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SUPERCOILING OF DNA AND NUCLEAR CONFORMATION DURING THE CELL-CYCLE

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SUMMARY

When cells are lysed in solutions containing high concentrations of salt and a non-ionic detergent, structures are released which retain many of the morphological features of nuclei. These nucleoids contain superhelical DNA but are depleted of nuclear protein. We have analysed DNA conformation in nucleoids derived from HeLa cells synchronized at different stages in the cell cycle. The gross differences in nuclear morphology seen during the cell cycle are reflected in the morphology of the nucleoids; for example, the individual chromosomes of mitotic cells remain identifiable and aggregated within the mitotic nucleoid. The sedimentation rate of nucleoids in sucrose gradients reflects the gross nuclear morphology; the small *S*-phase nucleoids sediment 9 times faster than the large mitotic nucleoids. Despite these large differences at the gross level of organization, both the degree of supercoiling and the size of the units in which supercoiling is maintained are roughly similar in the nucleoids derived from cells in the different phases. The protein content of the various nucleoids is also very similar. Like the nucleoids made from randomly growing cultures of cells, mitotic nucleoids are excellent templates for the RNA polymerase of *Escherichia coli*.

INTRODUCTION

Nuclear morphology changes as a cell progresses through the cell cycle (de la Torre & Navarrete, 1974). Conformational changes in interphase may be revealed by fusing interphase cells with mitotic cells to induce chromosome condensation prematurely (Johnson & Rao, 1970; Schor, Johnson & Waldren, 1975). The physical properties of DNA and its accessibility to enzymes and drugs also change during the cell cycle (Rigler, Killander, Bolund & Ringertz, 1969; Darżynkiewicz, Bolund & Ringertz, 1969; Pederson, 1972; Pederson & Robbins, 1972; Nicolini, Ajiro, Borun & Baserga, 1975; Moser, Müller & Robbins, 1975). Analysis of structure in nuclei, chromosomes or chromatin isolated by conventional techniques is hampered by the fragility of DNA (Burgi & Hershey, 1961; Levinthal & Davison, 1961; Wray, Stubblefield & Humphrey, 1972). Recently we have developed methods for separating nuclear DNA from the majority of the chromatin proteins using mild conditions which cause few breaks in the DNA (Cook & Brazell, 1975, 1976*a*, *b*; Cook, Brazell & Jost, 1976). Cells are lysed in solutions containing high concentrations of salt and a non-ionic detergent to release structures that retain many of the morphological features of nuclei and which are depleted of nuclear protein. We have analysed the conformation of the DNA in these nucleoids by sedimenting them through sucrose gradients containing the intercalating dye, ethidium bromide. The nucleoids sediment in the manner

characteristic of DNA that is intact, supercoiled and circular. In this paper we describe an analysis of DNA conformation in nucleoids derived from HeLa cells synchronized at different stages in the cell cycle.

MATERIALS AND METHODS

Cells

HeLa cells were grown in suspension (Cook & Brazell, 1975). Their generation time is about 21 h.

Chemicals

Chemicals were obtained from the following sources. RNase-free sucrose – Cambrian Chemicals Ltd., Croydon, Surrey; Ethidium bromide (3,8-diamino-6-phenyl-5-ethylphenanthridium bromide), Thymidine (2′-deoxythymidine) and Dithiothreitol (threo-2,3-dihydroxy-1,4-dithiolbutane) – Sigma, Kingston-upon-Thames, Surrey; Sodium metrizoate, 3-acetamido-2,4,6-triiodo-5-(*N*-methylacetamido)-benzoic acid (sodium salt as a 32·8 % w/v solution) – Nyegaard and Co. As., Oslo.

Radiochemicals

[*Me*-^{3}H]thymidine (40-60 Ci/mmol) and [5,6-^{3}H]uridine-5′-triphosphate (50 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks.

Cell synchronization

Cells were blocked in mitosis using thymidine and nitrous oxide (Rao, 1968). Mitotic indices of over 70 % were obtained routinely. Cell division is completed within 3 h after removal of the nitrous oxide block and the first cells enter *S*-phase after a further 1·5 h. Typically more than 60 % of the cells were in *S*-phase 10–13·5 h after release of the nitrous oxide. G_1- and *S*-phase cells were collected 3–4 h and 12·5–13·5 h after release of the nitrous oxide block. *S*-phase cells were also obtained after a single thymidine block. Synchronized cells have a fairly normal cell cycle with G_1, *S* and G_2 phases of 7, 10 and 3 h respectively. Mitotic indices were estimated from fixed and stained cells, and *S*-phase synchrony was assessed autoradiographically.

Sucrose gradients

Nucleoid conformation was analysed by sedimentation in sucrose gradients (Cook & Brazell, 1975). One modification was made to their procedure: cells were lysed at 4 °C. Centrifuge speeds and times are given in the legends to the figures.

γ-irradiation

Samples were γ-irradiated using a caesium 137 source (Cook & Brazell, 1976*a*).

Fluorescence microscopy

Photographs of nucleoids stained with ethidium (0·2 mg/ml) (Cook & Brazell, 1976*a*) were obtained using Ilford HP5 film, and exposure times of less than 1 s.

Analysis of proteins

Nucleoids from randomly growing cells and G_1- and *S*-phase cells were prepared in bulk using 'step' gradients (Cook *et al.* 1976). Nucleoids were prepared from mitotic cells using gradients made by layering 23 ml of the 15 % sucrose gradient solution over 8 ml of the 30 % sucrose gradient solution. Cells were resuspended in phosphate-buffered saline and added to 3 volumes of lysis mixture (1·95 M NaCl) and after 15 min at 4 °C, 4 ml of the mixture con-

taining about 2×10^7 cells were layered on the top of the step gradient. Gradients were spun at 5000 **g** for 30 min at 4 °C. The white aggregate containing the majority of the nucleoids was removed from the interface between the 15 and 30% sucrose, diluted to 0·2 M NaCl with 10 mM tris-(hydroxymethyl)-aminomethane (pH 8·0), and the nucleoids were pelleted at $1{\cdot}2 \times 10^5$ **g**. All solutions contained 0·1 mM phenylmethylsulphonylfluoride to inhibit proteases. Proteins were separated by electrophoresis on polyacrylamide gel slabs containing sodium dodecyl sulphate (Laemmli, 1970; Cook *et al.* 1976). Purified histone H1 – mol. wt. 21000 (a gift from Dr E. W. Johns), F-actin – mol. wt. 45000, tubulin – mol. wt. 56000 and bovine plasma albumin – mol. wt. 67000 (Armour Pharmaceutical Co. Ltd, Eastbourne) were used as references. Gels were stained with 0·2% Coomassie blue, photographed and analysed using a Joyce–Loebl densitometer.

