

Intracellular fragmentation of DNA

The observed pattern of DNA digestion in isolated nuclei was obtained under ionic conditions which are not necessarily physiological. In order to verify whether or not the chromatin in intact cells shows a similar pattern of distribution of nuclease-sensitive sites as the one in isolated nuclei, we took advantage of the observation that the ionophore A23187 causes an intracellular fragmentation of DNA in thymocyte primary cultures. This action of the antibiotic can be modulated by pre-treatment of the primary cultures with aurintricarboxylic acid (McConkey *et al.*, 1989) which is a general inhibitor of nucleases (Hallick *et al.*, 1977). Depending on the concentration of inhibitor, one can observe either the sites of initial fragmentation of chromatin or the size distribution of the fragments generated by more extensive digestion.

Freshly isolated thymocytes were pre-treated for 1 h with various concentrations of aurintricarboxylic acid and then treated with the ionophore. After preparation of agarose 'plugs', electrophoresis and staining, the gels were analysed as before (Figure 4). All the DNA from freshly isolated (control) thymocytes remained in the well indicating that it is essentially intact. The pre-incubation with aurintricarboxylic acid alone affects only slightly the integrity of the DNA. At low levels of enzyme activity (in cells pre-treated with high concentrations of aurintricarboxylic acid) the distribution of mol. wts exhibits distinct maxima at 50 and 300 kbp of DNA and as digestion progressed (in the thymocytes exposed to low concentrations of aurintricarboxylic acid) DNA fragments of 50 kbp accumulated.

Fragmentation of DNA by topoisomerase inhibitors

The third method of fragmenting the DNA consisted of the treatment of cells with inhibitors of topoisomerase II. This method is the most conservative of the three, as it does not modify the intracellular environment to any considerable extent. Furthermore, the cuts inflicted by some topoisomerase inibitors are reversible upon simple dilution of the inhibitor out of the medium (Zwelling *et al.*, 1981). When the primary thymocyte cultures were incubated with an increasing amount of VM26 or with *m*-AMSA [4′-(9-acridinylamino) methanesulfon-*m*-anisidide] the resulting fragments of DNA (Figure 5) exhibited a similar distribution of sizes as the DNA fragments produced by the ionophore-induced autodigestion of the chromatin in cells or in the experiments on isolated nuclei. The *m*-AMSA produced mainly the 50 kbp fragments while the VM26 mainly the 300 kbp fragments. In an experiment where the *m*-AMSA was removed from the medium after a 30 min incubation the breaks in the DNA were rapidly, although not completely, reversed. (Figure 5, lanes 7–10) confirming that the action is mediated by a topoisomerase.

