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DNA IS REPLICATED AT THE NUCLEAR CAGE

S. J. McCREADY,* J. GODWIN,† D. W. MASON, I. A. BRAZELL AND P. R. COOK

*Sir William Dunn School of Pathology, * Botany School, and † Department of Astrophysics, University of Oxford, Oxford, U.K.*

SUMMARY

Structures resembling nuclei are released when HeLa cells are lysed in a detergent and 2 M salt. These nucleoids, which lack any organized membrane structure, contain all the nuclear DNA packaged within a cage of RNA and protein. Their DNA is supercoiled so that the linear DNA must remain unbroken and looped during lysis. Following digestion with the restriction endonuclease, EcoRI, cages and associated DNA were filtered free of unattached DNA. Pulse-labelled (i.e. newly synthesized) DNA remains preferentially associated with the cages. This association has been confirmed by autoradiography. When nucleoids are prepared for electron microscopy by the Kleinschmidt procedure the DNA spills out to form a skirt around the flattened cage. Labelling, which is restricted to the region of the cage after short pulses, extends out into the skirt as the labelling time increases. A model, based on the premise that replication takes place at the nuclear cage, is presented in the Appendix. The results of the biochemical experiments and electron microscopy both indicate that the average size of the unit of replication is ~ 20 μm. This is about one-quarter the size of the average structural unit – the loop. Therefore sequences in the loop must become attached to the nuclear cage prior to the initiation of DNA synthesis.

INTRODUCTION

It is now widely accepted that nuclear DNA is organized into loops, possibly by attachment to a protein matrix (Cook & Brazell, 1975; Benyajati & Worcel, 1976; Igó-Kemenes & Zachau, 1977; Paulson & Laemmli, 1977). We now try to answer an important question relating to DNA synthesis: does the replication fork move freely around a fixed loop or does the DNA move through a fixed replication-complex attached to the matrix?

Whether or not DNA is replicated at a fixed site – for example, at the nuclear membrane or matrix – has been the subject of controversy for a number of years (Edenberg & Huberman, 1975; Pardee, Dubrow, Hamlin & Kletzein, 1978; Pardoll, Vogelstein & Coffey, 1980). Two approaches – biochemical and using autoradiography – have both given variable results. Biochemists have demonstrated association of more or less pulse-labelled DNA either with the nuclear membrane or with the matrix, and we believe that this variation is partly caused by the use of broken templates. DNA is extensively nicked when nuclei are prepared by any of the widely used methods (Warren, 1977) so that some inevitably becomes detached from the matrix and may artifactually associate with sticky membranes. The results from autoradiography are also conflicting. DNA synthesis is so extremely rapid (~ 1 μm/min) and the radius of the nucleus so small (~ 3 μm) that pulses long enough to give significant labelling

in the autoradiographs used for electron microscopy also permit ample time for the movement of the pulse-labelled DNA far from its site of synthesis (Edenberg & Huberman, 1975).

We have applied both approaches to the problem but have used novel methods for isolating and manipulating the DNA. We lyse living cells with a detergent in 2 M NaCl to release structures which retain many of the morphological features of nuclei. These nucleoids lack any organized membrane but contain naked DNA packaged within a flexible cage of RNA and protein. Their DNA is supercoiled so that the linear DNA must remain unbroken and looped during lysis. The cage, which is probably derived both from elements of the cytoskeleton and the proteins and RNA that underpin the nuclear membrane, protects the fragile DNA from breakage (Cook & Brazell, 1975, 1978; Cook, Brazell & Jost, 1976; McCready, Akrigg & Cook, 1979). We digest nucleoid DNA – which is now free of sticky membranes – with the restriction endonuclease, EcoRI, and filter the cages and any associated DNA free from unattached fragments. We find pulse-labelled DNA is preferentially associated with the cages. For autoradiography we spread the DNA – again without breaking it – over an area many times greater than that of the nucleus (the radius is $\sim$ 30 μm): the pulse-label is again cage-associated.

MATERIALS AND METHODS

Chemicals

Radiochemicals were obtained from the Radiochemical Centre, Amersham, the resistriction endonuclease EcoRI (10^5 units/ml) from Boehringer, cytosine arabinoside (1-β-*O*-arabinofuranosylcytosine) from Koch-Light Ltd, and hydroxyurea from Calbiochem Ltd.

Cells

HeLa cells were grown in suspension (Cook & Brazell, 1975) and synchronized by the nitrous oxide technique of Rao (1968) as described by Warren & Cook (1978).

Preparation and manipulation of nucleoids

HeLa nucleoids were isolated in bulk using 'step' gradients containing 1·95 M NaCl (Cook *et al.* 1976). Nucleoids were also obtained from small numbers of cells by modifying the basic procedure as follows. Cells in 50 μl phosphate-buffered saline were added to 150 μl of a lysis mixture (1·95 M) described by Cook *et al.* (1976). After 15 min the lysed cells were layered on a 'step' gradient and spun for 4 min in the Beckman Microfuge B. 'Step' gradients contained 0·2 ml of 30 % sucrose under 0·6 ml of 15 % sucrose. Both layers also contained 1·95 M NaCl, 1 mM EDTA and 10 mM Tris (pH 8·0). The nucleoids, which sedimented to the interface between the 2 layers of sucrose, were removed through a needle inserted through the side of the tube. Techniques for staining, counting and manipulating nucleoids have been described (Cook *et al.* 1976).

Spectrofluorometry

Procedures for monitoring the superhelical status of DNA by measuring the amount of ethidium bound to nucleoids by fluorometry have been described (Cook & Brazell, 1978).

Labelling cells

Cells ($0{\cdot}3 \times 10^6$/ml) were grown in suspension for 24 h in [*Me*-^{14}C]thymidine (56 mCi/mmol at 0·05 μCi/ml. [The generation time is 22 h (Warren & Cook, 1978)] .[^{14}C]-labelled cells were pelleted by centrifugation, resuspended in MEM medium (Gibco-Biocult) at 6×10^6/ml and incubated in suspension at 37 °C. In some experiments inhibitors were added to this stage; 10 min later, cells were pulse-labelled with [*Me*-^{3}H]thymidine (48 Ci/mmol) at 100 μCi/ml for various periods. The labelling regime for cells labelled with [^{3}H]thymidine for 24 h was slightly different: cells ($0{\cdot}3 \times 10^6$/ml) were labelled simultaneously with [^{14}C]thymidine (0·05 μCi/ml) and [^{3}H]thymidine (0·05 μCi/ml). At the end of the [^{3}H]-pulse, 0·5 ml cells were added to 10 ml ice-cold phosphate-buffered saline containing 2·5 mM thymidine, the cells pelleted, and washed twice in the thymidine-supplemented saline. The final pellet of doubly labelled cells was resuspended in 50 μl phosphate-buffered saline and nucleoids were then prepared as described above. Incorporation of [^{3}H]thymidine under these conditions was linear up to 60 min; subsequently the medium turned acid. Pulse-chase experiments were conducted in one of 2 ways. In both cases cells were labelled with [^{14}C] and [^{3}H] as described and then 0·5 ml cells were either diluted with 20 ml of warm medium and grown or washed 3 times with warm medium and then grown. After the chase, cells were washed with saline as described above. The 2 methods of removing the label (i.e. by dilution or washing) gave essentially similar results.

Using the conditions described, sufficient label was incorporated to be counted accurately. For example, filters bearing undigested nucleoids derived from cells labelled for 24 h with [^{14}C]thymidine and 2·5 min with [^{3}H]thymidine contained about 2000 and 5000 dpm of [^{14}C] and [^{3}H] respectively.

Digestion with EcoRI

Nucleoids ($1{-}2 \times 10^6$/ml) were digested with EcoRI in 0·2 M NaCl, 2 mM $MgCl_2$ and 10 mM Tris (pH 8·0) for 10 min at 37 °C. Different amounts of EcoRI were used, ranging from 1 to 10^3 units/ml. Digestions were stopped by adding 100 mM EDTA to give a final concentration of 20 mM.

Filtration

DNA remaining attached to nucleoid cages after digestion with EcoRI was removed from any free DNA by filtration through glass-fibre disks. Generally, 30-μl samples were applied to 2·5 cm GF/C glass-fibre disks (Whatman) and then 30 ml 2 M NaCl, 0·1 % tetrasodium pyrophosphate, 1 mM Tris (pH 8·0) were sucked through the disks at a flow rate of 0·5 ml/s. Any radioactivity associated with the cage was then measured using a Packard TriCarb liquid scintillation spectrometer after immersing the disk in 3 ml Unisolve I (Koch-Light Ltd).

The following control experiments showed that DNA associated with nucleoid cages remained attached to the disk after filtration whereas free DNA was removed. The amounts of label remaining associated with filtered cages derived from cells grown in [^{3}H]thymidine and digested with increasing amounts of deoxyribonuclease or EcoRI correlated with the amounts of DNA detected by fluorescence microscopy. Nucleoids were also prepared from cells grown in [^{3}H]thymidine or [^{3}H]leucine for 24 h and applied to the disks; after filtration > 90 % of the applied label remained associated with the disks. When a mixture of unlabelled nucleoids and pure HeLa [^{3}H]-DNA (derived from cells labelled for 10 min or 24 h) was applied to disks, > 90 % of the label was lost on filtration. This was true whether or not the DNA had been cut with EcoRI. When mixtures of unlabelled nucleoids and pure heat-denatured HeLa [^{3}H]-DNA were applied to disks, filtration removed 70 % of the label. (DNA of very high molecular weight released during digestion of nucleoids might be expected to be removed from the disks less efficiently than the pure DNA prepared by conventional procedures used in these controls. Unfortunately, as we cannot isolate pure DNA of equivalent molecular weight, we cannot determine this. However, an underestimate of the degree of digestion does not affect the principal conclusion of this study.)

