J. Cell Sci. 50, 209–224 (1981)
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RECONSTRUCTION OF COMPLEXES OF HISTONE AND SUPERHELICAL NUCLEAR DNA

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SUMMARY

When HeLa cells are lysed in solutions containing a non-ionic detergent and 2 M-NaCl, structures are released that retain many of the morphological features of nuclei. These nucleoids contain all the nuclear RNA and DNA but few of the proteins characteristic of chromatin. Their DNA is supercoiled and so intact. Using a simple and rapid procedure we have reconstructed nucleohistone complexes from nucleoids and the 'core' histones without breaking the DNA. We have probed the integrity and structure of the reconstructed complexes using a non-destructive fluorometric approach, which provides a general method for detecting agents that bind to DNA and alter its supercoiling. The superhelical status of the DNA in the reconstructed complexes is indistinguishable from that found in control nucleoids containing core histones. Experiments with micrococcal nuclease confirm that the DNA in the reconstructed complexes is organized into nucleosome-like structures. These, however, are spaced 145 base-pairs apart and not 200 base-pairs apart as is found in native chromatin.

INTRODUCTION

The nuclear DNA of higher cells may now be prepared free of histones and other chromatin proteins without being broken (Cook & Brazell, 1975, 1978; McCready, Akrigg & Cook, 1979). When living HeLa cells are lysed in solutions containing a non-ionic detergent and 2 M-NaCl, structures are released that resemble nuclei. These nucleoids contain naked nuclear DNA within a cage of fibrous protein. We are currently trying to reconstruct chromatin using these nucleoids. They have 3 major advantages for reconstruction studies. First, their DNA is supercoiled and so intact; supercoiling in small circles of DNA influences the binding of histones (Germond *et al.* 1975; Camerino-Otero & Felsenfeld, 1977; Vogel & Singer, 1975) and other proteins (Javaherian, Liu & Wang, 1978). Second, unlike free DNA, which is fragile and easily broken by shearing forces (Burgi & Hershey, 1961; Levinthal & Davison, 1961), nucleoids can be pipetted without breaking their DNA (Cook & Brazell, 1978). Third, we show here that complexes of histone and nucleoids can be prepared rapidly at physiological salt concentrations without aggregation, unlike those with pure nuclear DNA (Fredericq, 1971). The reconstructed complexes are stable and the intact DNA of the nucleoid remains unbroken by the procedure. (Procedures for rapidly reconstructing chromatin from much simpler templates have only recently been devised (Laskey, Honda, Mills & Finch, 1978; Germond, Rouvière-Yaniv, Yaniv & Brutlag, 1979; Ruiz-Carrillo, Jorcano, Eder & Lurz, 1979).) As we were

concerned with maintaining the integrity of the DNA, we had to develop a method to monitor this during reconstruction; such a fluorometric method, using the intercalating dye, ethidium, is described in the accompanying paper (Levin & Cook, 1981). We also analysed the fidelity of reconstruction by fragmenting the DNA using staphylococcal nuclease.

MATERIALS AND METHODS

Chemicals

Radiochemicals were obtained from the Radiochemical Centre, Amersham. Radioactivity was counted as described (Cook, Brazell & Jost, 1976). Chemicals and enzymes were obtained from the following sources: poly-L-lysine, type VI-B of approximate molecular weight 15000 (Sigma, Kingston-upon-Thames, Surrey); proteinase 'K' (Boehringer); pancreatic ribonuclease A (EC 3.1.4.22) and micrococcal nuclease (EC 3.1.4.7) (Worthington Biochemical Corp.); the restriction nuclease *Hae*III (a kind gift from Dr R. McKay); Pronase (B grade, Calbiochem).

Nucleoids and nuclei

Techniques for isolating nuclei and nucleoids from HeLa cells, for staining, counting, photographing and manipulating them, and for investigating the conformation of DNA by fluorometry, have been described (Cook & Brazell, 1978; Levin & Cook, 1981; Cook, Brazell & Jost, 1976). The DNA content of a HeLa nucleoid or nucleus was assumed to be 12 pg (Colman & Cook, 1977).

Histones

Histones were isolated from chromatin prepared from calf thymus (Zubay & Doty, 1959). All buffers contained 1 mM-phenylmethylsulphonylfluoride. Chromatin was solubilized by stirring in 10 mM-Tris (pH 8·0), and the histones separated from DNA by chromatography on agarose beads (Henson & Walker, 1970). Alternatively, histones were isolated by differential centrifugation (Beckman type 30 rotor, 30000 rev./min; 14 h; 0 °C) and solubilization of the chromatin pellet in buffers containing 0·65 M-NaCl (elutes H1), 1·2 M-NaCl (elutes H2A and H2B) and 2 M-NaCl (elutes H3 and H4).

'Core' histones and H2A and H2B were isolated free (> 97 %) of any contamination detectable on gels. They were stable on ice for at least 2 weeks and were used within this period. Samples of H3 and H4 prepared by centrifugation were contaminated by H2A and H2B (up to 20 % of total protein). As histone H3 degraded rapidly, samples of H3 and H4 were discarded 48 h after preparation. Histone concentrations were estimated from the absorbance at 230 and 280 nm (Johns, 1971; Camerino-Otero, Sollner-Webb & Felsenfeld, 1976).

Analysis of proteins and DNA

Proteins were separated by electrophoresis on polyacrylamide gel slabs containing sodium dodecyl sulphate (Kornberg & Thomas, 1975) and analysed by planimetry (Levin, Jost & Cook, 1978).