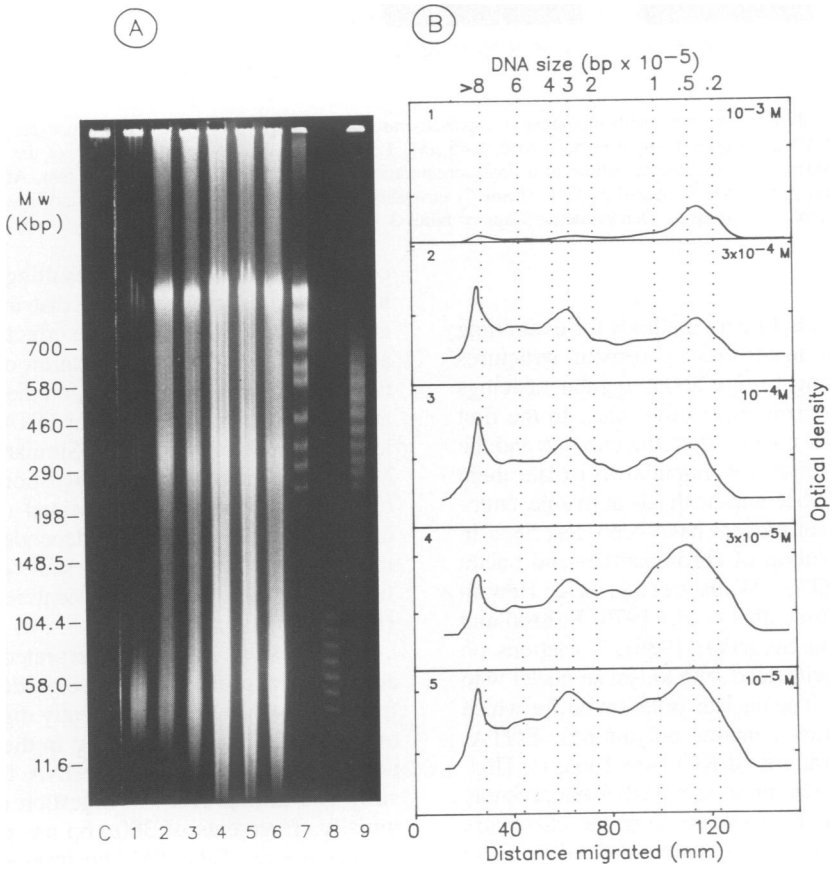

Fig. 4. Fragmentation of DNA in thymocytes induced by the ionophore A23187. (**A**) the primary culture of thymocytes was treated with the nuclease inhibitor aurintricarboxylic acid at decreasing concentrations and then incubated with 1 μM ionophore. **Lanes 1–6** correspond to 10^{-3} M, 3×10^{-4} M, 10^{-4} M, 3×10^{-5} M, 10^{-5} M and 0 M aurintricarboxylic acid concentration in the medium respectively. **Lanes 7–9** contain mol. wt standards. The control lane, **C**, shows that the DNA in freshly isolated cells is essentially intact. (**B**) **Lanes 1–5** were scanned to determine the size distribution of the DNA fragments.

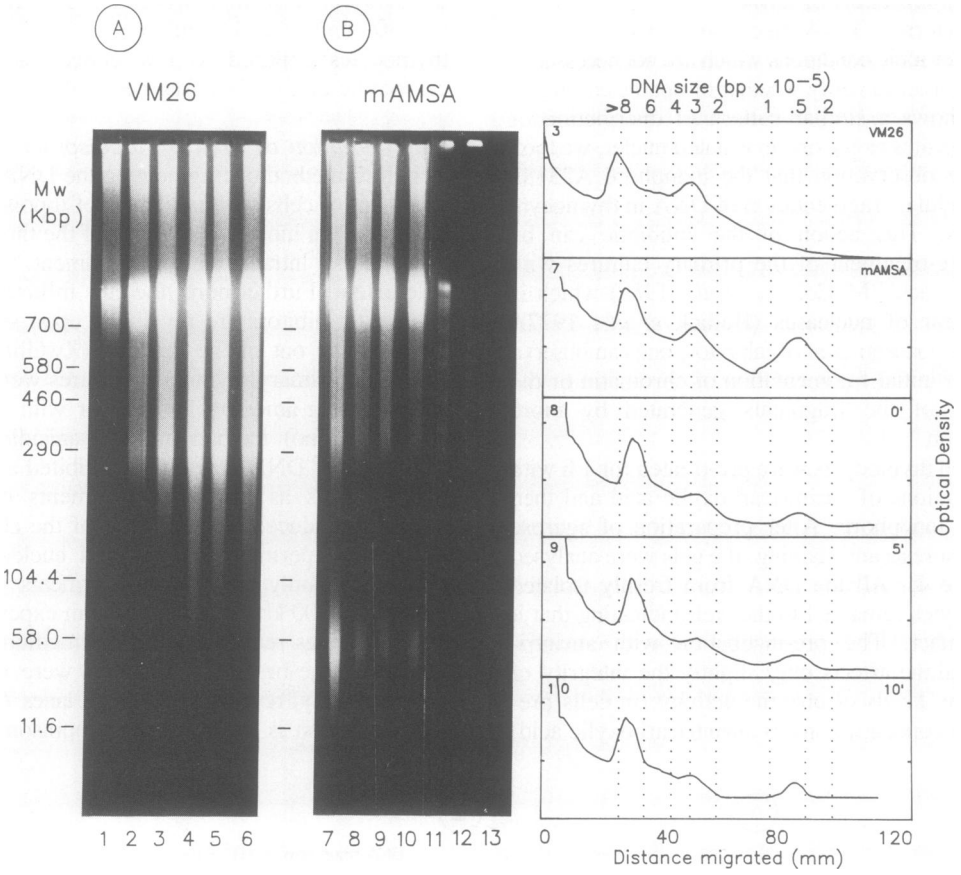

Fig. 5. Fragmentation of the DNA by treatment with inhibitors of topoisomerase II. **(A)** A primary culture of thymocytes was treated for 30 min with various concentration of VM26. **Lanes 1–6** correspond to 0, 0.45 μM, 1.5 μM, 4.5 μM, 15 μM and 45 μM of the inhibitor in the primary culture. **(B)** *m*-AMSA was added to the thymocyte culture at a final concentration of 20 μM and incubated for 30 min. After that time the cells were washed with the fresh medium and further incubated at 37°C. **Lane 7**, unwashed cells, **lanes 8–10**, cell washed and incubated for 0, 5 and 10 min, respectively. **Lanes 11–13**, mol. wt standards. Densitometric scans of **lanes 3** (VM26) and **7–10** (*m*-AMSA) are shown on the right.

