The EMBO Journal vol.4 no.4 pp.913–918, 1985

A general method for preparing chromatin containing intact DNA

D.A.Jackson and P.R.Cook

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

Communicated by H.Harris

A simple and general method is described for preparing chromatin from eukaryotic cells using isotonic conditions. First, cells are encapsulated in agarose microbeads and then lysed using Triton X-100 in the presence of a chelating agent and a physiological concentration of salt. Most cytoplasmic proteins and RNA diffuse rapidly out through pores in the beads to leave encapsulated chromatin which is nevertheless completely accessible to enzymes and other probes. This chromatin can be manipulated freely without aggregation in a variety of different salt and detergent concentrations. It also contains intact DNA since removal of the histones releases superhelical DNA. Conditions are described for incubating this chromatin at 37°C in the presence of Mg^{2+} ions without any nicking of the DNA. We illustrate the usefulness of this chromatin in investigations on the attachment of nascent RNA to the nucleoskeleton, the accessibility of the ribosomal locus to *Eco*RI and the properties of the endogenous RNA polymerase II. This type of chromatin preparation should prove useful for both structural and functional studies.

Key words: microbeads/nuclei/chromatin/supercoiling/method

Introduction

Nuclei and chromatin are rarely studied at a physiological salt concentration since they aggregate so readily (see, for example, Ohlenbusch *et al.*, 1967). As a result they are generally studied in the presence of 'stabilizing' divalent cations under hyper- or hypotonic conditions. Such conditions are unsatisfactory for several reasons. First, the 'stabilizing' cations activate degradative nucleases. Template integrity and supercoiling are essential prerequisites for efficient replication, recombination and transcription in simple templates (Gellert, 1981) so we might expect the same to be true of eukaryotic DNA. Second, unphysiological salt concentrations may introduce artefacts. Whether or not the nucleus of the living cell possesses the same skeletal substructure as that isolated after treatment with hypertonic salt concentrations remains controversial (for a review, see Hancock, 1982). Such structures, called variously the nuclear matrix, cage or scaffold, are often associated with replicating and transcribing DNA (for a review, see Jackson *et al.*, 1984b) but they are not seen in the micrographs of 'genes in action' obtained by Miller and colleagues using hypotonic conditions (Miller and Beattie, 1969; McKnight and Miller, 1979).

We now describe a method for isolating chromatin using a physiological salt concentration: the resulting preparation can be manipulated freely without aggregation. Our approach is an extension of a general method for isolating naked DNA from eukaryotic cells in a form that can be manipulated without breaking it (Cook, 1984). 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 protein as large as 1.5 x 10^8 daltons but not chromosomal DNA (Cook, 1984). Therefore, when the encapsulated cells are immersed in Triton X-100 at a physiological salt concentration, most cytoplasmic proteins and RNA diffuse out through the pores in the beads to leave encapsulated chromatin. If cells are lysed in the presence of EDTA, the resulting DNA remains intact. The procedure yields essentially a preparation of encapsulated nuclei. However, these nuclei dif-

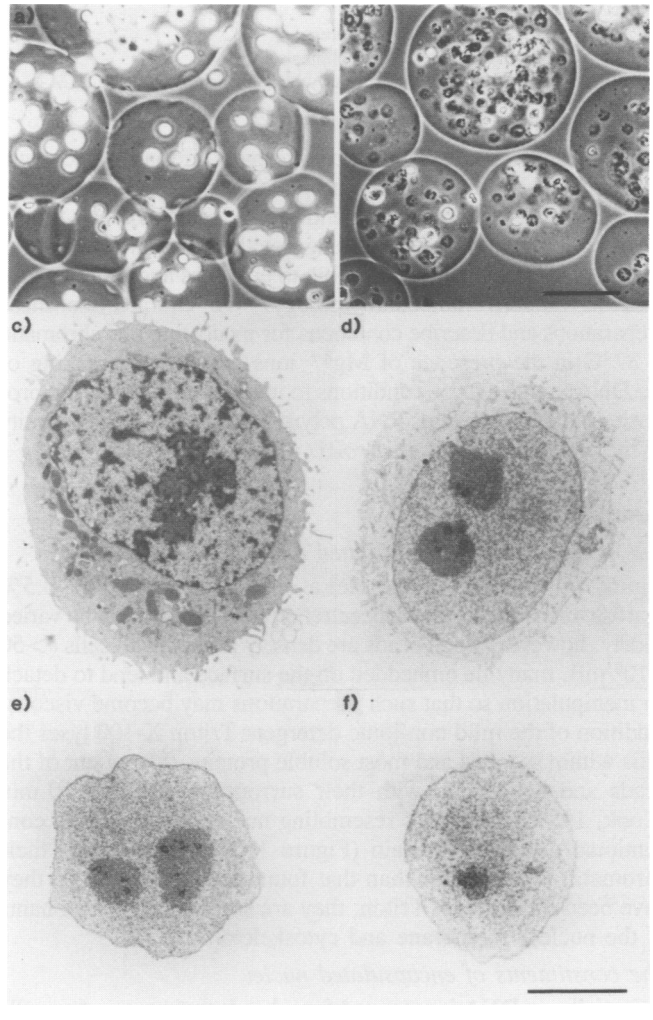

Fig. 1. Phase-contrast (**a,b**), and electron micrographs (**c–f**) of cells, nuclei and their lysed counterparts. (**a,c**) encapsulated cells, (**b,d**) encapsulated cells lysed and washed in the isotonic buffers, (**e**) nuclei made using a standard procedure and then encapsulated, (**f**) encapsulated nuclei from (**e**) lysed and washed in the isotonic buffers. Bars represent 100 (**a,b**) or 5 μm (**c–f**). About 75% of beads have diameters between 25 and 75 μm.