DNA was extracted from nuclei and nucleoids (Kornberg, 1977) and subjected to electrophoresis (6 V cm^{-1} or 10 V cm^{-1}) in slab gels containing 10 % acrylamide (Peacock & Dingman, 1967; Axel, Melchior, Sollner-Webb & Felsenfeld, 1974) or 2·25 % acrylamide, 0·75 % agarose (Loening, 1967). After electrophoresis the gels were stained with ethidium, photographed and the developed films analysed by densitometry. Size markers were obtained by digesting bacteriophage PM2 DNA (Boehringer GmbH, Mannheim) with the restriction enzyme *Hae*III (Noll, 1976).

Reconstruction

Nucleoids, isolated from 'step' gradients containing 1·95 M-NaCl were diluted to 5×10^6 ml^{-1} (60 μg DNA ml^{-1}) in 1·95 M-NaCl, 10 mM-Tris (pH 8·0), 1 mM-phenylmethylsulphonylfluoride and mixed with various amounts of histone at 0 °C. After 30 min histone was bound to the nucleoids by reducing the salt concentration progressively to 1·2 M, 1·0 M and finally 0·75 M-NaCl over 60–120 min using 10 mM-Tris (pH 8·0) and in some cases, 0·075 M-guanidine thiocyanate. Sometimes the reconstructed complexes were isolated free of unbound histone by sedimentation (2500 *g*; r_{av} = 10 cm; 2 °C; 25 min) onto a step of 42·5 % sucrose, 10 mM-Tris (pH 8·0), and the appropriate salt concentration. The reconstructed complexes were then diluted with 10 mM-Tris (pH 8·0) to 0·2 M-NaCl in steps (i.e. 0·6, 0·5, 0·4 and 0·2 M). Reconstructed complexes containing histone:DNA in a 1:1 ratio were prepared using an input ratio of 2·5:1 and were used in all experiments unless stated otherwise. The histones were present in such reconstructed complexes in equimolar amounts (results of planimetric analysis of sodium dodecyl sulphate/polyacrylamide gels not shown).

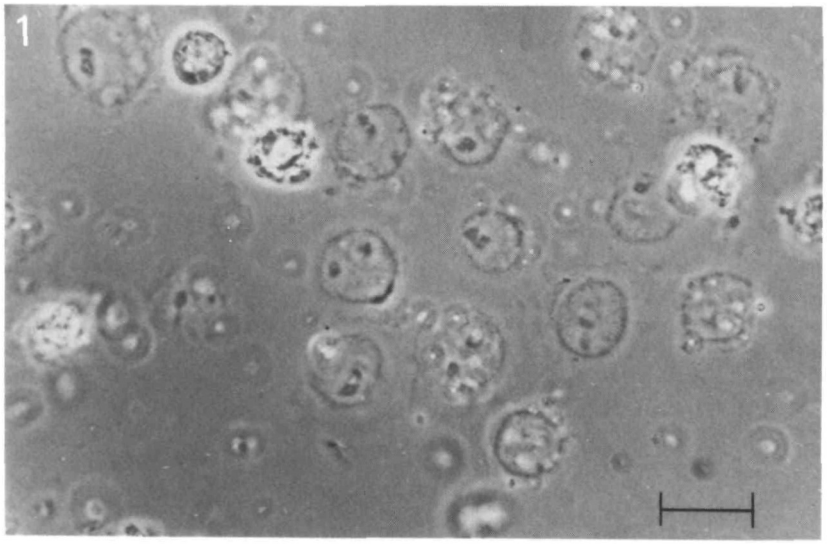
Digestion with micrococcal nuclease

The DNA of nucleoids and nuclei was cleaved *in vitro* using micrococcal nuclease (Noll & Kornberg, 1977). Nuclei or nucleoids were isolated from HeLa cells grown in the presence of [*methyl*-^{3}H]thymidine (56000 Ci mol^{-1}, 0·2 μCi ml^{-1}) for 24 h, diluted to between 0·5 and $2{\cdot}5 \times 10^6$ ml^{-1} in 10 mM-Tris (pH 8·0), 5 mM-$CaCl_2$ and either 0·175 M-NaCl or 0·1 M-NaCl and 0·075 M-guanidine thiocyanate. The mixtures were incubated at 37 °C for 15 min and digestion initiated by adding nuclease (final concn 5 to 200 units ml^{-1}; 1 unit of enzyme is defined by Heins, Taniuchi & Anfinsen, 1966), and stopped at various times by adding EDTA to a final concn of 10 mM. The amount of radioactivity insoluble in trichloroacetic acid was determined as described (Colman & Cook, 1977), and sizes of DNA fragments were determined after separation in polyacrylamide gels (Kornberg, 1977). Bandwidths were determined from densitometer tracings of the second fastest band by measuring the distance separating tangents to the leading and trailing sides of the peak at the level of the minimum between the second and third peaks.

RESULTS

Nucleoids isolated in 1·95 M-NaCl contain no histones and few other proteins characteristic of chromatin (Levin, Jost & Cook, 1978). We used these as reference nucleoids. Nucleoids isolated in 0·75 M-NaCl contain the core histones and we have compared these with reconstructed complexes made from nucleoids isolated in 1·95 M-NaCl and histones purified from calf thymus.