Transcription

The conditions for the transcription *in vitro* of nucleoids by the RNA polymerase of *Escherichia coli* (E.C. 2.7.7.6) have been described (Colman & Cook, 1977). RNA polymerase was obtained from Boehringer GmbH, Mannheim. It had a specific activity of 200–400 units/mg and contained sigma factor (manufacturer's data). (One unit is the enzyme activity which produces an incorporation in 10 min of 1 nmol AMP into acid-precipitable material under the conditions described by Burgess & Travers, 1971). Two kinds of experiments were performed. In the first, nucleoids were incubated with polymerase for different periods and the amounts of [^{3}H]-UTP incorporated into acid-insoluble material determined as described in the legend to fig. 3 of Colman & Cook (1977). In the second, the RNA synthesis directed by nucleoids in the presence or absence of rifampicin was determined and the maximum rates of RNA synthesis achieved in the presence of the drug are expressed as a percentage of the maximum rate in its absence (as described in fig. 13 and table 2 of Colman & Cook, 1977).

Spectrofluorometry

Fluorescence measurements were made using a Farrand Mark 1 spectrofluorometer (Kontron Instruments, Watford). 510 nm was chosen as the excitation wavelength and fluorescence was measured at 590 nm, the wavelength of maximum emission (Paoletti, Le Pecq & Lehman, 1971). 10-nm slit-widths were used throughout.

Nucleoids were prepared for fluorometry as follows: 1 vol. of HeLa cells (80×10^6/ml) in phosphate-buffered saline was added to 3 vol. of lysis mixture (1·95 M NaCl) and after 5 min at least 100 vol. of 2 M NaCl, 10 mM Tris (pH 8·0) were added to 1 vol. of the cells in lysis mixture. Measurements were made on ice-cold samples.

The amounts of ethidium bound to pure DNA are generally estimated directly by reference to the fluorescence of a known concentration of ethidium under conditions where all the ethidium is bound (i.e. in the presence of excess DNA or, in our case, excess nucleoids). However, we cannot obtain concentrations of nucleoids great enough to provide an excess over the whole range of ethidium concentrations that we use. It would also be inappropriate to determine the amounts of the dye bound to nucleoids by reference to binding isotherms constructed using an excess of pure DNA, since the nucleoids also contain RNA (Colman & Cook, 1977). The amount of ethidium bound to the nucleoids is therefore expressed as the equivalent concentration – the concentration of free ethidium (in μg/ml) which, on its own, fluoresces as brightly as the bound ethidium. The equivalent concentration is determined as follows. The fluorescence of a mixture of ethidium and nucleoids is measured as the output (μA) of a photomultiplier. Using a standard curve, the concentration of free ethidium giving the same output is determined. The total ethidium concentration and the concentrations of ethidium giving outputs equivalent to that of nucleoids and solvent alone are then subtracted to yield the concentration of free ethidium which fluoresces as brightly as the bound ethidium (the equivalent concentration). At low ethidium concentrations where nucleoids are in excess the equivalent concentration is underestimated. At high concentrations (> 20 μg/ml) the ethidium absorbs strongly both the exciting and emitted light, so complicating the analysis.

RESULTS

Fluorescence microscopy

When ethidium intercalates, its fluorescence is enhanced (Le Pecq & Paoletti, 1967): as a result the shape of stained nucleoids may be conveniently monitored using a fluorescence microscope (Fig. 1). Nucleoids prepared from HeLa cells at different stages in the cell cycle have very different conformations. Nucleoids from mitotic cells appear as aggregates of chromosomes (Fig. 1 A). Individual chromosomes within the aggregate are identifiable. Like those from interphase cells, such aggregates are sensitive to the illumination used in the fluorescence microscope (Cook *et al.* 1976); illumination disperses the fluorescent material so that the mitotic nucleoids become diffuse. Individual chromosomes can no longer be distinguished in the nucleoid after anaphase (Fig. 1 B). Telophase nucleoids are large and diffuse (Fig. 1 C) and as the cells enter G_1, small nucleoli appear (Fig. 1 D) and enlarge (Fig. 1 E); *S*-phase nucleoids are small and contain prominent nucleoli (Fig. 1 F). Some free chromosomes are released when mitotic cells which have been cooled in ice for 30 min are lysed (Fig. 1 G). A variety of agents (e.g. urea, sodium perchlorate, sarkosyl, sodium metrizoate) cause nucleoids to swell. When these agents are added to mitotic nucleoids the chromosomes are dispersed and individual chromatids are identifiable (Fig. 1 H). The chromosomes sometimes exhibit a banded appearance (Fig. 1 I). The DNA of mitotic nucleoids dispersed by sodium metrizoate remains supercoiled (unpublished observations).

Fig. 1. Photographs of fluorescent nucleoids stained with ethidium. Nucleoids in 1·95 M NaCl, 10 mM Tris (pH 8·0) were mixed with an equal volume of 100 mM dithiothreitol, 10 mM Tris (pH 8·0) before photography. The dithiothreitol slows the dispersion of fluorescent material that occurs on illumination. Scale bars in A–F and H represent 10 μm; in G, 20 μm; and in I, 3 μm.

A, nucleoids from metaphase cells. Individual chromosomes are identifiable.

B, nucleoid from an anaphase cell.

C, nucleoids from telophase cells.

D, nucleoids from telophase/early G_1-phase cells.

