Eur. J. Biochem. 76, 63–78 (1977)

Transcription of Superhelical DNA from Cell Nuclei

Alan COLMAN and Peter R. COOK

Department of Zoology and The Sir William Dunn School of Pathology, University of Oxford

(Received December 21, 1976)

Structures resembling nuclei may be released by gently lysing human or frog cells in solutions containing non-ionic detergents and 1.95 M NaCl. These structures, or nucleoids, sediment in sucrose gradients containing intercalating agents in the manner characteristic of DNA that is intact, supercoiled and circular. They are depleted of nuclear protein and contain no endogenous RNA polymerase activity. We describe conditions for the transcription *in vitro* of these nucleoids by the RNA polymerase of *Escherichia coli*. We compared the kinetics of RNA synthesis directed by nucleoids and by DNA prepared using conventional procedures: nucleoids direct RNA synthesis at three to four times the rate of an equivalent weight of pure DNA and under appropriate conditions the DNA of each nucleoid can direct the synthesis of twice its own weight of RNA. Most of the newly-synthesized RNA remains within the nucleoid. Experiments with the inhibitor, rifampicin, reveal that all RNA synthesis depends upon the initiation of new RNA chains and that nucleoids and pure DNA possess similar numbers of initiation sites. RNA synthesis directed by nucleoids has unusual kinetics: maximal rates are attained only after a lag of about ten minutes. Almost no lag is seen with pure DNA. The lag is due to the slow rate of formation of complexes of polymerase with nucleoid DNA that can initiate. Removal of supercoils by irradiation with γ-rays or by the addition of the unwinding ligand, ethidium bromide, decreases the numbers of polymerase molecules able to initiate synthesis rapidly and increases the lag before maximal synthetic rates are achieved. Loss of supercoiling does not alter the maximum synthetic rate. Supercoiling in eukaryotic DNA clearly influences the initiation of RNA synthesis. These results are discussed with reference to the effects of supercoiling on the transcription of prokaryotic templates.

Structures resembling nuclei may be released by lysing cells gently in solutions containing non-ionic detergents and high concentrations of salt. These structures, which we call nucleoids, sediment in sucrose gradients containing intercalating agents in a manner characteristic of DNA that is intact, supercoiled, and circular. These characteristic changes in sedimentation rate are abolished by irradiating the nucleoids with γ-rays, a procedure known to break DNA. We interpret these results as showing that nucleoids contain DNA that is subject to the same kind of topological constraint restricting rotation of one strand of the double helix of DNA about the other as that found in intact and circular DNA [1,2]. When the DNA of *Escherichia coli* is isolated using the procedures of Stonington and Pettijohn [3] similar constraints are found [4].

Supercoils are lost spontaneously from circles of DNA when only one phosphodiester bond is broken so that supercoiling is a sensitive index of DNA integrity. Since very low doses of γ-rays sufficient to introduce few breaks into DNA remove the majority of supercoils from nucleoid DNA [1] we believe that nucleoids contain DNA of great integrity. It is the lack of integrity of purified eukaryotic DNA which has been implicated in the failure to obtain faithful transcription in vitro [5–7]. In this paper we describe conditions for the transcription *in vitro* of human and amphibian nucleoids by the RNA polymerase of *Escherichia coli*. The nucleoids, which are complex templates containing both RNA and protein [8], might have been expected to direct RNA synthesis at the low rates seen when chromatin is transcribed by exogenous polymerase. Remarkably the nucleoids direct RNA synthesis at 3–4 times the rate of an equivalent weight of pure DNA prepared by conventional procedures.

The degree of supercoiling of circular DNA markedly affects its transcription *in vitro* [9–14]. We have therefore compared the kinetics of RNA synthesis directed by nucleoids containing superhelical or relaxed DNA. We find that template integrity clearly influences the initiation of RNA synthesis.

Enzymes. RNA polymerase (EC 2.7.7.6); ribonuclease A (EC 3.1.4.22).

MATERIALS AND METHODS

Reagents

Chemicals were obtained from the following sources. Nucleoside 5′-triphosphates (P. L. Biochemicals Ltd), dithiothreitol and actinomycin D (Calbiochem Ltd), ethidium bromide and Tris (Sigma Ltd), ribonuclease-free sucrose (Cambrian Chemicals Ltd), other chemicals (Analar grade, British Drug Houses Ltd). Rifampicin was a kind gift of Dr A. Vaciago.

The following radiochemicals were obtained from the Radiochemical Centre, Amersham, U.K.: [5,6-^{3}H]uridine (49000 Ci/mol), [5,6-^{3}H]uridine 5′-triphosphate (42000 Ci/mol), [U-^{14}C]uridine (515 Ci/mol), [*methyl*-^{14}C]thymidine (57 Ci/mol), adenosine 5′-[γ-^{32}P]triphosphate (16300 Ci/mol), guanosine 5′-[γ-^{32}P]-triphosphate (15400 Ci/mol).

Enzymes

E. coli RNA polymerase was obtained from Boehringer GmbH, Mannheim, and pancreatic ribonuclease A from Worthington Ltd. RNA polymerase had a specific activity of 200–400 units/mg and contained sigma factor (manufacturers 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 and Travers [15].

Cell Lines and Culture Conditions

Human HeLa cells and XTC-2 cells derived from tadpoles of *Xenopus laevis* [16] were grown in culture under the conditions described by Cook and Brazell [1,2].

Preparation and Irradiation of Nucleoids

Nucleoids were prepared in bulk in 'step' gradients [8]. Cells were lysed in 1.95 M NaCl with the non-ionic detergent Triton X-100 and the nucleoids which were released separated from cellular debris by sedimentation through 15% sucrose containing 1.95 M NaCl on to a step containing 30% sucrose. Procedures for manipulating, counting and irradiating nucleoids have been described [8].

Preparation of RNA and DNA

Unlabelled RNA was prepared from ovaries of *Xenopus laevis* [17] and ^{14}C-labelled cytoplasmic RNA from HeLa cells [18] cultured as described above. RNA (1 mg/ml in distilled water) was stored at − 70 °C and DNA (1 mg/ml in 88 mM NaCl, 10 mM Tris/HCl, pH 7.6) prepared from *Xenopus* liver [19] at − 20 °C. The molecular weight of this DNA, determined at different stages during this work by electrophoresis on agarose gels, was approximately 20×10^6. (We are grateful to A. Jones for making these measurements.)

Assay for RNA Synthesis

RNA synthesis was assayed in a mixture containing 17.5% glycerol, 2.2% sucrose, 6 mM magnesium acetate, 1 mM $MnCl_2$, 2.6 mM dithiothreitol, 15 mM $(NH_4)_2SO_4$, 0.195 M NaCl, 0.17 mM EDTA, 0.4 mM ATP, GTP and CTP, 0.05 mM UTP, 0.01–0.1 mCi/ml [^{3}H]UTP, 35 mM Tris/HCl, pH 7.9 (20 °C) and nucleoids or DNA at specified concentrations. The mixture was made by adding the following volumes at 0 °C; 1 vol. 10 mM $MnCl_2$, 1 vol. H_2O, 4 vol. 12.5 mM magnesium acetate, 1.25 mM EDTA, 3.1 mM dithiothreitol, 62.5 mM Tris/HCl pH 7.9 (20 °C), 1 vol. triphosphate mixture (0.5 mM UTP, 4 mM ATP, GTP, CTP and [^{3}H]UTP at 1.0–0.1 mCi/ml), 2 vol. of polymerase buffer containing *E. coli* RNA polymerase (3–10 units/ml) and 1 vol. nucleoids or DNA in 1.95 M NaCl, 22.5% sucrose, 1 mM EDTA, 10 mM Tris (pH 8.0). The polymerase buffer contained 1 mM magnesium acetate, 0.1 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, 75 mM $(NH_4)_2SO_4$, 50 mM Tris/HCl, pH 7.9 (20 °C). Any variations in the composition of the assay mixture are fully described in Results.

Reactions were usually started by transferring the mixture from 0 °C to a 30 °C water bath. Duplicate 20-µl or 30-µl aliquots were removed at regular intervals and deposited on 2.5-cm Whatman glass fibre (GF/C) filters. The filters were immediately plunged into ice-cold 5% trichloroacetic acid containing 0.1% tetrasodium pyrophosphate and left for 10 min. At 10-min intervals the trichloroacetic acid solution was changed, and after four changes was replaced by ethanol and finally acetone before the filters were dried and counted in 3 ml of a toluene-based fluor containing Nuclear Chicago scintillator (Amersham/Searle) [20] using a Beckman L 250 liquid scintillation spectrometer. Under these conditions 1000 counts/min represented an incorporation of 8.3 pmol of UMP. Filters carrying both ^{3}H and ^{14}C were counted in 3 ml Unisolve I (Koch-Light) using a Packard Tri-Carb liquid scintillation spectrometer (model 3390).

Analysis of Nucleoid Conformation

Nucleoid conformation was analysed in isokinetic sucrose gradients containing 1.0 or 1.95 M NaCl and various concentrations of ethidium bromide [1,2,8].

Determination of the Size of Transcripts

The size of the RNA transcripts made *in vitro* was determined by a modification of the method of Giorno *et al.* [21] which involves sedimenting the

denatured transcripts in sucrose gradients containing formaldehyde. 50-µl aliquots containing radioactive RNA were removed from the assay mixture at specified times and added to 10 µl containing 1.13% sodium dodecyl sulphate and 40 mM EDTA to stop the reaction. 15 µl of ^{14}C-labelled cytoplasmic RNA from HeLa cells (dissolved in 0.2 M phosphate buffer, pH 7.7) was added as a molecular weight marker, followed by 75 µl 0.04 M phosphate buffer (pH 7.7) containing 4 M formaldehyde. DNA was sheared by passing 40 times through a pipette tip 1.0-mm wide and the mixture incubated at 67 °C for 10 min to denature nucleic acids. 150 µl of 0.2 M phosphate buffer (pH 7.7) were mixed with the cooled solution. 200 µl from this final mixture were then layered on a 5–20% isokinetic sucrose gradient (4.6 ml) containing 0.1 M sodium phosphate (pH 7.7) and 1.1 M formaldehyde, and spun at 26000 rev./min for 14 h at 4 °C in a Beckman SW 50.1 rotor. Two-drop fractions from the gradient were collected on glass-fibre discs (Whatman GF/A). The discs were dried, washed and their radioactive content determined as described above. The molecular weights of the ^{3}H-labelled RNA molecules in each fraction of the sucrose gradient were determined by graphical interpolation using the ^{14}C-labelled ribosomal RNAs (28 S, 18 S) and transfer RNAs (4 S) as references. More than 70% (usually about 85%) of the ^{3}H and ^{14}C label applied to the gradients was recovered. Weight-average and number-average molecular weights were then calculated [22].