Like histone and DNA (Fredericq, 1971), very high concentrations of histones and nucleoids aggregate in 0·2 M-NaCl. Remarkably, the aggregates may be dispersed by gentle agitation or eliminated by using low concentrations of nucleoids (i.e. $< 5 \times 10^5$ ml^{-1}). Since fluorometric experiments are especially subject to aggregation artifacts, we have not only used low nucleoid concentrations (i.e. 2×10^5 ml^{-1}) but also repeated all the fluorometric experiments described using a chaotropic agent to suppress any undetected aggregation. Of the chaotropes tested, 0·075 M-guanidine thiocyanate or potassium thiocyanate prevented aggregation, but not sodium thiocyanate, ammonium thiocyanate or urea. Although at very much higher concentrations these agents denature DNA and proteins (von Hippel & Wong, 1964; Castellino & Barker, 1968), at low concentrations they only break weak hydrophobic bonds (Hatefi & Hanstein, 1969). Furthermore, 0·075 M-guanidine thiocyanate does not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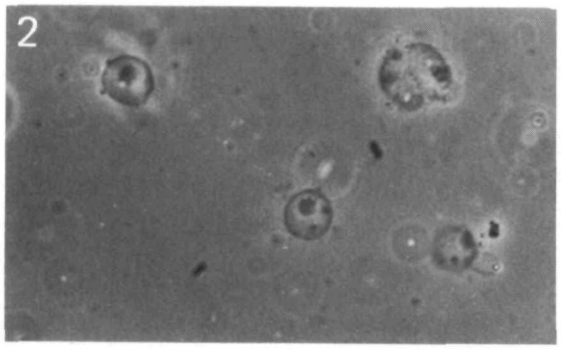
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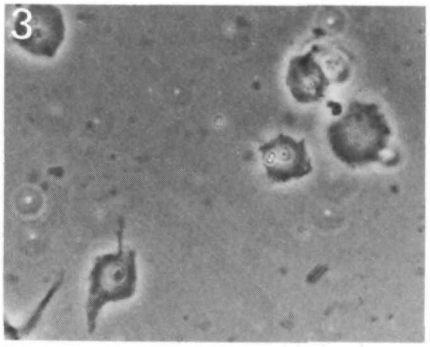
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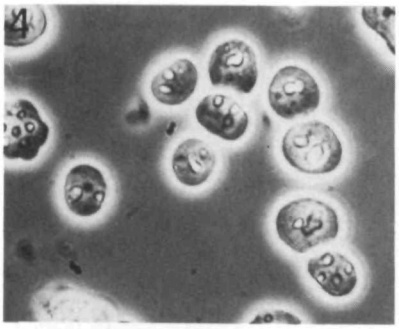
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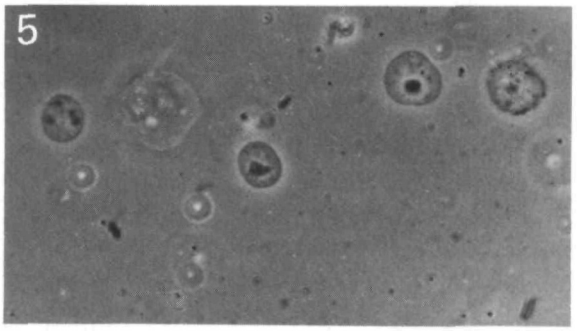
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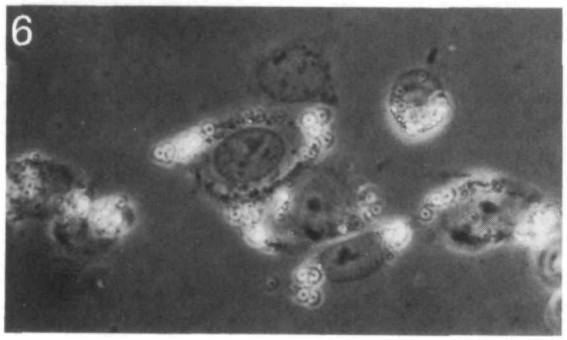
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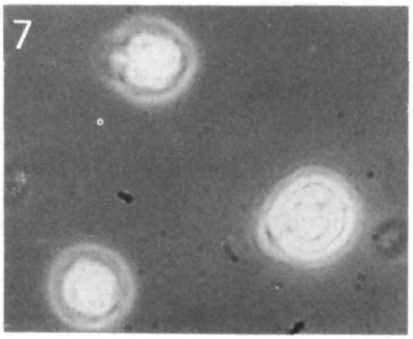
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alter nucleosome spacing (see below). In 0·2 M-NaCl, guanidine thiocyanate has little effect on the fluorescence of ethidium, but in 2 M-NaCl the fluorescence is slightly enhanced (results not shown). When the appropriate corrections are made for this enhancement, the chaotrope has little effect on the difference between intercalative binding to unirradiated and irradiated nucleoids (see accompanying paper).

Reconstructed complexes are prepared simply by mixing the nucleoids and purified histones in 2 M-NaCl and then reducing the salt concentration by dilution. As in the binding to pure DNA (Ohlenbusch, Olivera, Tuan & Davidson, 1967; Burton, Hyde & Walker, 1975; Wilhelm, Wilhelm, Erard & Daune, 1978), first the arginine-rich histones bind, then the slightly lysine-rich histones and finally histone H1 (results not shown). We first studied the reconstructed complexes by phase-contrast microscopy.

Nucleoids prepared in 1·95 M-NaCl are much paler than nuclei isolated by conventional procedures (cf. Figs. 1, 4) and they remain paler when the salt concentration is reduced to 0·2 M-NaCl (Fig. 2). Nucleoids isolated in 0·75 M-NaCl are darker and can be converted by vigorous pipetting to structures that resemble chromatin prepared by the procedure of Zubay & Doty (1959). Fig. 3 illustrates these nucleoids and some intermediate forms. Complexes of nucleoids isolated in 1·95 M-NaCl and core histones are refractile structures (illustrated in Fig. 7).

Nucleoids isolated in 1·95 M-NaCl are considerably larger than nuclei (cf. Figs. 1, 4). They shrink as the salt concentration is reduced (Table 1) and so do not behave as osmometers. Like pure DNA, which is condensed by histones, bound histones reduce the size of nucleoids (Table 1). However, excess histone alone cannot condense nucleoids to the size of nuclei.

