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A general method for preparing intact nuclear DNA

P.R.Cook

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

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Naked nuclear DNA is easily sheared. Two general methods are described for preparing intact DNA in a stable form that can be pipetted without breaking it. Cells are encapsulated in agarose microbeads and then lysed in a non-ionic detergent (i.e., Triton X-100) and 2 M NaCl or an ionic detergent (e.g., sodium or lithium dodecyl sulphate) in low salt. Most cellular protein and RNA then diffuse out through pores in the beads to leave encapsulated and naked DNA which is nevertheless accessible to enzymes and other probes. Remarkably, considerable structure is preserved since the DNA is supercoiled and chromosomes retain their shape.

Key words: microbeads/nucleoids/supercoiling/DNA loops/chromosome structure

Introduction

Nuclear DNA from eukaryotic cells has proved very difficult to isolate unbroken. Procedures for freeing DNA from most cellular material generally involve exhaustively treating cells with strong detergents and proteolytic enzymes (Gross-Bellard *et al.*, 1973). In some cases double-stranded molecules the size of chromosomes are released but it is not known whether such molecules contain single-strand breaks (Kavenoff and Zimm, 1973; Schwartz *et al.*, 1983). Such naked DNA molecules are so long and fragile that they cannot be pipetted without breaking them (Burgi and Hershey, 1961; Levinthal and Davison, 1961). To date, essentially intact DNA has been isolated in a pipettable form from only those cells that possess a nuclear 'cage' robust enough to protect it (Cook and Brazell, 1975, 1976a; Cook *et al.*, 1976). Unfortunately, most primary diploid cells possess weak cages that break easily, releasing the DNA which then shears (Cook and Brazell, 1976a). I now describe a general method for preparing essentially intact but naked DNA in a form that can be pipetted unbroken. Living cells are encapsulated in agarose microbeads by homogenizing an aqueous phase containing cells in molten agarose with an immiscible phase of liquid paraffin: on cooling, suspended agarose droplets gel into microbeads (Nilsson *et al.*, 1983). The pores in the beads are large enough to allow free exchange of quite big molecules but not chromosomal DNA. Therefore encapsulated cells can now be lysed and extracted to yield essentially pure DNA which is completely protected from shear.

Results

Lysis in 0.5% Triton and 2 M NaCl

Nucleoids isolated by lysing unencapsulated HeLa cells in Triton X-100 and 2 M NaCl contain all nuclear DNA packaged in a robust cage of RNA and protein. As these nucleoids have been well characterized (Cook *et al.*, 1976) I began by seeing whether nucleoids prepared from encapsulated cells had similar properties. Figures 1a–c and 2 show that their appearance is very similar: the nuclear region appears relatively empty except for nucleolar remnants and a diffuse 'matrix' (Berezney and Coffey, 1977). Figure 3 illustrates their protein content. Most proteins are lost rapidly on lysis so that within 10 min the protein pattern becomes almost indistinguishable from that found after 60 min or in free nucleoids. Studies with cells labelled for 24 h with [^{3}H]leucine show that only 10–15% of the cellular proteins remain in both encapsulated and free nucleoids (unpublished data). Clearly, the pores in the gel permit rapid egress of soluble proteins. In contrast – and in preparations containing few cells/bead – essentially all the DNA and ~50% of the cellular RNA is retained, just like free nucleoids (Colman and Cook, 1977). (This can be shown by prelabelling cells prior to lysis with [^{3}H]thymidine or uridine and then determining the retention of label after lysis.) When beads are packed with cells (e.g., 10^8/ml), cells embedded on the surface tend to be detached on manipulation so that the recovery of DNA falls initially to ~85% and then remains constant.