Protein, DNA and RNA Estimations

Nucleoids, nuclei or cells were precipitated from suspensions by addition of ice-cold trichloroacetic acid. Protein was estimated after dissolving the precipitate in 0.1 M NaOH, by the method of Lowry *et al.* [23] using bovine serum albumin (Armour, crystalline) as the standard.

For DNA estimations, precipitates were dissolved in 0.3 M KOH and incubated for 18 h at 37 °C. The solution was acidified with 100% trichloroacetic acid or 1 M perchloric acid and the resultant precipitate hydrolysed by heating at 90 °C for 10 min in 5% trichloroacetic acid. DNA was then assayed as described by Burton [24] using chick erythrocyte DNA (Calbiochem, grade A) as the standard.

For RNA determination, precipitates were incubated in 0.3 M KOH for 1 h at 37 °C, before adding perchloric acid to a final concentration of 0.1 M; the resulting precipitate was removed by centrifugation. The RNA content of the supernatant was determined from its absorbance at 260 nm in a Unicam SP800 spectrophotometer using known amounts of HeLa or *Xenopus* RNA which had been treated similarly as standards [25].

Autoradiography

0.2-ml aliquots containing nucleoids were removed from standard RNA synthesis assays, diluted with 10 mM Tris (pH 8.0), and spun at $3-15 \times g$ for 2 min in a cytocentrifuge (Shandon Scientific Co. Ltd, London) to attach the nucleoids to glass slides (3 in × 1 in, 76.2 mm × 25.4 mm). The slides were air-dried and any acid-soluble material removed by immersion for 30 min in ice-cold 5% trichloroacetic acid and 0.1% tetrasodium pyrophosphate. The slides were then washed, dried and dipped in liquid emulsion (Ilford K5 nuclear emulsion); the autoradiographs were exposed, developed and then stained with 10% Giemsa stain.

RESULTS

Characterization of Nucleoids

Appearance. Nucleoids are prepared in a lysis mixture that contains 1.95 M NaCl and a non-ionic detergent to disrupt membranes [8]. In the phase-contrast microscope nucleoids resemble nuclei depleted of protein; nucleoli are clearly visible and membrane ghosts and cytoplasmic material adhere to the nucleoids (Fig. 1).

DNA, RNA and Protein Content of Nucleoids. The DNA, RNA and protein content of nucleoids prepared in 1.95 M NaCl is given in Table 1. Nucleoids contain as much DNA as the cells from which they are derived but less than 10% of the total cellular protein. Nucleoids are depleted of nuclear proteins and contain no histones; five to seven different polypeptides constitute 30–50% of their protein [8]. Cytoplasmic debris attached to the nucleoids probably constitutes a substantial fraction ($\approx$ 50%) of nucleoid protein (Colman and Levin, unpublished observations).

Supercoiling in Nucleoid DNA. The rate of sedimentation of circular and superhelical DNA is affected by intercalating agents (*e.g.* ethidium bromide) in a characteristic manner [26–28]. Nucleoids made from a variety of cells of different species sediment in this characteristic manner [1, 2]. The original experiments concerned nucleoids prepared from HeLa and XTC cells in 1.0 M NaCl; we have since shown that HeLa nucleoids may be isolated freer of histones in 1.95 M NaCl, and that they can be pipetted without loss of supercoiling [8]. When XTC cells are applied to gradients containing 1.95 M NaCl and ethidium, the released nucleoids sediment in the manner characteristic of superhelical DNA (Fig. 2B). XTC nucleoids may be isolated in 1.95 M NaCl and the isolated nucleoids may be pipetted freely whilst retaining the properties characteristic of superhelical DNA (Fig. 2A). The shape of the curves in Fig. 2A and B ob-

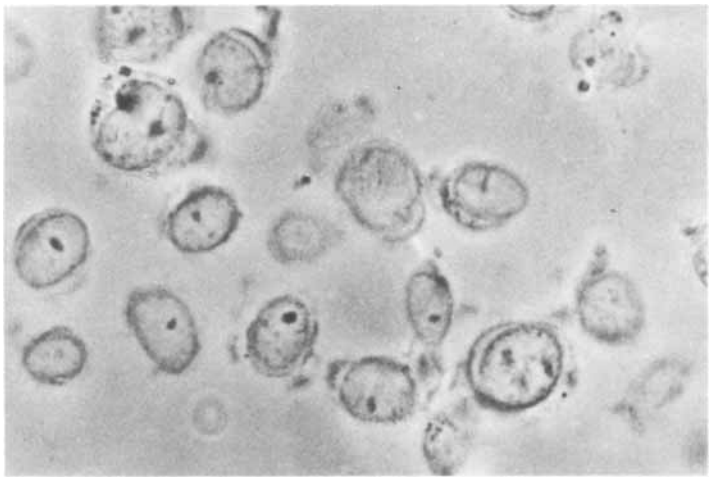

Fig. 1. *Photomicrograph of XTC-2 nucleoids.* XTC-2 nucleoids were isolated in 1.95 M NaCl and photographed using Ilford Pan F film and a phase-contrast microscope (magnification 380x)

Table 1. *Protein, DNA and RNA content of nucleoids*
Protein, DNA and RNA contents of suspensions of nucleoids, cells or nuclei were determined as described in Materials and Methods. Nuclei were prepared by the method of Marzluff [35]. Each figure represents an average of three determinations

Material	Protein	DNA	RNA
	pg		
HeLa cell	266	11.9	–
HeLa nucleus	62	12.6	–
HeLa nucleoid	20.1	12.9	6.9
XTC cell	323	11.1	–
XTC nucleoid	21.3	10.0	8.4

tained with XTC nucleoids resemble curves obtained with HeLa nucleoids and so will not be discussed further [8]. We have also estimated that there are between 1 and 2 supercoils every 200 base pairs in nucleoid DNA in 1.95 M NaCl [8a]. The concentration of salts surrounding a double helix affects its supercoiling [29–32]. These salt effects are so small that in 0.2 M NaCl (the concentration used in the assays described below) the DNA of nucleoids is likely to remain supercoiled [8].

Nucleoid DNA is a Very Efficient Template for RNA Polymerase

When nucleoids are incubated in 0.2 M NaCl with the appropriate triphosphates no RNA synthesis is detected; therefore nucleoids possess no active endogenous RNA polymerase. When RNA polymerase from *E. coli* is added to the assay mixture, substantial amounts of RNA are made (Fig. 3). After an initial lag, the rate of RNA synthesis remains constant for about 70 min and in many experiments RNA synthesis continues for at least 120 min. Equal concen-

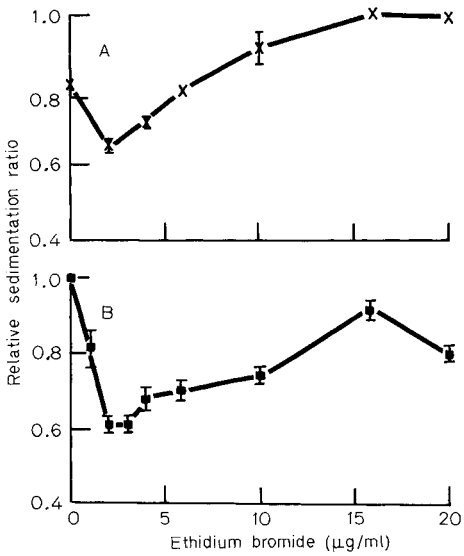

Fig. 2. *Supercoils in nucleoid DNA.* (A) Sedimentation of XTC-2 nucleoids, isolated in 1.95 M salt, through gradients containing 1.0 M NaCl and different concentrations of ethidium. XTC-2 nucleoids were isolated in 1.95 M NaCl, counted and diluted to 1.0 M NaCl with 10 mM Tris (pH 8.0). 200-μl aliquots containing between $1-3\times10^5$ nucleoids were applied to six sucrose gradients (15–30% sucrose; pH 8.0; 4.6 ml) containing 1.0 M NaCl and different concentrations of ethidium. One of the six gradients contained 16 μg/ml ethidium and served as a reference. Gradients were spun at 5000 rev./min for 10 min at 20 °C in the SW 50.1 rotor in a Beckman L2-65b ultracentrifuge and the position of the nucleoids in the gradient determined. The distance sedimented by the nucleoids in the gradients is expressed as a ratio relative to that of nucleoids in the reference tube. (B) Sedimentation of XTC-2 nucleoids in gradients containing 1.95 M NaCl. Sucrose gradients (15–30% sucrose; pH 8.0; 4.6 ml) containing 1.95 M NaCl and various concentrations of ethidium were overlaid with 150 μl lysis mixture (1.95 M NaCl). 50 μl of phosphate-buffered saline containing $1-3\times10^5$ XTC-2 cells were then added to the lysis mixture, and 15 min later the gradients spun at 5000 rev./min for 30 min and analysed as above. The distance sedimented by the nucleoids in gradients containing ethidium is expressed as a ratio relative to that of nucleoids sedimenting in the absence of ethidium

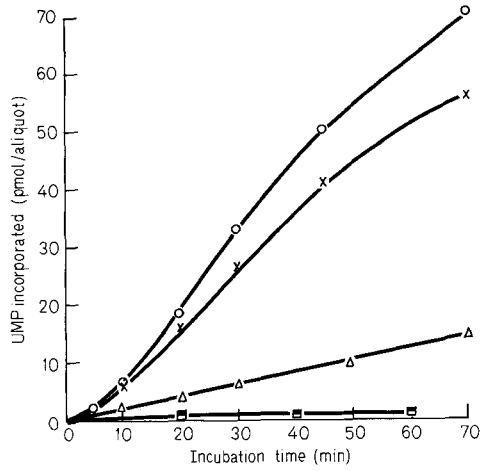

Fig. 3. *RNA synthesis in nucleoids.* 30-µl aliquots containing XTC nucleoids (4×10^6/ml), HeLa nucleoids (5×10^6/ml) or DNA (50 µg/ml) were added at 0 °C to 270 µl of standard assay mixture containing 0.6 unit of *E. coli* RNA polymerase. The mixture was then incubated at 30 °C. At various times, 20-µl aliquots were removed and the amount of [^{3}H]UMP incorporated into RNA determined. XTC nucleoids (×); HeLa nucleoids (○); *Xenopus* DNA (△); XTC nucleoids with no added polymerase (□); HeLa nucleoids with no added polymerase (■)