Nuclei isolated by conventional procedures and the reconstructed complexes both have a clearly defined periphery; on the other hand nucleoids isolated in 1·95 M-NaCl are generally surrounded by an irregular 'halo' containing membrane blebs and other adherent material (Fig. 1). The halo is cytoplasmic in origin since 'nucleoids' prepared from isolated nuclei lack it (Fig. 5). Nucleoids also contain proteins with the

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Figs. 1–7. Phase-contrast micrographs of nuclei and various types of nucleoids. Bar, 20 μm. All figures are at the same magnification.

Fig. 1. Nucleoids isolated in 1·95 M-NaCl from cells grown in suspension and photographed in 1·95 M-NaCl.

Fig. 2. Nucleoids similar to those in Fig. 1 diluted to 0·2 M-NaCl.

Fig. 3. Nucleoids isolated in 0·75 M-NaCl from cells grown in suspension and photographed after dilution to 0·2 M-NaCl. They have been vigorously pipetted; note the partially extended form resembling chromatin.

Fig. 4. Nuclei isolated by a conventional procedure.

Fig. 5. Nuclei similar to those illustrated in Fig. 4 mixed with the lysis mixture containing 1·95 M-NaCl and Triton X-100 used to prepare the nucleoids illustrated in Fig. 1.

Fig. 6. Nucleoids in 1·95 M-NaCl prepared from HeLa cells growing as a monolayer, using the same lysis mixture as that used in Figs. 1 and 5.

Fig. 7. A histone–nucleoid complex formed by diluting to 0·2 M-NaCl mixtures of core histones and nucleoids isolated in 1·95 M-NaCl.

Table 1. *The sizes of various nuclear derivatives containing different amounts of core histones*

Nuclear derivative	Content of core histone (%)	Mean diameter (μm) (± S.D.)
Nucleoids isolated in 1·95 M-NaCl	0	12 ± 2
Nucleoids isolated in 0·75 M-NaCl	95	6 ± 1
Reconstructed complexes with core histone	100	11 ± 2
Reconstructed complexes with excess core histone	113	8 ± 2
Nuclei	100	5·5 ± 1

Nucleoids, reconstructed complexes and nuclei were diluted to 0·15 M-NaCl using 10 mM-Tris (pH 8·0) and the appropriate salt concentration, attached to glass slides by incubation for 10 min at 20 °C, fixed in methanol, stained with Giemsa and the average diameter measured using a microscope fitted with a graticule. The means are of at least 100 measurements. Core histone contents of equal numbers of nucleoids, reconstructed complexes and nuclei were determined using polyacrylamide gels and are expressed as a percentage of the content found in nuclei. Reconstructed complexes with a core histone content of 113 % were prepared using an input ratio (histone:DNA) of 5:1.

characteristics of actin and intermediate filaments (Levin, 1978). Furthermore, when prepared *in situ* from cells growing as a monolayer nucleoids retain the outline of the cytoplasm (Fig. 6). During reconstruction, these cytoplasmic remnants must condense onto the nuclear cage.

We next used ethidium to probe the structure of the resulting complexes by fluorometry. The binding of ethidium can be described in 2 ways. The relative fluorescence is the fluorescence of ethidium bound at one salt concentration expressed as a percentage relative to that bound by the same number of unirradiated reference nucleoids in 2·0 M-NaCl; it is a direct measure of the amount of bound dye. Since histone-binding prevents the binding of the dye, the relative fluorescence inversely reflects the amount of histone bound. The ratio is the relative fluorescence obtained with unirradiated nucleoids divided by that obtained with nucleoids that have been γ-irradiated and so contain broken DNA; it reflects the sense and degree of supercoiling in the DNA of unirradiated nucleoids since only intercalative binding is affected by γ-irradiation. Ratios greater than unity indicate the presence of free energy of supercoiling associated with negative supercoiling (i.e. $\tau < 0$, using the nomenclature of Bauer & Vinograd (1974)).

Reconstruction with core histones

We have established how DNA folding is altered by the *dissociation* of histones in the accompanying paper (Levin & Cook, 1981). Here we describe the changes on *reassociation*. The NaCl concentration of mixtures of core histones and nucleoids was reduced from 2 M (Fig. 8A). The histones lessen the increase in relative fluorescence seen in their absence; the histones bind to the DNA so preventing ethidium from binding. At all salt concentrations studied, more ethidium binds to unirradiated nucleoids that have been freed of all histones than it does to their γ-irradiated counter-