Discussion

We have devised three independent methods for examining the folding of DNA into higher order chromatin structures assuming that this folding brings about regular spacings between the nucleolytic enzyme-sensitive sites. In the first method the isolated nuclei contain both the enzyme and the substrate of the reaction and it is essential to isolate them with both their DNA intact and with an activable endonuclease. Several different buffers have been described in the literature for the isolation of chromosomes and nuclei containing high mol. wt DNA (Wallace *et al.*, 1971; Hewish and Burgoyne, 1973; Blumenthal *et al.*, 1979; Filipski and Kohn, 1982; Walker and Sikorska, 1986). Variations on these cocktails were experimented with to isolate nuclei with the above characteristics. The nuclear isolation buffer which satisfies both requirements contains polyamines, EGTA, EDTA and low concentrations of KCl (see Table I). High salt in the isolation buffer also protects the DNA but probably does this by dissociating the nuclease from the chromatin (see Table I) since autodigestion was not observed in nuclei isolated using the buffer containing 150 mM KCl.

Autodigestion of the chromatin in isolated nuclei at 37°C for 15 min in a digestion buffer of low salt concentration (Figure 1) produced fragments 20–60 kbp long while a digestion in 150 mM KCl produced mainly the 300 kbp long fragments. When the digestion buffer of intermediate salt concentration was used the resulting fragments were more heterogeneous in size and the distribution showed maxima at 50, 100 and 300 kbp. These effects of monovalent cation are probably due to the relaxation of the chromatin structures at the low ionic strength, on the one hand, and a lower affinity of the endonuclease for DNA in solutions of higher ionic strength on the other. Similarly, an increase in the $MgCl_2$ concentration in the digestion buffer stimulated the digestion (Figure 2), as expected (since the endogenous endonuclease is magnesium dependent) also producing, at moderate concentrations, the same characteristic distribution of the DNA fragments centered at 50 and 300 kbp (Figure 2B).

These results could be interpreted in two ways; either autodigestion proceeds stepwise producing first the 300 kbp fragments which are subsequently digested to 50 kbp ones, or chromatin regions differing in the degree of condensation simultaneously produce DNA fragments differing in size. The time courses of digestion (Figure 3) show that, initially, fragments of 300 kbp are produced and that the disappearance of the 300 kbp fragments from the mixture as the digestion proceeds is accompanied by the appearance of the 100 kbp and 50 kbp fragments. There is also a significant pause before the 50 kbp fragments are degraded. Thus the first interpretation of the observed results is most likely accurate, although chromatin condensation-related effects probably also contribute to the size distribution of

DNA fragments. The optimal pH value for digestion (pH 7.4), the dependence of the reaction on Ca^{2+} and Mg^{2+} as well as the effect of the monovalent cations are all consistent with the properties of the nuclear Ca/Mg-dependent endonuclease, indicating that this enzyme is responsible for the cutting.

The results presented so far were all obtained by autodigestion of the chromatin in the isolated rat or mouse liver nuclei using a buffer which may not necessarily preserve the native, biologically relevant, chromatin folding. In order to follow digestion of the chromatin in intact cells we exposed the primary cultures of thymocytes to ionophore A23187 which brings about the fragmentation of cellular DNA. This fragmentation is probably due to the activation of the endogenous nuclease *in nucleo* by an increase in the concentration of intracellular calcium ions (McConkey *et al.*, 1989).