Table I. How various treatments affect the protein content of encapsulated chromatin

Treatment	% Protein remaining[a]	
	Without electrophoresis	With electrophoresis
1. Isotonic	100	86 (83–90)
2. 2 M NaCl	40 (38–42)	29 (26–32)
3. Hypotonic	76 (72–79)	60 (53–65)
4. + *Eco*RI[b]	84 (72–92)	72 (64–81)
5. + DNase[c]	71 (56–81)	58 (50–62)
6. + RNase[d]	80 (75–90)	71 (67–76)
7. + 0.5% sarkosyl	8 (6–10)	3 (1–3)
8. + 0.5% Lithium dodecylsulphate	3 (1–4)	1 (1–2)

Cells in methionine- or leucine-free medium supplemented with 5% dialyzed newborn calf serum were labelled for 15 h with [^{35}S]methionine (5 μCi/ml) and [^{3}H]thymidine (0.25 μCi/ml or [^{3}H]leucine (2.5 μCi/ml) and [^{14}C]-thymidine (0.01 μCi/ml), encapsulated and lysed in the isotonic buffer. This sample (1) retained 27% of cell protein compared with unencapsulated nuclei with 30%. Other identical samples were treated, washed in isotonic buffer and half of each sample subjected to electrophoresis. Finally they were counted and the amount of label in protein expressed as a percentage of the label present in sample 1.

[a]Average of four experiments with the two labelling regimens, range in brackets.

[b]50% DNA remained after electrophoresis.

[c]<10% DNA remained after electrophoresis.

[d]90% RNA labelled in a 2.5 min pulse with [^{3}H] uridine became TCA-soluble.

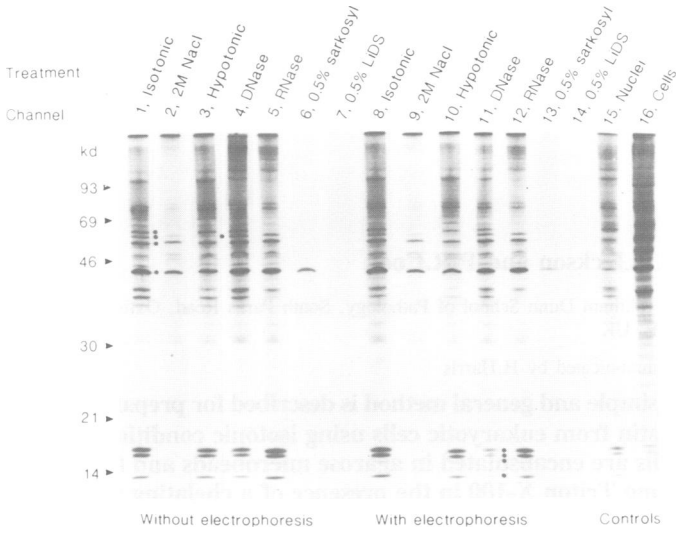

Fig. 2. The protein composition of encapsulated nuclei following various treatments. Cells were grown in [^{35}S]methionine, encapsulated, lysed in the isotonic buffer, washed and treated as indicated. Half of each sample was stored on ice and the other half subjected to electrophoresis to remove any detached material. Proteins remaining within the beads were analysed by electrophoresis in an acrylamide gel and finally an autoradiograph was prepared. Channels 15 and 16 show the proteins from an equal number of nuclei isolated by a conventional procedure and from 1/3 the number of cells, respectively. The positions of marker proteins are indicated.

fer from their unencapsulated counterparts in that they contain unbroken DNA and can be manipulated freely in a variety of different salt or detergent concentrations. The chromatin within the bead is well-protected from aggregation and shearing but is nevertheless completely accessible to enzymes and other probes used in modern molecular biology. We characterize these preparations and describe conditions for incubating this chromatin at 37°C in the presence of Mg^{2+} ions without any nicking of the DNA and use these conditions to investigate some of the properties of the endogenous RNA polymerase II and the accessibility of the ribosomal locus to *Eco*RI.

Results

The morphology of encapsulated nuclei

Figure 1a illustrates some HeLa cells encapsulated in 0.5% agarose microbeads. The concentration of cells/bead can be varied widely: however, when beads are densely packed with cells (>50 x 10^6/ml), many lie embedded on the surface and tend to detach on manipulation so that such preparations may become viscous. Addition of the mild non-ionic detergent Triton X-100 lyses the cells within seconds and most soluble proteins diffuse out of the beads and equilibrate with their surroundings within 10 min (Cook, 1984). Structures resembling nuclei prepared by a conventional procedure remain (Figure 1b–f). Like nuclei their chromatin is less dense than that found in cells and since they have been treated with Triton, they are surrounded by remnants of the nuclear membrane and cytoskeleton.