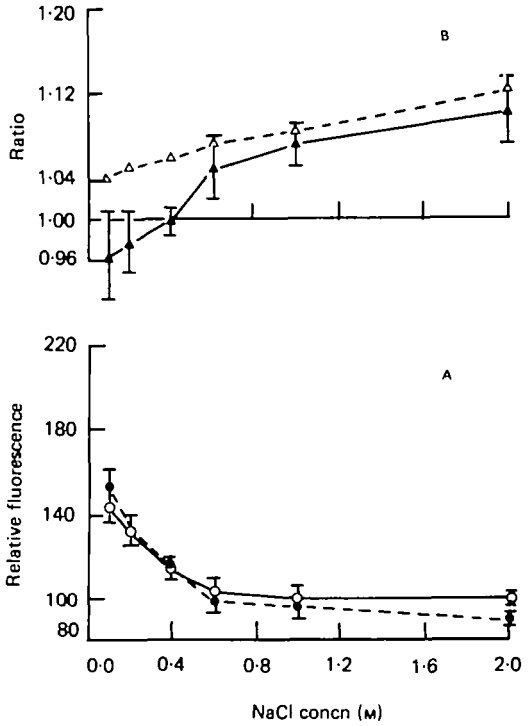

Fig. 8. Binding of core histones to irradiated and unirradiated nucleoids isolated in 1·95 M-NaCl. Histones were bound to nucleoids by reducing the NaCl concn from 2 M to a variety of different concentrations; after the addition of ethidium, the extent and nature of the histone binding was monitored by fluorometry. Nucleoids, isolated from step gradients containing 1·95 M-NaCl were diluted to $2{\cdot}0 \times 10^6$ ml^{-1} using 2 M-NaCl, 10 mM-Tris (pH 8·0). Some nucleoids were irradiated. Core histones in 2 M-NaCl, 10 mM-Tris (pH 8·0), 0·1 mM-phenylmethylsulphonylfluoride were added to a final concn of 30 μg ml^{-1} to give a histone to DNA ratio (w/w) of 1·25:1. Mixtures were then incubated for 30 min on ice. The nucleoids were diluted over a period of 60 min to $0{\cdot}2 \times 10^6$ ml^{-1} and various salt concentrations using 10 mM-Tris (pH 8·0) and the appropriate NaCl concn. Ethidium was added and the fluorescence measured. 0·075 M-guanidine thiocyanate was present in all dilution buffers. A. The fluorescence of mixtures of core histones and unirradiated (○) and irradiated (●) nucleoids is expressed as a percentage relative to that of unirradiated reference nucleoids in 2 M-NaCl. B. The ratio (▲) for each salt concn is the relative fluorescence of unirradiated nucleoids divided by that of irradiated nucleoids. Error bars give the standard deviation of the means obtained from at least 4 different experiments like that described in A. The corresponding ratios (△) obtained in the absence of the core histones are taken from fig. 1 in the accompanying paper (Levin & Cook, 1981).

parts with relaxed DNA; the ratio is always greater than unity, indicative of negative supercoiling. In contrast to such histone-free nucleoids, irradiated nucleoids complexed with histones bind more ethidium below 0·4 M than their unirradiated counterparts (Fig. 8A); the ratio falls below unity (Fig. 8B). The histones remove free energy of supercoiling, presumably by folding the DNA, so that none remains to assist dye-binding.

These effects are reversible and the binding does not break the DNA. This can be

demonstrated by reconstructing complexes of nucleoids and histones, disrupting them in 2 M-NaCl, then freeing the nucleoids from the histones by sedimentation and finally measuring their ethidium-binding capacity. It is just like that of freshly prepared histone-free nucleoids (Table 2). Remarkably, the reconstructed complexes may be stored unaggregated for up to a week with their DNA largely intact since some supercoiling remains when the histones are removed.

Table 2. *The stability of the DNA in complexes of nucleoids and core histones*

	Ratio of relative fluorescence	
Time (h)	Reconstructed nucleoids freed of histones	Freshly isolated nucleoids
1	1·11	1·12
24	1·10	—
168	1·04	—

Core histones were bound to nucleoids by dilution from 2 M-NaCl to 0·1 M-NaCl, 0·075 M-guanidine thiocyanate and 10 mM-Tris (pH 8·0). The final concentration of unirradiated nucleoids and core histones was $1{\cdot}2 \times 10^6$ ml^{-1} (14·4 μg DNA ml^{-1}) and 36 μg ml^{-1}, respectively. The mixture was stored at 4 °C, and at various times reconstructed complexes were freed of histones by raising the salt concentration to 2 M-NaCl, using 10 mM-Tris (pH 8·0) and the appropriate salt concentration. Some samples were irradiated, ethidium was added and the fluorescence measured. The ratio is the relative fluorescence of unirradiated nucleoids divided by that of irradiated nucleoids. Nucleoids that were freshly prepared from cells in 2 M-NaCl served as controls; such nucleoids aggregated in the absence of histones after 24 h.

If the histones compete with ethidium for free energy of supercoiling, the binding of more and more histone to nucleoid DNA should progressively reduce dye intercalation. The addition of increasing amounts of core histone to nucleoids in 2·0 M-NaCl, followed by a reduction in the salt concentration to 0·75 M, increases histone binding (see legend to Fig. 9). The increased histone binding reduces the fluorescence of both irradiated and unirradiated nucleoids (Fig. 9A) and drives the ratio below unity (Fig. 9B). An approximately equal weight of core histone to that found in control nucleoids (isolated in 0·75 M) needs to be bound in the reconstructed complex to achieve a similar degree of positive supercoiling (cf. fig. 2A in the accompanying paper, Levin & Cook, 1981, and Fig. 9A here).

Reconstruction with histones H2A and H2B or H3 and H4

H2A and H2B bind to histone-free nucleoids at salt concentrations below 1·2 M-NaCl (results not shown) preventing ethidium from binding and so decreasing the relative fluorescence (Fig. 9C). The ratio falls and reaches a minimum value when equal weights of histone and nucleoid DNA are mixed (Fig. 9D). This reduction is very much smaller than that induced by a full complement of the core histones, but nevertheless it is quite reproducible (it cannot be due to contaminants as the protein samples used contained >97% H2A and H2B).