Figure 4 shows the results of this experiment and clearly demonstrates that the cells treated with ionophore have most of their DNA fragmented to sizes of 50 and 300 kbp. If they were pre-treated with an inhibitor of the endogenous nuclease, aurintricarboxylic acid, the proportion of fragmented DNA diminishes. At its highest concentration the aurintricarboxylic acid almost entirely inhibits the nuclease and the 300 kbp fragments are absent from the gel (lane 1). The small amount of 50 kbp DNA visible in this lane probably results from the breakage of very relaxed chromatin in a subset of the thymocytes and can be considered to be background. If this background is disregarded, it appears that the ionophore treatment first causes fragmentation of the DNA to 300 kbp long segments which are subsequently split into 50 kbp fragments during more extensive digestions (see lane 6, for example), proceeding through a set of intermediate size fragments (lanes 4 and 5). Thus digestion of the chromatin in cells *in situ* produces the same size fragments as the chromatin digested in isolated nuclei. Furthermore, since ionophore-induced, *in situ* DNA cleavage eventually proceeds to completion (i.e. DNA is fragmented to nucleosome-sized pieces, McConkey *et al.*, 1989) the endonuclease must have free access to all of the DNA. Thus, the initial generation of the 300 and 50 kbp fragments reflects a periodicity in the structure of chromatin rather than a periodicity in the location of the endogenous nuclease.

In the third series of experiments we employed inhibitors of eucaryotic topoisomerases some of which [such as *m*-AMSA (Nelson *et al.*, 1984; Minford *et al.*, 1986) and VM26 (Ross *et al.*, 1984)] stimulate the formation of DNA breaks in the treated cells, using a mechanism (Ross *et al.*, 1979; Filipski, 1983) that is independent of the intracellular Ca^{2+} concentration. A brief treatment of cells with increasing concentrations of VM26 caused an accumulation of 300 kbp DNA fragments (Figure 5). Interestingly, the treatment with *m*-AMSA mainly caused the accumulation of 50 kbp fragments. The fast reversal of the *m*-AMSA-induced breaks upon the removal of the drug from the incubation mixture suggests that they are not the products of autodigestion of the chromatin in a small fraction of dead or dying cells in which no such reversal (but rather further DNA degradation) would be expected.

At present we cannot determine whether the topoisomerase inhibitor-induced breaks are inflicted by enzyme molecules which anchor the chromatin loops in the protein matrix. The cuts could result mainly from the interaction of free enzyme with exposed regions of the DNA in other parts of the loop.

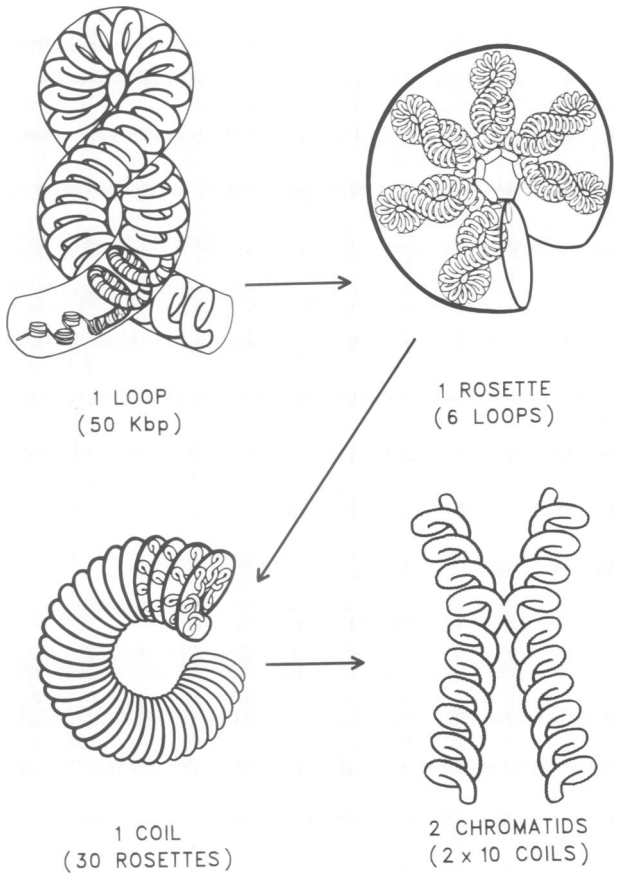

Fig. 6. Proposed model of the higher order folding of the chromatin into chromosomes.

Mapping experiments are in progress to try to resolve this issue. The finding that two different inhibitors produce different distributions of sizes of the fragmented chromatin may be related to the sequence heterogeneity of the sites attacked by the enzyme as well as to the heterogeneity of the enzyme itself.