DNA in free HeLa nucleoids is supercoiled; nicking with γ-rays relaxes it (Cook and Brazell, 1975). Therefore this DNA is intact and probably organised into a series of loops of ~220 kb. I next showed that encapsulated DNA is similarly supercoiled using the intercalating dye, ethidium. The fluorescence of ethidium is enhanced when it binds so binding can be monitored fluorometrically (Bauer and Vinograd, 1974; Cook and Brazell, 1978). At low concentrations, more dye binds to a negatively supercoiled DNA than to its relaxed counterpart. At high concentrations, where binding induces positive supercoiling, less dye binds to the intact molecule. (This difference forms the basis of the widely-used method for purifying supercoiled plasmid DNA free of relaxed plasmid or chromosomal DNA in caesium chloride density gradients.) The concentration where binding is reversed reflects the degree of supercoiling. Below 0.8 μg/ml, unencapsulated and unirradiated HeLa nucleoids bind more dye in 2 M NaCl than their relaxed counterparts and above they bind less: relative binding (unirradiated:irradiated) is >1 below 0.8 μg/ml and <1 above it (Figure 4, curve 1). Encapsulated nucleoids bind similarly so their DNA must be similarly supercoiled (i.e., their relative binding lies on curve 1).

The average size of supercoiled loops in nucleoids can be determined by progressively relaxing loops with increasing γ-ray dose: one nick/loop relaxes that loop and large loops are more likely to be 'hit' than small loops. At dye concentrations >1 μg/ml more ethidium binds as more loops relax (Cook and Brazell, 1978). The dose-response is similar in 2 M NaCl for both free and encapsulated nucleoids (Figure 4b, curve 4): encapsulation does not alter loop size.

These results show that the morphology, constitution and structure of DNA in free and encapsulated nucleoids are very similar. However, they concern HeLa nucleoids which have