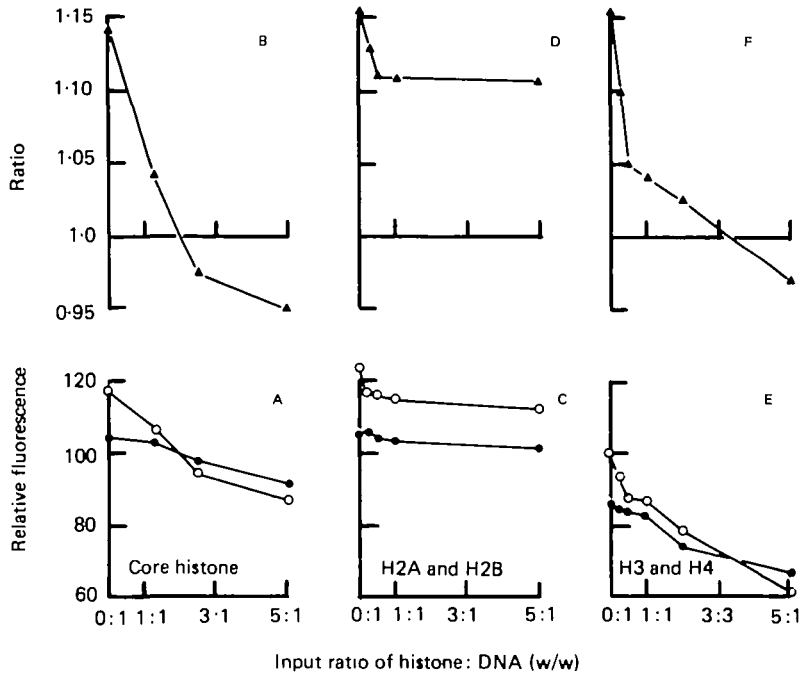

Fig. 9. The binding of ethidium to unirradiated and irradiated nucleoids complexed with various amounts and types of histone. Various types and amounts of histone were mixed with nucleoids in 1·95 M-NaCl and the histones bound by reducing the salt concn to 0·75 M-NaCl; after the addition of ethidium the extent and nature of the histone binding was monitored by fluorometry. Three different histone mixtures were used: A, B, core histones; C, D, H2A and H2B; E, F, H3 and H4. Nucleoids isolated from step gradients containing 1·95 M-NaCl were diluted to about $0{\cdot}5 \times 10^6$ ml^{-1} with 1·95 M-NaCl, 10 mM-Tris (pH 8·0), 0·1 mM-phenylmethylsulphonylfluoride. Some nucleoids were irradiated. The mixtures were incubated for 30 min on ice and diluted over a period of 60 min to $0{\cdot}2 \times 10^6$ nucleoids ml^{-1} in 0·75 M-NaCl using 10 mM-Tris (pH 8·0) and 0·1 M-phenylmethylsulphonylfluoride. Ethidium was added and the fluorescence measured. A, C, E. The fluorescence of unirradiated (○) and irradiated (●) nucleoids is expressed as a percentage relative to that of unirradiated reference nucleoids in 2 M-NaCl. B, D, F. The ratio (▲) for each histone:DNA combination is the relative fluorescence of unirradiated nucleoids divided by that of their irradiated counterparts. The results are the means from at least 4 different experiments. The input histone:DNA ratios (w/w) are given on the abscissa. The amount of histone bound to the unirradiated nucleoids was also determined using polyacrylamide gels as described in Materials and methods. In A the histone:DNA ratio (w/w) of the core histone bound was 0·67, 1·0 and 1·13 for the input histone:DNA ratios (w/w) of 1·25, 2·5 and 5·0, respectively. In C the corresponding ratios for H2A and H2B bound were 0·18, 0·33, 0·55, 1·1 and 1·4 at input ratios of 0·25, 0·5, 1, 2·5 and 5, respectively. The histones bound in equimolar amounts.

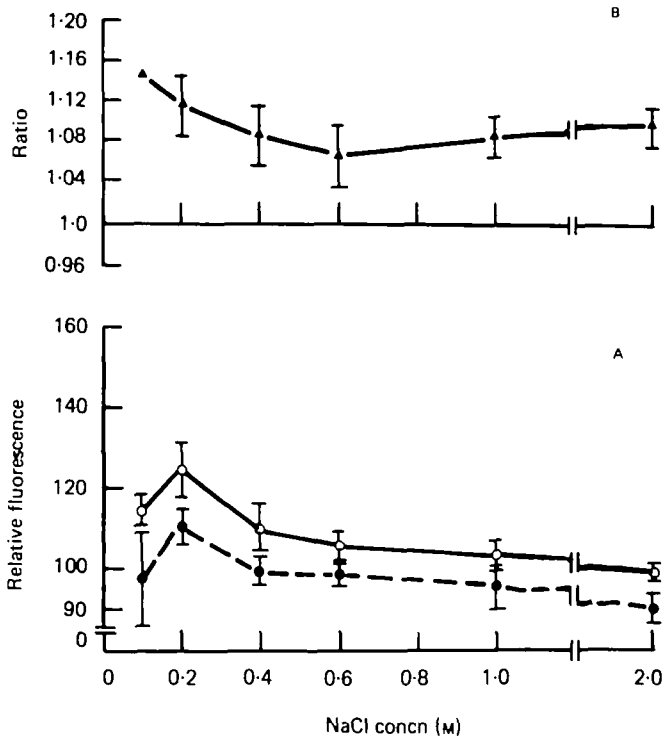

Fig. 10. Binding of polylysine to unirradiated and irradiated nucleoids isolated in 1·95 M-NaCl. Polylysine was bound to nucleoids by reducing the NaCl concentration from 2 M to a variety of different concentrations; after the addition of ethidium, the extent and nature of the polylysine binding was monitored by fluorometry. Conditions for isolating nucleoids in 1·95 M-NaCl, preparing mixtures of nucleoids, polylysine and ethidium, and for measuring their fluorescence are similar to those described in the legend to Fig. 9. The final polylysine:DNA ratio (w/w) was 0·44:1. A. The fluorescence of unirradiated (○) and irradiated (●) nucleoids is expressed as a percentage relative of that of unirradiated reference nucleoids in 2 M-NaCl. B. The ratio (▲) is the relative fluorescence of unirradiated nucleoids divided by that of irradiated nucleoids. Error bars give the standard deviations of the means obtained from at least 5 different experiments.

In contrast, the binding of H3 and H4 drives the ratio below unity (Fig. 9E, F); it is clearly these histones that remove the free energy. (Contamination by H2A and H2B cannot account for the large effects that we see; see Materials and methods.) More H3 and H4 is needed in the reconstructed complexes to remove all negative supercoiling than is found in control nucleoids isolated in 0·75 M-NaCl and which contain undissociated core histones (cf. fig. 2B in the accompanying paper and Fig. 9F here).