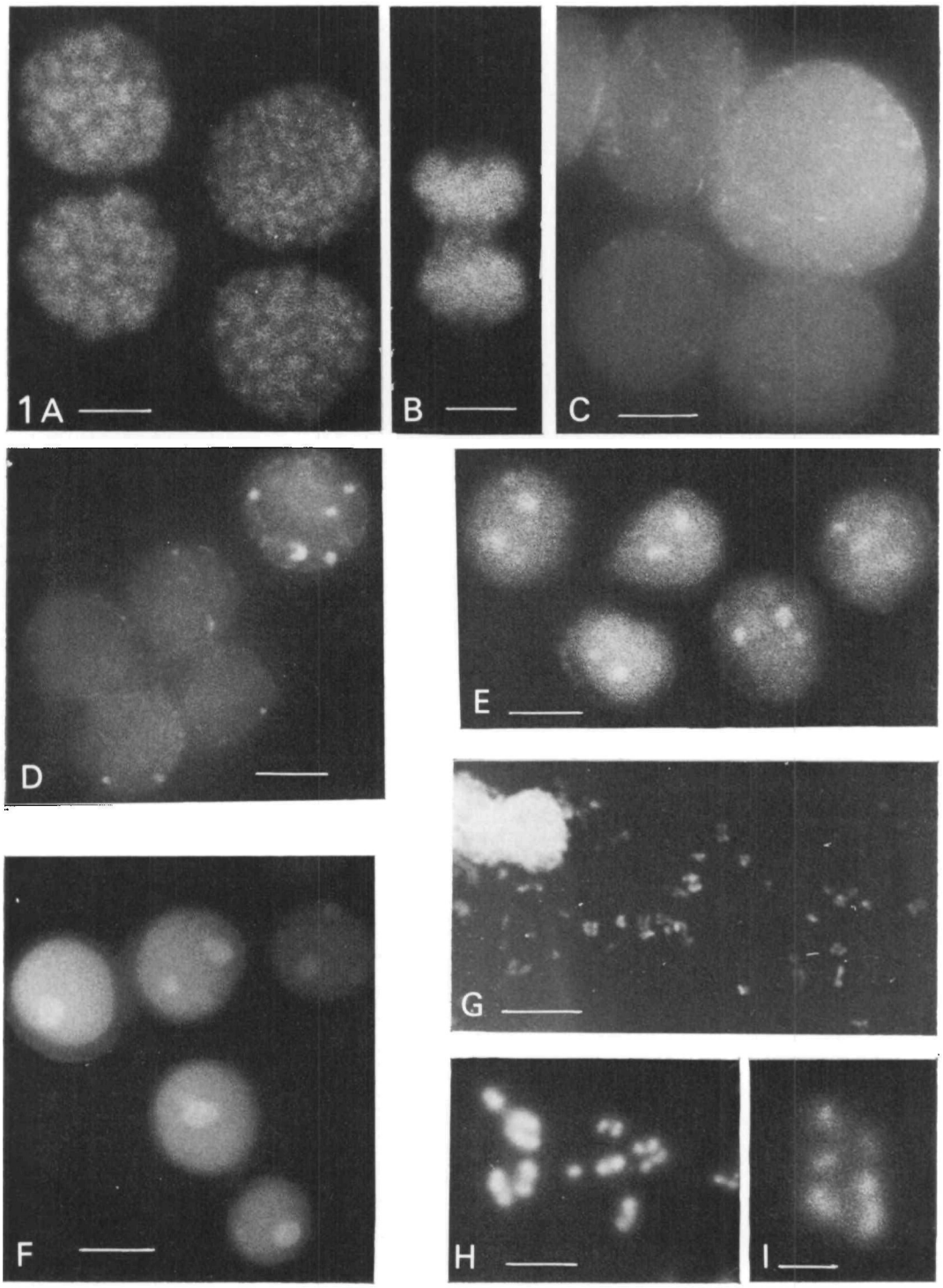
E, nucleoids from cells in mid-G_1

F, nucleoids from *S*-phase cells containing prominent nucleoli.

G, photograph of fluorescent chromosomes stained with ethidium. Mitotic cells in phosphate-buffered saline were cooled on ice for 40 min, and lysed by addition of 3 vol. of lysis mixture. An equal volume of 100 mM dithiothreitol, 10 mM Tris (pH 8·0) was added and photographs taken after staining with ethidium.

H, photograph of fluorescent chromosomes stained with ethidium. The free chromosomes were released from mitotic nucleoids by sodium metrizoate. An equal volume of sodium metrizoate solution (32·8 % w/v) was added to mitotic nucleoids. An equal volume of 100 mM dithiothreitol, 10 mM Tris (pH 8·0) was then added to this mixture and photographs taken in the fluorescence microscope after staining with ethidium.

I, photograph of a fluorescent chromosome stained with ethidium. Free chromosomes were released from mitotic nucleoids as described in H. The individual chromatids are banded.


1A
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Analysis of nucleoid conformation by sedimentation in sucrose gradients

The integrity and conformation of DNA may be monitored by sedimenting nucleoids in sucrose gradients lacking ethidium; nucleoids containing broken DNA sediment more slowly than their counterparts containing intact and supercoiled DNA (Cook & Brazell, 1975, 1976*a*, *b*; Cook *et al.* 1976). Nucleoids derived from cells in different phases sediment at very different rates (Table 1). Remarkably, mitotic nucleoids derived from cells with the most highly condensed DNA sediment most slowly, whereas the *S*-phase nucleoids, which might have been expected to contain DNA broken at the replication fork, sediment most rapidly.

Table 1. *Relative sedimentation rates of nucleoids from different phases of the cell cycle*

Phase	Centrifuge speed, rev/min	Centrifuge time, min	Relative sedimentation rate* ± S.E.
S	4300	30	1·7 ± 0·1
G_1	4300	38	1·3 ± 0·1
R	4300	60	1·0 ± 0·1
M	12500	40	0·2 ± 0·1

* The sedimentation rate is expressed as a ratio relative to the sedimentation rate of nucleoids from unsynchronized cells (R).