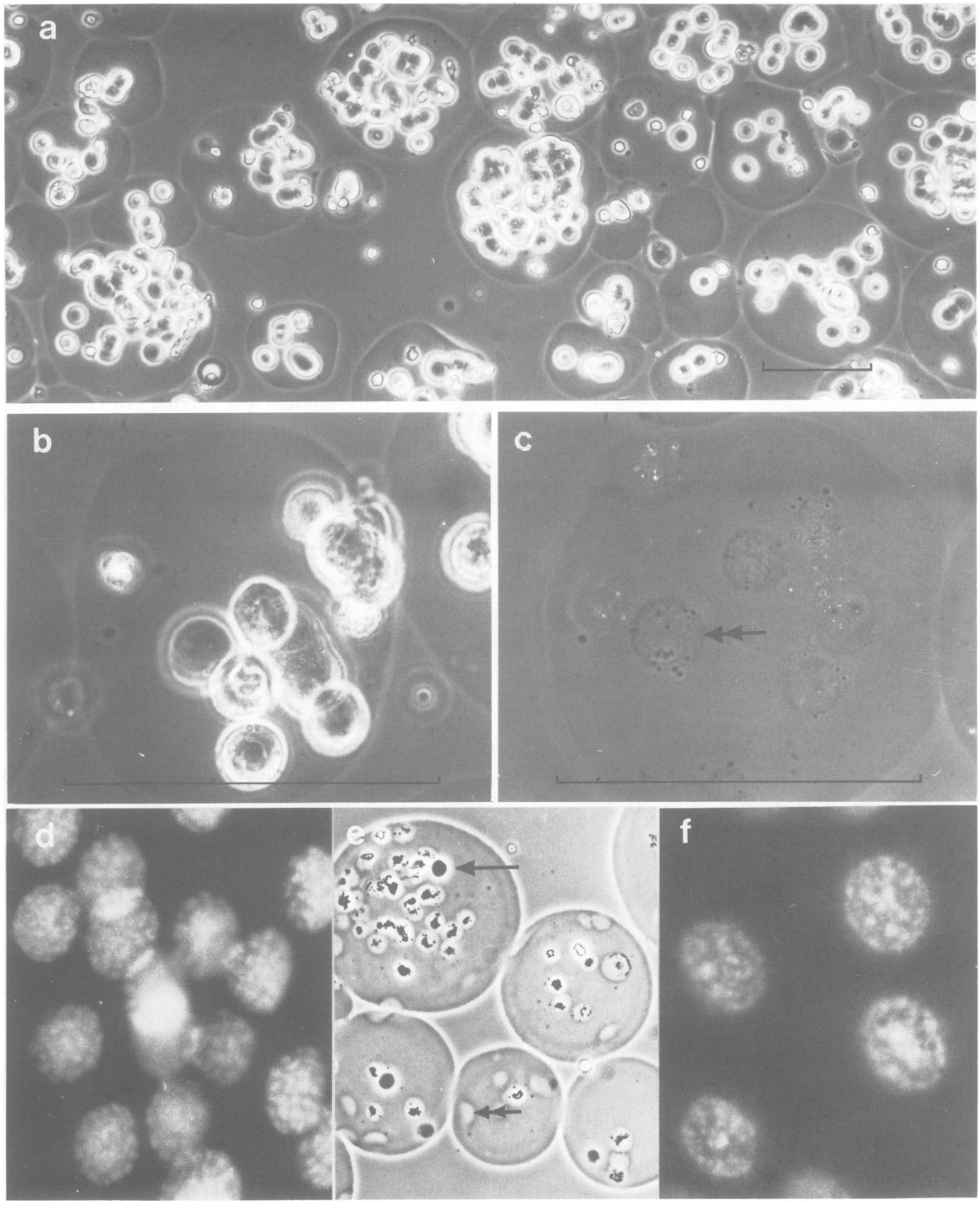

Fig. 1. Phase contrast **(a–c, e)** and fluoresence **(d,f)** micrographs of agarose beads containing HeLa cells **(a,b)** or nucleoids prepared in Triton and 2 M NaCl **(c,d)** or LiDS **(e,f)**. Mitotic cells were used in **d** and **f** and stained with 50 μg/ml ethidium: mitotic chromosomes from a number of different single cells can be seen as fluorescent clusters. Bars, 100 μm: **a,e** and **b,c,d,f** are at the same magnification, respectively. Nucleoids appear phase-light (double-headed arrows) and incompletely extracted cells dark (arrow). Note chromosomes **(f,d)** surrounded by faint punctate fluorescence in **f** but not **d**. Measurements of bead size show that 99 and 73% of beads have diameters < 125 μm and $25-75$ μm. respectively.

robust cages: can the agarose capsule protect DNA of cells that have fragile cages? Lysing human white blood cells in Triton and 2 M NaCl releases superhelical DNA: on pipetting the cage breaks so that the DNA spills out and gets sheared so that supercoiling cannot be demonstrated now by fluorometry (Cook and Brazell, 1976b). When cells from whole blood are encapsulated and then lysed, the red cells dissolve and supercoiling is readily demonstrated in the remaining encapsulated white cells even after the many manipulations required for fluorometry. (Figure 4a curve 1, b curve 4: manipulations include repeated pelleting and resuspension to remove the Triton and pipetting into cuvettes).

Stability of supercoiling

The difference in ethidium-binding of irradiated and unirradiated nucleoids reflects the proportion of loops that are supercoiled and so intact. Encapsulated nucleoids can be stored for 24 h without affecting supercoiling (Table I). On