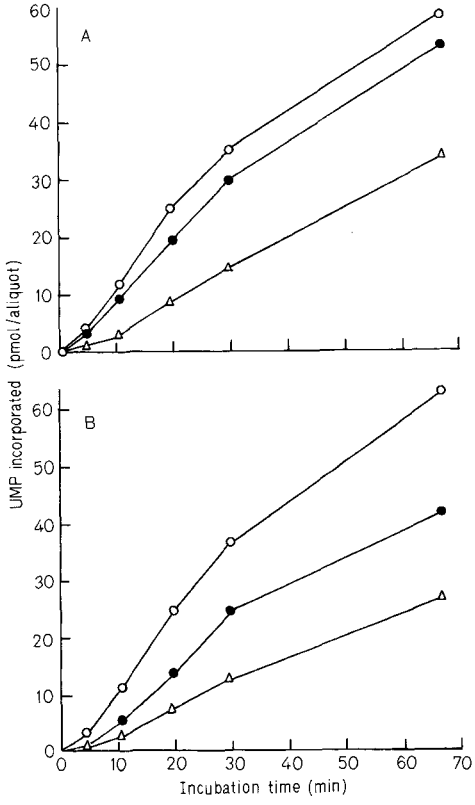

Fig. 4. *Effect of temperature on RNA synthesis in nucleoids.* The amounts of RNA synthesis directed by (A) HeLa nucleoids (3.9×10^6/ml) and (B) XTC nucleoids (4×10^6/ml) were assayed as described in the legend to Fig. 3 except that the assay temperatures were 25 °C (△), 30 °C (●) or 37 °C (○)

trations of HeLa and XTC nucleoids support similar rates of RNA synthesis. At limiting concentrations of polymerase, nucleoids are 3–4 times more efficient as templates for RNA synthesis than equivalent concentrations of DNA purified from *Xenopus* liver (Fig. 3) or from calf thymus. Even with saturating concentrations of polymerase, nucleoids direct the synthesis of 2–3 times more RNA than an equivalent weight of DNA. Maximal rates of RNA synthesis are attained after an undetectable (or shorter) lag with pure DNA than with nucleoids. HeLa nuclei prepared and assayed at 30 °C for endogenous polymerase activity by the methods of Sarma *et al.* [33] synthesize 200-fold less RNA than equivalent numbers of nucleoids assayed as described in Fig. 3; furthermore, the addition of *E. coli* RNA polymerase to the nuclei only slightly stimulates RNA synthesis. These comparisons illustrate the high template activity of nucleoid DNA and the following experiments characterize the synthesis further.

Effect of Temperature on RNA Synthesis

RNA synthesis in nucleoids is stimulated by raising the incubation temperature (Fig. 4). In this respect nucleoids resemble DNA [34] and bacterial nucleoids [21] rather than isolated nuclei in which RNA is synthesized most rapidly at 25 °C [33, 35–37].

Effect of Salt on RNA Synthesis

The kinetics of RNA synthesis *in vitro* critically depend on the salt concentration and template origin and conformation [12, 38–40]. Transcription of nucleoids and pure DNA is affected differently by the salt concentration (Fig. 5). The duration of the lag which precedes maximal RNA synthesis depends on the ionic strength. With all three templates little synthesis occurs in 0.45 M NaCl. Since chains are elongated but not generally initiated at concentrations greater than 0.45 M [39, 41, 42] this suggests that RNA is made in the nucleoids by the initiation and synthesis of new chains and not by the elongation of pre-existing chains.

Nature of RNA Synthesis

We measure RNA synthesis by the incorporation of [^{3}H]UTP into acid-precipitable material. The following experiments confirm that this assay measures RNA synthesis. Actinomycin D (1 µg/ml) completely inhibits the incorporation and omission of ATP, GTP or CTP reduces it by over 95%. No radioactivity insoluble in trichloroacetic acid is recovered after treatment with pancreatic ribonuclease A. If rifampicin (10 µg/ml), an inhibitor of chain initiation but not chain growth [43, 44], is added to the assay mixture before or with RNA polymerase no RNA is subse-

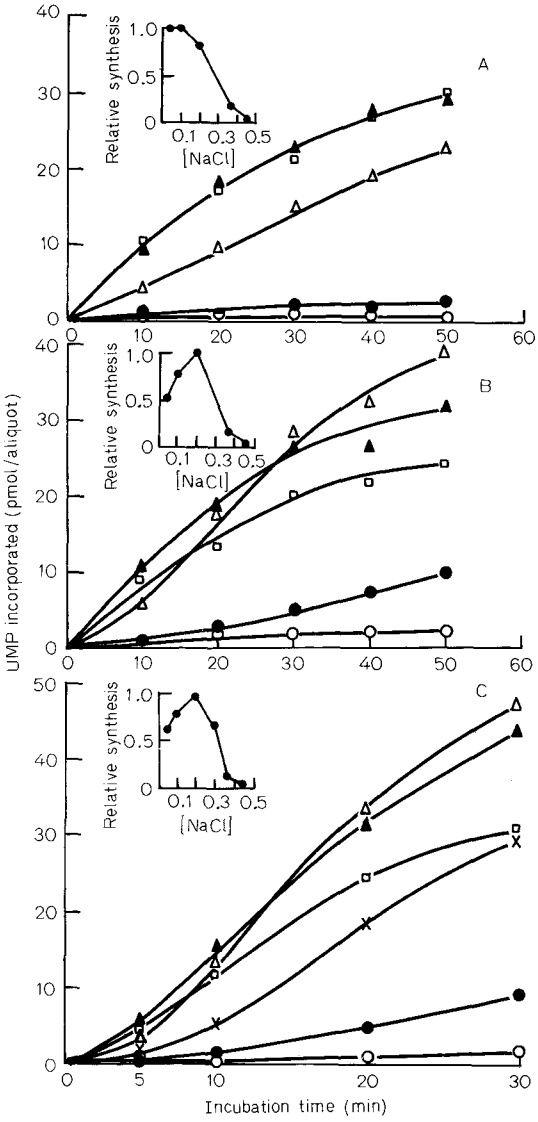

Fig. 5. *The effect of salt concentration on RNA synthesis.* The amounts of RNA synthesized by (A) *Xenopus* DNA (70 μg/ml), (B) HeLa nucleoids (2.9×10^6/ml) and (C) XTC nucleoids (3.2×10^6/ml) were assayed as described in the legend to Fig. 3 with the following exceptions. Assays contained 1.2 units of *E. coli* RNA polymerase and the concentrations of NaCl in the assays were 0.05 M (□), 0.1 M (▲), 0.2 M (△), 0.3M (×), 0.375 M (●), and 0.45 M (○). The inserts relate the amounts of RNA synthesis occurring between the 10th and 30th min of incubation to NaCl concentration

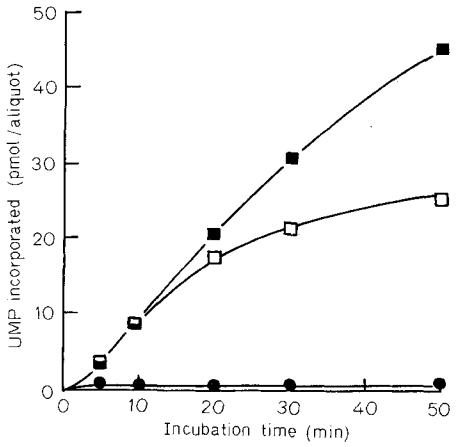

Fig. 6. *Effect of rifampicin on RNA synthesis in nucleoids.* RNA synthesis directed by XTC nucleoids (0.6×10^6/ml) added to an assay mixture containing 1.8 units of *E. coli* RNA polymerase was measured as described in the legend to Fig. 3. Rifampicin (1 mg/ml in H_2O) was added to make its final concentration 10 μg/ml at 0 min (●) or 10 min (□); control contained no rifampicin (■)

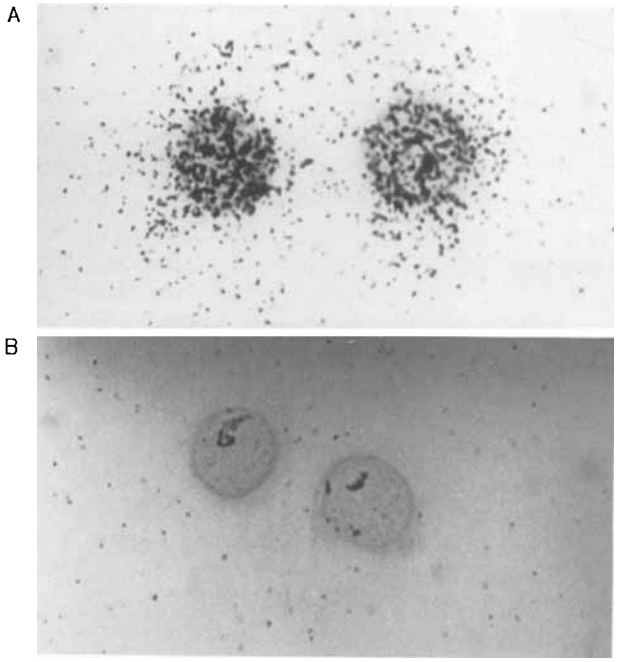

Fig. 7. *Intact nucleoids synthesize RNA.* HeLa nucleoids (10×10^6/ml) were incubated as described in the legend to Fig. 3 with (A) 1.0 unit or (B) no *E. coli* RNA polymerase. After 30 min at 30 °C, 0.2-ml aliquots were removed and autoradiographs prepared. Autoradiographs were exposed for 3 days and photomicrographs taken. Magnification 855×

quently made; on the other hand the addition of rifampicin to nucleoids already making RNA slows down, but does not stop, RNA synthesis (Fig. 6). Therefore all RNA synthesis in the nucleoids requires the initiation of new chains and the considerable amount of endogenous RNA found in nucleoids (Table 1) cannot be used as a primer for elongation.

