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The size of chromatin loops in HeLa cells

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It is widely believed that the chromatin fibre is organized into loops during interphase, with the loop being implicated as an important unit of nuclear function. However, there remains little direct evidence for looping, with estimates of loop size varying widely. This has led to the suggestion that some loops, or even all of them, arise artefactually during isolation as chromatin aggregates so easily. We have now investigated the effect of isolation procedure on loop size using HeLa cells encapsulated in agarose to allow easy manipulation. Loop size in various derivatives (i.e. nuclei, nucleoids, matrices and scaffolds) critically depended on procedure; some (or all) of their loops are artefacts. The loop size in derivatives isolated using the most 'physiological' conditions was 86 kb; this remained unchanged throughout the cell cycle. This loop size is probably an average of a range of loops of between 5 and 200 kb.
Key words: chromatin domain/nuclear matrix/nuclear scaffold/nucleoskeleton/supercoiling

Introduction

It is widely believed that the chromatin fibre is organized into loops during interphase, with the loop being implicated as an important unit of nuclear function (see, for example, Pardoll *et al.*, 1980; Mirkovitch *et al.*, 1984; Gasser and Laemmli, 1987; Cook, 1988). The best evidence for looping remains the direct observation of lateral loops in meiotic lampbrush chromosomes of living cells (Callan, 1977). Evidence for looping in interphase is mainly derived from studies on fixed (DuPraw, 1970; Paulson and Laemmli, 1977) or extracted material such as nuclear matrices, scaffolds and nucleoids (Cook and Brazell, 1975; Igo-Kemenes and Zachau, 1977; Mirkovitch *et al.*, 1984). However, whether any of these structures have counterparts *in vivo* is controversial (Cook, 1988). Chromatin is so highly concentrated in the nucleus and aggregates so easily that it would seem likely that loops might well form artefactually in the unphysiological conditions used during preparation. Then we would expect to get different results with different preparations and this is exactly what is found. For example, transcribed genes are generally found at the base of loops in nucleoids (Cook *et al.*, 1982) but not in scaffolds (Mirkovitch *et al.*, 1984; Gasser and Laemmli, 1986). Furthermore, estimates of loop size vary from 10 to 220 kb (Cook and Brazell, 1975; Benyajati and Worcel, 1976; Paulson and Laemmli, 1977; Igo-Kemenes and Zachau, 1977; Cook and Brazell, 1978; Mirkovitch *et al.*, 1984).

Nuclei are generally isolated using non-isotonic salt concentrations because chromatin and nuclei aggregate in physiological conditions (MacGillivray and Birnie, 1986; Verheijen *et al.*, 1988). We have recently been able to sidestep the problem of aggregation by encapsulating cells in microbeads of agarose before lysis (Jackson and Cook, 1985a). As protein complexes as large as 1.5×10^8 daltons can diffuse through the agarose, encapsulated cells are completely accessible to molecular probes. Embedded within the microbead they are protected from shear and can be transferred from one buffer to another simply by pelleting. This has allowed us to lyse cells in a range of different buffers and then to compare the resulting loop sizes, which we measure as follows. Cells are labelled with [^{3}H]thymidine, encapsulated and lysed. Digestion with a restriction enzyme detaches some chromatin so that it can then be removed electrophoretically, leaving the base of the loops still attached (Jackson and Cook, 1988). Loop size is calculated from the percentage of chromatin (i.e. ^{3}H) remaining in beads and the size of the attached fragments (determined by gel electrophoresis; Igo-Kemenes and Zachau, 1977).

To establish a bench-mark for comparison, we lyse cells using Triton in a buffer that is as close to the physiological as is conveniently possible and maintain the lysed cells in it throughout all procedures. This 'physiological' buffer (pH 7.4) contains 22 mM Na^+, 130 mM K^+, 1 mM Mg^{2+}, <0.3 μM free Ca^{2+}, 132 mM Cl^-, 11 mM phosphate, 1 mM ATP and 1 mM dithiothreitol (Jackson *et al.*, 1988). Whilst the precise ionic constitution *in vivo* remains unknown, we cannot be certain that the resulting *in vitro* preparation is free of artefact, but we do know that it initially contains intact DNA and essentially all the replicative and transcriptional activities of the living cell. Most chromatin can be removed by a combined nucleolytic and electrophoretic treatment without reducing these activities (Jackson *et al.*, 1988) so that if loops are generated by the procedure, the resulting artefacts cannot interfere with vital functions.