Conclusions

The considerable literature on chromatin structure (reviewed by Paulson, 1988; see also Van Holde, 1989) suggests that there are five levels of folding of the DNA in the chromosome of eucaryotic cells. The nucleosomes, at the first level, are organized into solenoids (the second level of folding) which form the 30 nm fiber observed in the electron microscope. The exact number of nucleosomes in one solenoid is probably 12 (Walker *et al.*, 1986; Walker and Sikorska, 1987) although alternative models have also been proposed (reviewed by Felsenfeld and McGhee, 1986; Van Holde, 1989). At the third level, the 30 nm fiber is folded to form a loop of chromatin. The fourth level of folding is the organization of chromatin loops into the 200−300 nm thick chromatin fiber observed in the electron microscope (Comings, 1977). The oganization of this fourth level of folding and its relationship to the fifth and final level of DNA folding, the chromatid, is controversial and remains poorly defined. Based primarily on studies of the non-histone protein component of the chromatin fiber a radial loop model, in which many DNA loops radiate out of a central protein core

of the chromatid, was proposed (Laemmli *et al.*, 1978; Pienta and Coffey, 1984). However this model is not consistent with the helical coiling of the chromonema seen in many electron micrographs (Ohnuki, 1968). Our data are more in agreement with the idea first suggested by Comings (1977) and later supported by the electron micrographic studies of Haapala and Nokkala (1982), Rattner and Lin (1985) and Boy de la Tour and Laemmli (1988) that the DNA loops radiate from a protein core that follows a helical path along the chromatid axis of the chromosome. The evidence presented here suggests that the DNA loops of 50 kbp are folded into hexameric rosettes containing 300 kbp of DNA which form a fiber of 200–300 nm in diameter (see Figure 6). A 'medium size' human chromosome contains 10 such turns which is similar to the average number observed in the electron microscope (Boy de la Tour and Laemmli, 1988). Furthermore, this model agrees well with all known features of chromatin and chromosomes.

Materials and methods

Preparation and treatment of thymocytes

Thymocytes, excised from 3 week old rats, were washed to remove excess blood, minced with scissors in 5 ml of Hank's balanced salt solution (HBSS) without phenol red (Whitfield *et al.*, 1968). The concentration of divalent cations was set to 140 mg/l $CaCl_2$ and 100 mg/l $MgCl_2 \cdot 6H_2O$. The minced thymuses were strained through gauze and the resulting suspension was diluted 10-fold with the above HBSS to $\sim 50 \times 10^6$ cells/ml. The cells were rotated at 37°C for 1 h in 10 ml aliquots in an atmosphere of 95% air and 5% CO_2 before treatments. Aurintricarboxylic acid (Sigma, St Louis, MO) from a 100 mM stock solution in dimethyl sulphoxide (DMSO) was added to final concentrations of 0, 10^{-5} M, 3×10^{-5} M, 10^{-4} M, 3×10^{-4} M and 10^{-3} M for 1 h and the cells were then supplemented with 1 μM calcium ionophore A23187 (Sigma, St Louis, MO) from a 1 mM stock in ethanol.

In a separate experiment the thymocyte primary cultures were treated with 0 μM, 0.45 μM, 1.5 μM, 4.5 μM, 15 μM and 45 μM VM26 (Teniposide, Bristol Laboratories, Ottawa, Ontario), for 30 min at 37°C or with 20 μM *m*-AMSA (Chemistry Branch, NCI, NIH, Bethesda, MD) from a 5 mM stock solution in DMSO for 30 min at 37°C. The reversal of the *m*-AMSA induced DNA fragmentation was achieved by washing the cells and incubating in fresh buffer for the various periods of time indicated in the figure legends.

Preparation of agarose plugs from cells

About 10^7 cells were resuspended in 1.0 ml of nuclear buffer containing 0.15 M NaCl, 2 mM KH_2PO_4/KOH (pH = 6.4) 1 mM EGTA and 5 mM $MgCl_2$ (Filipski *et al.*, 1983). In the experiments involving treatment with topoisomerase inhibitors the *m*-AMSA or VM26 were also present in the nuclear buffer at the same concentrations as in the medium in order to prevent the reversal of the DNA breaks during isolation. Cells were then centrifuged at 2000 *g* for 2 min at 4°C, resuspended in 250 μl of nuclear buffer, transferred to Eppendorf tubes and centrifuged for 10 s at 4°C in a microfuge, resuspended once more in 250 μl of nuclear buffer and mixed with 250 μl of molten 1.5% LMP agarose prepared in nuclear buffer containing 0.4 mg/ml proteinase K. The mixture was injected with a hypodermic needle into 70 mm × 5 mm × 1 mm glass moulds made from microscope slides and left at 4°C for 30 min. The agarose blocks were placed in 3 ml of 10 mM NaCl, 10 mM Tris–HCl pH 9.5, 25 mM EDTA, 10% *N*-lauroyl sarcosine (Sigma, St Louis, MO) supplemented with 50 μl of 2 mg/ml proteinase K and incubated at 37°C for 18 h with gentle mixing on a multipurpose rotator. The incubation was followed by rinsing in several changes of 10 mM Tris–HCl pH 8, 1 mM EDTA at 4°C for several hours. The agarose blocks could then be stored in this buffer for several weeks. About 3 mm long slices of these blocks ('the plugs') were used for electrophoresis. They contained 2–5 μg of DNA.