Location of Newly-Synthesized RNA

Autoradiographs of nucleoids which had been directing the synthesis of RNA for 30 min indicate that some newly-synthesized RNA remains associated with the nucleoids (Fig. 7). The following double-labelling experiment confirms that most of the RNA is made in intact nucleoids and is retained within them. Nucleoids were incubated in our standard assay mixture for 10 min before sedimenting them in sucrose gradients (Fig. 8). The majority of the newly-synthesized

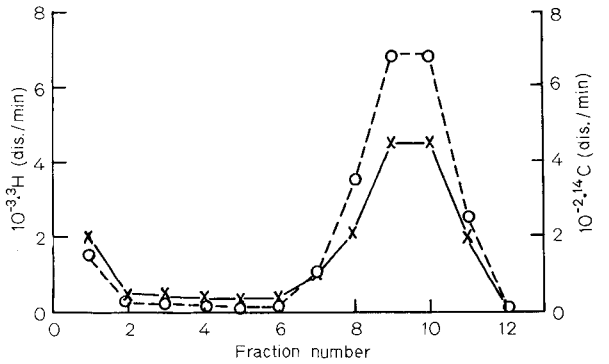

Fig. 8. *Newly-synthesized RNA remains associated with nucleoids.* Nucleoids were prepared from HeLa cells which had been cultured in the presence of [^{14}C]thymidine (0.02 μCi/ml) for the previous 24 h. 150 μl nucleoids (20×10^6/ml) were added at 0 °C to 1350 μl standard assay mixture containing 1.5 units of *E. coli* RNA polymerase. When the mixture had been incubated at 30 °C for 10 min, the salt concentration was raised to 1.0 M NaCl by the addition of 5 M NaCl and the nucleoid concentration adjusted to 10^6 nucleoids/ml with 1.0 M NaCl and 10 mM Tris (pH 8.0). 200 μl of this solution were layered on an isokinetic sucrose gradient (15–30% sucrose; 4.6 ml; pH 8.0) containing 1.0 M NaCl [8]. Gradients were spun at 5000 rev./min for 25 min at 20 °C in the SW 50.1 rotor in a Beckman L2-65b ultracentrifuge. 8-drop fractions of the gradient were collected on glass-fibre discs and the radioactive content of each disc determined. (○) ^{14}C; (×) ^{3}H. More than 60% of the label applied to the gradient was recovered. The use of radioactively-labelled nucleoid DNA is not entirely satisfactory for studies on nucleoid conformation since labelling affects DNA conformation, presumably by inducing breaks in DNA [8a, 45]

RNA (labelled with ^{3}H) co-sediments with the nucleoid DNA (labelled with ^{14}C). A similar result was obtained after 30-min incubation (results not shown). A small amount of RNA and DNA from disrupted nucleoids remains at the top of the gradient but we do not know whether or not this RNA is associated with DNA.

The Effect of Transcription on the Integrity of Nucleoids

The integrity of nucleoids stained with ethidium may be monitored using a fluorescence microscope [8]. Nucleoids which had been directing the synthesis of RNA for 30 min at 30 °C remain intact and do not aggregate. However, when nucleoids are incubated at 30 °C in the absence of polymerase they slowly unfold and so sediment more slowly. After 10 min their rate of sedimentation is reduced to 60% of that of controls maintained on ice [8]. Nucleoids retaining supercoils sediment more rapidly in the presence of 16 μg/ml ethidium than in its absence, whereas the rate of sedimentation of irradiated nucleoids lacking supercoils is reduced by ethidium. We therefore determined what effect transcription had on the sedimentation behaviour of the nucleoids. Nucleoids which had been making RNA for 10 min in our complete assay mixture sediment no differently from controls incubated in 10 mM Tris (pH 8.0); both unfold to the same degree and sediment more slowly than controls maintained on ice in 10 mM Tris, and both sediment about 1.2 times as rapidly in the presence of ethidium as in its absence. Transcription *per se* does not, therefore, alter nucleoid integrity although some unfolding takes place during the assay.

Since it is the initiation of RNA synthesis that proves to be most sensitive to template conformation and not later events like chain growth and termination, the unfolding of the nucleoid DNA during the assay should not greatly affect the interpretation of studies on initiation.

Composition of the Assay Medium

Since we hope eventually to study RNA synthesis directed by templates constructed from nucleoids and selected proteins, we chose assay conditions known to be favourable for the initiation and elongation of RNA chains in isolated nuclei and chromatin [35, 46, 47]. Fig. 9 illustrates how variations in the composition of the assay mixture affect transcription of HeLa nucleoids and *Xenopus* DNA. With the exception of dithiothreitol, the omission of any component tested in this experiment increases RNA synthesis directed by both nucleoids and DNA (Fig. 9).

The Effect of Nucleoid Concentration on RNA Synthesis

The dependence of RNA synthesis on template concentration was studied at constant polymerase concentration by varying the number of nucleoids in the assay mixture. The initial rate of RNA synthesis is not directly proportional to template concentration (Fig. 10); the rate of RNA synthesis per nucleoid is greater the lower the nucleoid concentration. Below concentrations of 10^5 nucleoids/ml, a weight of RNA two times greater than the total weight of template DNA is made in 30 min. (DNA and polymerase are present in a ratio by weight of 1:11 at a concentration of 10^5 nucleoids/ml.) At high nucleoid concentrations increasing the template concentration does not further increase the rate of RNA synthesis, presumably because the template is in excess. (This occurs at DNA/polymerase ratios greater than 3:1.) At low and limiting concentrations of template, the initial rate of RNA synthesis also depends on polymerase concentration. A similar dependence of initial rate on polymerase concentration has been found with T4 DNA; it has been explained by a differential affinity of the polymerase for binding sites on the template [48]. Maximal rates of synthesis are reached at a four-fold lower concentration of pure *Xenopus* DNA than with the DNA of XTC nucleoids.

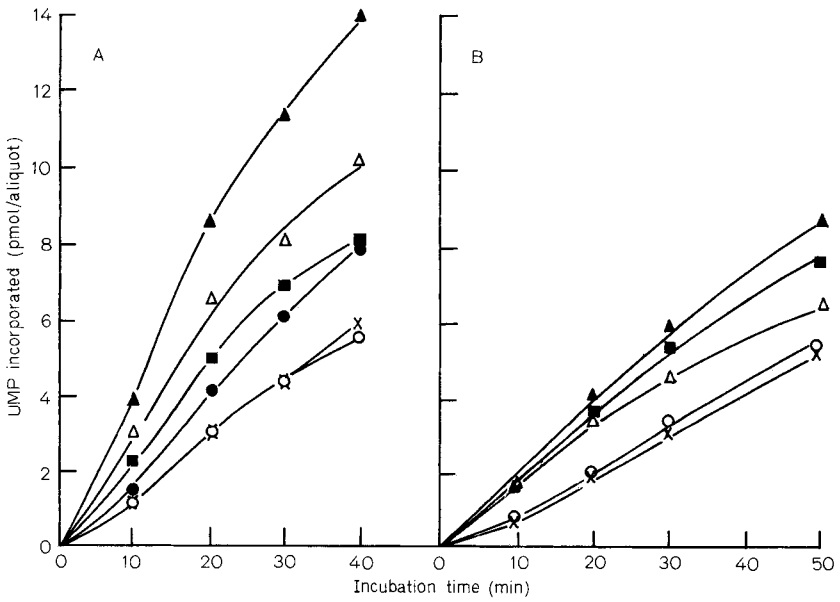

Fig. 9. *Composition of the assay medium affects transcription.* The amounts of RNA synthesis directed by (A) HeLa nucleoids (10^7/ml) or (B) *Xenopus* DNA (200 µg/ml) were determined using 0.45 unit of *E. coli* RNA polymerase as described in the legend to Fig. 3. The following constituents were omitted from the assay mixture: Mn^{2+} ions (▲), glycerol (△), Mg^{2+} ions (■), EDTA (●) or dithiothreitol (×). Control, no omissions (○). Although it is not immediately apparent from (A), where we did not assay RNA synthesis at times shorter than 10 min, the lag remains unaffected by any of the omissions (unpublished results)

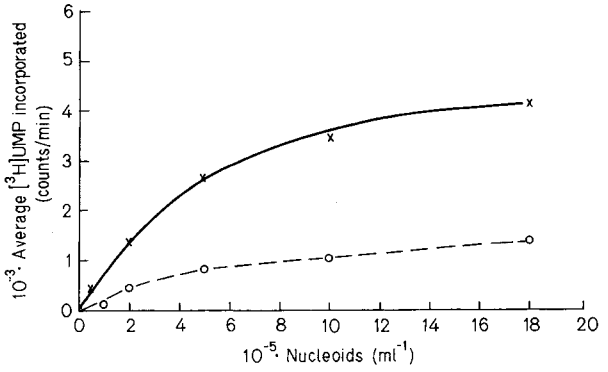

Fig. 10. *The effect of nucleoid concentration on RNA synthesis.* RNA synthesis directed by different concentrations of nucleoids was determined using 0.78 unit (×) or 0.21 unit (○) of *E. coli* RNA polymerase. The amounts of labelled RNA in 20-µl aliquots removed from the assay mixture after 5, 10, 15, 20 and 30 min of incubation were determined. The ordinate represents the amounts of [^{3}H]UMP incorporated into RNA during the 10–30 min period. The amounts incorporated during the first 10 min are lower due to the lag period (*cf.* Fig. 3)

However, even at low and limiting template concentrations, nucleoid DNA is a four-fold more effective template.

Binding of Polymerase to Nucleoid DNA

When polymerase is added to nucleoids a completed molecule of RNA is formed only after the diffusion of the polymerase into the nucleoid, the binding of the polymerase to the appropriate site on the DNA to form a complex capable of initiating an RNA chain (a productive complex) and chain initiation, elongation and termination [49, 50]. Diffusion of the polymerase into the nucleoids, enzyme binding or chain initiation might limit the incorporation of [^{3}H]-UTP into acid-insoluble material so producing the lag that precedes the attainment of the maximal rate (see Fig. 3).

We have investigated the lag by incubating nucleoids in the presence of polymerase for varying times at 30 °C. During this time the polymerase is free to diffuse into the nucleoid and bind to the DNA to give a productive complex. The reaction is then started and the formation of further productive complexes prevented by the simultaneous addition of triphosphates and rifampicin. The amount of RNA synthesized in the next 20 min reflects the number of polymerase molecules productively bound to nucleoid DNA during the pre-incubation. Maximal rates of synthesis occur after 17, 20, and 5 min pre-incubation with HeLa and XTC nucleoids and *Xenopus* DNA respectively (Fig. 11); therefore productive complexes are formed more slowly with nucleoid DNA than with pure DNA. Thus a slow rate of formation of productive complexes might account for the lag.