Results

Loop size under physiological conditions

Figure 1 illustrates a typical experiment for determining loop size. Beads containing encapsulated cells were lysed in Triton and the 'physiological' buffer and incubated with different amounts of *Hae*III. Some beads were subjected to electrophoresis in the buffer; detached chromatin migrated out of the beads and was lost. The percentage of chromatin remaining in beads was determined and this attached DNA purified. Attached DNA and total DNA purified from beads that had not been subjected to electrophoresis were applied to a second gel and the size range of DNA fragments visualized (Figure 1). As more nuclease was used, the size of total DNA progressively decreased (lanes 2–5) except

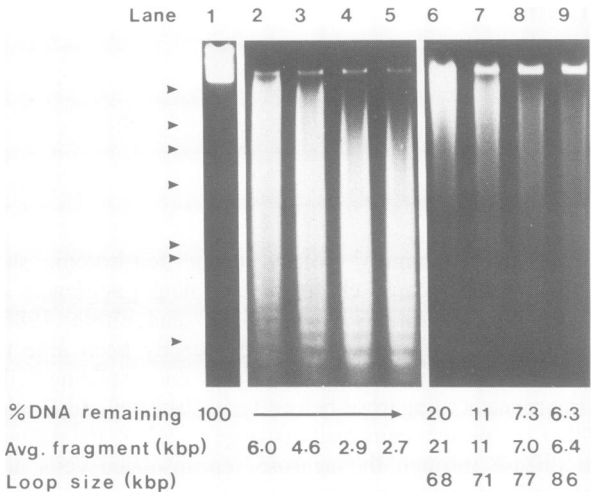

Fig. 1. Loop size in HeLa nuclei determined by nuclease digestion. HeLa cells were labelled with [^{3}H]thymidine for one generation, encapsulated and lysed with Triton in the 'physiological' buffer. Beads were washed, resuspended in an equal volume, incubated with *Hae*III (30 min at 32°C), split and half subjected to electrophoresis to remove detached chromatin. Beads were recovered, protein removed, applied to a 0.8% agarose gel and their DNA sized electrophoretically. After ethidium staining and photography, gel tracks were sliced and the ^{3}H in the slices counted and weight average mol. wts determined. The photograph of the ethidium-stained gel illustrates the range of DNA fragments in total chromatin and chromatin resisting electroelution. **Lane 1**, no *Hae*III or electrophoresis; **lanes 2–5** (total chromatin), *Hae*III (100, 250, 500 or 750 U/ml), no electrophoresis; **lanes 6–9** (attached chromatin), *Hae*III (100, 250, 500 or 750 U/ml), with electrophoresis. The percentage of DNA remaining in beads, average fragment and weight average mol. wts are given below each track. Arrowheads: size markers (λ/*Hin*dIII fragments).

for 2.05% (±SD = 0.25; n = 10) of the total which remained at the top of the gel. This results from a satellite DNA devoid of *Hae*III sites which is also found when naked DNA is digested (not shown). As analysis depends on random scattering of restriction sites, we exclude this satellite from subsequent analysis, but it does provide a useful internal control that loadings are correct. At high levels of digestion, a clear nucleosomal repeat is seen in total chromatin, up to 2 kb in size (lanes 3–5). At the highest level of digestion, only 6.3% chromatin remains in beads (lane 9). Of this 6.3%, 4.3% is non-satellite and has a weight average of 6.4 kb (calculated after slicing the gel and counting the ^{3}H in each slice). A number average of 3.7 kb can be calculated from this weight average using a standard procedure (Botchan *et al.*, 1973), so the average loop size is then 100/4.3 × 3.7 = 86 kb.

When beads are treated with less *Hae*III, less chromatin electroelutes; DNA fragments are correspondingly larger (lanes 6–8) so that calculated loop size remains much the same. Average loop sizes determined from different experiments are given in Table I.