The degree of supercoiling in nucleoid DNA can be determined by sedimenting nucleoids in sucrose gradients containing ethidium. The intercalating agent affects the sedimentation of supercoiled DNA in a characteristic manner (Crawford & Waring, 1967; Cook & Brazell, 1975). Like those from unsynchronized cells, nucleoids derived from G_1-, *M*- and *S*- phases are affected in this way (Fig. 2). Low concentrations of ethidium reduce the sedimentation rate; at higher concentrations the rate increases. The concentration of ethidium which minimizes the sedimentation rate reflects the degree of supercoiling (Crawford & Waring, 1967). This concentration is roughly the same for the nucleoids derived from cells in the various phases of the cell cycle, indicating that the DNA in each has roughly the same superhelical density. The large differences in sedimentation rate of the various nucleoids in the absence of ethidium are unlikely, therefore, to be explained by differences in the degree of supercoiling.

Quite large differences in the degree of supercoiling might not be detected by our sedimentation technique. Recently we have developed a more sensitive method for detecting such differences and we shall publish the method in detail elsewhere. Using this method we have confirmed that nucleoids from mitotic and randomly growing cells possess the same degree of supercoiling (Fig. 3). At low concentrations ethidium binds more avidly to negatively supercoiled DNA than to its broken and so relaxed counterpart: at higher concentrations, where binding induces the formation of supercoiling of sense opposite to that initially present, the ethidium binds less tightly to the intact molecule. At an intermediate concentration the ligand binds equally to the

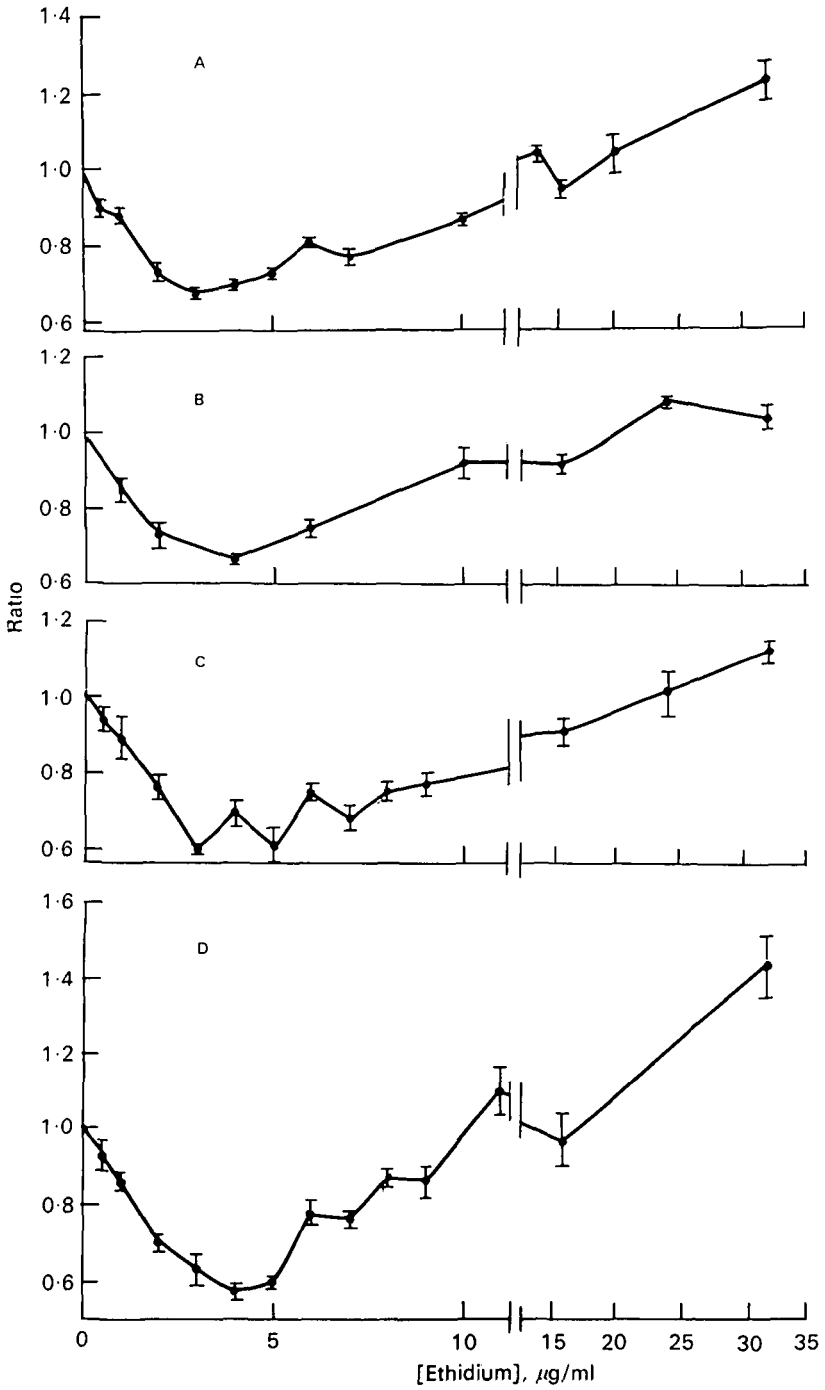

Fig. 2. The effect of ethidium bromide on the sedimentation of nucleoids. The distance sedimented by nucleoids in gradients containing different concentrations of ethidium is expressed as a ratio relative to nucleoids sedimenting in the absence of ethidium under the same conditions. Between 0·5 and $1{\cdot}0 \times 10^5$ HeLa cells were applied to each gradient. Nucleoids were obtained from *S*-phase (A), G_1-phase (B), randomly growing (C) and mitotic cells (D). *S*-phase cells were obtained by reversal of a nitrous oxide block. Gradients in A, B and C were spun at 4300 rev/min for 10, 38 and 60 min respectively. Gradients in D were spun at 12500 rev/min for 40 min. Error bars give the standard error of the mean.