Reconstruction with poly-L-lysine

These effects of the histones on supercoiling might be non-specific in the sense that they might also be caused by other positively charged molecules. Therefore as a

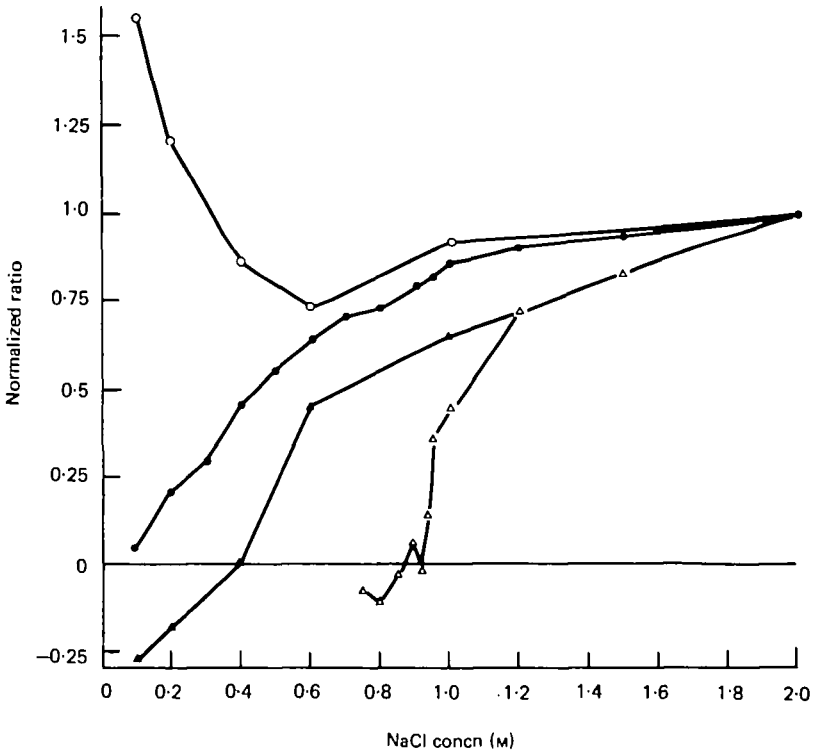

Fig. 11. A comparison of the effects of salt on the availability of free energy of supercoiling in the DNA of mixtures of nucleoids and the core histones or poly-L-lysine. The ratio of the relative fluorescence of unirradiated and irradiated nucleoids reflects the free energy of supercoiling in nucleoid DNA that is available to assist the binding of ethidium. The normalized ratios presented here summarize results illustrated in Figs. 8, 9 and 10 and in the accompanying paper. In each case, the difference from unity in the ratio obtained in 2·0 M-NaCl has been assigned the value of unity; ratios obtained at lower salt concentrations have been normalized with respect to this value. Ratios above and below unity yield positive and negative normalized ratios, respectively. (●) Histone-free nucleoids; (▲) mixtures of histone-free nucleoids and core histones; (△) nucleoids isolated in 0·75 M-NaCl and containing the core histones; (○) mixtures of histone-free nucleoids and poly-L-lysine. Salt concentrations were progressively reduced from 2 M, except in the experiment with nucleoids isolated in 0·75 M-NaCl, in which the salt concentration was progressively increased from 0·75 M to 2 M.

control we studied the binding of the histone analogue, poly-L-lysine, a molecule that has been studied extensively (Akinrimisi, Bonner & Ts'o, 1965; Tsuboi, Matsuo & Ts'o, 1966). Poly-L-lysine was mixed with histone-free nucleoids and the salt concentration reduced (Fig. 10). The relative fluorescence initially rises only slightly as polypeptide-binding limits dye-binding. Below 0·2 M-NaCl the relative fluorescence falls as the poly-L-lysine–nucleoid complexes precipitate (precipitation occurs even in the presence of guanidine thiocyanate). The analogue suppresses dye-binding to a very much greater extent than an equivalent weight of histone (cf. Figs. 9, 10) but has

little effect on the ratio (Fig. 10B); although it binds to the nucleoid DNA, the binding has little effect on supercoiling.

The effects of the core histones and poly-L-lysine on the binding of ethidium by nucleoids are summarized in Fig. 11. The normalized ratios reflect the availability of free energy of supercoiling in the various mixtures – the greater the ratio, the more positive free energy. It is clear that the core histones remove free energy on binding, unlike poly-L-lysine.

Table 3. *The size of polynucleotide fragments released from nucleoids digested with micrococcal nuclease*

	Nucleoids isolated in 0·75 M-NaCl (base-pairs)				Nuclei (base-pairs)
Band no.	5 %	11 %	20 %	33 %	13 %
1	165	165	165	155	165
2	360	320	210	200	330
3	560	560	310	290	540
4	800	790	490	360	740
5	1000	990	530	410	950
6	1210	1200	570	460	1150
7	1420	1380	620	530	
8	1650		740	630	
9	1850		930	710	
Repeat length	204				204 ± 6

Nucleoids were isolated from step gradients containing 0·75 M-NaCl, diluted to $0{\cdot}5 \times 10^6$ ml^{-1} in 0·1 M-NaCl, 0·075 M-guanidine thiocyanate, 5·0 mM-$CaCl_2$ and 10 mM-Tris (pH 8) and digested with micrococcal nuclease (50 units ml^{-1}) for various periods. Samples of DNA from nucleoids (digested until various percentages of the DNA were reduced to acid-soluble oligonucleotides) were separated by electrophoresis in polyacrylamide/agarose gels and the size of the fragments in the bands determined. Bands were numbered from the one that had migrated the furthest. The sizes of fragments produced by digestion of HeLa nuclei are included for comparison. All methods are described in Materials and methods. Control experiments indicated that the presence of guanidine thiocyanate did not affect the fragmentation pattern (see also Fig. 12).