This analysis requires that *all* detached fragments are removed. Although chromatin fragments containing DNA of 150 kb can electroelute through agarose (Jackson and Cook, 1985b), a fraction might be so entangled that they cannot. Therefore we periodically inverted the electric field during electrophoresis (0.3 s forward and 0.1 s backward, linearly ramped to 9 s forward and 3 s backward over 14 h at 2 V/cm) to untangle such complexes, but with the same results (not shown). Fragments might also fail to electroelute because membranes were insufficiently disrupted. Indeed,

Table I. Loop sizes (±SD; n = number of determinations) in HeLa derivatives

Derivative		Loop size (kb)
'Physiological'		
Nuclei	U	85.5 (±7.2; n = 20)
	M	81.1 (±2.5; n = 4)
	G_1	79.3 (±3.9; n = 4)
	S	78.4 (±3.8; n = 4)
Hypertonic		
Nucleoids	U	123.0 (±11.6; n = 6)
Hypotonic		
Nuclei	U	33.8 (±2.9; n = 8)
Scaffolds	U	15.0 (±2.4; n = 6)
Hypotonic then hypertonic		
Matrix	U	48.4 (±3.6; n = 6)

Cells were unsynchronized (U), or from mitosis (M), G_1 (3 h post mitosis) or S-phase (9 h post mitosis, 3 h into S). Derivatives were isolated and loop sizes determined using high concentrations of *Hae*III where DNA fragments could be measured most accurately.

decreasing the amount of Triton or shortening the exposure time to it during lysis, decreased the calculated loop size (not shown); presumably cells were not sufficiently well extracted to allow all detached chromatin to escape. Initially we lysed encapsulated cells with different amounts of non-ionic detergents, added sufficient *Hae*III to cut the chromatin completely and then determined how much chromatin remained. Five washes in isotonic buffer containing 0.5% Triton X-100, NP40 or Tween 20 gave the same limit retention, suggesting that all were completely disrupting membranes; subsequently the minimum exposure giving this limit was used (see Materials and methods). The fact that similar loop sizes are calculated from different degrees of detachment (e.g. Figure 1, lanes 7–9) also suggests that inefficient chromatin removal is not a problem. Furthermore, different enzymes (i.e. *Mbo*I, *Hin*fI) each gave characteristic digestion profiles, satellites, partial digestion products and limit retentions but gave the same loop size (not shown).

The analysis also requires that DNA attachments do not rearrange during analysis. Although we cannot be certain that this does not happen (see Discussion), we do know that wholesale rearrangements of nucleosomes do not occur since even at the highest levels of digestion some sites remain uncut, presumably because they are covered by chromatin proteins; these sites remain uncut irrespective of the length of incubation (not shown). If proteins (e.g. histones) were redistributing, sites would become exposed and the partial digestion products would disappear.

Loop size remains unchanged during the cell cycle

Loop size remains unchanged during mitosis, G_1 and S (Table I). Note that this does not necessarily mean that loops are not dynamic structures; for example, changes occurring in a minor replicating fraction would be undetectable by this assay.

Loop size in nucleoids, nuclei, matrices and scaffolds

We next investigated how isolation procedure affected loop size. Encapsulated cells were treated in various ways and then returned to the 'physiological' buffer for nuclease treatment and electroelution (Figure 2). The different

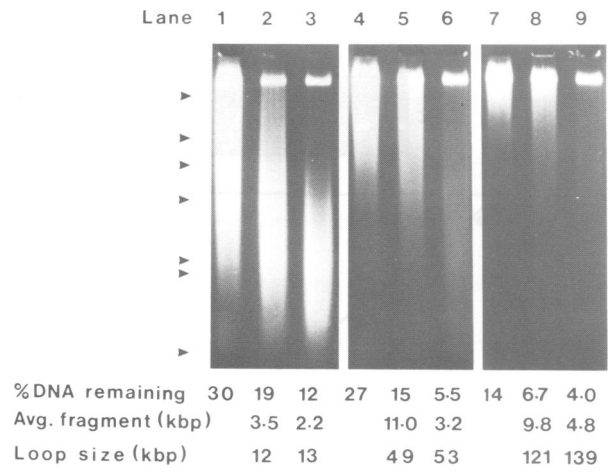

Fig. 2. Loop sizes in scaffolds, matrices and nucleoids. Encapsulated HeLa cells were lysed using various procedures, returned to the 'physiological' buffer for digestion with *Hae*III (25, 100, or 250 U/ml, left to right) and the DNA fragments in chromatin remaining in beads visualized as described in Figure 1. **Lanes 1–3**, 'scaffolds'; **lanes 4–6**, 'matrices'; **lanes 7–9**, 'nucleoids'.