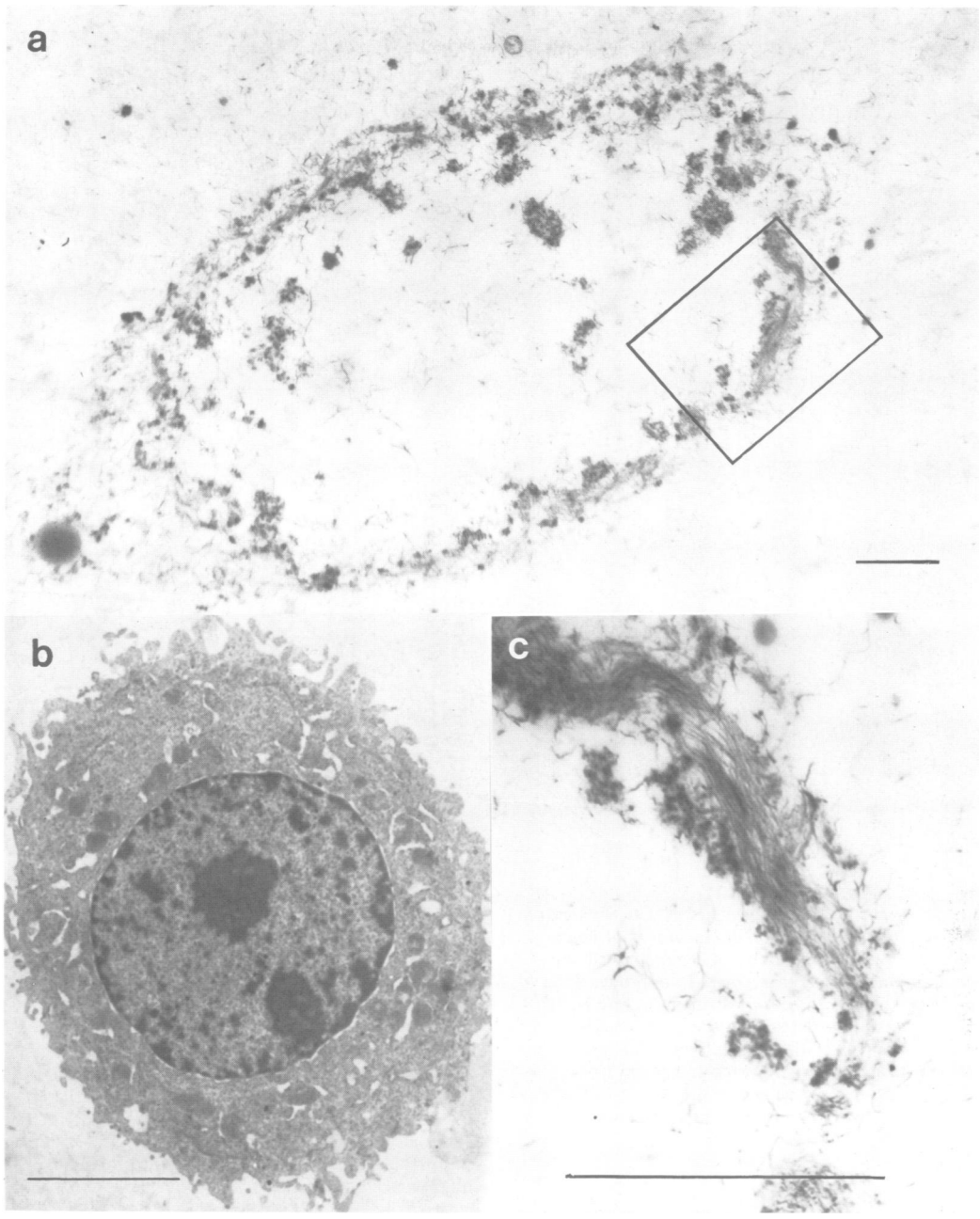

Fig. 2. Electron micrographs of sections of an encapsulated HeLa cell (**b**) and nucleoid prepared in Triton and 2 M NaCl (**a**). (**c**) shows a magnified area of (**a**). Bars, 1 μm. Nucleic acids are not stained by the procedure used.

prolonged storage some relaxation occurs but well-washed preparations of HeLa or white blood cell nucleoids retain all supercoiling for 6 days (unpublished data). Supercoiling is stable to ribonuclease, high temperatures, alkaline pH (Cook and Brazell, 1978), SDS, and electrophoresis, but not to proteinase K (Table I). Note that supercoils remain after treatments similar to those used to solubilize total cell protein for SDS-gel electrophoresis (Laemmli, 1970).

Lysis in lithium dodecyl sulphate (LiDS)

Nearly all protein and RNA can be stripped from DNA using ionic detergents. Therefore encapsulated HeLa cells were extracted with LiDS in low salt: they appear under phase-contrast as empty regions in beads (Figure 1e; double-headed arrow) and contain 100% of the cellular DNA, ~12% of the RNA and < 5% of the protein (results using cells pre-labelled for 24 h with [^{3}H]thymidine, uridine or leucine and few cells/bead). High levels of residual protein correlate with incomplete extraction of cells (one such cell is arrowed in Figure 1e). Complete extraction is achieved by immersing beads in LiDS for 3 h; then no staining material is detected in electron micrographs or gels like those in Figures 2 and 3 and < 1% of the protein remains (results using cells pre-labelled for 24 h with [^{3}H]leucine). Any residual RNA can be removed by electrophoresis as described in Table I. Remarkably, mitotic cells yield structures in which discrete chromosomes remain visible (Figure 1f) and which are stable for days on storage in LiDS (unpublished data). However, their structure is not irreversibly fixed since irradiation (9.6 J/kg) converts them into a diffuse mass of DNA that completely fills the hole in the bead. The encapsulated DNA (from both randomly-growing and mitotic HeLa) is supercoiled because it binds ethidium in