We have confirmed this conclusion by pre-incubating HeLa nucleoids or *Xenopus* DNA for 17 min to maximise the formation of productive complexes and then we added triphosphates and followed the kinetics of RNA synthesis. In this experiment (Fig. 12), which does not involve rifampicin, RNA is made at the maximum rate almost immediately on addition

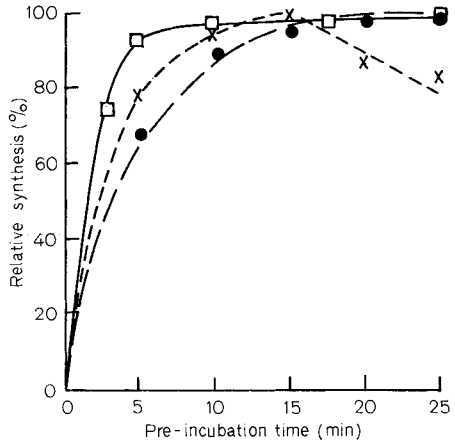

Fig. 11. *The effect of pre-incubation on the initial rate of synthesis.* 50 µl of XTC nucleoids (9×10^6/ml), HeLa nucleoids (10×10^6/ml) or *Xenopus* DNA (200 µg/ml) were added to 390 µl standard assay mixture containing 1.0 unit of *E. coli* RNA polymerase but lacking triphosphates. 88-µl aliquots of this mixture were incubated at 30 °C for various times, and then the reaction started by the simultaneous addition of 10 µl containing [^{3}H]UTP and triphosphates and 2 µl rifampicin (0.5 mg/ml in H_2O). 30-µl aliquots were removed after 20 min and their content of radioactive RNA determined. The incorporation of [^{3}H]UTP into RNA observed in the 20-min period following the pre-incubation periods of varying duration is expressed as a percentage of the maximum incorporation. Maximum incorporations (*i.e.* 100%) occurred after pre-incubation periods of 5, 15 or 17 min with *Xenopus* DNA, HeLa nucleoids or XTC nucleoids respectively. HeLa nucleoids (×), XTC nucleoids (●), *Xenopus* DNA (□)

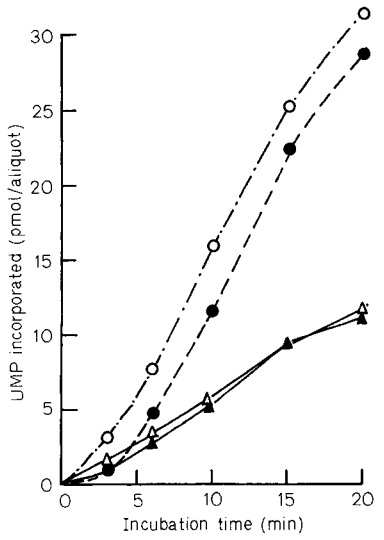

Fig. 12. *The effect of pre-incubation on the kinetics of RNA synthesis.* 30 µl of HeLa nucleoids (12.5×10^6/ml) or *Xenopus* DNA (0.34 mg/ml) were added to 240 µl of standard assay mixture containing 1.5 units of *E. coli* RNA polymerase but lacking triphosphates. The resultant mixture was either not pre-incubated or pre-incubated for 17 min at 30 °C before the addition of 30 µl triphosphates containing [^{3}H]UTP (0.1 µCi/ml) to start the reaction. At the times indicated in the figure, 20-µl aliquots were removed and their content of radioactive RNA determined. HeLa nucleoids: pre-incubated (○), no pre-incubation (●). *Xenopus* DNA: pre-incubated (△), no pre-incubation (▲)

of the triphosphates. Thus the lag almost completely disappears after pre-incubating the nucleoids with polymerase.

The following experiment indicates that a slow diffusion of polymerase into nucleoids cannot alone explain the slow rate of formation of productive complexes. Nucleoids were pre-incubated with polymerase in 1.0 M NaCl for 17 min at 30 °C; under these conditions the polymerase is free to diffuse into the nucleoid but cannot bind to DNA. When the salt concentration is lowered to permit binding and the reaction started by adding triphosphates, maximal rates of RNA synthesis are again attained only after a lag. When a similar experiment was performed by preincubating polymerase with pure DNA at 1.0 M NaCl maximum rates were achieved immediately on dilution to 0.2 M NaCl; this observation excludes the possibility that the polymerase is adversely affected by pre-incubation in 1.0 M NaCl. If diffusion into the nucleoids were limiting the rate of synthesis, no lag should be seen. Some event occurring after diffusion of polymerase into the nucleoid but before the formation of the first phosphodiester bond is therefore rate-limiting.

The processes preceding the initiation of the synthesis of new RNA chains have been analysed using purified DNA templates and the polymerase of *E. coli* [49, 50]. The polymerase bound to promoters probably exists in two interconvertible states; in one the polymerase probably cannot initiate RNA synthesis and in the other synthesis is rapidly initiated [51]. Whilst the enzyme is more or less sensitive to rifampicin in both states, polymerase molecules that initiate an RNA chain become insensitive to the drug so that the proportion of synthesis insensitive to rifampicin reflects the proportion of polymerase in a complex capable of initiating synthesis rapidly and so able to escape inactivation. Differences in the binding of polymerase to allomorphic DNAs can then be demonstrated by comparing the rifampicin sensitivity of RNA synthesis directed by the allomorphs [9, 10]. We have therefore used rifampicin to investigate the binding of polymerase to the DNA of nucleoids (Fig. 13). The drug was added before or after pre-incubating the templates with polymerase and rates of RNA synthesis were then determined. If rifampicin is present before the addition of polymerase to nucleoids no RNA is made; all enzyme is inactivated. On the other hand, when rifampicin is added after pre-incubating nucleoids with polymerase for 17 min, considerable amounts of RNA are made at a rate which is 63% of that of controls to which no

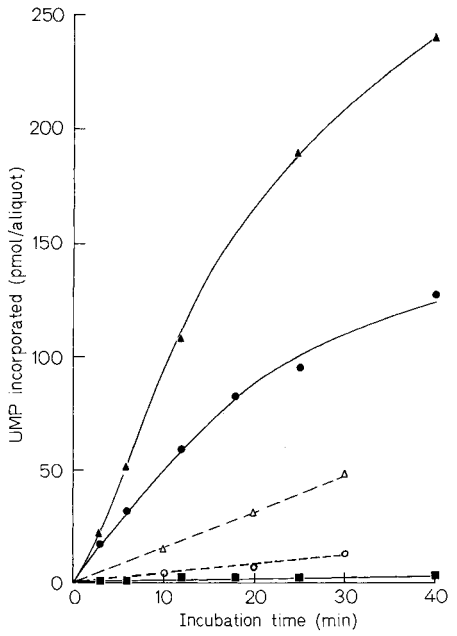

Fig. 13. *The effect of rifampicin on RNA synthesis directed by templates pre-incubated with polymerase.* 30-µl aliquots of XTC nucleoids (20×10^6/ml) or *Xenopus* DNA (200 µg/ml) were added to 230 µl standard assay mixture containing 1.8 units of *E. coli* RNA polymerase but lacking triphosphates. The mixture was incubated for 17 min at 30 °C before the simultaneous addition of 30 µl containing triphosphates and [^{3}H]UTP (0.1 mCi/ml) and 10 µl H_2O (± rifampicin at 300 µg/ml) to start the reaction. At different times 20-µl aliquots were removed and their content of radioactive RNA determined. XTC nucleoids with (●) and without (▲) rifampicin. DNA with (○) and without (△) rifampicin. As a control, rifampicin was added to nucleoids before pre-incubating them with polymerase (■)

rifampicin had been added; much of the polymerase is able to escape inactivation by the drug. In a similar experiment with pure DNA the rate of RNA synthesis after addition of rifampicin is only 25% that of the controls. Therefore after pre-incubation a greater proportion of the nucleoid · polymerase complexes are able to initiate synthesis rapidly (and so become resistant to rifampicin) than are the complexes of polymerase with pure DNA.

Effect of Sonication and γ-Radiation on Transcription in Nucleoids

Nucleoid DNA reduced to a molecular weight of $0.1-0.6 \times 10^6$ by shearing and sonication directs the synthesis of RNA at one fifth the rate of intact DNA (Fig. 14B). The large reduction in synthetic rate probably results from non-productive binding of polymerase at single-stranded and double-stranded breaks produced by sonication [52]. Any subtler effects due to loss of supercoiling will be obscured. We therefore removed supercoiling by γ-irradiating nucleoids with doses that introduce very few breaks into DNA [8, 53]. Increasing the dose of γ-radiation increases the lag before the maximum rate of RNA synthesis is reached but it does not alter the maximum rate (Fig. 14A). The longer lag cannot result from the longer time taken by polymerase to diffuse into irradiated nucleoids, which are disrupted and larger [8], since the lag is not shortened by pre-incubating the irradiated nucleoids with polymerase in 1.0 M NaCl before starting the reaction. We therefore examined

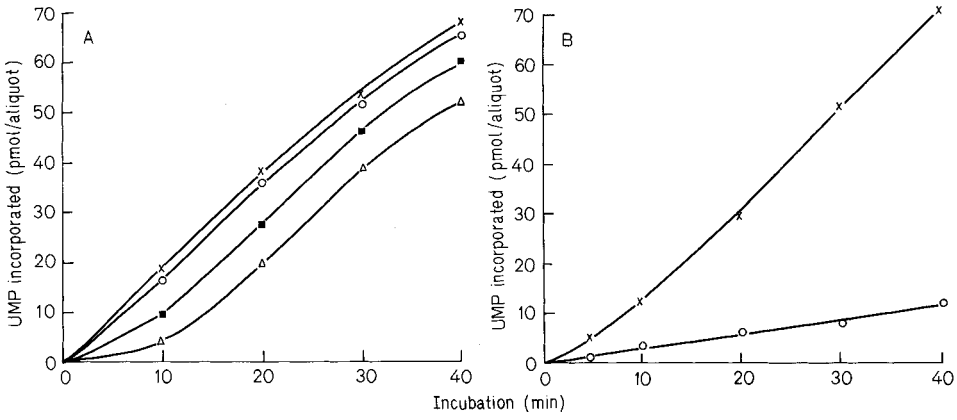

Fig. 14. *Effects of (A) γ-irradiation and (B) shearing and sonication on transcription directed by nucleoids.* (A) 30 µl of HeLa nucleoids (8×10^6/ml) were added to 180 µl standard assay mix which lacked both RNA polymerase and triphosphates. The mixtures were then γ-irradiated (dose rate $1.2-5.44$ J · kg^{-1} min^{-1}) at 0 °C with the following doses of radiation: (×) no irradiation, (○) 5.9 J · kg^{-1}, (■) 163.2 J · kg^{-1}, (△) 272 J · kg^{-1}. 1.8 units of *E. coli* RNA polymerase in 60 µl polymerase buffer were added and the resultant mixtures pre-incubated for 17 min at 30 °C. 30 µl containing triphosphates and [^{3}H]UTP (0.1 mCi/ml) were then added to start the reaction. 20-µl aliquots were removed at various times and their content of radioactivity determined. (B) The experiment described in (A) was repeated except that (a) no pre-incubation period was included and (b) nucleoids were sheared and sonicated instead of being irradiated. Nucleoid suspensions were sheared by passing them 20 times through a 25-gauge needle attached to a syringe using maximum thumb pressure. The sheared DNA of the nucleoids was then broken further by ten 15-s periods of sonication at 0 °C (M.S.E. sonicator, maximum power). Each period of sonication was followed by a 30-s cooling period. The shearing and sonication reduced the DNA of nucleoids to a molecular weight of between 10^5 and 5×10^5. (×) No shearing or sonication; (○) sheared and sonicated