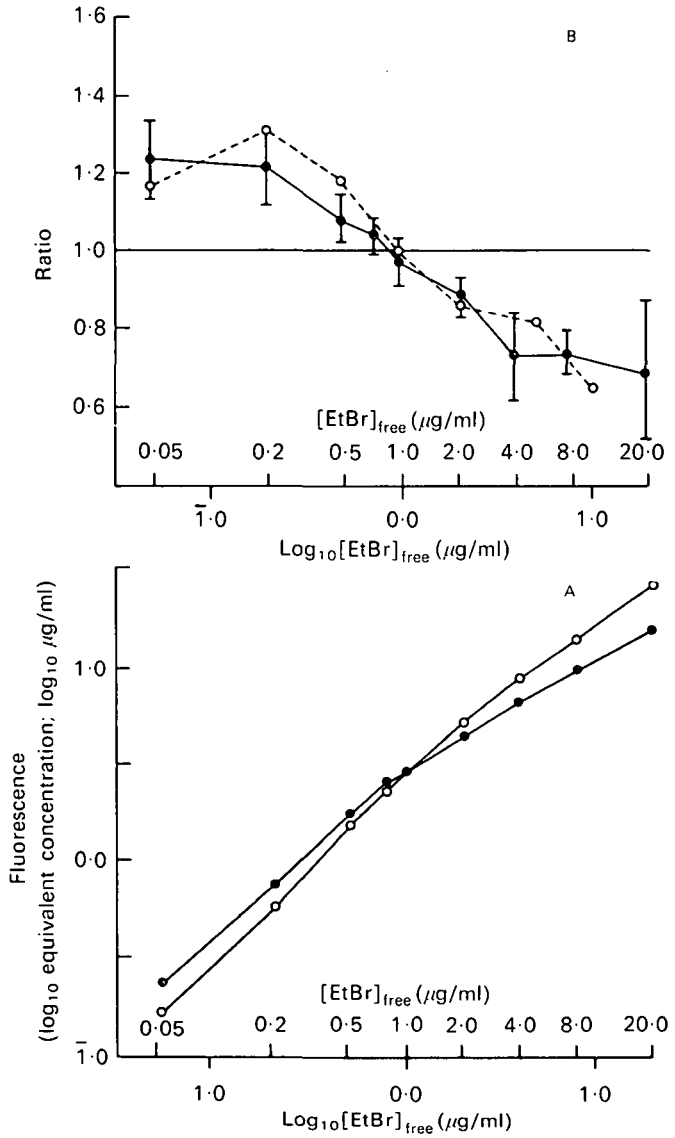

Fig. 3. Ethidium binding to nucleoids from mitotic and randomly growing cells. The amounts of ethidium (EtBr) bound to nucleoids from mitotic and randomly growing cells in the presence of varying amounts of free ethidium were determined spectrofluorometrically as described in Materials and methods.

A, the amount of ethidium bound to unirradiated and irradiated (9·6 J kg^{-1}) nucleoids from randomly growing cells is expressed as the equivalent concentration – the concentration of free ethidium (in μg/ml) which fluoresces as brightly as the bound ethidium. The free ethidium and the equivalent concentration are expressed as the logarithm to condense the axes. ●—●, unirradiated nucleoids; ○—○, irradiated nucleoids.

B, the relative binding capacities of unirradiated and irradiated (9·6 J kg^{-1}) nucleoids from mitotic and randomly growing cells. The ratio is derived from curves like those in A by dividing the equivalent concentration of ethidium bound to unirradiated nucleoids by the equivalent concentration of ethidium bound to irradiated nucleoids. Mean ratios from a large number of experiments with unirradiated and irradiated nucleoids isolated from randomly growing cells: ●—●. Error bars give the standard error of the mean. Ratios for unirradiated and irradiated nucleoids from mitotic cells: ○ – – – ○.

intact and broken forms and this concentration reflects the degree of supercoiling in the intact form (Bauer & Vinograd, 1968). We have measured by fluorometry (Paoletti *et al.* 1971) the binding of ethidium to irradiated and unirradiated nucleoids from mitotic and randomly growing cells. Ethidium is bound to nucleoids in rough proportion to the amount of free ethidium (Fig. 3A). In the range 0·05–1·0 μg/ml the irradiated nucleoids bind less than their unirradiated counterparts; at about 1 μg/ml they bind an equal amount and at high concentrations they bind more. Small variations in nucleoid concentration markedly affect the fluorescence; increasing concentrations shift the 2 curves in Fig. 3A upwards but do not change the concentration of ethidium at which the irradiated and unirradiated nucleoids fluoresce similarly. Small differences in nucleoid concentration therefore complicate comparison of one experiment with another. This difficulty may be overcome by considering the binding capacity of unirradiated nucleoids relative to those of their unirradiated counterparts (Fig. 3B). The average ratios indicate that the irradiated and unirradiated bind equal amounts of ethidium at about 1 μg/ml, and this is true for nucleoids from both mitotic and randomly growing cells.

The effect of γ-radiation on the sedimentation of nucleoids

Supercoiling can be maintained in a length of DNA only if free rotation about the ends of the duplex is restricted. We have suggested that nucleoid DNA is *quasi*-circular and organized into loops in such a way that rotation of one strand of the duplex about the other within the loop is restricted. Breaking the DNA in one loop would then release supercoiling within that loop but not in adjacent loops. The size of the loop can be estimated by applying target theory to curves relating the dose of γ-radiation to the reduction in sedimentation rate of irradiated nucleoids (Cook & Brazell, 1975). Changes in the size of such loops might alter the compaction of the nucleoids and so account for the large differences in conformation of the various nucleoids. We therefore measured the effects of γ-radiation on the sedimentation rate of the various nucleoids (Fig. 4). Increasing the radiation dose decreases the sedimentation rate: small doses have a marked effect, larger doses have a progressively smaller effect. About 4·8 J kg^{-1} halves the sedimentation rate of G_1-, M- and S-phase nucleoids, as it does with nucleoids from unsynchronized cells (Cook & Brazell, 1975). The radiation-sensitive targets (i.e. the *quasi*-circles) must therefore have roughly the same size in the different nucleoids.