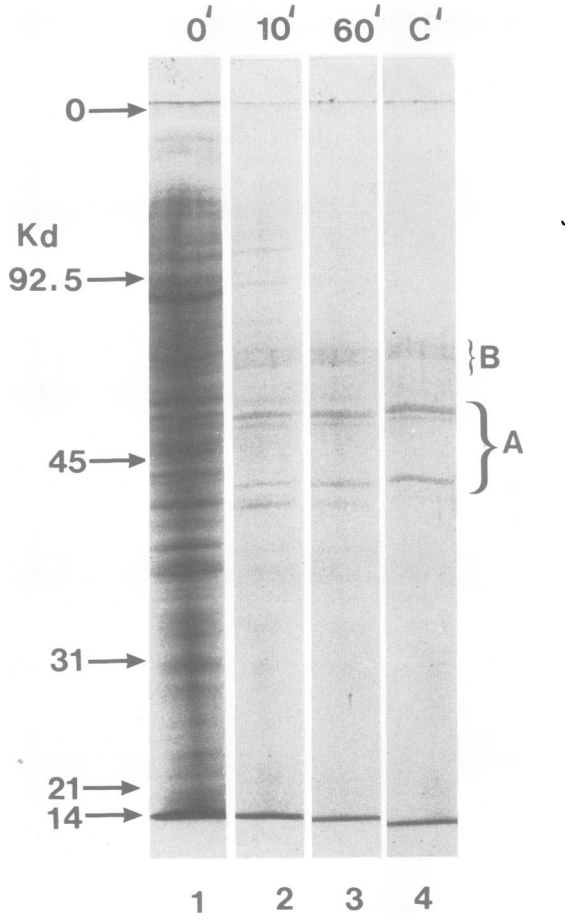

Fig. 3. Protein loss from encapsulated cells. Encapsulated HeLa cells were lysed and 0 **(track 1)**, 10 **(2)** and 60 min **(3)** later, equal numbers of beads subjected to electrophoresis in a 10% acrylamide gel (Laemmli, 1970) and the gel stained with Coomassie blue and photographed. Total protein for **1** was prepared by pelleting beads through PBS, solubilizing in sample buffer (Laemmli, 1970), boiling and shearing. Alternatively, encapsulated cells were lysed in Triton and 2 M NaCl. After 10 or 60 min the beads were washed in 0.2 M NaCl and proteins solubilized. **Track 4**, proteins from unencapsulated nucleoids. O, the top of the separating gel. Group A and B proteins are from the cytoskeleton and nuclear pore complex/matrix, respectively (Cook *et al.*, 1976).