Table 2. *Irradiation and ethidium bromide affect the proportion of RNA synthesis directed by nucleoids that is insensitive to rifampicin*
The RNA synthesis directed by HeLa 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. In assays (a–e) 30-µl aliquots containing HeLa nucleoids (16×10^6/ml) were added to 170 µl of the standard assay mixture lacking *E. coli* polymerase and triphosphates. This mixture was then irradiated with various doses of γ-rays (dose rate 5.44 J · kg^{-1} · min^{-1}) before the addition of 60 µl of polymerase buffer containing 1.8 units of *E. coli* RNA polymerase. The resulting mixture was then incubated at 30 °C for 17 min (assays a–d) or 36 min (assay e) before the reaction was started by the simultaneous addition of 30 µl containing triphosphates and [^{3}H]UTP (0.1 mCi/ml) and 10 µl H_2O (± rifampicin at 300 µg/ml). At different times, 20-µl aliquots were removed and their content of radioactive RNA determined. In assays (f–i) mixtures were not irradiated; instead ethidium bromide was added before pre-incubation. The final concentration of ethidium in the assay mixture is given. The maximum rates of RNA synthesis achieved in the presence of rifampicin are expressed as a percentage of the maximum rates in its absence

Assay	Radiation dose	Ethidium bromide	Relative synthesis
	J · kg^{-1}	µg/ml	%
a	–	–	70
b	9.6	–	65
c	81.6	–	39
d	163.2	–	31
e	163.2	–	32
f	–	–	74
g	–	0.5	68
h	–	1.5	35
i	–	3.0	33

whether radiation alters the binding of polymerase to nucleoid DNA by using rifampicin in a manner analogous to that previously described (*cf.* Fig. 13). Irradiated nucleoids were pre-incubated with polymerase. Triphosphates and rifampicin were then added to start the reaction and to prevent formation of any additional productive complexes. The amounts of rifampicin-insensitive synthesis measured in this way are expressed as a percentage of the synthesis obtained with controls without rifampicin. The percentage of synthesis insensitive to the drug was reduced by irradiation in proportion to dose (Table 2, a–d).

The pre-incubation period of 17 min used in this experiment is evidently long enough to maximise formation of productive complexes (Table 2, compare assays d and e) and RNA is made immediately at the maximum rate when rifampicin and triphosphates are added (unpublished observations; a similar effect is also seen with unirradiated nucleoids, Fig. 13). Therefore neither diffusion of the triphosphates nor any event occurring after the formation of the first phosphodiester bond can be rate-limiting. The extended lag that precedes the attainment of maximum

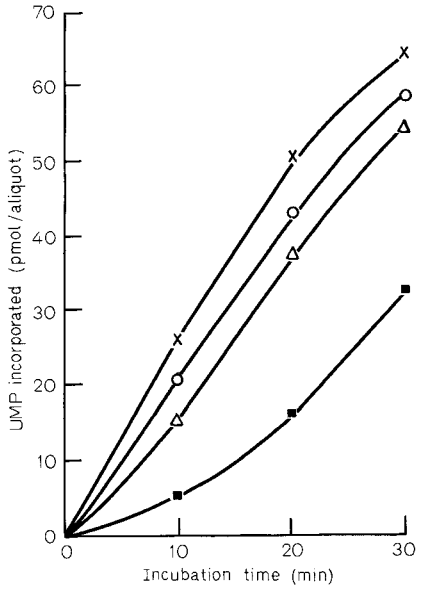

Fig. 15. *Effect of ethidium bromide on transcription directed by nucleoids.* RNA synthesis directed by HeLa nucleoids (12×10^6/ml) was assayed as described in Fig. 14A except that nucleoids were not irradiated; instead ethidium bromide at the indicated concentrations was present in the assay during pre-incubation and the subsequent periods of RNA synthesis. Ethidium bromide 0 µg/ml (×), 0.5 µg/ml (○), 1.5 µg/ml (△), 3.0 µg/ml (■)

synthetic rates directed by irradiated nucleoids in the absence of rifampicin but following the same preincubation procedure (Fig. 14A) must therefore be caused by some event occurring after complex formation. Irradiation thus has two effects on nucleoids: it increases the rifampicin sensitivity of the synthesis directed by polymerase · DNA complexes and it affects a rate-limiting step occurring after formation of productive complexes but before the formation of the first phosphodiester bond.

Effect of Ethidium on Transcription in Nucleoids

Like γ-irradiation, intercalating agents (*e.g.* ethidium bromide) remove supercoils from nucleoid DNA [1, 2]. Ethidium has two major effects on RNA synthesis directed by nucleoids. It increases the lag that precedes the attainment of the maximal rate (Fig. 15) and it increases the rifampicin sensitivity of the RNA synthesis directed by polymerase · nucleoid-DNA complexes (Table 2, f–i). Similar effects of ethidium on the rate of RNA synthesis directed by superhelical viral DNA have been noted by Richardson [54] and result from a lowered rate of initiation. Although ethidium's effects are probably very complicated our results are sufficiently similar to those obtained after irradiation (Fig. 14A; Table 2, a–e) to suggest that both agents exert their effects by altering the superhelical conformation of nucleoid DNA.

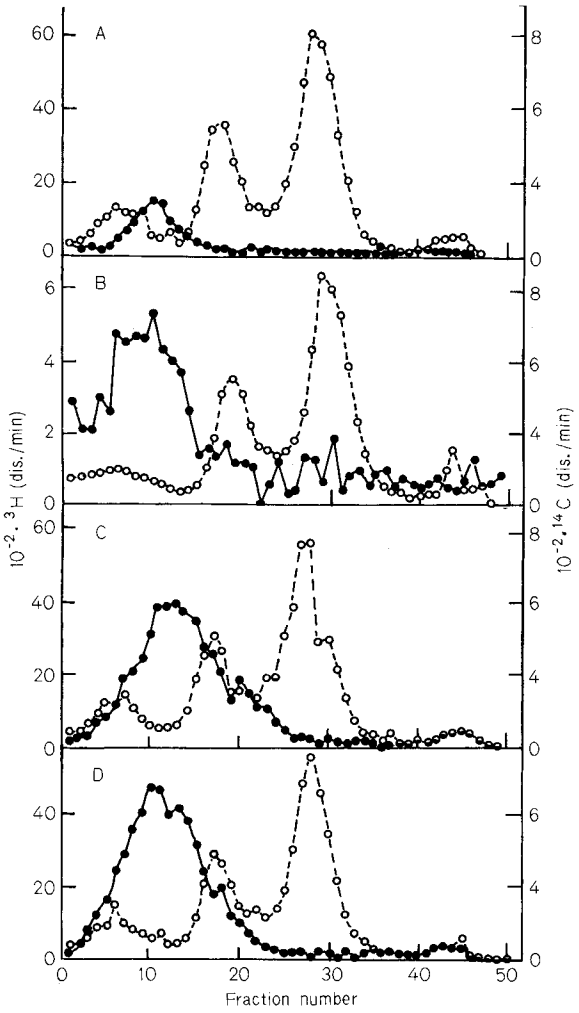

Fig. 16. *Sucrose-gradient analysis of transcripts of nucleoid and pure DNA of* Xenopus. 50-µl aliquots of XTC nucleoids (17×10^6/ml) or *Xenopus* DNA (200 µg/ml) were added to 395 µl standard assay mixture lacking triphosphates but containing 3 units of *E. coli* RNA polymerase, and pre-incubated at 30 °C for 17 min. 50 µl triphosphates containing [^{3}H]UTP (0.1 mCi/ml and 0.4 mCi/ml for nucleoids and DNA respectively) and 5 µl rifampicin (1 mg/ml) were then added simultaneously to start the reaction. 50-µl aliquots were removed after 3 and 30 min and the sizes of the radioactively-labelled RNA determined using sucrose gradients containing formaldehyde. The weight-average and number-average molecular weights of the newly-synthesized RNA labelled with ^{3}H (●——●) in each gradient were calculated by reference to the marker RNAs labelled with ^{14}C (○——○). Transcripts of XTC nucleoids incubated for 3 (A) and 30 min (C) and of *Xenopus* DNA incubated for 3 (B) and 30 min (D). The weight-average and number-average molecular weights calculated were (A) 165000 and 93000, (B) 150000 and 86000, (C) 380000 and 172000 and (D) 350000 and 160000. Fraction 1 was from the top of the gradient

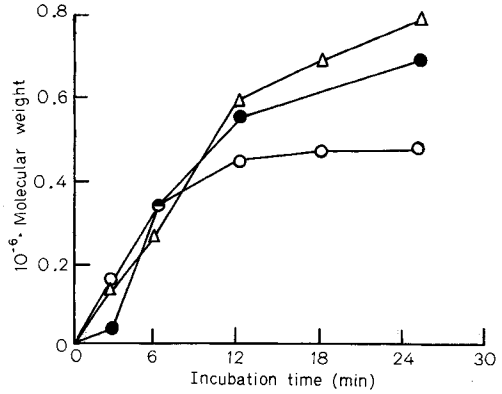

Fig. 17. *Initial rates of chain elongation.* 50-µl aliquots of HeLa nucleoids (22.5×10^6/ml), XTC nucleoids (6.7×10^6/ml) or *Xenopus* DNA (200 µg/ml) were added to 395 µl standard assay mixture lacking triphosphates but containing 3 units of *E. coli* RNA polymerase. The mixture was pre-incubated at 30 °C for 17 min. 50 µl triphosphates containing [^{3}H]UTP (0.1 mCi/ml) and 5 µl rifampicin (1 mg/ml) were then added simultaneously to start the reaction. 50-µl aliquots were removed at various times and the sizes of the radioactively-labelled RNA determined using sucrose gradients containing formaldehyde. Weight-average molecular weights were calculated by reference to marker RNAs labelled with ^{14}C. The weight-average molecular weights of transcripts of HeLa nucleoids (△), XTC nucleoids (○), and *Xenopus* DNA (●) are given at different times. Initial rates of chain elongation are derived from the above curves by assuming that an average nucleotide residue has a molecular weight of 300

Sucrose-Gradient Analysis of RNA

The difference in rates of transcription of pure DNA and nucleoids could also result from differences in the numbers of initiation sites, or from differences in rates of chain elongation or termination. If the two templates direct synthesis with different rates of chain elongation or termination then the size-distributions of RNA molecules synthesized after a given time should be different. We therefore compared the sizes of the RNA molecules synthesized on XTC nucleoids and *Xenopus* DNA (Fig. 16). Polymerase was added to the templates, the mixtures incubated for 17 min to permit complex formation and then the reaction started by adding triphosphates. Rifampicin was added simultaneously with the triphosphates to prevent reinitiation. After 3 or 30 min, samples were removed and the size of the newly-synthesized and labelled RNA determined by centrifugation in sucrose gradients containing formaldehyde [21]. As the weight-average and number-average molecular weights of the RNA synthesized on the two templates after identical periods are similar (Fig. 16 legend) the rates of chain elongation and termination must also be similar and so cannot account for the differences in transcriptional efficiencies of nucleoids and pure DNA. In similar experiments with unirradiated and irradiated ($163.2\ J \cdot kg^{-1}$) HeLa nucleoids no differences were found in the weight-average and number-average molecular weights of the newly-synthesized RNA.