Fluorescence microscopy

Methods for staining nucleoids with ethidium and photographing them using the fluorescence microscope have been described (Cook *et al.* 1976).

Electron microscopy

Nucleoids were spread using Kleinschmidt's procedure as already described (McCready *et al.* 1979) and picked up onto 200-mesh nickel grids. For each spreading some grids were stained and some not; all were rotary shadowed with gold and it was found to be necessary to add a layer of carbon before exposure to emulsion to avoid chemography. Grids were coated with Ilford L4 nuclear research emulsion using the loop method of Caro & van Tubergen (1962), except that 10 g of emulsion were diluted with 15 ml 1% glycerol in distilled water. After exposure autoradiographs were developed using Kodak D19 developer and fixed with 20% sodium thiosulphate. Silver grains were counted in a 30° sector extending from the centre of the cage region to 30 μm from the cage edge (the average extent of the DNA skirt) for each of 12 to 15 nucleoids for each set of labelling conditions. Exposure times (2–21 days) were chosen so that between 100 and 200 grains were counted for each nucleoid. The percentage of grains lying over the cage and in 2·5-μm sections of the sector were plotted.

RESULTS

Experimental design of biochemical experiments

For the biochemical experiments, cells were grown for more than one generation (i.e. 24 h) in [^{14}C]thymidine to label their DNA uniformly and then for a much shorter period in [^{3}H]thymidine. After nucleoids had been isolated, they were digested with different amounts of the restriction endonuclease, EcoRI, and the nucleoid cages with associated DNA filtered free of any unattached fragments. Finally the amounts of cage-associated radioactivity on the filters were determined and expressed as a percentage of radioactivity in undigested controls. We need make no assumptions as to how DNA is attached to the cage: the association is defined operationally in the sense that more or less DNA is collected on the filter.

The release of DNA from nucleoid cages can be monitored by fluorescence microscopy after staining with ethidium or acridine orange. Treatment with EcoRI removes DNA from the nucleoplasmic region, leaving the RNA of the nucleolus and the perinuclear rim (Figs. 1, 2). Digested nucleoids which have been spread using the Kleinschmidt procedure also lack a surrounding skirt of DNA (Figs. 3, 4). Instead, an empty cage remains, to which only a few strands of DNA remain attached.

We first showed that all loops could be cut by EcoRI using a sensitive fluorometric assay (Table 1). [Each loop is, on average, so large (i.e. 2×10^5 base-pairs) that it would be expected to contain many ($\sim$ 50) potential cutting sites.] In 8 μg/ml ethidium, unirradiated nucleoids containing intact superhelical DNA bind less of the intercalating dye, ethidium, than γ-irradiated nucleoids with relaxed loops (Cook & Brazell, 1978). Pretreatment of unirradiated nucleoids with EcoRI increased the amount bound to the level found with irradiated nucleoids, indicating that all supercoiling had been removed (i.e. that all loops had been cut at least once).

If replication takes place at sites scattered at random throughout the DNA, then the [^{14}C]- and [^{3}H]-labels will be removed equally from the cages as the DNA in the

loops is cut with the endonuclease. For the cage-associated DNA, the normalized ratio of pulse label to bulk label [i.e. (% [^{3}H]) ÷ (% [^{14}C])] will remain at unity independently both of pulse-length and degree of digestion. On the other hand, if DNA is replicated at sites close to the cage, then after digestion relatively more pulse label will remain associated with the cage. The ratio will be greater than unity and will critically depend upon the length of the pulse and the degree of digestion. It will only approach unity when the length of the pulse enables all the DNA in the loops to become labelled with [^{3}H]. (The average loop contains $\sim 2 \times 10^5$ base-pairs (Cook & Brazell, 1978) or 75 μm of DNA and would be replicated in 75 min if the net rate of

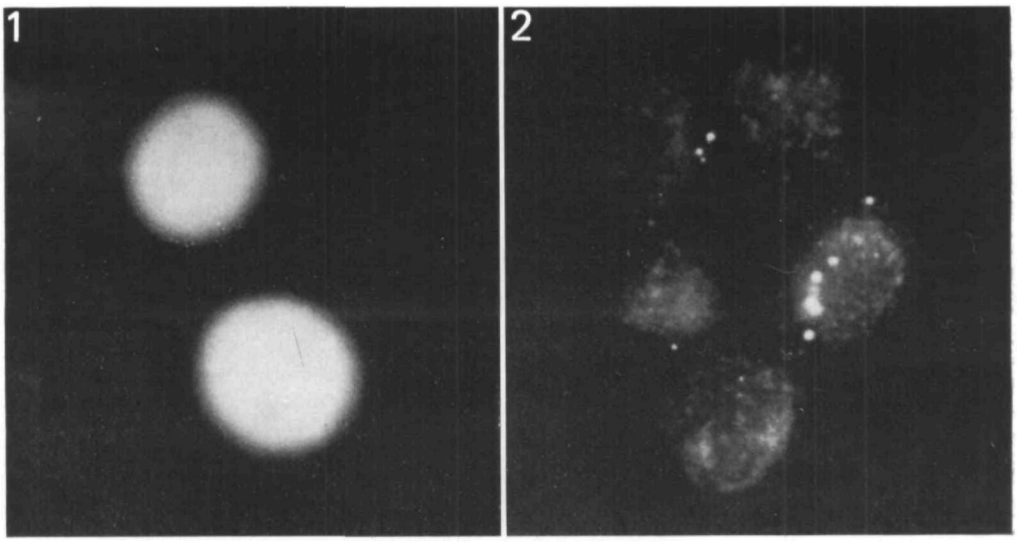


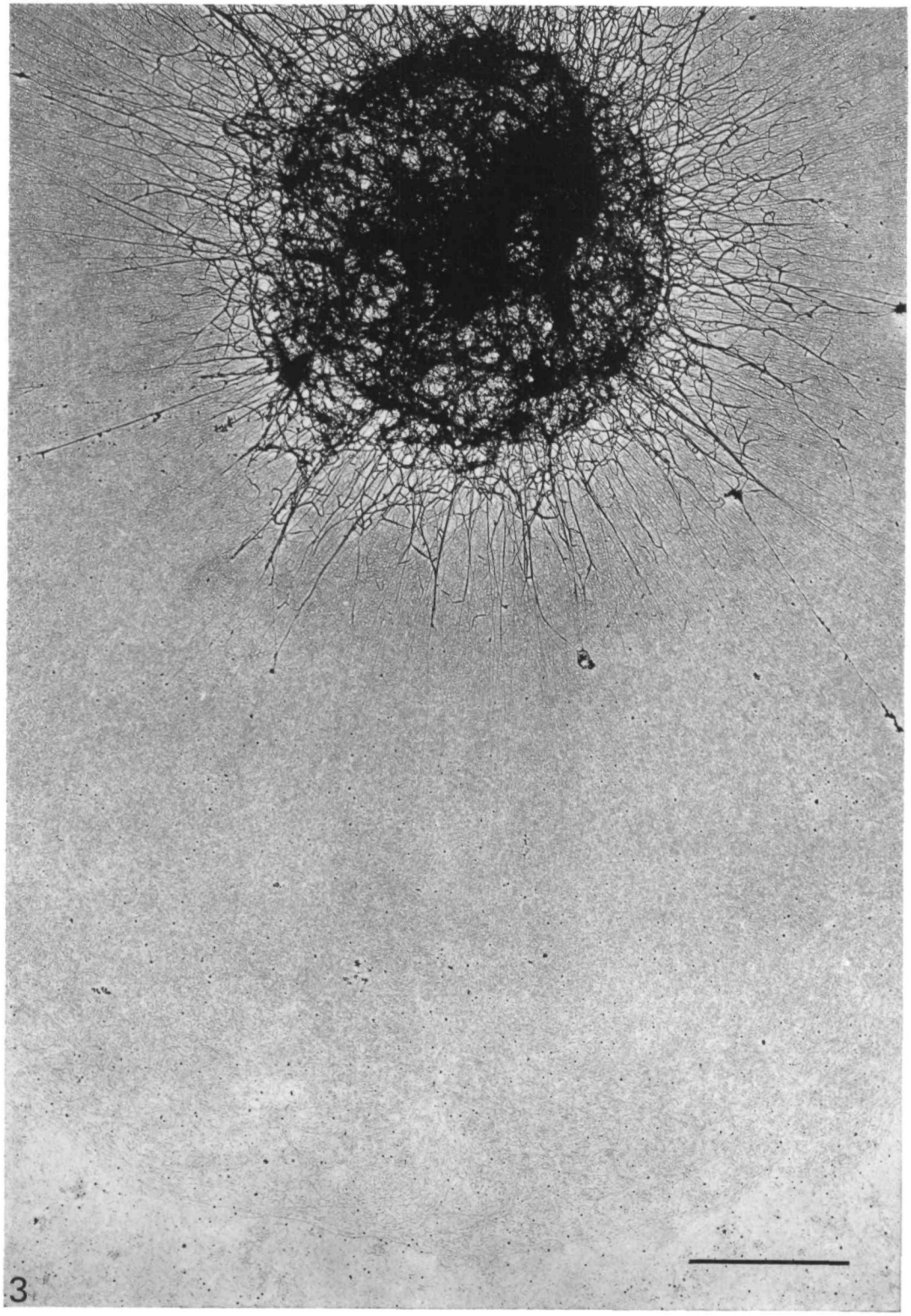
Fig. 1. Fluorescence micrograph of undigested nucleoids stained with ethidium. × 2050.