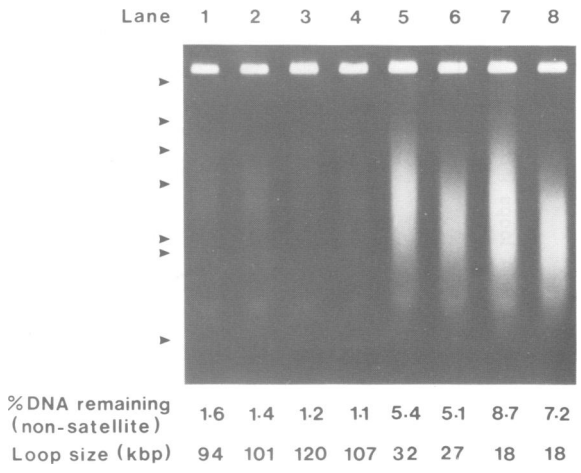

Fig. 3. Changes in loop size during isolation of scaffolds. Samples of encapsulated cells were withdrawn at different stages of the procedure of Mirkovitch *et al.* (1984), extracted with lithium diiodosalicylate and returned to the 'physiological' buffer for digestion with *Hae*III (odd numbered lanes, 100 U/ml; even numbers, 250 U/ml), electroelution and visualization of DNA fragments remaining in beads as in Figure 1. **Lanes 1** and **2**, lysis with digitonin in buffer B (see Materials and methods); **lanes 3** and **4**, lysis with digitonin and washing in buffer C; **lanes 5** and **6**, lysis, washing in buffer C and incubation at 25°C for 20 min; **Lanes 7** and **8**, as for lanes 5 and 6 but incubation at 37°C.

procedures gave strikingly different results; this is most clearly seen simply by comparing the amount of DNA remaining in beads (i.e. in the various tracks in Figure 2). Treatment with the 2 M NaCl used to isolate 'nucleoids' (Cook and Brazell, 1975, 1976) yielded larger loops, presumably because some attachments were destroyed (Figure 2, lanes 7–9; Table I). Surprisingly, exposure to 1/10 the physiological salt concentration, which is commonly used in the preparation of nuclei (MacGillivray and Birnie, 1986), reduced loop size to 34 kb (Table I). Subsequent exposure to 2 M NaCl, like that used to prepare nuclear matrices, increased it from this lower value (Figure 2, lanes 4–6; Table I).

Another popular procedure uses the detergent lithium diiodosalicylate ('LIS') to generate scaffolds (Mirkovitch *et al.*, 1984). These have even smaller loops of 15 kb (Figure 2, lanes 1–3; Table I). The stage in this prolonged procedure that causes this dramatic reorganization was investigated by withdrawing beads at different stages in the procedure and treating them with 'LIS' before returning them to the 'physiological' buffer for digestion and analysis. Immediately after lysis with digitonin in a hypotonic buffer, LIS treatment gave loops of ~100 kb (Figure 3, lanes 1 and 2). Subsequent washing in a Mg^{2+}-free buffer had little further effect (lanes 3 and 4), but loop size fell dramatically as a result of the mandatory incubation required to 'stabilize' the scaffolds (lanes 5–8).

These results show that almost any loop size can be generated by exposure to an appropriate set of conditions and provide an explanation for most of the discrepancies in loop size that have been seen to date.

The distribution of fragments in total and attached DNA provides some insight into the nature of attachments in the different preparations. Total chromatin prepared using the 'physiological' buffer is cut to completion with *Hae*III into fragments averaging 2.7 kb, but the attached fragments are larger (i.e. 6.4 kb). Presumably the attached region extends over a number of *Hae*III sites. The other preparations have quite different attachment sites with the LIS-extracted scaffolds lying at one extreme. Their attached fragments have an average size of 2.2 kb, only slightly bigger than the 'total' fragments of 1.7 kb; fewer sites are protected from the nuclease so their points of attachment must be smaller.