The constituents of encapsulated nuclei

Essentially no DNA is extracted from beads that contain few cells (<10^7/ml). However, when beads are densely packed with cells (e.g., 10^8/ml), those on the surface may detach so that the recovery of DNA falls, initially to ~85% and then remains constant. On lysis, ~70% of the total cell protein immediately diffuses out of beads (Table I, legend). Very large complexes (e.g., chromatin fragments generated by restriction endonuclease treatment) are lost much more slowly (unpublished data) but can be removed conveniently by electrophoresis. All but the very largest macromolecular complexes can escape. For example, chromatin containing DNA fragments the size of T5 DNA (i.e., 125 kb) can readily be removed electrophoretically. Such a particle has a weight of >1.5 x 10^8 daltons (Jackson and Cook, accompanying paper). Table I and Figure 2 illustrate how a variety of different treatments affect the protein content of encapsulated nuclei, both before and after electrophoresis. (There is some variation from experiment to experiment and this is indicated in Table I. This is largely due to the effects of cell concentration on the efficiency of extraction.)

When analyzed by one-dimensional gel electrophoresis, the proteins of encapsulated nuclei are essentially similar in amount and type to those of nuclei isolated by a conventional procedure (Figure 2, channels 1 and 15), with the obvious addition of cytoskeletal elements (channel 1, dots). [These differences are seen more clearly by 2-D electrophoresis (unpublished data).] 2 M NaCl removes most chromatin proteins, leaving salt-insoluble proteins characteristic of 'nucleoids' [i.e., the nuclear pore complex/lamins (60–75 kd) and cytoskeletal elements (40–56 kd; channel 2)]. Hypotonic treatment is less disruptive but at least one high mol. wt. protein (channel 3, dot) and some ribonucleoprotein (35–41 kd) are specifically extracted. [The high mol. wt. protein is also missing from unencapsulated nuclei (channel 15).] After incubation with nucleases, chromatin fragments and ribonucleoprotein particles become small enough to be removed from the beads. Few histones are lost following DNase treatment even when only 30% of the DNA remains (channel 4); subsequent electrophoresis leaves <5% of the DNA and removes most of the histones (channel 11, dots). Presumably the enzyme cuts both inter- and intra-nucleosomal DNA but some histones and DNA remain associated with the rest of the nucleus until removed by electrophoresis. In contrast, treatment with ribonuclease results in specific protein loss (channel 5, 25–41 kd and 64 kd) even without electrophoresis. Ionic detergents extract

Table II. The effects of various treatments on the RNA content of nuclear derivatives

Nuclear derivative	Pre-treatment	Treatment	% Label remaining	
			Labeling time	
			24 h[a]	2.5 min
1. Nuclei			15	>95
2. 'Nucleoids'			13	>95
3. 'Isotonic'	Isotonic		16	>95
	1.5 mM NaCl		10	40
	2 M NaCl		13	90
	Isotonic	0.5% Sarkosyl	4	28
	1.5 mM NaCl	0.5% Sarkosyl	<1	15
	2 M NaCl	0.5% Sarkosyl	<1	19
	Isotonic	0.5% LiDS[b]	3	15
	1.5 mM NaCl	0.5% LiDS[b]	<1	8
	2 M NaCl	0.5% LiDS[b]	4	30
	Isotonic	6 M urea		10
	Isotonic	RNase[c]		<5
	Isotonic	DNase[c]		95
	Isotonic	Proteinase K[d]		12

Cells were labelled with [^{14}C]thymidine for 24 h and [^{3}H]uridine for 24 h or 2.5 min then (1) nuclei isolated by the conventional procedure and encapsulated, (2) cells encapsulated and lysed in 2 M NaCl ('nucleoids'), (3) cells encapsulated and lysed in isotonic salt ('isotonic'). Beads were pre-treated in hyper-, iso- or hypotonic salt, rewashed in isotonic buffer with the supplement indicated, subjected to electrophoresis and the amount of ^{3}H remaining expressed as a percentage of that initially present in the same number of cells.

[a]Corrections were made for the incorporation of [^{3}H]uridine into DNA.

[b]Lithium dodecyl sulphate.

[c]Incubated at 37°C for 15 min with 50 μg/ml.

[d]Incubated at 37°C for 15 min with 500 μg/ml.