Fig. 2. Fluorescence micrograph of nucleoids digested with EcoRI and stained with ethidium; 10 % of the DNA remains associated with the nucleoid cage (measured by filtration). × 2050.

Table 1. *The ethidium-binding capacity of nucleoids digested with EcoRI*

Template	Ethidium bound, %
Unirradiated nucleoids	100
Irradiated nucleoids	133
Unirradiated nucleoids + EcoRI	132
Irradiated nucleoids + EcoRI	132

Nucleoids, isolated in 1·95 M NaCl, were adjusted to $0{\cdot}4 \times 10^6$ nucleoids/ml in 0·2 M NaCl, 10 mM $MgSO_4$, 10 mM Tris (pH 8·0). Some nucleoids were γ-irradiated (9·6 J kg^{-1}). Nucleoids were then incubated with and without EcoRI (10 units/ml) for 10 min at 37 °C, the reaction was stopped by the addition of 1/10 volume of EDTA and ethidium to give final concentrations of 50 mM and 8 μg/ml respectively. The amount of ethidium bound to nucleoids was determined by fluorometry and expressed as a percentage of that bound to undigested unirradiated nucleoids.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replication is 1·0 μm/min (Edenberg & Huberman, 1975; Sheinin & Humbert, 1978; Pardee *et al.* 1978).)

Newly synthesized DNA resists detachment from nucleoid cages

Fig. 5 shows that the ratio is sensitive to both pulse-length and degree of digestion. For example, after a very short [^{3}H]-pulse of 2·5 min all but 20% of the [^{14}C] is removed, whereas 50% of the [^{3}H] remains: therefore, the ratio is 50% ÷ 20% or 2·5. As the pulse-time increases the maximum ratio decreases and is reached when less [^{14}C] has been removed. When pulsed for 24 h both labels become equally digested and the ratio is unity at all degrees of digestion (Fig. 5F).

The preferential association of pulse label with cages is not due to a labelling artifact since newly synthesized DNA can be chased away from the cage to give a ratio approaching unity at all degrees of digestion, quite unlike the pattern obtained with a

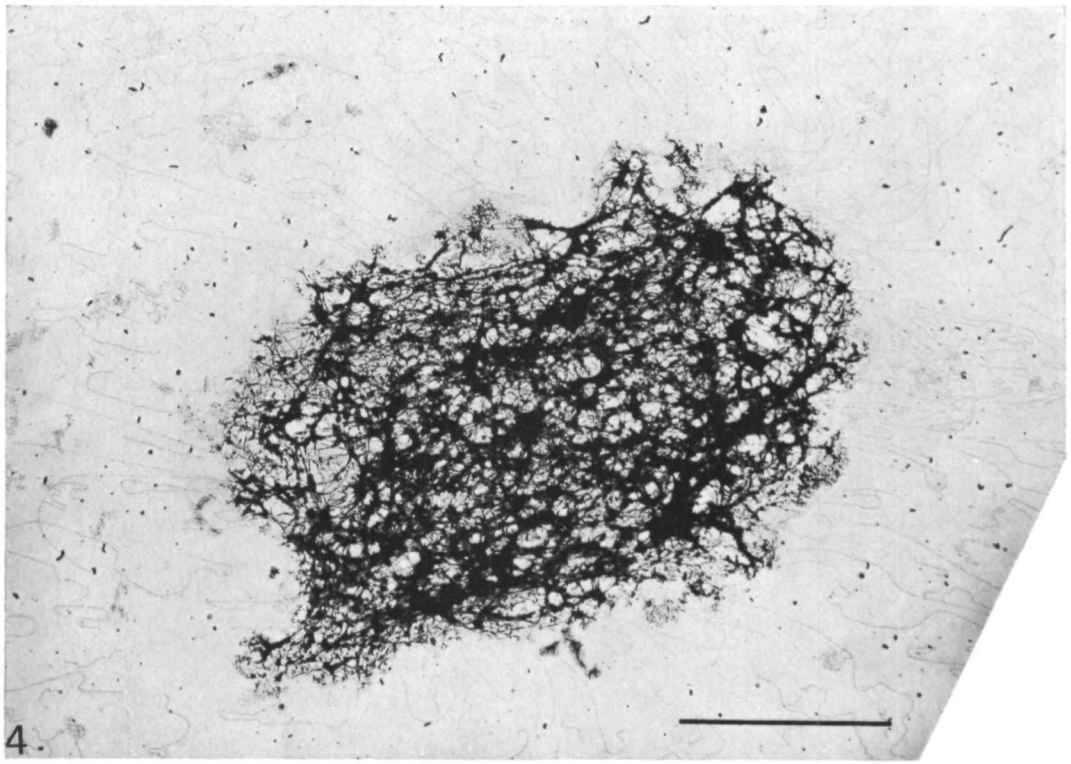


Fig. 4. Electron micrograph of a nucleoid spread after extensive digestion with EcoRI restriction endonuclease. Cut DNA fibres remain attached to the cage. The bar represents 5 μm.

Fig. 3. Electron micrograph of an intact nucleoid, stained and shadowed. The central 'cage', from which the DNA has spilled out at an air-aqueous interface, contains a protein network which includes elements of the cytoskeleton and residual nuclear proteins. The denser fibrils radiating from the cage disappear on nuclease digestion and are probably aggregates of DNA fibres. The bar represents 5 μm.

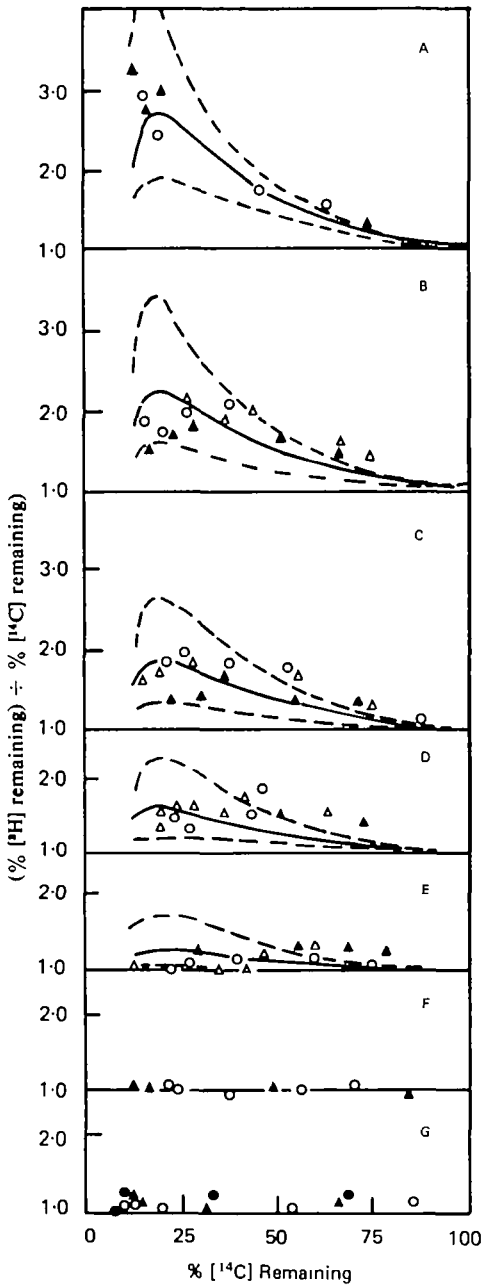


Fig. 5. The effect of digestion with EcoRI on the DNA remaining associated with nucleoid cages. Cells were labelled with [^{14}C]thymidine for 24 h and then for different periods with [^{3}H]thymidine A–E, 2·5, 5, 10, 20 and 60 min, respectively; F, 24 h; G, 5-min pulse + 55-min chase. Nucleoids were isolated, digested with varying amounts of EcoRI and the DNA remaining associated with nucleoid cages filtered free of detached DNA. The radioactivity remaining associated with cages was measured and expressed as a percentage of that associated with undigested nucleoids. In any graph identical symbols refer to the same experiment. The lines were generated by computer (see Discussion and Appendix).