The range of loop sizes

These experiments give only the *average* size of the range of loops that we might expect to find in nuclei. The only practical method that can be used to determine the extent of this range is a fluorometric one (Cook and Brazell, 1978; Cook, 1984) but, unfortunately, it involves intercalation of ethidium into naked superhelical DNA so it cannot be applied to chromatin directly. As both matrices and scaffolds have broken DNA, the method can only be applied to 'nucleoids'. Fortunately, their loop size is closest to that found under 'physiological' conditions so they probably have accumulated the fewest artefacts. Irradiating nucleoids with increasing doses of γ-rays progressively breaks loops, releasing supercoils so that more ethidium binds; the fluorescence therefore increases to a maximum when all loops are nicked (Cook, 1984). After subtraction of appropriate blanks, the difference in fluorescence of dye bound to unirradiated and irradiated nucleoids gives an estimate of the percentage of loops remaining intact at the particular radiation dose (Figure 4). Increasing doses have progressively less effect (Cook and Brazell, 1975, 1978). This is clearly illustrated by the non-linearity of dose against response when plotted on a semi-logarithmic scale; nucleoids must contain loops of different sizes and inspection shows that there are two broad populations, with the majority of DNA in large loops and a minority in much smaller ones.

The sizes of these loops can be determined by comparison with plasmid DNA circles of known length nicked with equivalent doses. Nicking plasmid DNA is most accurately detected using gels; irradiation progressively converts supercoiled form I to relaxed form II (Figure 5A) and, unlike the relaxation of nucleoid DNA, this conversion appears

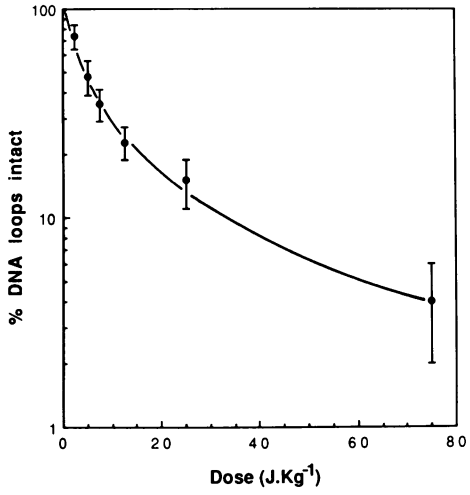

Fig. 4. Loop sizes in nucleoids. Relation between dose and loop integrity in encapsulated 'nucleoids', measured fluorometrically. Mean and SD of five different experiments.

linear on the appropriate semi-logarithmic plot (Figure 5B; cf. Figure 4). This is to be expected for one plasmid circle. Comparison of plasmids of different sizes allows us to establish the relation between plasmid size and the dose relaxing half the circles (Figure 5C) and then nucleoid loop size can be determined by linear extrapolation. The curve in Figure 4 is fitted by assuming nucleoids contain a mixture of two populations of loops, with 20% centred around 12.5 kb and the other 80% broadly distributed between 50 and 250 kb (i.e. with four further sets of 20% centred around 75, 100, 150 and 250 kb). Then the weight average (118 kb) is satisfactorily close to that obtained by nuclease digestion (Table I). [Note also that the gel and fluorometric assays give comparable results for plasmids (Figure 5C).] Of course, it must be remembered that nucleoid loops are slightly larger than those found under 'physiological' conditions and that loops of a particular size might be more susceptible to disruption by 2 M NaCl.

Discussion

Four major conclusions can be drawn from these results. First, measured loop size critically depends on isolation procedure and any size between 15 and 125 kb can be obtained by appropriate choice of conditions (Table I). Even the 'mild' hypotonic conditions generally used to isolate nuclei halve loop size, with even slight variations in procedure having significant effects (not shown). This means that for every attachment existing *in vivo*, one *new* attachment is created *in vitro* as nuclei are prepared. Structures like matrices and scaffolds—which are derived from such nuclei—have accumulated additional rearrangements. Even though it has been argued that binding of specific sequences to these structures implies that attachments are not created artefactually (Cook and Brazell, 1980; Robinson *et al.*, 1982; Mirkovitch *et al.*, 1984) it seems that most of them arise specifically *after* lysis. For example, five out of every six loops in 'LIS'-extracted scaffolds are seen only after thermal 'stabilization' (Figure 3). This 'stabilization' may be related to the *in vitro* 'heat-shock' response (Evan and Hancock, 1985; Littlewood *et al.*, 1987; McConnell *et al.*, 1987; Berrios and Fisher, 1988). [Note that we digest at 32°C in