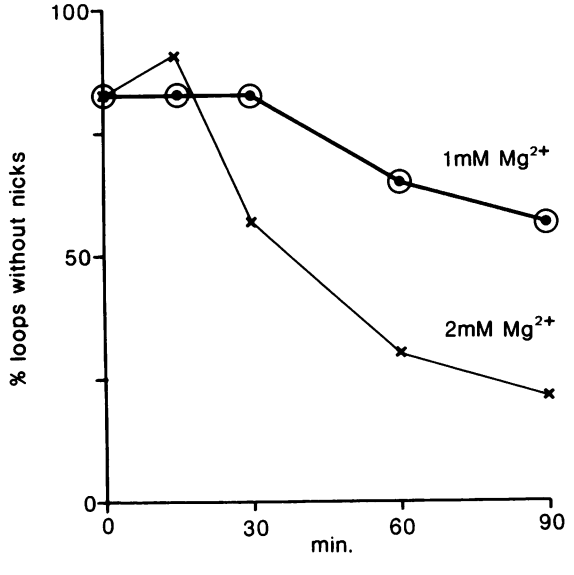

Fig. 3. Nicking DNA during incubation in Mg^{2+}. Cells were encapsulated (10^7/ml agarose), lysed in the isotonic buffer, washed and incubated at 37°C in 100 mM KCl, 25 mM $(NH_4)_2SO_4$, 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP and 1 (o) or 2 mM (x) $MgCl_2$. At different times 10 volumes of ice-cold 2 M NaCl, 10 mM Tris (pH 8.0) and 1 mM EDTA were added, half of each sample was γ-irradiated, ethidium added to 8 μg/ml to both and the fluorescence measured. After subtraction of appropriate blanks, the fluorescence of dye bound to unirradiated nuclei was subtracted from that of irradiated nuclei. This difference is expressed as a percentage of the difference (measured under similar conditions) found between control nuclei derived by lysing the encapsulated cells directly in Triton and 2 M NaCl. This percentage primarily reflects the proportion of loops that remains without nicks and supercoiled: it is relatively insensitive to changes in the degree of supercoiling.

nearly all protein, although sarkosyl is noticeably less severe than lithium dodecyl sulphate (channels 6, 7; a longer exposure of the autoradiograph illustrates this more clearly).

The RNA content of encapsulated nuclei is also very similar to that of nuclei prepared by conventional procedures: both retain ~15% of the RNA labelled in 24 h and essentially all that in 2.5 min (unpublished data). We have examined how various procedures affect the attachment of radiolabelled RNA in encapsulated nuclei by treating them and then removing any detached RNA by electrophoresis (Table II). Some of the RNA labelled in 24 h is detached by hyper- or hypotonic treatment and nearly all by ionic detergents. Perhaps surprisingly, the kind of hypotonic treatment (1.5 mM NaCl) that is widely used for the preparation of transcription complexes (i.e., in 'Miller spreads') must disrupt the complex so that some nascent RNA can be removed. Hypertonic treatment with 2 M NaCl detaches little nascent RNA and probably artefactually generates new attachments so that some become less easily extracted by lithium dodecyl sulphate. Even though sarkosyl extracts nearly all nuclear proteins (Table I), 28% nascent RNA resists extraction: presumably this is the fraction that is associated with the polymerase in the transcription complex (Gariglio *et al.*, 1974, 1981). Even after treatment with lithium dodecyl sulphate or proteinase K, some nascent RNA remains trapped in the bead, perhaps because it remains hydrogen-bonded to the superhelical template.

The integrity of the encapsulated DNA

We next investigated the integrity of DNA in encapsulated and extracted cells using the intercalating dye, ethidium. Supercoils can only be maintained in intact circles of pure DNA; nicking anywhere in the circle releases all supercoiling. At high ethidium concentrations less dye binds to a negatively supercoiled DNA than to its nicked or relaxed counterpart. (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.) As the fluorescence of ethidium is enhanced when it binds, binding – and hence integrity – can be monitored conveniently by fluorometry (LePecq, 1971).

When unencapsulated or encapsulated cells are lysed in Triton and 2 M NaCl, most nuclear proteins are removed, leaving naked DNA loops of ~220 kb attached to the nuclear cage (Cook and Brazell, 1975, 1978; Cook *et al.*, 1976). If such nucleoids are γ-irradiated, their ethidium-binding increases from a normalized value of 1.0 to ~1.25, indicating that essentially all their DNA is initially supercoiled and can be relaxed by irradiation (Cook and Brazell, 1978; Cook, 1984). The dye-binding of encapsulated cells extracted in Triton and isotonic salt and then subsequently treated with 2 M NaCl increases from a value of 1.0 to 1.21. This increase is only 83% of that found with cells lysed directly in 2 M NaCl (Figure 3). This shows that although their DNA remains mostly intact, presumably some nicking has occurred during the lysis and the extensive washing.

Such encapsulated nuclei can be incubated on ice in 1 mM EDTA for hours without further nicking. Since we wish to use such preparations for functional studies we investigated their stability at 37°C in the presence of ATP and Mg^{2+} ions (Figure 3). Incubation in 1 mM EDTA, 1 mM ATP and 1 mM $MgCl_2$ for up to 30 min did not reduce the difference in dye-binding of unirradiated and irradiated samples (determined after raising the salt concentration to 2 M): in other words, no nicking could be detected. However, in 2 mM $MgCl_2$ the DNA became slowly nicked and at higher concentrations, more rapidly so (unpublished data). Since this assay is so sensitive – one nick per