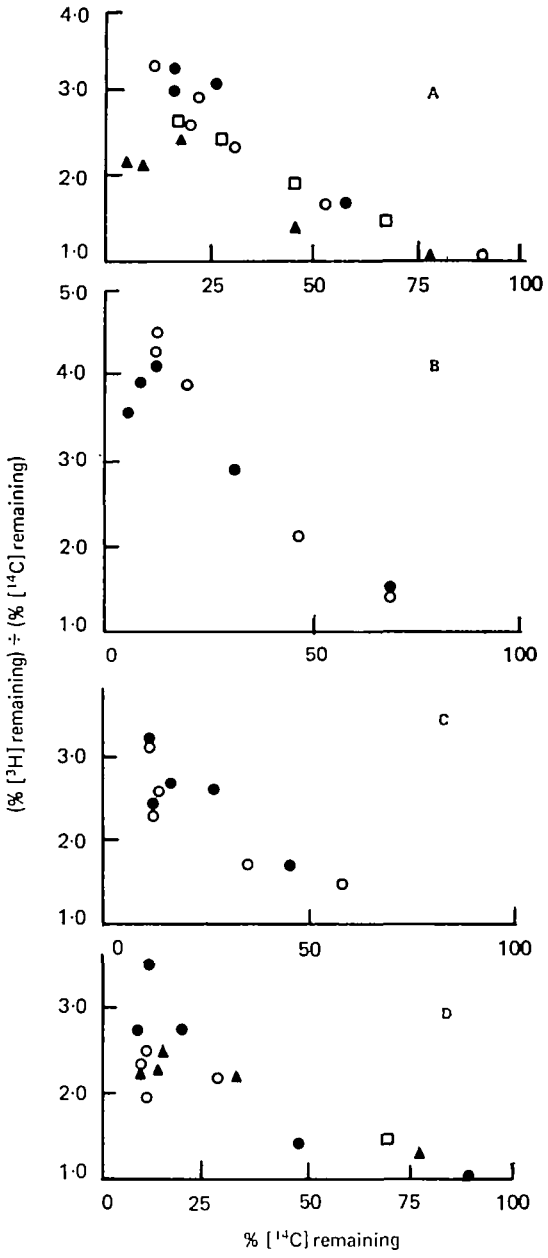


Fig. 6. The effects of slowing the rate of synthesis on the DNA remaining associated with cages after digestion with EcoRI. Cells were labelled with [^{14}C]thymidine for 24 h and then with [^{3}H]thymidine for: A, 90 min in the presence of 10^{-5} M cytosine arabinoside; B, 20 min in 10^{-5} M cytosine arabinoside; C, 60 min in 10^{-3} M hydroxyurea; D, 60 min at 24° C. Nucleoids were isolated, digested, and the radio-activity remaining associated with cages measured and expressed as a percentage of that associated with undigested nucleoids. Identical symbols refer to the same experiment. 10^{-5} M cytosine arabinoside and 10^{-3} M hydroxyurea reduce the rate of incorporation of [^{3}H]thymidine into DNA by 16-fold and 18-fold respectively.

pulse alone (Fig. 5). Furthermore it cannot simply reflect the geometry of the cell and the effects of diffusion – the DNA at the outside of the nucleus being labelled first. Cytosine arabinoside – an inhibitor of DNA synthesis (Cozzarelli, 1977) – reduces the amount of DNA made in 90 min to the level found after 7 min in the absence of the drug. What little DNA is made resists digestion – the maximum ratio is > 3 – and so must be closely associated with the cage, even though the label has had plenty of time during the 90 min to diffuse to the centre of the nucleus (Fig. 6).

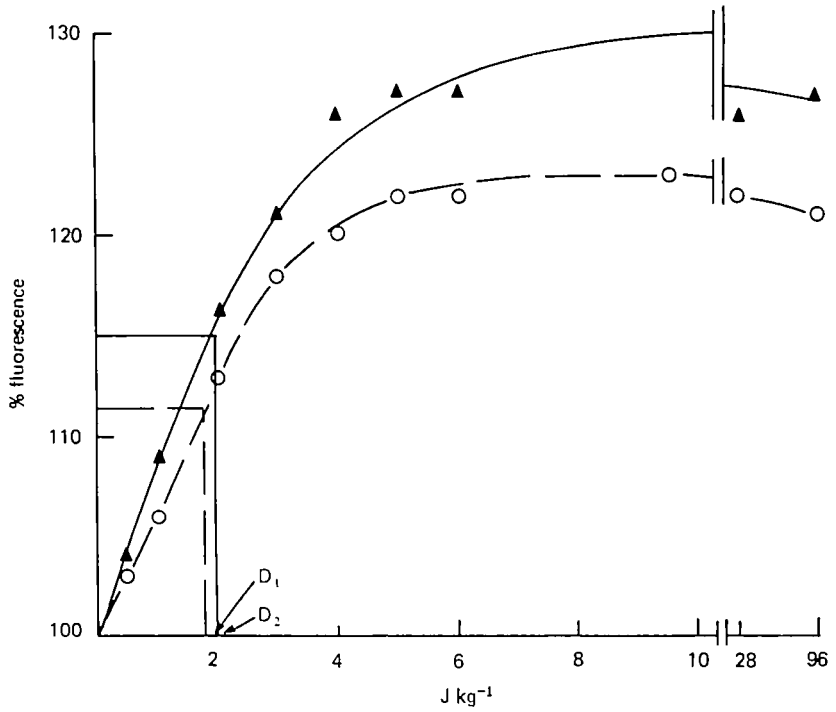


Fig. 7. Loop-size of nucleoids derived from G_1- and S-phase HeLa cells. Nucleoids derived from G_1- and S-phase HeLa cells were isolated in 1·95 M NaCl, diluted to 0·2 × 10^6/ml using 2 M NaCl and 10 mM Tris (pH 8·0). Samples were irradiated with different doses of γ-rays and ethidium was added to a final concentration of 8 μg/ml. The amount of bound ethidium was determined by fluorometry and expressed as a percentage of the amount bound by unirradiated nucleoids. The doses (arrows D_1, D_2) giving half the maximum increase were 1·8 and 1·9 J kg^{-1} for G_1- (○--○) and S-phase (▲--▲) nucleoids respectively.

When very small amounts of DNA are pulse-labelled (e.g. during a short pulse of 20 min in the presence of cytosine arabinoside) the 2 labels become digested away from the cages at strikingly different rates. Up to 70% of the [^{14}C] can be removed without detachment of *any* [^{3}H] (i.e. the ratio is > 3 when 30% of the [^{14}C] remains). This must reflect a reduced synthetic rate rather than some other unknown side-effect, since another inhibitor, hydroxyurea (Cozzarelli, 1977), or a reduced temperature have similar effects (Fig. 6).

The size of loops during S-phase

Nuclear DNA might be looped by tying the linear duplex to the nucleoid cage. If these ties are maintained whilst DNA is replicated, then the additional ties at replication forks should lead to a reduction in *average* loop size during *S*-phase. Using a rather insensitive sedimentation technique we could not detect such a reduction as cells progressed around the cell cycle (Warren & Cook, 1978), nor can we do so using a more sensitive fluorometric technique (Fig. 7). Large loops of DNA are nicked and

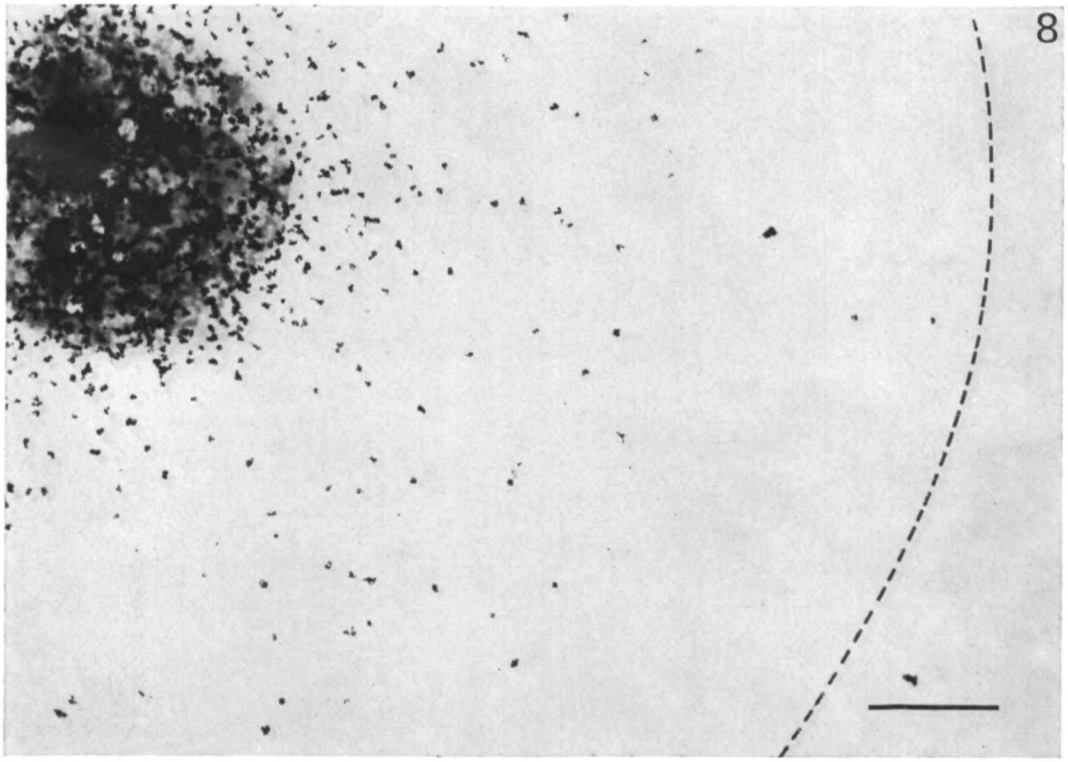


Fig. 8. Autoradiograph of a nucleoid labelled for 24 h with [^{3}H]thymidine (i.e. randomly labelled). In order that silver grains can be easily seen and counted, DNA is shadowed but not stained and so can be seen only at higher magnifications than represented here. The bar represents 5 μm, and the dotted line the edge of the skirt.

so lose supercoiling more easily than do small loops. Therefore loop size can be estimated from curves relating the amount of ethidium bound by nucleoids to dose of γ-rays (Cook & Brazell, 1978). Similar doses release supercoiling from nucleoids isolated from G_1- and *S*-phase cells. This result is perhaps not surprising for we calculate that only about 1 in 10 of the loops in a nucleus is being replicated at any time, and our techniques are too insensitive to detect changes in such a small proportion.