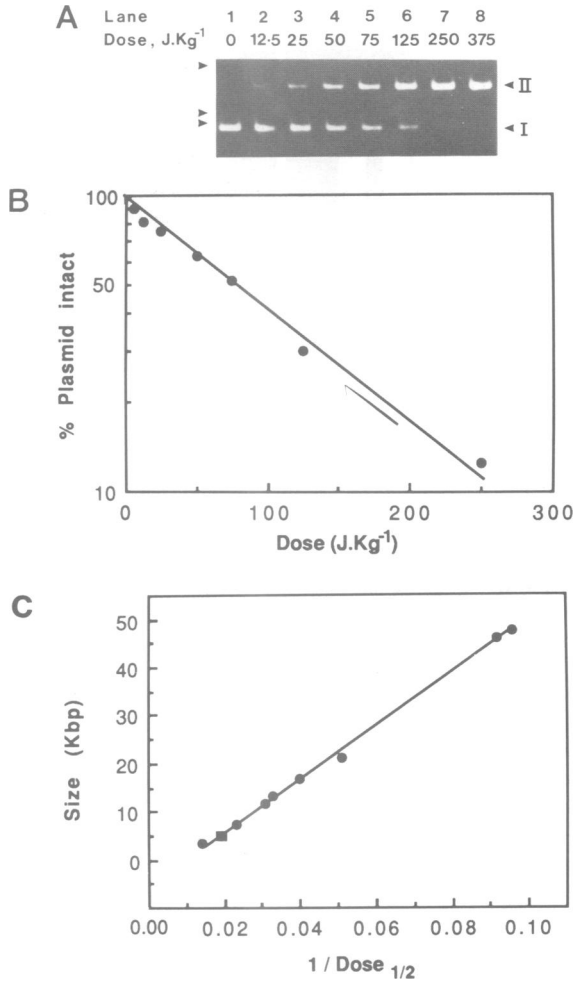

Fig. 5. Loop sizes in plasmids. (**A**) Relaxation of supercoiled plasmid DNA by γ-rays. Supercoiled gel-purified plasmid DNA (I) of 3.4 kb was irradiated with various doses (**lanes 2–8**), subjected to electrophoresis and the gel stained (0.5 μg/ml ethidium) and photographed. The percentage remaining supercoiled was estimated by densitometry by reference to different loadings of unirradiated DNA (**lane 1**). Size markers as Figure 1. (**B**) Relation between dose and plasmid integrity determined by quantitative densitometry of photographs like that in (A). (**C**) Relation between plasmid size (range 3.5–50 kb) and γ-ray dose (J/kg) nicking half the supercoiled molecules, determined from graphs like that in (B). Each point is the average of two or three analyses. The value given by the square was determined fluorometrically as in Figure 4.

the 'physiological' buffer—conditions that do not induce protein aggregation (Jackson *et al.*, 1988).] Whether attachments seen in such scaffolds ever exist *in vivo* remains to be demonstrated.

Secondly—bearing in mind that these results show that chromatin is poised in a metastable state—we cautiously suggest that the average of 86 kb obtained with HeLa cells lysed in the 'physiological' buffer (Table I) is the most likely of the sizes determined to date to reflect the size *in vivo*. These loops *are* stable to variations in conditions, provided they remain isotonic; for example, similarly sized loops are found (Jackson and Cook, 1985a) using a simpler isotonic buffer containing EDTA at pH 8.0. As stable partial digestion products persist throughout long incubations with high enzyme concentrations, nucleosomal 'sliding' does not occur. Although it is impossible to be certain that any preparation is free of artefact, these derivatives are isolated

using conditions that are closest to the physiological. They are free of nicks in DNA, retain gross nuclear morphology and nearly all the authentic replicational and transcriptional activity of the living cell (Jackson *et al.*, 1988). In addition, transcribing and replicating DNA are specifically and quantitatively attached (Jackson and Cook, 1985b, 1986a, 1988; Jackson *et al.*, 1988).

Thirdly, average loop size remains unchanged during the cell cycle (Table I). The basic structure of a nucleoskeleton and attached loops probably persist during the gross structural changes occurring during mitosis.