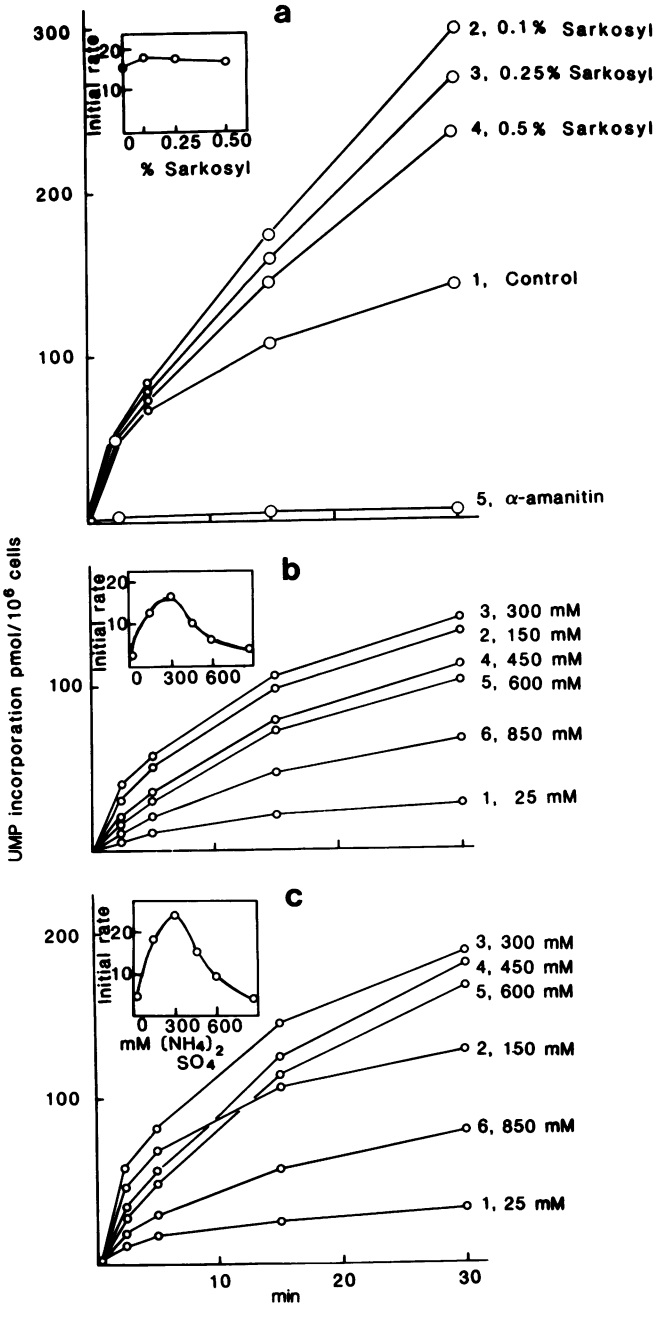

Fig. 4. The effect of (**a**) sarkosyl, (**b**) $(NH_4)_2SO_4$ and (**c**) $(NH_4)_2SO_4$ and $MnCl_2$ on transcription. The incorporation of [^{32}P]UTP into RNA was determined using the standard conditions described in Materials and methods with the modifications shown: (**a**) addition of sarkosyl or α-amanitin (10 μg/ml), (**b**) $(NH_4)_2SO_4$ concentration varied, (**c**) $(NH_4)_2SO_4$ concentration varied and 2 mM $MgCl_2$ replaced by 1 mM $MgCl_2$ and 1 mM $MnCl_2$. The insets show the effect of the various modifications on initial rates (pmol UMP incorporated/10^6 cells/min).

220 kb giving the maximum effect – this means either that there is very little nucleolytic activity in these preparations, or that any nicking is efficiently reversed prior to the high salt treatment.

Transcription

The encapsulated nuclei contain a very active RNA polymerase which can be demonstrated by incubating the beads in the presence of appropriate precursors and radiolabelled UTP (Figure 4a). As expected from Figure 3, little nicking occurs during the first 30 min of such an incubation (unpublished data). The activity is sensitive to α-amanitin, a specific inhibitor of RNA