Autoradiography

Autoradiography confirms the association of newly synthesized DNA with the nucleoid cage (Figs. 8–10). Nucleoids were labelled for different periods, spread using Kleinschmidt's procedure and autoradiographs prepared. The radial distribution of label was then found by counting silver grains in 2·5-μm sections of a 30° sector extending from the centre of the cage (Figs. 11, 12). After 24-h labelling, the radial distribution reflects the radial concentration of the uniformly labelled DNA. 45% of

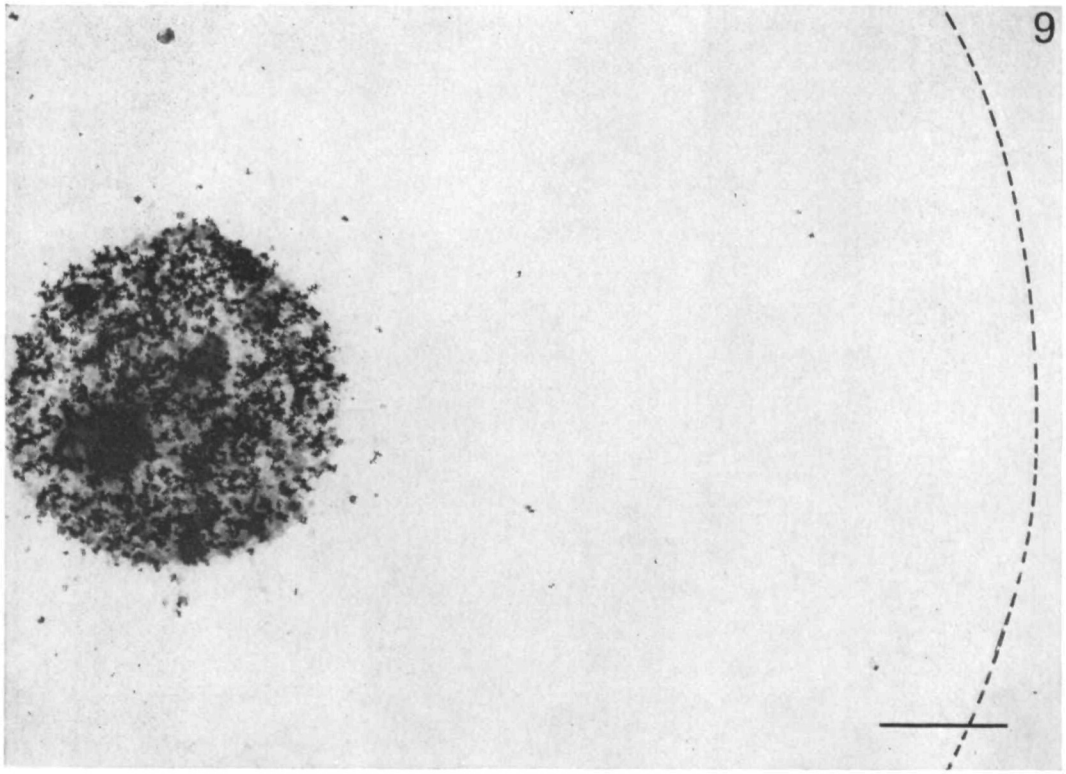


Fig. 9. Autoradiograph of a nucleoid spread after a 5-min pulse label. Label is concentrated at the cage. The bar represents 5 μm, and the dotted line the edge of the skirt.

the grains in the sector lie over the cage; the remainder are almost entirely within 15 μm from the cage edge, with < 2% between 15 μm and the edge of the skirt (~ 30 μm). After a 5-min pulse, 90% of the grains lie over the cage. As the pulse time increases this proportion progressively falls and the grains spread out over the skirt. Few grains are found beyond 5 μm after a 5-min pulse or beyond 10 μm after a 10-min pulse, consistent with a rate of elongation of 1 μm/min. Again 2 controls rule out trivial explanations of these results. Label, which after a 5-min pulse is over the cage, can be chased out into the skirt (Fig. 12B). In contrast, when replication during a 60-min pulse is slowed by cytosine arabinoside, 90% of the label remains cage-associated (Fig. 12A).

We can derive the time taken to replicate a loop from the pulse time needed to approach the radial distribution of randomly labelled DNA. The proportion of label over the cage declines as the pulse time increases. The actual rate of decline is illustrated in Fig. 13, together with curves derived using a simple model and various theoretical loop replication times. A replication time of about 10 min best fits the actual rate of decline. Assuming an elongation rate of 1 μm/min and bidirectional replication, this gives an average replicon size of about 20 μm.

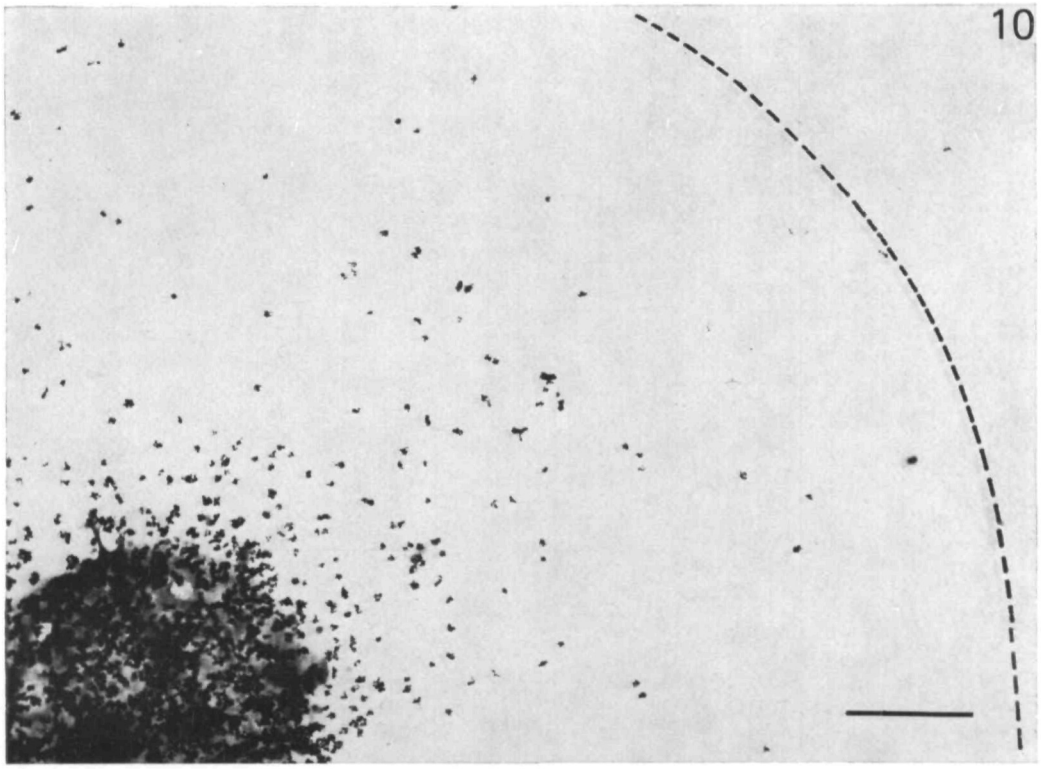
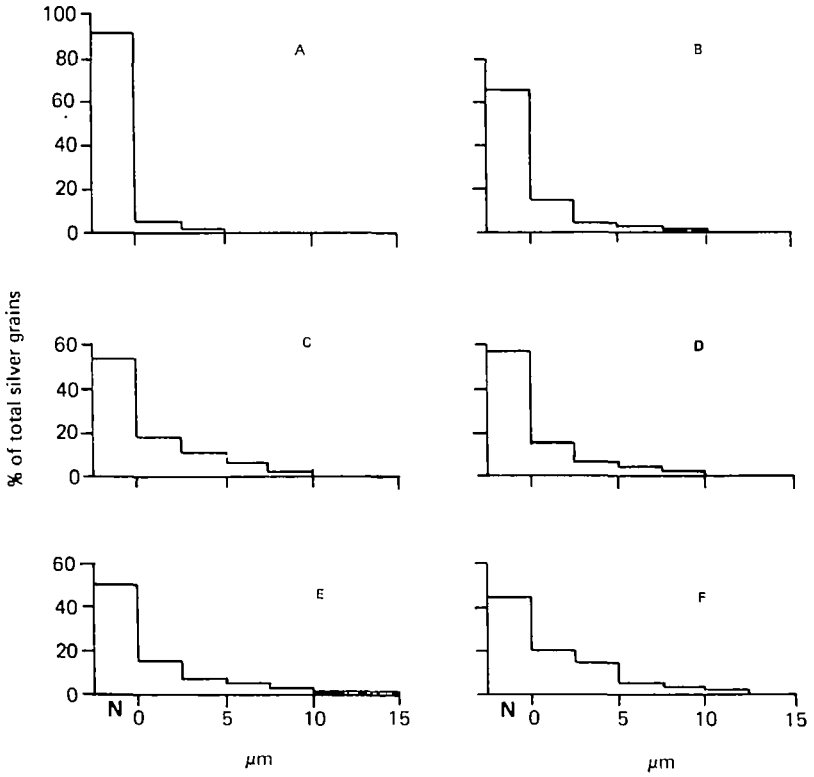


Fig. 10. Autoradiograph of nucleoid spread following a 5-min pulse of [^{3}H]thymidine and a 55-min cold chase. Label extends away from the cage. The bar represents 5 μm, and the dotted line the edge of the skirt.