Digestion of nucleoids with micrococcal nuclease

Micrococcal nuclease cleaves the DNA between the subunits in native chromatin to yield a characteristic series of DNA fragments, which are multiples of 200 base-pairs, and so this enzyme is the probe used most commonly to monitor the fidelity of reconstruction (Kornberg, 1977). Micrococcal nuclease digests the DNA of nucleoids isolated in 0·75 M-NaCl 3–5 times more rapidly than that of nuclei (results not shown). The DNA of such nucleoids containing core histones was digested with micrococcal nuclease until 5 % and 11 % became acid-soluble, and the remaining DNA was subjected to electrophoresis on polyacrylamide/agarose gels. After the gels had been stained with ethidium, up to 10 bands could be seen, the average difference in size between successive bands being 204 base-pairs (Table 3). The same value was obtained with nuclei, suggesting that DNA–histone complexes are similarly organized

in both templates despite the absence of H1. Remarkably, the band-widths of the fragments from nuclei and these nucleoids are similar (i.e. about 60 base-pairs), unlike the broad bands obtained by digesting H1-depleted chromatin prepared by conventional procedures (Noll & Kornberg, 1977). At later stages during digestion the fragmentation pattern of the DNA of these nucleoids is extremely complex.

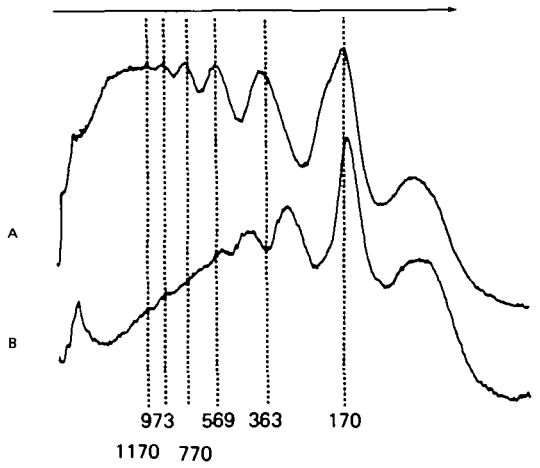

Fig. 12. The patterns of DNA fragments released from nuclei and reconstructed complexes by partial digestion with micrococcal nuclease. Nuclei (A) or complexes (B) of core histones with nucleoids isolated in 1·95 M-NaCl were digested with micrococcal nuclease in the absence of guanidine thiocyanate until 10% and 15%, respectively, of their DNA became acid-soluble. The undigested DNA was isolated and subjected to electrophoresis on a polyacrylamide/agarose gel. After staining, the gels were photographed and densitometer tracings prepared. All procedures are described in Materials and methods. The sizes (in base-pairs) of the fragments in the peaks were determined by reference to fragments of known size run in an adjacent channel. The arrow indicates the direction of migration.

Next we digested reconstructed complexes made from core histones and nucleoids isolated in 1·95 M-NaCl until 10% of their DNA became acid-soluble (the reconstructed complexes contained histone:DNA in a 1:1 ratio). Up to 5 bands of discretely sized fragments can sometimes be resolved superimposed upon a high background of polydisperse fragments (Fig. 12). The yield of discretely sized fragments is as high as the yields obtained with reconstructed complexes prepared by conventional procedures (see Steinmetz, Streeck & Zachau, 1978; and Tatchell & van Holde, 1977, for reviews). However, as others have found, the average difference between successive bands is 145 base-pairs and not the 200 base-pairs found in nuclei or in nucleoids isolated in 0·75 M-NaCl.

Despite these differences in the fragmentation pattern of partial digests, more complete digests show similarities. Micrococcal nuclease degrades only about half the DNA of chromatin since the released complexes are precipitated (Noll & Kornberg, 1977). The precipitated DNA is double-stranded and about 50–160 base-pairs long; the larger fragments predominate (Camerino-Otero *et al.* 1976; Axel *et al.* 1974). The

same range of resistant fragments is obtained with the reconstructed complexes but the smaller fragments predominate, i.e. fragments of 65, 80, 93, 105, 120 and 140 base-pairs. This is not surprising since up to three-quarters of the DNA of reconstructed complexes is digested, making comparisons difficult. However, 0·075 M-guanidine thiocyanate prevents precipitation so that nuclei can be digested to the same extent as fully digested reconstructed complexes; their patterns of resistant fragments are identical (results not shown).

DISCUSSION

Procedures for reconstructing chromatin from nuclear DNA are laborious and generally involve a lengthy dialysis to reduce the salt concentration from 2 M to 0·15 M (see Steinmetz *et al.* 1978; and Tatchell & van Holde, 1977, for reviews). We show here that reconstructed complexes can be prepared rapidly by diluting mixtures of nucleoids and purified core histones. The reconstructed complexes are stable for up to a week and their DNA remains unbroken by the procedure. The fluorometric experiments with ethidium show that the superhelical status of the DNA in these reconstructed complexes is indistinguishable from that of control nucleoids containing undisrupted complexes. However, the nucleosome-like structures are spaced 145 base-pairs apart and not 200 base-pairs apart as is found in native chromatin. Nevertheless, we hope that these reconstructed complexes will prove useful templates for transcriptional studies since we have shown that nucleoids, with their intact superhelical DNA, are excellent templates for RNA polymerases (Colman & Cook, 1977; Akrigg & Cook, 1980).

We thank Professor Henry Harris, F.R.S. for his continued encouragement, Iris Brazell for her help, Dr E. W. Johns for purified histone fractions and Dr R. McKay for the restriction enzyme *Hae*III. This research was supported by the Cancer Research Campaign. J.M.L. was supported by a grant from the Medical Research Council.

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(*Received* 13 *January* 1981)