Nuclei probably contain a range of loops of different sizes, but our nuclease-digestion assay only gives the average. However, we can assess the extent of the range by extrapolation from the results using the fluorometric assay with nucleoids. Obviously, we should be especially cautious in interpreting results obtained with structures made using such unphysiological conditions (i.e. 2 M NaCl) but, fortunately, nucleoid loop size is reasonably similar to that found under 'physiological' conditions. Nucleoids contain a mixture of two populations of loops, with 20% centred around 12.5 kb and the other 80% broadly distributed between 50 and 250 kb (Figure 4). As loops under 'physiological' conditions are 0.7 times (i.e. 86/123, see Table I) the size of those in nucleoids, nuclei in 'physiological' conditions would then have 20% of their loops centred around 7.5 kb, with the other 80% broadly distributed between 50 and 175 kb, well within the range that has been found. It is obviously tempting to speculate that the large loops constitute the inactive chromatin fraction.

Materials and methods

Cells and encapsulation

HeLa cells were labelled with [^{3}H]thymidine for one generation (0.2 μCi/ml; ~50 Ci/mmol) and encapsulated (2.5 $\times$ 10^6 cells/ml agarose) as described by Jackson and Cook (1985a). Cells were synchronized using thymidine and nitrous oxide (Jackson and Cook, 1986b).

Isolation procedures

Beads were washed in the appropriate buffer and then lysed using various procedures.

(i) Triton in 'physiological' buffer (Jackson *et al.*, 1988). Cells were lysed by washing in three changes (15 min each) 10 vol 0.5% Triton X-100 in the buffer.

(ii) Nuclear scaffolds were prepared using the 'LIS' procedure exactly as described in Mirkovitch *et al.* (1984) with omission of the cell homogenization step as the cells are encapsulated. This involves lysing cells with digitonin in a hypotonic buffer (buffer B of Mirkovitch *et al.*, 1984), washing in buffer C, incubation at 37°C for 20 min, and dilution and incubation in lithium diiodosalicylate (buffer D).

(iii) Nucleoids were isolated by lysing living cells with Triton and 2 M NaCl (Cook, 1984).

(iv) 'Nuclei'. Many different procedures have been used conventionally, but most involve lysis in hypotonic buffers. Therefore a 'consensus' procedure was adopted. Encapsulated cells were washed (2×, 15 min) in 20 mM Tris–HCl (pH 7.5), 10 mM KCl, 2 mM $MgCl_2$, 200 mM sucrose, 0.1 mM phenylmethylsulphonyl fluoride, incubated for 15 min on ice in this buffer supplemented with 0.5% Triton X-100 and washed twice in this buffer to release 'hypotonic nuclei'.

(v) 'Matrices'. Again a 'consensus' procedure was adopted. 'Hypotonic nuclei' were incubated at 20°C for 30 min (the usual DNase treatment was omitted as it prevents subsequent analysis) and NaCl added to 2 M.

Determination of loop size

By nuclease digestion. A typical procedure is given using the 'physiological' buffer, which is used from lysis to final sample analysis. Samples were also kept at 4°C subsequently except during nuclease digestion. Following lysis, beads were washed in buffer (3 $\times$ 5 min), resuspended in an equal volume of buffer, incubated with *Hae*III (30 min at 32°C), split, and half subjected to electrophoresis in the buffer to remove detached chromatin (1 V/cm, 15 mA, 15 h; buffer recirculated to prevent pH drift). Beads were recovered, protein removed (0.2% SDS plus 50 μg/ml proteinase K, 37°C, 5 h), applied to a 0.8% agarose gel and their DNA sized electrophoretically (Jackson *et al.*, 1988). After ethidium staining and photography, gel tracks were sliced and slices (0.25 cm) dissolved in 0.5% SDS, 1 M HCl (90°C, 5 min), [^{3}H] counted and weight average mol. wts determined. From these, number averages (Botchan *et al.*, 1973) and loop sizes were calculated (Igo-Kemenes and Zachau, 1977).

Structures isolated using the different procedures were washed three times in the 'physiological' buffer, prior to digestion with *Hae*III and electroelution.

By gel electrophoresis. Supercoiled plasmid DNA (200 ng) was irradiated with γ-rays, subjected to electrophoresis and the gel stained (0.5 μg/ml ethidium) and photographed. The percentage remaining supercoiled was estimated by densitometry by reference to different loadings of unirradiated DNA (Cook and Brazell, 1978).

By fluorometry. Loop sizes in encapsulated 'nucleoids' were measured as described by Cook (1984). A dose of 250 J/kg gave the maximum fluorescence and was assumed to relax all loops (Jackson *et al.*, 1988).

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