DISCUSSION

Our two approaches, biochemical and using autoradiography, are in general agreement and clearly indicate that newly synthesized DNA is closely associated with the nucleoid cage. However, the site of replication has been the subject of controversy for so long that we have sought alternative explanations for our results. Two kinds of control experiments rule out a number of trivial explanations (e.g. labelling artifacts and the effects of diffusion and pool size). The pulse label, initially associated with the cage, can be chased away from the cage. In addition, when DNA synthesis is reduced by the addition of inhibitors (i.e. cytosine arabinoside or hydroxyurea) or by lowering the temperature, what little DNA is made during the extended pulse remains cage-

associated. We have also repeated the majority of the biochemical experiments using deoxyribonuclease instead of EcoRI with essentially similar results. Nevertheless, there remains one trivial explanation that can explain all our results: perhaps replication forks, which are randomly scattered throughout the nucleus *in vivo*, stick to the cage when nucleoids are prepared. Since these forks would have to be much stickier than pure single or double-stranded DNA, which have little affinity for nucleoids (see Materials and methods), and since nearly all the forks would have to stick and remain stuck in 2 M NaCl during filtration or spreading for electron microscopy, we believe this explanation to be extremely unlikely.



Figs. 11, 12. The radial distribution of silver grains over pulse-labelled nucleoids. Histograms showing distribution of silver grains after various pulse times over the nucleoid cage (N) and in 2·5-μm sections of a 30° sector extending from the centre of the cage. o marks the edge of the cage. In Fig. 11, A, B, C, D, E, F represent 5, 10, 20, 30 and 60 min, and 24 h respectively. In Fig. 12, A = Ara C, 60 min; B = 5-min pulse +55-min chase.

We have compared our biochemical results with computer-generated predictions based on a number of models that involve a constant rate of replication within a loop, random initiation and termination amongst loops and a random cutting and consequent detachment of DNA (see Appendix, p. 384). Only models involving replication at, or close to, the cage are consistent with our results. The basic model described in the Appendix leads to predictions that fit the data moderately well. However, in this

model we would expect digestion to increase the ratio to a maximum, but we observe that extreme digestion reduces the ratio from the maximum (Figs. 5, 6). Any one of 3 modifications leads to a better fit, but only one – which takes into account the inefficient filtration – seems plausible. Up to 10% (on average 7%) of free DNA is not removed by filtration (see Materials and methods) and this extra detached DNA –

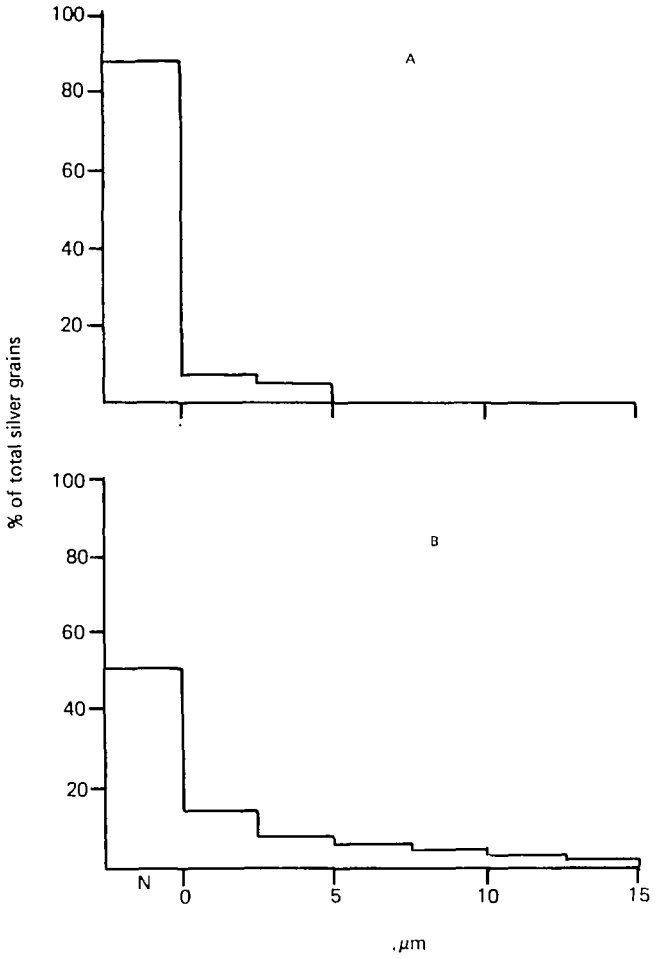
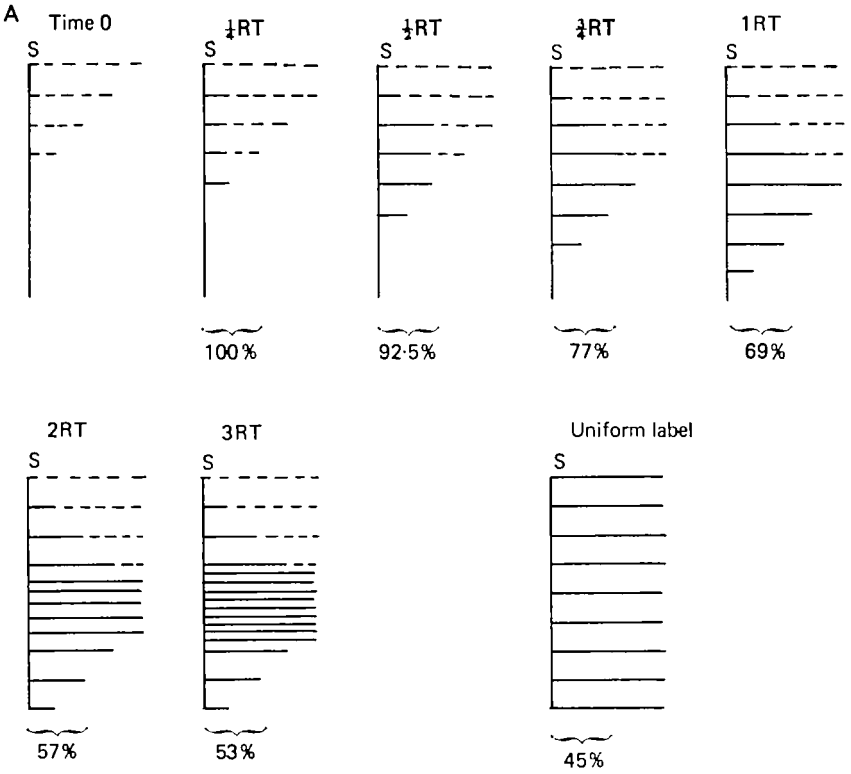


Fig. 12. For legend see facing page.

being mainly [^{14}C]-labelled – reduces the ratio of the DNA on the filter. Curves of best fit were obtained using this modification of the model and are presented in Fig. 5. These curves require the time taken to replicate the loop to be ~ 11 min when 7% of detached DNA is retained on the filter.

Autoradiography is consistent with a rate of elongation of ~ 1 μm/min and suggests a loop replication time of ~ 10 min (Fig. 13), in excellent agreement with the biochemical results. Assuming bi-directional replication, this gives an average replicon



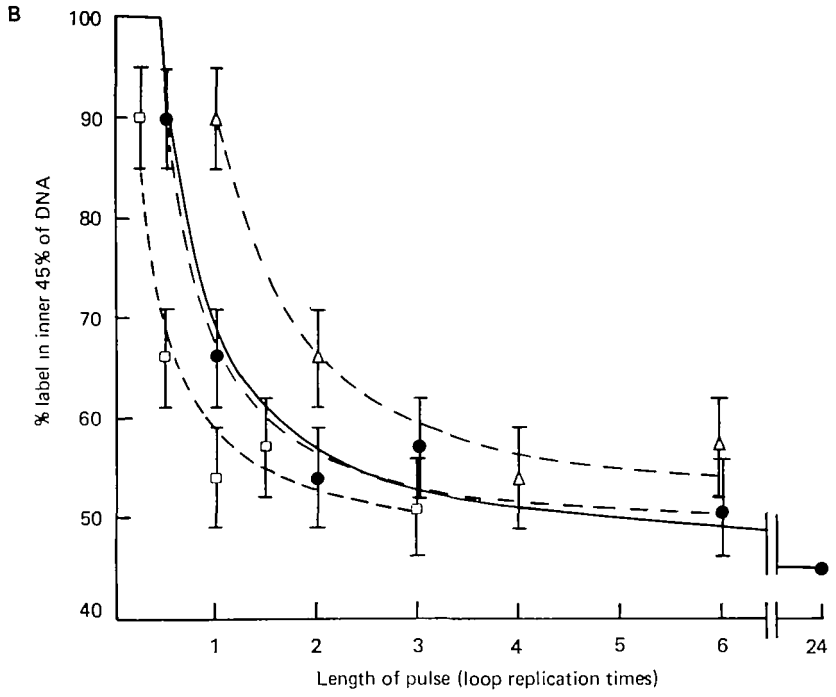


Fig. 13A and B. For legend see facing page.