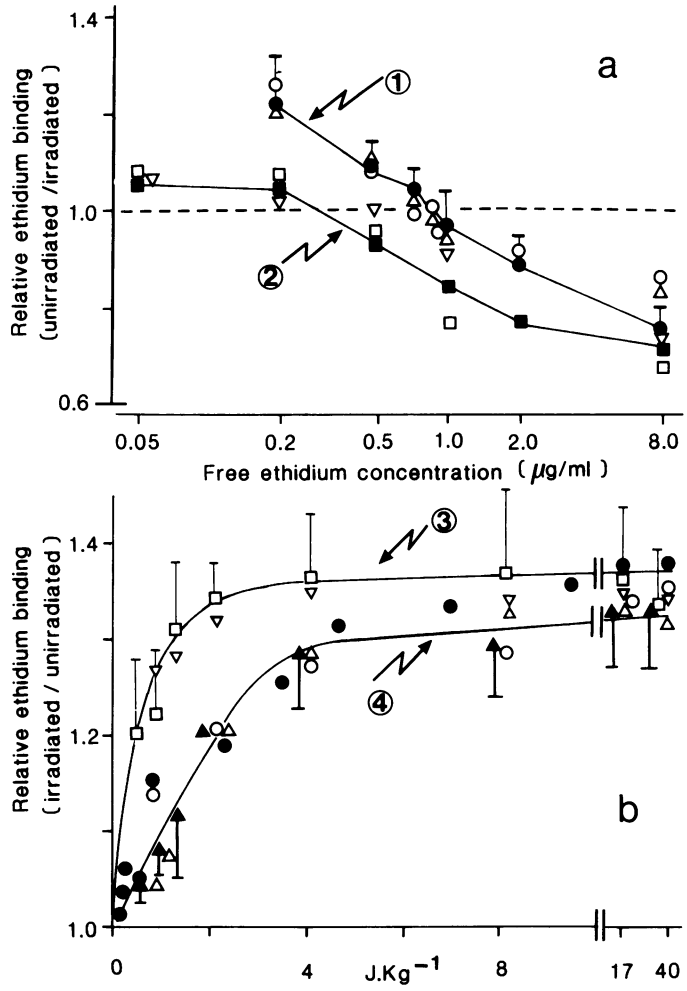

Fig. 4. Supercoiling in nucleoid DNA. **(a)** Ethidium binding of unirradiated and γ-irradiated (9.6 J/kg) nucleoids was determined by fluorometry in 2 M **(curve 1)** or 0.2 M NaCl **(curve 2)** at various dye concentrations (plotted logarithmically to condense the axis). Dye is in excess over the whole range. **Curve 1:** unencapsulated HeLa lysed in Triton and 2 M NaCl and measured in 2 M (●, from Cook and Brazell, 1978); encapsulated HeLa (○) and encapsulated white cells (△), similarly lysed and measured, both lie on this curve. **Curve 2:** unencapsulated HeLa lysed in Triton and 2 M NaCl and diluted to 0.2 M (■, from Cook and Brazell, 1978); encapsulated HeLa (□) and encapsulated mitotic HeLa (▽), both lysed in LiDS and diluted to 0.2 M, lie on this curve. **(b)** The relative binding of nucleoids isolated in LiDS **(curve 3)** or Triton **(curve 4)** was determined in 8 μg/ml dye, after irradiation with different doses. **Curve 3:** encapsulated HeLa lysed in LiDS and diluted to 0.2 M (□); encapsulated mitotic HeLa (▽), treated similarly. **Curve 4:** encapsulated HeLa lysed in Triton and 2 M NaCl and diluted to 0.2 M (▲); unencapsulated HeLa (●), encapsulated HeLa (○) and encapsulated white cells (△), all lysed in Triton and 2 M NaCl and measured in 2 M, all lie on this curve. Error bars; + or − the SD.

the characteristic manner (i.e., in Figure 4a the points lie on curve 2). Since $<1/3$ the γ-ray dose relaxes DNA in these nucleoids compared with their counterparts isolated in Triton and 2 M NaCl (Figure 4b, curves 3, 4), their loops must be $\sim 3-4$ times as big (i.e., ~770 kb). (The great size of these loops gives us a very sensitive assay for breaks in DNA.) Small loops stable in Triton and 2 M NaCl are unstable in the ionic detergent. However, the big loops can be relaxed by proteinase K (Table I).