The proteins of nucleoids

Nucleoids isolated in 1·95 M NaCl contain few of the proteins characteristic of chromatin; they lack the histones but contain some proteins with molecular weights greater than 38000 (Cook *et al.* 1976; Levin, Jost & Cook, 1978). We compared the proteins of the various nucleoids using polyacrylamide gels containing sodium dodecyl sulphate: their contents of the major proteins were very similar (Fig. 5). All lacked the histones and most of the chromatin proteins and all contained the proteins with molecular weights greater than 38000 that are characteristic of nucleoids. Some nucleoid proteins with molecular weights between 60000 and 70000 have mobilities

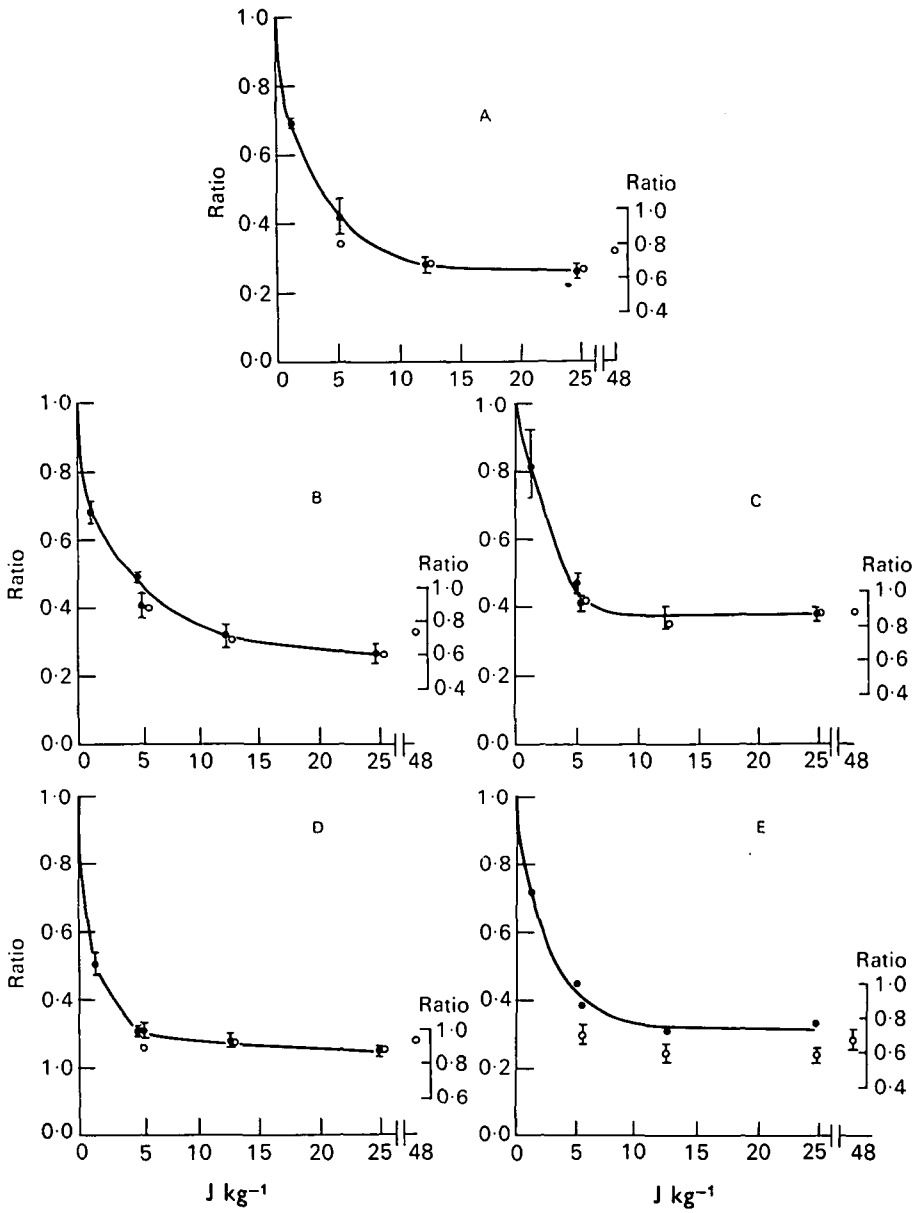

Fig. 4. The effect of radiation dose on the sedimentation of nucleoids from cells in different stages of the cell cycle. The data presented in each curve are derived from 2 sets of experiments: one (●—●) relates to the left-hand ordinate and represents the distance sedimented by irradiated nucleoids, in gradients lacking ethidium, expressed as a ratio relative to the distance sedimented by unirradiated nucleoids sedimenting under the same conditions. The other (○) relates to the right-hand ordinate and represents the distance sedimented by irradiated nucleoids, in gradients lacking ethidium, expressed as a ratio relative to the distance sedimented by nucleoids irradiated with 4·8 J kg^{-1}. In each case the scales and alignment of the ordinates permit a rough comparison of the sedimentation rates of nucleoids determined in the

[*continued on facing page*

in our gels similar to the proteins of isolated nuclear envelopes (Cook *et al.* 1976; E. Jost, unpublished observations). Since the nuclear membrane disappears during mitosis it is perhaps surprising that these proteins are present in mitotic nucleoids: perhaps the envelope is associated with the individual chromosomes of the mitotic nucleoid and so maintains their structure.

Transcription of nucleoids

Nucleoids made from randomly growing cells are excellent templates for the RNA polymerase of *Escherichia coli*; they direct RNA synthesis at 3–4 times the rate of an equivalent weight of pure DNA (Colman & Cook, 1977). Since mitotic chromosomes do not synthesize RNA *in vivo* we investigated whether mitotic nucleoids could direct the synthesis of RNA by incubating them with the appropriate triphosphates and the RNA polymerase of *E. coli*. Like nucleoids made from randomly growing cells, the mitotic and *S*-phase nucleoids are excellent templates (Fig. 6). After an initial lag, equal weights of DNA from the various nucleoids support RNA synthesis at roughly equal rates. (The kinetics of the RNA synthesis have been discussed elsewhere (Colman & Cook, 1977).) If rifampicin, an inhibitor of chain initiation but not chain growth (di Mauro *et al.* 1969; Sippel & Hartmann, 1970), is added to the assay mixture before the addition of RNA polymerase no RNA is subsequently made. All the RNA synthesis must therefore require the initiation of new chains.