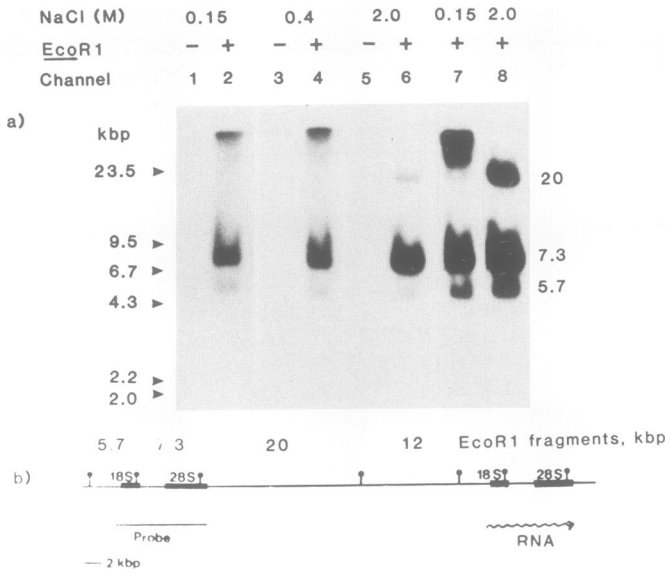

Fig. 5. The 5′ end of each ribosomal locus is sensitive to *Eco*RI digestion. (**a**) Encapsulated cells, lysed in 0.15, 0.4 or 2 M NaCl were incubated ± *Eco*RI and DNA prepared in beads, subjected to electrophoresis, 'blotted', hybridized with a ribosomal DNA probe and an autoradiograph prepared. The position of size markers is indicated on the left and the sizes of the three fragments detected by the probe on the right. Channels 7 and 8 are overexposures of channels 2 and 6, respectively. (**b**) A map of two ribosomal loci (Jackson *et al.*, 1984a), showing the coding regions (18 and 28S), *Eco*RI sites, fragment sizes, a scale of 2 kbp and the positions (in one locus only) of the transcribed region and the probe.

polymerase II. Preliminary experiments show that encapsulated nuclei synthesize RNA at a rate slightly greater than their unencapsulated counterparts prepared by conventional procedures and that neither preparation initiates the synthesis of new chains to any measurable degree (unpublished data).

Figure 4 illustrates the effects of various conditions on transcription. Sarkosyl, which is known to stimulate polymerase activity (Gariglio *et al.*, 1974, 1981), has little effect on the initial rate but allows it to be maintained for longer (Figure 4a), presumably because it strips histones from the template enabling the polymerase to transcribe further. Increasing the salt concentration has an additional effect: it increases the initial rate up to a maximum at 300 mM $(NH_4)_2SO_4$ (Figure 4b). If 2 mM $MgCl_2$ is replaced by 1 mM $MgCl_2$ and 1 mM $MnCl_2$, then the initial rate is further stimulated (Figure 4b,c). We have as yet no complete explanation for these complex kinetics: we have included these experiments to illustrate how the beads might facilitate such studies. [These hypertonic concentrations of salt lyse and jellify unencapsulated nuclei.]

Analysis of chromatin structure

Figure 5 illustrates how these beads may be used to compare the accessibility of *Eco*RI sites in chromatin and DNA. Cells were encapsulated, lysed in Triton and 0.15, 0.4 and 2 M NaCl, incubated with *Eco*RI and then DNA prepared in the beads, subjected to electrophoresis, blotted and hybridized with a ribosomal DNA probe (Figure 5b). Figure 5a illustrates the resulting autoradiograph. As expected, the essentially naked DNA present in the beads treated with 2 M NaCl is almost completely cut and gives three bands. [Uncut DNA is too large to leave the beads and so is lost (channels 1, 3, 5).] In contrast, chromatin prepared in 0.15 M NaCl gives only two bands and a smear at the top. Comparison of the relative intensities of the bands show that the 5.7-kb band is almost as intense in channel 2 as in chan-

nel 6 but the 20-kb band is almost completely missing in channel 2, even when the autoradiogram is overexposed (channel 7). Thus *Eco*RI sites at the 5′ end of the locus are more accessible than those at the 3′ end. The inaccessibility of the 3′ sites persists after treatment with 0.4 M NaCl (channel 4). When the same filter was rehybridized with a γ-globin probe, strong bands were obtained in channel 6, but only faint bands – due mainly to partial digestion products – were seen in channels 2 and 4 (unpublished data). Thus non-transcribed chromatin from both the 3′ end of the ribosomal locus and the inactive γ-globin gene proves relatively inaccessible, in keeping with results from many other systems (Weisbrod, 1982).

Discussion

Nuclei are generally isolated by immersing cells in a hypotonic buffer and then breaking the swollen cells by homogenization. This hypotonic treatment is generally considered to be a mild one, but it can extract more than a fifth of the protein and half the nascent RNA (Tables I and II). [Uncontrolled hypotonic treatments in the initial step in the isolation of nuclear matrices and scaffolds may underlie some of the conflicting results obtained with these structures. Nucleoid cages – prepared directly from cells without hypotonic treatment – will differ from analogous structures prepared from nuclei.] The nuclei prepared by conventional procedures aggregate if restored to isotonic conditions unless 'stabilized' by divalent cations. This activates nucleases so that template integrity is lost. Our procedure utilizes isotonic conditions throughout and maintains template integrity. Fortunately the template can be incubated at 37°C for 30 min in the presence of Mg^{2+} ions wthout any net nicking of the DNA (Figure 3). Most importantly, the encapsulated nuclei can be freely pipetted and pelleted using a bench microcentrifuge, enabling them to be transferred from one buffer to another. Some of the experiments described here involve several enzyme digestions or assays, treatment with hypotonic solutions or detergents and electrophoresis overnight – manipulations that would be impossible using free nuclei or chromatin which aggregate and jellify so readily.

The nuclear cage, matrix and scaffold are all prepared by treating cells with hypertonic salt but such structures are not seen using hypotonic conditions. As a result, it has been suggested that they are artefacts that have no counterparts *in vivo* (see earlier). We believe that the availability of isotonically-prepared chromatin will help resolve this controversy. We show here that nascent RNA – unlike bulk RNA – cannot be removed electrophoretically from the encapsulated nucleus (Table II). The attachments of the nascent RNA are destabilized by hypotonic treatment and stabilized by hypertonic treatment. Therefore both treatments probably generate artefacts, low salt concentrations detaching nascent RNA and higher ones creating additional attachments. We have extended this study to show that two other elements of the transcription complex (i.e., the polymerase and template) are associated with a 'nucleoskeleton' present under isotonic conditions and that this association is disrupted by hypotonic treatment (Jackson and Cook, see accompanying paper).