Discussion

Two simple and general methods are described for preparing naked and intact eukaryotic DNA in a stable form that preserves considerable structural organization. Both require the encapsulation of cells in agarose microbeads so that long DNA strands are protected from shearing. The first using Triton and 2 M NaCl gives structures similar to the unencapsulated nucleoids that have been characterized previously. These are devoid of most cytoplasmic and chromatin proteins but contain residual cytoskeletal and nuclear pore-complex and matrix proteins (Cook *et al.*, 1976; Berezney and Coffey, 1977). Their DNA and pulse-labelled nuclear RNA are specifically associated with a residual cage (Cook and Brazell, 1980; Pardoll *et al.*, 1980; McCready *et al.*, 1980; Jackson *et al.*, 1981; Cook *et al.*, 1982; Robinson *et al.*, 1982). The second method using LiDS in low salt yields essentially pure DNA: the few remaining proteins (<1%) must be tightly bound or contaminants from unlysed cells and nearly all residual RNA can be removed electrophoretically (Table I). We have extended these two methods using different extraction conditions (e.g., by varying the detergent and salt concentration or by using chaotropic agents) and have prepared

Table I. Stability of supercoiling in encapsulated HeLa nucleoids

Treatment	Relative ethidium binding (irradiated/unirradiated)	
	'Triton' nucleoids[a]	'LiDS' nucleoids[b]
None	1.30	1.32
Storage for 24 h	1.30[c]	1.32[d]
Electrophoresis (16 h)[e]	1.29	1.32
+ 1% SDS (30 min: 37°C)	1.27[f]	–
+ 1% SDS, 80 mM dithiothreitol (10 min: 70°C)	1.34[f]	–
+ proteinase K (10 mg/ml; 0.5 h: 37°C)[g]	1.00	1.01

Nucleoids prepared in Triton and 2 M NaCl or LiDS were treated and then the ethidium binding of unirradiated and irradiated (9.6 J/kg) samples compared by fluorometry in 8 μg/ml ethidium. Values >1.01 indicate supercoiling.

[a]Beads were treated in 50 mM NaCl, washed in 50 mM NaCl (2x) and resuspended in 2 M NaCl for fluorometry.

[b]Beads were treated in 50 mM NaCl, washed 2x in 50 mM, 1x in 0.2 M and resuspended in 0.2 M for fluorometry.

[c]Stored in 2 M NaCl on ice.

[d]Stored in 1% LiDS at 20°C.

[e]Beads were washed 2x in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, layered on a vertical 0.8% agarose gel in the same buffer and subjected to electrophoresis at 1.3 V/cm. A bromophenol blue marker and a 23-kb DNA fragment ran 10 and 2 cm, respectively, under these conditions and the nucleoid DNA becomes concentrated on the anodal side of beads

Pulse-labelled nuclear RNA is removed from 'LiDS' but not 'Triton' nucleoids.

[f]Loop size increased 3–4x.

[g]Proteinase K (Boehringer) had been self-digested (50 mg/ml:37°C:19 h) to inactivate nucleases. A 50-kb supercoiled cosmid was not nicked during 24 h incubation under conditions used with nucleoids.

encapsulated DNA associated with different amounts of protein from a wide range of cells (e.g., HeLa, frog red cells, yeast spheroplasts). After washing to remove detergents, the resulting naked DNA remains intact and yet is accessible to proteins and other probes used in modern molecular biology. For example, restriction enzymes, polymerases and ligases all diffuse freely into the beads and can act enzymatically on the encapsulated DNA: the resulting fragments or transcripts can then be recovered by electrophoresis (unpublished data).

The survival of both supercoiling and chromosome morphology in LiDS was very surprising and begs a number of questions. For example, how are loops formed and does the structure have a counterpart *in vivo*? Loops could be generated artefactually on lysis. If so, the more tightly packed the DNA initially, the more tightly packed the resulting loops: however, mitotic and interphase loops are similarly sized (Figure 4b, curve 3) and do not progressively untangle and relax on storage or electrophoresis. Encapsulated chromosomes even retain their shape when subjected for 2 h to an electric field as described in the legend to Table I in which the polarity is reversed every 30 s (unpublished data). If these are not artefactual tangles, chromosomes – unlike chromosomal 'scaffolds' and nuclear 'matrices' (Berezney and Coffey, 1977; Adolph *et al.*, 1977) – must be maintained by forces resistant to strong ionic detergents.

In conclusion, it seems likely that the availability of intact DNA in an accessible yet stable and manipulable form will prove useful for studies on higher-order structure in the nucleus.