Under the conditions used in the experiment described in Fig. 6, where the concentration of polymerase was limiting the rate of synthesis, the maximum rate reflects the rate of elongation of RNA chains. We have shown elsewhere that the conformation of nucleoid DNA has little influence on the maximum rate under these conditions (Colman & Cook, 1977). A comparison of the rates of initiation directed by the various nucleoids would be of more interest, since it is the initiation of the synthesis of new RNA chains that is influenced by supercoiling in the DNA (Colman & Cook, 1977).

Polymerase bound to DNA probably exists in 2 interconvertible states; in the one the polymerase probably cannot initiate synthesis and in the other synthesis is rapidly initiated (Chamberlin, 1974). The proportions of polymerase bound in the 2 states can be determined using rifampicin. Polymerase engaged in elongating an RNA chain is insensitive to the drug, so that any enzyme that can initiate synthesis rapidly can

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2 sets of experiments. Error bars represent the standard error of the mean. Between 0·5 and $1{\cdot}0 \times 10^5$ HeLa cells were applied to each gradient and irradiated for different times before the gradients were spun.

A, randomly growing cells were applied to gradients which were spun for 1 h at 4300 (●—●) or 10000 (○) rev/min.

B, *S*-phase HeLa cells, obtained by release of a thymidine block, were applied to gradients which were spun at 4300 (●—●) or 10000 (○) rev/min for 30 min.

C, HeLa cells, blocked in *S*-phase by excess thymidine, were applied to gradients which were spun at 4300 (●—●) or 10000 (○) rev/min for 30 min.

D, mitotic HeLa cells were applied to gradients which were spun at 12500 (●—●) or 30000 (○) rev/min for 40 min.

E, G_1-phase HeLa cells were applied to gradients which were spun at 4300 (●—●) or 14000–15000 (○) rev/min for 38 min.

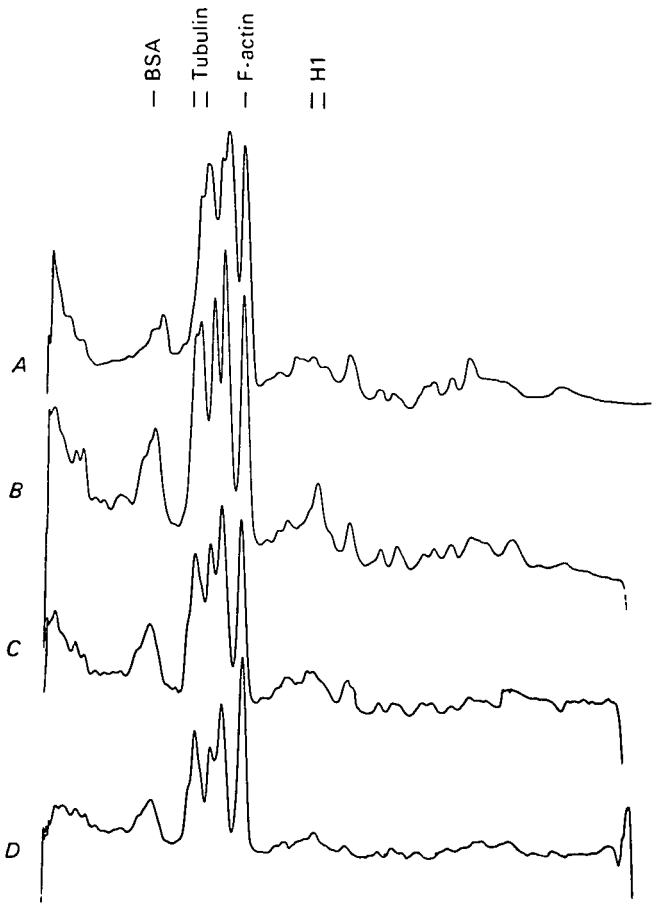

Fig. 5. Proteins of nucleoids derived from cells synchronized at different stages of the cell cycle. The traces represent microdensitometer scans of proteins separated on polyacrylamide gels.

The proteins are from mitotic nucleoids (*A*), nucleoids from *S*-phase cells obtained by reversal of a thymidine block (*B*), nucleoids from G_1-phase cells (*C*), and nucleoids from unsynchronized cells (*D*). The direction of migration is from left to right. Reference proteins bovine plasma albumin (BSA), tubulin, F-actin and histone (H1) migrated to the positions indicated in a separate channel.

escape inactivation. The drug was therefore added before or after preincubating nucleoids with polymerase to permit binding, and then the rates of RNA synthesis were determined. If rifampicin is present before the addition of polymerase to nucleoids no RNA is made (Fig. 6); all enzyme is inactivated. On the other hand, when rifampicin is added after preincubating the nucleoids with polymerase considerable amounts of RNA are made at a rate which is about 70% that of controls to which no rifampicin had been added; 70% of the polymerase is bound to DNA in a form able to initiate rapidly and escape inactivation by the drug (Table 2). The proportion bound in this way by nucleoids from mitotic and randomly growing cells is very similar. It is reduced to about 30% when the supercoiling in the nucleoid DNA is

removed by γ-irradiation. Since the DNA of the various nucleoids is supercoiled to the same extent, and since it is the degree of supercoiling that influences the proportion of polymerase bound in the form able to initiate rapidly, it is again not surprising that the different nucleoids possess similar properties. Although nucleoids from mitotic and randomly growing cells have grossly different morphologies, such differences affect neither the rates of initiation nor the growth of RNA chains.