We have estimated the rates of elongation of the RNA chains synthesized on the various DNA templates by measuring their lengths at different times (Fig. 17). The average elongation rate (measured during the first 10 min) is similar for all three templates and is approximately 3 nucleotides/s. Although the average chain length does not increase after 30-min synthesis on XTC nucleoids and *Xenopus*

Table 3. *Initiation sites in nucleoids and DNA*
HeLa and XTC nucleoids (0.2×10^6/ml) or *Xenopus* DNA (2.4 μg/ml) were assayed in the presence of the indicated amounts of RNA polymerase as described in the legend for Fig. 16, except that the triphosphate mixture contained [^{3}H]UTP at 2 mCi/ml. 50-μl aliquots were removed after 20-min incubation at 30 °C and the sizes of radioactively-labelled RNA determined in sucrose gradients containing formaldehyde. The number-average molecular weights of the newly-synthesized RNA (labelled with ^{3}H) were calculated by reference to the marker RNAs (labelled with ^{14}C) in the same gradient. As the rates of elongation are low, a proportion of the RNA molecules synthesized during the 20-min-incubation period are smaller than the smallest molecular-weight marker (*i.e.* 4 S RNA) and this affects the accuracy with which the number-average molecular weight is determined. However, as the molecular weights of the transcripts of the different templates are estimated in the same way, estimates of the relative numbers of initiation sites are unlikely to be affected by these inaccuracies. The number of base pairs per assay in the template was calculated by assuming that one mole of nucleotide pairs weighs 600 g. The chain length was calculated from the number-average molecular weight (M_n) (the molecular weight of a nucleotide residue was assumed to be 300). Irradiated HeLa nucleoids were irradiated with 163.2 J · kg^{-1} as described in the legend to Fig. 14A

Template (base pairs)	RNA polymerase	Nucleotides incorporated	$10^{-3} \times M_n$	Chain length (nucleotides)	Initiations	Base pairs DNA per initiation site
(pmol)	units				pmol	
HeLa nucleoids (40)	4	36	186	560	0.064	625
HeLa nucleoids (40)	2	30	234	703	0.043	930
Irradiated HeLa nucleoids (40)	4	14.5	217	650	0.022	1818
Irradiated HeLa nucleoids (40)	2	14.9	211	632	0.024	1666
XTC nucleoids (40)	4	38	249	746	0.051	784
XTC nucleoids (40)	2	38	219	656	0.058	690
Xenopus DNA (80)	7.6	22	237	710	0.031	2580
Xenopus DNA (80)	3.8	25	210	630	0.040	2000

DNA, we can still detect net RNA synthesis. This is presumably because some of the smaller molecules are still growing. The rates of chain elongation are low but might be increased by omitting Mn^{2+} ions [55]. We have shown that such an omission increases the rate of RNA synthesis (*cf.* Fig. 9).

Determination of the Number of Initiation Sites

The number of initiation sites used by the RNA polymerase of *E. coli* in nucleoids and pure DNA can be determined by measuring the number of chains synthesized under conditions where each initiation gives rise to one RNA chain [56]. Excess polymerase is incubated with DNA to saturate all binding sites and then RNA synthesis is started by adding triphosphates. Simultaneously rifampicin is added to prevent further initiation so that only one chain is synthesized at each initiation site. The number-average molecular weight of the newly-synthesized RNA is determined from its distribution in sucrose gradients. The number of chains made is equal to the total weight of the newly-synthesized RNA divided by the number-average molecular weight. XTC and HeLa nucleoids possess 4-fold more initiation sites than an equivalent quantity of pure DNA (Table 3). The value of about 600 initiation sites estimated for HeLa nucleoids is probably an under-estimate since the polymerase was not quite in excess. (It proves very difficult to completely saturate nucleoids with polymerase.) Irradiation halves the number of intiation sites in nucleoids (Table 3).

DISCUSSION

Nucleoids are complex templates containing nuclear RNA and some protein and unknown quantities of lipid (Table 1); they might have been expected to direct RNA synthesis at the low rates seen when chromatin is transcribed by exogenous RNA polymerase. Remarkably, they are very efficient templates. Under certain circumstances the intact DNA of each nucleoid can direct the synthesis of twice its own weight of RNA (see Results). Nucleoids direct RNA synthesis at three to four times the rate of an equivalent weight of DNA (Fig. 3). The transcription of nucleoid DNA also differs from that of pure DNA in its kinetics (Fig. 3) and in its requirements for ions (Fig. 5) and other factors (Fig. 9). These differences might reflect differences in template conformation (*e.g.* supercoiling) or integrity (*e.g.* number of breaks in DNA), in accessibility of initiation sites to added polymerase or in differences in the chemical composition of the two templates. The gradual unfolding of the nucleoids complicates the analysis. Despite these complexities, we are encouraged to discuss the template properties of the intact and broken DNA of nucleoids because they resemble so strikingly those of much simpler viral templates [9–14].

When the DNA of nucleoids is broken by shearing and sonication the rate of transcription is reduced fivefold (Fig. 14B). The reduction probably results mainly from a reduction in the effective concentration of polymerase by breaks in the template binding non-

productively the polymerase [52]. However, since the DNA of intact nucleoids directs the synthesis of more RNA than an equivalent weight of pure DNA even when polymerase is present in excess, this non-productive binding cannot be the sole reason for the difference in transcriptional efficiency.

Whereas the rates of chain elongation and termination directed by pure DNA and nucleoids are similar (Fig. 16, 17), pure DNA has one-third the number of initiation sites of *Xenopus* or HeLa nucleoids (Table 3). Our rough estimate of about 1 site every 2200 base pairs in *Xenopus* DNA is close to the estimates of one site every 1500 base pairs (calf thymus DNA) and 1300 base pairs (chick oviduct DNA) obtained by others [56, 57]. Since we used rifampicin in these experiments, the values displayed in Table 3 only reflect the number of polymerase molecules bound to DNA in a form able to initiate rapidly and so escape inactivation by the drug; additional polymerase bound at other initiation sites is inactivated by the drug so that these sites are not measured by our assay. Thus, although we measure three times more initiation sites in nucleoids than in pure DNA, RNA synthesis directed by nucleoids is three times more resistant to rifampicin inactivation. As a result the total numbers of initiation sites on both templates are probably similar. This conclusion is only valid if we assume that very little re-initiation occurs in the absence of rifampicin. This assumption is justified since rifampicin reduces the initial rate of RNA synthesis in nucleoids by only 30%; the reduction would be greater if much re-initiation occurred. Furthermore, since nucleoids and DNA direct the synthesis of RNA chains of similar size in similar times (Fig. 16), chains are probably re-initiated as infrequently with pure DNA as with nucleoids.

If nucleoids and DNA possess the same numbers of initiation sites the greater rate of transcription of nucleoids relative to that of pure DNA in the presence of saturating concentrations of polymerase must result from an increased rate of initiation per initiation site (*cf.* [10–14]). We have attempted to confirm our estimates of the number of initiation sites by a different method. One γ-phosphorous atom of ATP or GTP is incorporated into each RNA chain so that if this atom is labelled with ^{32}P the incorporation of label directly reflects the number of initiated chains. However, we were unable to obtain nucleoid concentrations great enough to permit sufficient incorporation.

The DNA to which a polymerase binds probably exists in two inter-convertible states, closed and open [49–51, 58–60]. Polymerase can only initiate synthesis after the DNA has been 'opened' by unwinding or melting the DNA [61]. Supercoiling facilitates unwinding [62–64] and so enhances binding of polymerase to open DNA; irradiation, by releasing supercoiling, favours complexing in the closed conformation. We find more polymerase bound to open sites in nucleoids containing supercoiled DNA than to open sites in pure DNA or in the relaxed DNA of irradiated nucleoids. Like irradiation the unwinding agent, ethidium, reduces the proportion bound to open DNA (Table 2).

RNA synthesis directed by nucleoids has unusual kinetics. Maximal rates of synthesis are attained only after a lag of about 10 min; no (or very little) lag is seen with pure DNA (Fig. 3 and 11). The lag is extended by raising the ionic strength (Fig. 5), by irradiating nucleoids (Fig. 14A), or by ethidium (Fig. 15). Pre-incubation experiments and experiments with rifampicin suggest that the rate-limiting step occurs at or after binding but before the formation of the first phosphodiester bond in RNA. The kinetics of RNA synthesis can be explained using the model illustrated above if we assume that the rate of opening and closing nucleoid DNA is slow and rate-limiting (*cf.* [65]). (The opening of sites in pure DNA cannot be rate-limiting since no lag precedes the attainment of maximal synthetic rates). When nucleoids are preincubated with polymerase in 0.2 M NaCl the polymerase binds and is distributed between open and closed sites in proportions determined by DNA conformation. When triphosphates are added, only polymerase at open sites initiates immediately. Equilibrium proportions are slowly restored by further opening of the DNA. This slow opening limits initial rates of synthesis and only when all initiation sites have been opened can maximal rates be attained. Irradiation increases the proportion of polymerase bound to closed DNA and so extends the lag but does not alter the maximal rate. (Irradiation may also decrease the rate of equilibration.) If further equilibration of polymerase between the closed and open conformations is prevented by effectively removing the polymerase at closed sites by adding rifampicin, RNA is synthesized immediately at the maximal rate. Thus supercoiling in nuclear DNA, as it does in circular DNA, influences the initiation of RNA synthesis *in vitro* [10–12, 14]. It is therefore possible that *in vivo* supercoiling affects the opening of initiation sites [11, 60, 66–68]. Indeed, the DNA of nucleoids prepared from cells active in RNA synthesis is supercoiled, whereas that of nucleoids prepared from inactive cells is relaxed [2].