Materials and methods

Cells

HeLa cells were grown (Cook and Brazell, 1975), labelled (Cook *et al.*, 1976) and >90% blocked in mitosis using N_2O (Warren and Cook, 1978). The percentage of DNA, RNA or protein remaining in beads was determined using cells labelled for 24 h with [^{3}H]thymidine, uridine or leucine by comparing the acid-insoluble label (Cook *et al.*, 1976) before and after extraction. The concentration of beads in such samples can be determined by measuring their packed volume after sedimentation in 1 mm diameter capillary tubes (i.e., as in a haematocrit).

Encapsulating cells in 0.5% agarose (Nilsson et al., 1983)

2.5% agarose (Sigma types I, VI, VII or IX used where appropriate) in phosphate buffered saline (PBS) was melted, cooled to 39°C (for type VII) and 1 vol was mixed in a round-bottomed flask with 4 vol whole blood or cells in PBS at 39°C. After adding 2 vol liquid paraffin B.P. (The Boots Co., Nottingham, UK) at 39°C to 1 vol cells in molten agarose, the mixture was shaken (800 cycles/min; 30 s; 20°C), cooled in ice and after 5 min excess PBS added and beads pelleted on a bench centrifuge. After removal of paraffin and excess aqueous phase, encapsulated cells are ready for growth or lysis. The concentration of cells/bead can be varied widely; however when beads are packed with cells (e.g., 10^8/ml), many lie embedded on the surface and these tend to be detached on manipulation: on extraction such preparations become viscous since so much DNA lies unprotected on bead surfaces. If cells are to be cultured in beads, medium should replace the saline at all stages.

Nucleoids

Unencapsulated nucleoids were prepared by mixing 1 vol cells in PBS with 3 vol 2.6 M NaCl, 133 mM EDTA, 2.7 mM Tris (pH 8.0) and 0.67% Triton X-100 and isolated in 'step' gradients containing 2 M NaCl (Cook *et al.*, 1976). Encapsulated nucleoids were prepared from encapsulated cells using Triton and 1.95 M NaCl as above or by adding 3 vol 100 mM EDTA, 10 mM Tris (pH 8.0) and 1% LiDS. After 1 h at 20°C, beads were washed at least twice with 10 vol of the appropriate salt concentration to remove detergents. All solutions contained 10 mM Tris, 1 mM EDTA (pH 8.0) and the indicated NaCl concentrations.

Microscopy

Phase contrast and fluorescent microscopy were performed as described by Cook *et al.* (1976). For electron microscopy, beads were fixed in 2.5% glutaraldehyde, then 2% osmium and stained in 0.5% uranyl acetate, then lead citrate.

Fluorometry

The ethidium-binding capacity of both encapsulated and free nucleoids was determined on ice-cold samples, using 10 nm slit-widths as described by Cook and Brazell (1978). The excitation wavelength chosen was 510 nm since it is the isosbestic wavelength for bound and free ethidium: however excitation at 350 nm gives qualitatively similar results and as it gives a much brighter fluorescence is more convenient for routine use. Fluorescence was measured at 590 nm, the wavelength of maximum emission.

Lysed cells (whether encapsulated or not) were diluted using the appropriate salt concentration to give roughly equivalent DNA concentrations (i.e., 0.2 x 10^6 HeLa nucleoids/ml; 10^6 white blood cell nucleoids/ml). Such low DNA concentrations ensure that dye is in excess at ethidium concentrations >0.05 μg/ml: higher DNA concentrations (e.g., 10^7 HeLa nucleoids/ml) give stronger signals and so can be used at dye concentrations >1 μg/ml. Surprisingly, as much as 0.25 ml packed beads/ml solvent give satisfactory fluorometric readings, provided the beads are resuspended prior to reading. Nucleoids were γ-irradiated (Cook *et al.*, 1976) before the addition of ethidium since irradiation reduces the dye's fluoresence. As dye is in excess, the free ethidium concentration equals the total dye concentration.

Every experiment involved a comparison of the fluorescence of irradiated and unirradiated samples. Nucleoid preparations were divided, one irradiated, dye added to both and their fluorescence measured. Fluorescence due to bound dye was determined by subtracting that of dye alone and nucleoids alone.

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