In conclusion, it seems likely that the availability of chromatin containing intact DNA, in an accessible yet stable and manipulable form, will prove useful for studies on higher-order structure in the nucleus and its relation to function. We would stress that the use of isotonic conditions does not necessarily guarantee the isolation of 'native' nuclei or chromatin, only that the resulting preparation is likely to be less prone to artefacts than other preparations that use non-isotonic conditions.

Materials and methods

Radiochemicals

Labels (Amersham International) included [methyl-^{14}C] thymidine (~60 Ci/mmol), [methyl-^{3}H]thymidine (~50 Ci/mmol), [5,6-^{3}H]uridine (~50 Ci/mmol), uridine-5′-[α-^{32}P]triphosphate (~400 Ci/mmol), deoxyadenosine 5′-[α-^{32}P]triphosphate (~3000 Ci/mmol), L-[^{35}S]methionine (~500 mCi/mmol).

Cells

HeLa cells were grown (Cook and Brazell, 1975) and the percentage of DNA, RNA and protein remaining in beads was determined using the appropriate radiolabels (Cook, 1984).

Encapsulating cells in 0.5% agarose (Nilsson et al., 1983; Cook, 1984)

2.5% agarose (Sigma type VII) in phosphate buffered saline (PBS) was melted, cooled to 39°C and 1 volume mixed in a round-bottom flask with 4 volumes of cells in PBS at 39°C. After adding 2 volumes of liquid paraffin B.P. (The Boots Co., Nottingham, UK) at 39°C to 1 volume of 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 lysis. If cells were cultured in beads, medium replaced the saline at all stages. The experiments described here used beads containing $5-50 \times 10^6$ cells/ml beads. The packed volume of beads in a sample can readily be determined after sedimentation in 1 mm diameter capillary tubes (i.e., as in a haematocrit).

Some batches of agarose contain impurities that inhibit restriction endonucleases and ligases. These were removed by extraction with DEAE-cellulose (Whatman). 100 ml of molten 2.5% agarose in PBS were mixed (30 min; 50°C) with 10 ml (packed slurry) DEAE-cellulose (pre-equilibrated with PBS). The DEAE-cellulose was removed by centrifugation and the process repeated.

Cell lysis

Encapsulated cells were lysed by mixing 1 volume of beads in PBS or growth medium with 3 volumes of various lysis mixtures at 4°C. The isotonic lysis mixture contained 0.5% Triton X-100, 100 mM KCl, 10 mM Tris (pH 8.0), 25 mM Na_2 EDTA, 1 mM dithiothreitol. After 20 min on ice, beads were washed in isotonic buffer [100 mM KCl, 25 mM $(NH_4)_2SO_4$, 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol] at least three times. An alternative lysis mixture contained 0.67% Triton, 2.6 M NaCl, 133 mM EDTA, 2.7 mM Tris (pH 8.0) (for lysis in 0.5% Triton and 2 M NaCl): subsequent washes contained 10 mM Tris (pH 8.0), 1 mM EDTA and the appropriate salt concentration.

Nuclei were prepared by swelling cells in 5 mM Tris (pH 8.0), 1 mM EDTA, 1 mM EGTA, 10 mM NaCl, 0.5 mM dithiothreitol for 15 min on ice, breaking them by homogenization and washing twice in the above buffer.

Microscopy

Phase contrast and electron microscopy were performed as described by Cook (1984).

Fluorometry

The ethidium-binding capacity of both encapsulated and free nucleoids was determined on ice-cold samples (Cook and Brazell, 1978; Cook, 1984). Every experiment involved a comparison of the fluorescence of γ-irradiated (0.6 J/kg) and unirradiated samples. Preparations were divided, one irradiated, dye added to both (8 μg/ml final concentration) and their fluorescence measured. Fluorescence due to bound dye was determined by subtracting that of dye alone and nucleoids alone.

Transcription

Beads were washed in isotonic buffer, an equal volume of a twice concentrated transcription mixture added, pre-incubated (37°C; 10 min) and synthesis initiated by addition of triphosphates, S-adenosyl methionine and $MgCl_2$. At various times samples were mixed with 3 volumes of 2.5% SDS and then spotted on to glass fibre discs (Whatman GF/C). After washing in 5% trichloroacetic acid, the discs were counted (Colman and Cook, 1977). During transcription the final concentrations were 50 mM Tris (pH 8.0), 25 mM KCl, 100 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EDTA, 5 mM spermidine, 5% glycerol, 50 μM S-adenosyl methionine, 500 μM ATP, 500 μM CTP, 500 μM GTP, 50 μM UTP (supplemented with 10 μCi/ml [^{32}P]UTP at ~400 Ci/mmol).