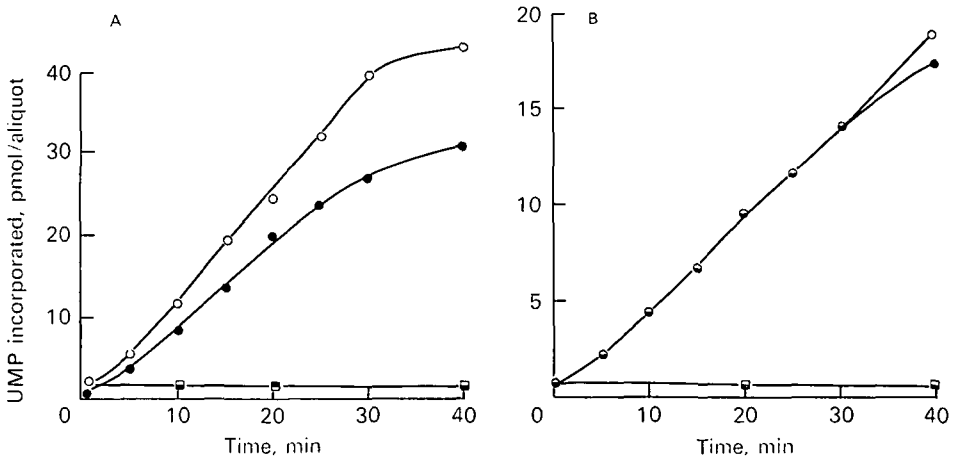

Fig. 6. RNA synthesis directed by mitotic and *S*-phase nucleoids.

A. Nucleoids from randomly growing cells or from mitotic cells were added to a standard assay mixture containing 1·8 units of the RNA polymerase of *E. coli* and 0·1 mCi/ml ^{3}H-UTP (see figs. 3 and 6, Colman & Cook, 1977). The final concentration of nucleoids was $1{\cdot}6 \times 10^6$/ml (random) or $0{\cdot}8 \times 10^6$/ml (mitotic). ○, nucleoids from randomly growing cells; ●, from mitotic cells; □, from randomly growing cells incubated in the presence of rifampicin; ■, from mitotic cells incubated in the presence of rifampicin.

B. Nucleoids from randomly growing cells, or *S*-phase cells obtained by reversal of a thymidine block, were added to a standard assay mixture containing 0·8 units of the RNA polymerase of *E. coli* and 0·1 mCi/ml of ^{3}H-UTP. The final concentration of nucleoids was $2{\cdot}3 \times 10^6$/ml (random) or $2{\cdot}4 \times 10^6$/ml (*S*-phase). ○, nucleoids from randomly growing cells; ●, from *S*-phase cells; □, from randomly growing cells incubated in the presence of rifampicin; ■, from *S*-phase cells incubated in the presence of rifampicin.

The relative amounts of DNA in the various nucleoids may be calculated from the known durations of the different phases of the cell cycle, assuming that the cells progress regularly through the cycle. They are:–random:*S*-phase:mitotic = 1·4:1·5:2. The relative maximum rates of transcription directed by the same weight of DNA were:–A, *S*-phase:random = 1·0:1·0 and B, mitotic:random = 1·2:1·0.

DISCUSSION

We have studied at least 3 different levels of organization of nucleoid DNA. The gross differences in the morphology of nucleoids made from cells in the different phases of the cell cycle reflect the gross differences seen in nuclear morphology; for example, the individual chromosomes of mitotic cells remain identifiable and form an aggregate. Nucleoli disappear during mitosis and reappear at telophase in most cell

Table 2. *Irradiation affects rifampicin-insensitive RNA synthesis in nucleoids*

Nucleoids	Radiation dose, J kg^{-1}	Relative synthesis, %
Random	—	70
Random	163·2	31
Mitotic	—	69
Mitotic	163·2	30

RNA synthesis directed by HeLa nucleoids in the presence or absence of rifampicin was determined, and the maximum rate of RNA synthesis achieved in the presence of the drug is expressed as a percentage of the maximum rate in its absence (see Table 2, Colman & Cook, 1977). Nucleoids were added to a standard assay mixture lacking *E. coli* RNA polymerase and triphosphates. The mixture was then irradiated with γ-rays before the addition of polymerase (1·2 units). The resulting mixture was incubated at 30 °C for 17 min before the reaction was started by the simultaneous addition of triphosphates (containing ^{3}H-UTP, 0·1 mCi/ml final concentration) and water (± rifampicin). At different times, aliquots were removed and their content of radioactivity determined. The maximum rates of RNA synthesis achieved in the presence of rifampicin are expressed as a percentage of the maximum rates in its absence.

types; small and isolated nucleoli aggregate during G_1 to form the prominent nucleoli of *S*-phase cells (Lafontaine & Chouinard, 1963; Stevens, 1965; Erlandson & de Harven, 1971). These changes in nucleolar morphology can also be seen in nucleoids. Remarkably, the constraints that maintain the basic nuclear conformation and even the banding pattern of the chromosomes remain stable in the presence of 1·95 M NaCl and the non-ionic detergent Triton X-100. Since many protein–nucleic acid interactions are disrupted in high concentrations of salt, proteins may not be involved in maintaining nucleoid structure, and our failure to find any differences in protein content is consistent with this view. The sedimentation rate of the nucleoids also reflects the gross morphology; for example the small *S*-phase nucleoids sediment nine times faster than the larger mitotic nucleoids.

Despite these large differences at the gross level of organization we have been unable to find any differences at lower levels: both the degree of supercoiling and the size of the units of DNA in which supercoiling is maintained are roughly similar in nucleoids derived from cells in the different phases of the cell cycle. These levels of organization seem to reflect some fundamental structure that remains invariant throughout interphase. Any additional condensation or decondensation of chromatin that accompanies mitosis or replication must be superimposed upon this basic structure.

It is perhaps surprising that the DNA of *S*-phase nucleoids is supercoiled. The semi-conservative replication of topologically closed molecules of DNA requires that at least one of the backbone strands of the double helix is broken to permit strand separation. During such breaking it is generally assumed that any constraints retaining supercoils within the molecule would be lost. As the 12 pg of DNA in the HeLa nucleus (Colman & Cook, 1977) are replicated at a rate of about 0·5 μm min^{-1} or less (Cairns, 1966), within the 10 h of *S*-phase, at least 3000 replication sites must be

active simultaneously. Little supercoiling would be retained even if only one break were permanently associated with each replication point, as the number of breaks would be approximately equal to the number of loops. Therefore constraints might be maintained in the DNA by proteins or other molecules even during strand breakage and separation (Cook, 1973, 1974).

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