Isolation, autodigestion and preparation of agarose blocks from rat and mouse liver nuclei

Nuclei were isolated from livers of 200 g male specific-pathogen-free Sprague–Dawley rats or BALB/c nude mice bred at this facility as described previously (Sikorska *et al.*, 1980; Walker *et al.*, 1986) using the buffers described below. Briefly, animals were sacrificed and 1 g of tissue was homogenized by eight strokes of a glass–Teflon Potter homogenizer in 5 ml of isolation buffer containing 50 mM Tris–HCl pH 7.4, 0.25 mM sucrose, 20 mM KCl, 0.5 mM EGTA, 2 mM EDTA, 0.15 mM spermine 0.5 mM spermidine and 0.2 mM phenylmethanesulfonyl fluoride. In some experiments various concentrations of KCl were present in the buffer, the EGTA was omitted and, instead of spermine–spermidine–EDTA, 5 mM $MgCl_2$ was used to stabilize the nuclei (see Table I). The homogenate was centrifuged at 2000 g for 10 min at 5°C. The pellet was resuspended in 5 ml of isolation buffer and centifuged again. The cells were then resuspended in 5 ml of the same buffer containing 1% w/v Triton X-100. The Triton step was repeated and the pellet was resuspended in 5 ml of isolation buffer, centrifuged, washed once more in the isolation buffer without EGTA, split into two aliquots, centrifuged and resuspended in 2 ml of digestion buffer containing 10 mM Tris–HCl pH 7.4, 70 mM KCl and 5 mM $MgCl_2$. The concentrations of KCl and $MgCl_2$ varied in some experiments. One aliquot was supplemented with $CaCl_2$ to a final concentration of 0.1 mM and the other aliquot was used as a control. They were incubated for 0–60 min at 37°C.

The digestions were stopped by addition of EGTA to a final concentration of 2 mM and chilling on ice. Aliquots of 125 or 150 μl were diluted to 250 μl with nuclear buffer and mixed with 250 μl of molten 1.5% LMP agarose in the same buffer containing 0.4 mg/ml proteinase K. Plugs were formed and processed as described above.

Pulsed field gel electrophoresis

This was performed using a horizontal gel electrophoresis tank, a model 200/20 power supply and Pulswave 760 field inverting accessory, all purchased from BioRad Laboratories Richmond, CA. The mol. wt calibration was performed by three sets of standards with overlapping mol. wt ranges: the commercially available yeast chromosomal DNA (250–1100 kbp) purchased from BioRad Laboratories, Richmond, CA, polymerized λ phage DNA (50–700 kbp) purchased from Clontech Labs Inc. Palo Alto, CA and a polymerized plasmid DNA (11.6–200 kbp) made in this laboratory. Electrophoreses were run at 200 V in 0.25 × TAE buffer (TAE is 0.04 M Tris-acetate pH 8.5, 0.004 M EDTA) with the ramping rate changing from $T_1 = 0.5$ s to $T_2 = 10$ s for the first 20 h and from $T'_1 = 10$ to $T'_2 = 60$ for the next 20 h, with the forward to backward ratio r = 3, in the cold room, at 5°C, using 1.5% electrophoresis grade agarose purchased from Bethesda Research Laboratories, Gaithersburg, MD. Under these conditions, DNA fragments ranging in size from < 10 kbp to ~750 kbp are resolved and all DNA fragments >750–800 kbp that enter the gel migrate at the same rate. Therefore, in all the gels the apparent band at ~800 kbp does not indicate an additional periodicity of chromatin.

After staining in ethidium bromide the gels were photographed in the UV light with a Polaroid camera using Polaroid positive/negative film 55. The negatives were scanned and analysed by a Biorad Model 620 computerized video Densitometer and data analysis software.

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