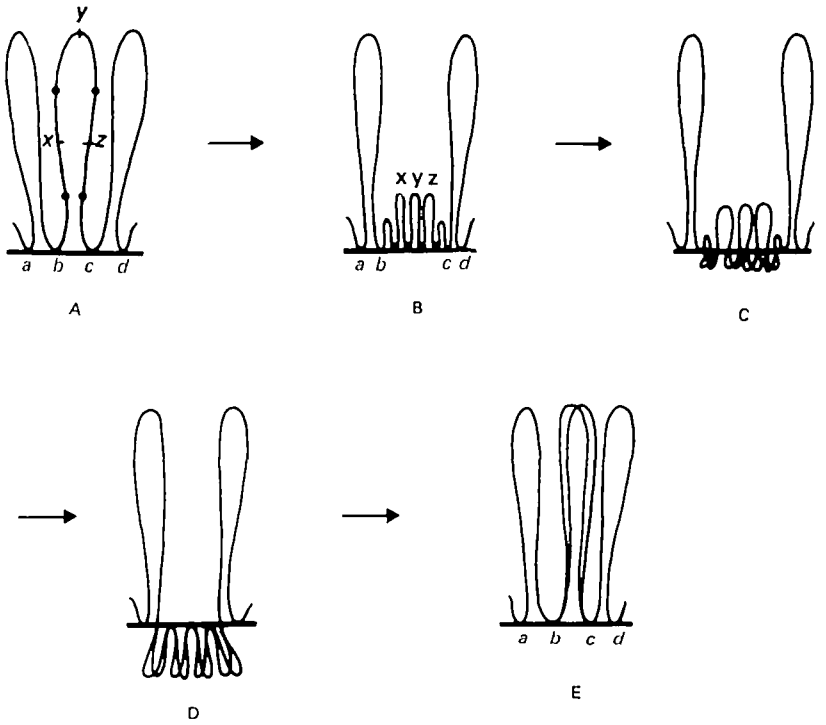


Fig. 14. A model of the replication of nuclear DNA. (A) Three adjacent structural loops (each approx. 75 μm) are attached to the nuclear cage by specific base sequences (*a*, *b*, *c*, *d*). (B) Prior to initiation, there is synchronous attachment of (in this case) 4 sequences (-●-) to form 4 replicons (*bx*, *xy*, *yz*, and *zc*) each of about 20 μm. (C, D) Bi-directional replication from each of the initiation points takes place as replicon loops move through the replication complex at the nuclear cage. (E) When DNA synthesis is complete, the DNA detaches to form 2 newly replicated structural loops.

Fig. 13. The time taken to replicate a loop. (A) The upper diagram shows the theoretical distribution of label amongst nucleoid DNA assuming a fixed site of DNA synthesis (S) and a constant rate of initiation. At time o (some time in *S*-phase) some chromosomal loops will have completed DNA synthesis and will remain unlabelled (-----) whilst others will be at various stages of completion or not have begun replication. After pulses equivalent to increasing proportions of loop replication time (RT) label (——) will extend away from the site of synthesis as illustrated until, after a complete cell cycle, DNA becomes uniformly labelled. Only half the replication fork is illustrated.

(B) The observed proportion of silver grains found over the cage in uniformly labelled nucleoids (24-h label) was 45 %. Using the model above, the theoretical rate of decrease in proportion of label in this portion of the DNA can be calculated relative to the number of loop replication times and an ideal curve plotted (solid line). This can then be compared to the actual results obtained, substituting various possible replication times (5 min, △--△; 10 min, ●--●; and 20 min, □--□ are plotted above) to determine what actual replication time best fits the theoretical curve. Error bars represent 10 % error in grain counts.

size of ~ 20 μm – again this agrees with published results (Edenberg & Huberman, 1975; Hand, 1978). How is the unit of replication – the replicon – related to the unit of structure, the loop? We estimate the average loop to be ~ 75 μm or $2{\cdot}2 \times 10^5$ base-pairs (Cook & Brazell, 1975, 1978) and this basic structure remains throughout the cell cycle (Warren & Cook, 1978) (Fig. 14). [Others estimate smaller loop sizes (Benyajati & Worcel, 1976; Igó-Kemenes & Zachau, 1977; Paulson & Laemmli, 1977). However, these estimates are for a ***minimum*** loop size. In addition we feel that our fluorometric estimate, determined by comparing the effects of radiation on nucleoid loops (as in Fig. 7) and a circular DNA of known length, is the most accurate.] When replication is initiated, sequences remote from the cage in one structural unit (~ 75 μm) must become attached to the cage to form about 4 smaller loops or replicons (~ 20 μm). Results from autoradiography of DNA-fibres, differential staining of late-replicating regions and inhibition of synthesis by very low doses of radiation, all point to *synchronous* initiation in adjacent replicons (Edenberg & Huberman, 1975; Jasny & Tamm, 1979; Willard & Latt, 1976; Painter & Young, 1975, 1976; Povirk, 1977), suggesting that different sites in one structural loop attach together. Since replication is bi-directional, each attachment and replication complex must involve 2 replication forks.

Our results do not answer the questions: what is the nature of the association of DNA with the cage and what is the relationship of the cage to the nuclear membrane? Nucleoid cages contain proteins with electrophoretic mobilities in SDS gels similar to those from structures variously called the nuclear 'matrix', 'envelope' or 'pore complex' (Berezney & Coffey, 1974; Riley, Keller & Byers, 1975; Aaronson & Blobell, 1975; Comings & Okada, 1976; Cook *et al.* 1976; Adolph, Cheng & Laemmli, 1977; Levin, Jost & Cook, 1978). (In addition they contain cytoskeletal elements (i.e. actin and intermediate filaments) which have condensed on to the nuclear cage (Levin, 1978).) We – like many others – are attracted by the notion that specific sequences are tied to specific sites on the nuclear cage (Cook, 1973, 1974; Cook & Brazell, 1975). Recently we have been able to map the position of globin genes relative to the attachment site, showing that structural loops are indeed attached by *specific* sequences (Cook & Brazell, 1980). However, there is as yet no direct evidence as to the precise nature of the attachment. Labelling in autoradiographs of cell sections prepared after very short pulses of [^{3}H]thymidine is often over the centre of the nucleus, implying that replication is *not* associated with the nuclear membrane (Edenberg & Huberman, 1975; Pardoll *et al.* 1980). These results can be reconciled with ours if elements of the nucleoid cage run through the centre of the nucleus forming a skeleton to which the DNA is attached and at which the DNA is replicated. If so, the structure of the nucleus is much more complex than has hitherto been imagined.

Perhaps the most surprising result of these experiments is not that replication takes place at the nuclear cage but that the mechanism that holds the replicating fork is stable in the detergent and 2 M salt used during isolation of the nucleoids. Therefore nucleoids, with their intact superhelical DNA held at the site of replication, should prove useful in studies on replication *in vitro*.

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REFERENCES

AARONSON, R. P. & BLOBELL, G. (1975). Isolation of nuclear pore complexes in association with a lamina. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1007–1011.

ADOLPH, K. W., CHENG, S. M. & LAEMMLI, U. K. (1977). Role of nonhistone proteins in metaphase chromosome structure. *Cell* **12**, 805–816.

BEREZNEY, R. & COFFEY, D. S. (1974). Identification of a nuclear protein matrix. *Biochem. biophys. Res. Commun.* **60**, 1410–1417.

BENYAJATI, C. & WORCEL, A. (1976). Isolation, characterization, and structure of the folded interphase genome of *Drosophila melanogaster*. *Cell* **9**, 393–407.

CARO, L. G. & VAN TUBERGEN, R. P. (1962). High resolution autoradiography. *J. Cell Biol.* **15**, 173–188.

COMINGS, D. E. & OKADA, T. A. (1976). Nuclear proteins. III. The fibrillar nature of the nuclear matrix. *Expl Cell Res.* **103**, 341–360.

COOK, P. R. (1973). Hypothesis on differentiation and the inheritance of gene superstructure. *Nature, Lond.* **245**, 23–25.

COOK, P. R. (1974). On the inheritance of differentiated traits. *Biol. Rev.* **49**, 51–84.

COOK, P. R. & BRAZELL, I. A. (1975). Supercoils in human DNA. *J. Cell Sci.* **19**, 261–279.

COOK, P. R. & BRAZELL, I. A. (1978). Spectrofluorometric measurement of the binding of ethidium to superhelical DNA from cell nuclei. *Eur. J. Biochem.* **84**, 465–477.

COOK, P. R. & BRAZELL, I. A. (1980) Mapping sequences in loops of nuclear DNA by their progressive detachment from the nuclear cage. *Nucleie Acids Res.* **8**, 2895–2906.

COOK, P. R., BRAZELL, I. A. & JOST, E. (1976). Characterization of nuclear structures containing superhelical DNA. *J. Cell Sci.* **22**, 303–324.

COZZARELLI, N. R. (1977). The mechanism of action of inhibitors of DNA synthesis. *A. Rev. Biochem.* **46**, 641–648.

EDENBERG, H. J. & HUBERMAN, J. A. (1975). Eukaryotic chromosome replication. *A. Rev. Genet.* **9**, 245–284.

HAND, R. (1978). Eukaryotic DNA: organization of the genome for replication. *Cell* **15**, 317–325.

IGÓ-KEMENES, T. & ZACHAU, H. G. (1977). Domains in chromatin structure. *Cold Spring Harb. Symp. quant. Biol.* **42**, 109–118.

JASNY, B. R. & TAMM, I. (1979). Temporal organization of replication in DNA fibres of mammalian cells. *J. Cell Biol.* **81**, 692–697.

LEVIN, J. M. (1978). D.Phil. Thesis, University of Oxford.

LEVIN, J. M., JOST, E. & COOK, P. R. (1978). The dissociation of nuclear proteins from superhelical DNA. *J. Cell Sci.* **29**, 103–116.

MCCREADY, S. J., AKRIGG, A. & COOK, P. R. (1979). Electron microscopy of intact nuclear DNA from human cells. *J. Cell Sci.* **39**, 53–62.

PAINTER, R. B. & YOUNG, B. R. (1975). X-ray induced inhibitions of DNA synthesis in Chinese hamster ovary, human HeLa and mouse L cells. *Radiat. Res.* **64**, 648–656.

PAINTER, R. B. & YOUNG, B. R. (1976). Formation of nascent DNA molecules during inhibition of replicon initiation in mammalian cells. *Biochim. biophys. Acta* **418**, 146–153.