$$\text{Free polymerase} \rightarrow \begin{matrix}\text{Polymerase}\\ \text{bound to}\\ \text{closed DNA}\end{matrix} \rightleftharpoons \begin{matrix}\text{Polymerase}\\ \text{bound to}\\ \text{open DNA}\end{matrix} \rightarrow \begin{matrix}\text{Polymerase}\\ \text{synthesizing}\\ \text{RNA}\end{matrix}$$

We should like to emphasize some of the properties of nucleoids that make them attractive templates. They may be prepared in bulk from a wide variety of cells (*i.e.* fibroblasts, lymphocytes, erythroblasts, teratocarcinoma and epithelial cells of men, mice, birds, frogs and insects [2] (and Cook, P. R. and Brazell, I. A. unpublished results). The DNA packaged within nucleoids is largely intact and its supercoiling provides a sensitive index of DNA integrity. Unlike pure DNA which is fragile and easily broken by pipetting [69,70], nucleoid DNA can be pipetted without breakage [8]. Unfortunately nucleoid DNA slowly unfolds on incubation. However, the unfolding of nucleoid DNA takes place much less rapidly than the DNA of whole nuclei (Warren, A. and Cook, P. R., unpublished results). Nucleoids lack endogenous RNA polymerases and histones and contain few non-histone proteins characteristic of chromatin [8]. They are excellent templates for the RNA polymerase of *E. coli*. Whereas the transcription *in vitro* of nuclei and chromatin generally involves the elongation of pre-existing chains [35,46,47,71] all synthesis in nucleoids depends upon the initiation of new RNA chains. Since transcription *in vivo* is likely to be controlled at initiation these features make nucleoids attractive for studies on the control of transcription.

We thank Alan Jones for determining the sizes of various samples of DNA, Professor A. Vaciago for a gift of rifampicin, Professor Henry Harris for his continued support and encouragement and Drs J. B. Gurdon and A. Travers for reading the manuscript. This work was supported by a grant to P.R.C. from the Cancer Research Campaign.

REFERENCES

1. Cook, P. R. & Brazell, I. A. (1975) *J. Cell. Sci. 19*, 261–279.
2. Cook, P. R. & Brazell, I. A. (1976) *J. Cell Sci. 22*, 287–302.
3. Stonington, O. G. & Pettijohn, D. E. (1971) *Proc. Natl Acad. Sci. U.S.A. 68*, 6–9.
4. Worcel, A. & Burgi, E. (1972) *J. Mol. Biol. 71*, 127–147.
5. Hecht, R. M. & Birnstiel, M. L. (1972) *Eur. J. Biochem. 29*, 489–499.
6. Beebee, T. J. C. & Butterworth, P. H. W. (1974) *Eur. J. Biochem. 44*, 115–122.
7. Chambon, P. (1974) *The Enzymes*, 3rd edn, vol. 10 (Boyer, P. D., ed.) pp. 261–331, Academic Press, New York.
8. Cook, P. R., Brazell, I. A. & Jost, E. (1976) *J. Cell. Sci. 22*, 303–324.
8a. Cook, P. R. & Brazell, I. A. (1977) *Eur. J. Biochem. 74*, 527–531.
9. Westphal, H. (1970) *2nd Lepetit Colloquia on Biology and Medicine* (Silvestri, L., ed.) pp. 77–87, North Holland, Amsterdam.
10. Hayashi, Y. & Hayashi, M. (1971) *Biochemistry, 10*, 4212–4218.
11. Botchan, P., Wang, J. C. & Echols, H. (1973) *Proc. Natl Acad. Sci. U.S.A. 70*, 3077–3081.
12. Richardson, J. P. (1974) *Biochemistry, 13*, 3164–3169.
13. Wang, J. C. (1974) *J. Mol. Biol. 87*, 797–816.
14. Zimmer, S. G. & Milette, R. L. (1975) *Biochemistry, 14*, 300–307.
15. Burgess, R. R. & Travers, A. A. (1971) in *Procedures in Nucleic Acid Research* (Cantoni, G. L. & Davies, D. R., eds) vol. 2, pp. 851–863, Harper and Row, New York.
16. Pudley, M., Varma, M. G. R. & Leake, C. J. (1973) *Experientia (Basel) 29*, 466–467.
17. Knowland, J. S. (1970) *Biochim. Biophys. Acta, 204*, 416–429.
18. Bramwell, M. E. & Harris, H. (1967) *Biochem. J. 103*, 816–830.
19. Miller, L. & Knowland, J. (1970) *J. Mol. Biol. 53*, 329–338.
20. Benbow, R. M., Mayol, R. F., Picchi, J. C. & Sinsheimer, R. L. (1972) *J. Virol. 10*, 99–114.
21. Giorno, R., Stamato, T., Lydersen, B. & Pettijohn, D. (1975) *J. Mol. Biol. 96*, 217–237.
22. Palmiter, R. O. (1972) *J. Biol. Chem. 247*, 6770–6780.
23. Lowry, D. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
24. Burton, K. (1956) *Biochem. J. 62*, 315–323.
25. Fleck, A. & Munro, H. N. (1962) *Biochim. Biophys. Acta, 55*, 571–583.
26. Crawford, L. V. & Waring, M. J. (1967) *J. Mol. Biol. 25*, 25–30.
27. Wang, J. C. (1969) *J. Mol. Biol. 43*, 263–272.
28. Bauer, W. & Vinograd, J. (1971) *Progr. Mol. Subcell. Biol. 2*, 181–215.
29. Wang, J. C. (1969) *J. Mol. Biol. 43*, 25–39.
30. Upholt, W. B., Gray, H. B. & Vinograd, J. (1971) *J. Mol. Biol. 61*, 21–38.
31. Hinton, D. M. & Bode, V. C. (1975) *J. Biol. Chem. 250*, 1061–1070.
32. Shure, M. & Vinograd, J. (1976) *Cell, 8*, 215–226.
33. Sarma, M. H., Feman, E. R. & Baglioni, C. (1976) *Biochim. Biophys. Acta, 418*, 29–38.
34. Fuchs, E., Millette, R. L., Zillig, W. & Walter, G. (1967) *Eur. J. Biochem. 3*, 183–193.
35. Marzluff, W. F., Murphy, E. C. & Huang, R. C. (1973) *Biochemistry, 12*, 3440–3446.
36. Busiello, E. & Di Girolamo, M. (1975) *Eur. J. Biochem. 55*, 61–70.
37. Udvardy, A. & Seifart, K. H. (1976) *Eur. J. Biochem. 62*, 353–363.
38. Takanami, M., Okamoto, T. & Sugiura, M. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 179–187.
39. Matsukage, A. (1972) *Mol. Gen. Genet. 118*, 11–22.
40. Travers, A., Baillie, D. L. & Pedersen, S. (1973) *Nat. New Biol. 243*, 161–163.
41. Anthony, D. D., Zeszotek, E. & Goldthwait, D. A. (1966) *Proc. Natl Acad. Sci. U.S.A. 56*, 1026–1033.
42. Richardson, J. P. (1966) *J. Mol. Biol. 21*, 83–114.
43. Di Mauro, E., Synder, L., Marino, P., Lamberti, A., Coppo, A. & Tocchini-Valentini, G. P. (1969) *Nature (Lond.) 222*, 533–537.
44. Sippel, A. E. & Hartmann, G. R. (1970) *Eur. J. Biochem. 16*, 152–157.
45. Cook, P. R. & Brazell, I. A. (1976) *Nature (Lond.) 263*, 679–682.
46. Marzluff, W. F., Murphy, E. C. & Huang, R. C. (1974) *Biochemistry, 13*, 3689–3696.
47. Marzluff, W. F. & Huang, R. C. (1975) *Proc. Natl Acad. Sci. U.S.A. 72*, 1082–1086.
48. Bremer, H., Konrad, M. & Bruner, R. (1966) *J. Mol. Biol. 16*, 104–117.
49. Chamberlin, M. J. (1974) *The Enzymes*, 3rd edn, vol. 10 (Boyer, P. D., ed.) pp. 333–374, Academic Press, New York.
50. Chamberlin, M. J. (1974) *Annu. Rev. Biochem. 43*, 721–775.
51. Mangel, W. F. & Chamberlin, M. J. (1974) *J. Biol. Chem. 249*, 2995–3001.
52. Hinkle, D. C. & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 197–207.

53. Dean, C. J., Ormerod, M. G., Serianni, R. W. & Alexander, P. (1969) *Nature (Lond.) 222*, 1042–1044.
54. Richardson, J. P. (1973) *J. Mol. Biol. 78*, 703–714.
55. Richardson, J. P. (1970) *J. Mol. Biol. 49*, 235–240.
56. Cedar, H. & Felsenfeld, G. (1973) *J. Mol. Biol. 77*, 237–254.
57. Tsai, M., Schwartz, R. J., Tsai, S. Y. & O'Malley, B. (1975) *J. Biol. Chem. 250*, 5165–5174.
58. Mangel, W. F. & Chamberlin, M. J. (1974) *J. Biol. Chem. 249*, 3002–3006.
59. Mangel, W. F. & Chamberlin, M. J. (1974) *J. Biol. Chem. 249*, 3007–3013.
60. Travers, A. (1974) *Cell, 3*, 97–104.
61. Saucier, J. M. & Wang, J. C. (1972) *Nat. New Biol. 239*, 167–170.
62. Vinograd, J., Lebowitz, J. & Watson, R. (1968) *J. Mol. Biol. 33*, 173–197.
63. Davidson, N. (1972) *J. Mol. Biol. 66*, 307–309.
64. Hsieh, T. & Wang, J. C. (1975) *Biochemistry, 14*, 527–535.
65. Domingo, E., Escarmis, C. & Warner, R. C. (1975) *J. Biol. Chem. 250*, 2872–2877.
66. Puga, A. & Tessman, I. (1973) *J. Mol. Biol. 75*, 83–97.
67. Cook, P. R. (1973) *Nature (Lond.) 245*, 23–25.
68. Cook, P. R. (1974) *Biol. Rev. 49*, 51–84.
69. Burgi, E. & Hershey, A. D. (1961) *J. Mol. Biol. 3*, 458–472.
70. Levinthal, C. & Davison, P. F. (1961) *J. Mol. Biol. 3*, 676–683.
71. Price, R. & Penman, S. (1972) *J. Mol. Biol. 70*, 435–450.

A. Colman, Department of Biological Sciences, University of Warwick, Coventry, Warwickshire, Great Britain, CV4 7AL

P. R. Cook*, The Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, Great Britain, OX1 3RE

* To whom correspondence should be addressed.