Electrophoresis

DNA was subjected to electrophoresis (2 V/cm for 6 h or 0.7 V/cm for 16 h) through 0.8% agarose gels in 40 mM Tris (pH 8.3), 2 mM EDTA and 20 mM sodium acetate (TEA). Encapsulated chromatin was electrophoresed similarly except that the sample and electrophoresis buffers were equal volumes of isotonic

buffer and TEA. After electrophoresis beads were recovered and washed in the appropriate buffer.

Proteins were prepared for electrophoresis (Figure 2) as follows. Cells (5 x 10^7) were labelled with 1 mCi [^{35}S]methionine for 18 h at 37°C in 100 ml methionine-free minimal essential medium for suspension cultures (Gibco) supplemented with 5% dialyzed newborn calf serum and 2% minimal essential medium. The cells were collected, encapsulated, lysed and washed in isotonic buffers containing 0.1 mM phenylmethylsulphonylfluoride, which was present at all subsequent stages. Samples were treated for 15 min as follows: (i) isotonic control stored on ice; (ii) 2 M NaCl treatment at 4°C [two washes with 10 volumes of 2 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA]; (iii) hypotonic treatment at 4°C [two washes with 10 volumes 100 μM borate buffer (pH 8.5)]; (iv) DNase treatment at 37°C [50 μg/ml DNase I in 2.5 mM $MgCl_2$]; (v) RNase treatment at 37°C [33 μg/ml RNase A]; (vi) 0.5% sarkosyl treatment at 20°C; (vii) 0.5% lithium dodecyl sulphate at 20°C [Na^+ replaced K^+ in the buffer]. Following treatment, samples were washed in isotonic buffer and divided: one half was stored on ice, the other subjected to electrophoresis. These beads were collected, washed in 30 volumes of 5 mM Tris (pH 8.0), 10 mM NaCl, 2 mM $MgCl_2$ and incubated with DNase (100 μg/ml) and RNase A (50 μg/ml) for 30 min at 37°C, mixed with sample buffer, heated and subjected to electrophoresis in a 12% acrylamide gel (Laemmli, 1970). Finally an autoradiograph was prepared using 'preflashed' Fuji RX film and intensifying screens.

Mapping sensitive sites in chromatin (Figure 5)

Encapsulated cells were lysed in 0.5% Triton, 10 mM Tris (pH 8.0), 100 mM EDTA and 0.15, 0.4 and 2 M NaCl, washed in 10 mM Tris (pH 8.0), 1 mM EDTA and 0.15 M NaCl, and 30 μl samples containing 3 μg DNA incubated with 0 or 160 units *Eco*RI for 1 h. Next beads were treated with 1% sarkosyl and RNase A (25 μg/ml for 30 min at 56°C) and then proteinase K (125 μg/ml) for 60 min at 56°C before applying a final volume of 40 μl to a gel. After electrophoresis, blotting and hybridization with a 'nick-translated' ribosomal probe, an autoradiograph was prepared (Jackson *et al.*, 1984a).

Acknowledgements

We thank Mike Simpkins and Jill Lang for their help and the Cancer Research Campaign for support.

References

Colman,A.C. and Cook,P.R. (1977) *Eur. J. Biochem.*, **76**, 63-78.
Cook,P.R. (1984) *EMBO J.*, **3**, 1837-1842.
Cook,P.R. and Brazell,I.A. (1975) *J. Cell Sci.*, **19**, 261-279.
Cook,P.R. and Brazell,I.A. (1978) *Eur. J. Biochem.*, **84**, 465-477.
Cook,P.R., Brazell,I.A. and Jost,E. (1976) *J. Cell Sci.*, **22**, 303-324.
Gariglio,P., Buss,J. and Green,M.H. (1974) *FEBS Lett.*, **44**, 330-333.
Gariglio,P., Bellard,M. and Chambon,P. (1981) *Nucleic Acids Res.*, **9**, 2589-2598.
Gellert,M. (1981) *Annu. Rev. Biochem.*, **50**, 879-910.
Hancock,R. (1982) *Biol. Cell*, **46**, 105-122.
Jackson,D.A., Cook,P.R. and Patel,S.B. (1984a) *Nucleic Acids Res.*, **12**, 6709-6726.
Jackson,D.A., McCready,S.J. and Cook,P.R. (1984b) *J. Cell Sci.*, Suppl. **1**, 59-79.
Laemmli,U.K. (1970) *Nature*, **227**, 680-683.
LePecq,J.-B. (1971) *Methods Biochem. Anal.*, **20**, 41-86.
McKnight,S.L. and Miller,O.L. (1979) *Cell*, **17**, 551-563.
Miller,O.L. and Beattie,B.R. (1969) *Science (Wash.)*, **164**, 955-957.
Nilsson,K., Scheirer,W., Merton,O.W., Ostberg,L., Liehl,E., Katinger,H.W.D. and Mosbach,K. (1983) *Nature*, **302**, 629-630.
Ohlenbusch,H.H., Olivera,B.M., Tuan,D. and Davidson,N. (1967) *J. Mol. Biol.*, **25**, 299-315.
Weisbrod,S. (1982) *Nature*, **297**, 289-295.

Received on 2 January 1985; revised on 6 February 1985