PARDEE, A. B., DUBROW, R., HAMLIN, J. L. & KLETZEIN, R. F. (1978). Animal cell cycle. *A. Rev. Biochem.* **47**, 715–750.

PARDOLL, D. M., VOGELSTEIN, B. & COFFEY, D. S. (1980). A fixed site of DNA replication in eukaryotic cells. *Cell* **19**, 527–536.

PAULSON, J. R. & LAEMMLI, U. K. (1977). The structure of histone-depleted metaphase chromosomes. *Cell* **12**, 817–828.

POVIRK, L. F. (1977). Localization of inhibition of replicon initiation to damaged regions of DNA. *J. molec. Biol.* **114**, 141–151.

RAO, P. N. (1968). Mitotic synchrony in mammalian cells treated with nitrous oxide at high pressure. *Science, N.Y.* **160**, 774–776.

RILEY, D. E., KELLER, J. M. & BYERS, B. (1975). The isolation and characterization of nuclear ghosts from cultured HeLa cells. *Biochemistry, N.Y.* **14**, 3005–3013.

SHEININ, R. & HUMBERT, J. (1978). Some aspects of eukaryotic DNA replication. *A. Rev. Biochem.* **47**, 277–316.

WARREN, A. C. (1977). D.Phil. Thesis, University of Oxford.

WARREN, A. C. & COOK, P. R. (1978). Supercoiling of DNA and nuclear conformation during the cell cycle. *J. Cell Sci.* **30**, 211–226.

WILLARD, H. F. & LATT, S. A. (1976). Analysis of deoxyribonucleic acid replication in human X chromosomes by fluorescence microscopy. *Am. J. hum. Genet.* **28**, 213–227.

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Appendix

A mathematical model was set up to simulate the labelling and subsequent cutting of DNA. Good agreement was found between model predictions and experiment.

Consider a strand of DNA which has been labelled uniformly with [^{14}C]thymidine (Fig. A 1 A): it is attached to the cage at the left-hand end, whilst the right-hand end can be imagined, for simplicity, attached to the right-hand end of another similar strand to form a closed loop.

Now it is assumed that replication takes place at the cage so that [^{3}H]-DNA grows outward from the cage at a uniform rate (Fig. A 1 B). Only half the replication fork is shown. Any cut detaches DNA from the cage since, to a good approximation, we may assume that the strand to which the right-hand end is attached has also been cut (Fig. A 1 C). A cut at ① detaches [^{3}H], whereas a cut at ② does not.

In Fig. A 1 D, the length of [^{14}C]-DNA, which is half the loop length, is unity. l is the length of [^{3}H]-DNA; and x is the distance from the cage. It is assumed that cuts occur with equal probability over the range $0 < x < 1$. For one cut, the probability that the remaining piece has length at least x is

$$P_1 = 1 - x, \tag{A 1}$$

for n cuts

$$P_n = (1-x)^n, \tag{A 2}$$

whence the probability $-dp_n$ that the length of DNA remaining attached to the cage lies between x and $x+dx$ is given by

$$-dp_n = n(1-x)^{n-1}dx. \tag{A 3}$$

Let the activity due to [^{3}H] be $(Cx)/l$ for $x \leqslant l$ and C for $x > l$; it follows that the mean activity is

$$\begin{aligned} \bar{C} &= \int_0^l \frac{Cxn}{l}(1-x)^{n-1}dx + \int_l^1 Cn(1-x)^{n-1}dx \\ &= \frac{C}{l(n+1)}[1-(1-l)^{n+1}]. \end{aligned} \tag{A 4}$$

Similarly, the activity due to [^{14}C] gives a mean

$$\bar{K} = \frac{K}{n+1}. \tag{A 5}$$

Combining (A 4) and (A 5), and making provision for a fraction α of the material which has been cut off remaining in the system after filtration, it can be shown that for an ensemble of strands

$$\frac{F(^3\mathrm{H})}{F(^{14}\mathrm{C})} = \sum_{n=0}^{\infty} \int_{l=0}^{l=1} \left\{ \frac{[(1-\alpha)/l(n+1)]\,[Z-(1-l)^{n+1}]+\alpha(n+1)}{1+n\alpha} \right\} P(n)\,Q(l)dl. \qquad \text{(A 6)}$$

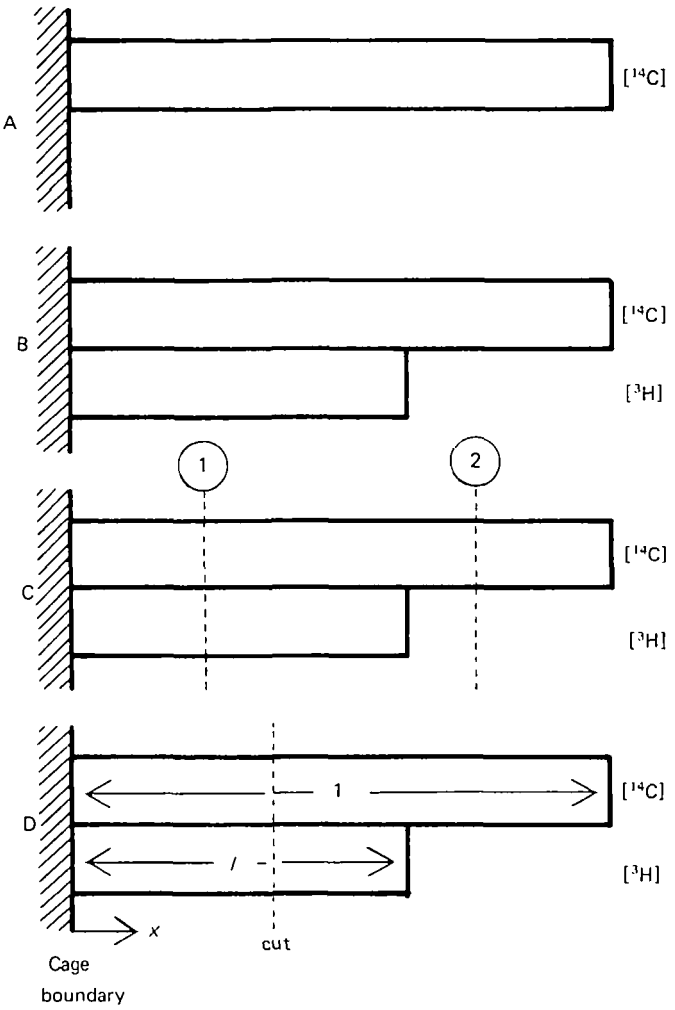

Fig. A 1. A model for labelling and cutting DNA. See text for discussion.

Here $F(^3\mathrm{H})$ is the fraction of the initial activity, due to [^{3}H] remaining after cutting; $F(^{14}\mathrm{C})$ is the fraction due to [^{14}C] remaining; $Z = 1$; $P(n)$ is the Poisson distribution; and $Q(l)$ is a distribution function which takes account of the fact that loops replicate at random during the cell cycle.

Using the data of Fig. 5 (excluding those from the pulse-labelled experiments) a maximum-likelihood solution (solid line) was made for t_L, the time taken for the complete labelling of a strand with [^{3}H], and α. The solid line represents (t_L = 10·5

min, $\alpha = 0{\cdot}1$) and the dotted lines show the effect of varying t_L by a factor of 4 (i.e. t_L 42 min or 2·6 min) (Fig. 5).

The basic model with $\alpha = 0$ leads to predictions that fit the data moderately well. However, digestion should increase the ratio to a maximum, but we observe that extensive digestion *reduces* the ratio from the maximum. Any one of 3 modifications leads to a better fit.

(1) Perhaps a proportion of unreplicating DNA (e.g. in heterochromatin) is condensed and so resists cleavage: it would be enriched by extensive digestion, reducing the ratio. Although a model based on this premise gives an excellent fit it is difficult to imagine how a fraction of the DNA would remain condensed in the nucleoids in the absence of protein.

(2) Pulse-label might be incorporated close to, but not quite at, the cage (i.e. at distance λ from the cage). This stable incorporation might arise because DNA was indeed replicated at this slight distance away from the cage or because Okazaki fragments, synthesized at the cage, were subsequently displaced – and so lost – by branch migration of parental strands during isolation or digestion of nucleoids. Some branch migration would be expected to occur under our conditions (Radding, Beattie, Holloman & Wiegand, 1977). Only when the Okazaki fragments had been ligated to form longer nascent strands would the label become stably incorporated and this would be at a point a little way away from the cage. In this case $Z = (1-\lambda)^{n+1}$ in (A 6) and Q changes correspondingly. With $\alpha = 0$, a good fit to the data can be obtained with $\lambda = 0{\cdot}09$, $t_L = 18{\cdot}8$ min; assuming a rate of elongation of 1 μm/min, $\lambda = 1{\cdot}6$ μm, requiring extensive branch migration. As α increases t_L and λ become correspondingly smaller.

(3) Under 10% (on average 7%) of detached DNA is not removed by filtration (see Materials and methods). As the detached DNA is richer in [^{14}C] the ratio will be reduced by this non-specific binding to the filter. If this imperfection in the filtration procedure is incorporated into the mathematical model a very good fit is obtained. This is illustrated in Fig. 5 in the main paper.

REFERENCE

RADDING, C. M., BEATTIE, K. L., HOLLOMAN, W. K. & WIEGAND, R. C. (1977). Uptake of homologous single-stranded fragments of superhelical DNA. IV. Branch migration. *J. molec. biol.* **116**, 825–839.