Nucleosomes from Normal and Regenerating Rat Liver

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Micrococcal-nuclease digestion of rat liver nuclei selectively released mononucleosomes associated with ADP-ribosylated [Caplan, Ord & Stocken (1978) *Biochem. J.* 174, 475–483] histone H1. Two classes of mononucleosome were detected, those that leaked out during digestion and those that were subsequently released by 5 mM-sodium phosphate buffer (pH 6.8)/0.2 mM-NaEDTA. The former, from which histone H1 had been dissociated, contained 140-base-pair-length DNA and core histones; the latter contained core particles and mononucleosomes with histone H1 and 200-base-pair-length DNA. When normal liver nuclei were phosphorylated with [γ - ^{32}P]ATP, dissociated histone H1, which could be separated from core particles with Sephadex G-200, showed ^{32}P uptake. ^{32}P uptake into histones H2A and poly(ADP-ribosylated) H3 was appreciable in core particles, but was less evident in nucleosomes still containing histone H1. When [^3H]-thymidine was given to partially hepatectomized rats in S-phase, 5–10 min pulses in animals of over 300 g body wt. showed the presence of high-specific-radioactivity DNA in released core particles and mononucleosomes compared with DNA retained in the nuclear pellets. Mononucleosomes from rat livers in S-phase with new, [^3H]lysine-containing histones, had higher ^{32}P incorporation in histones H1 and their core histones, than for di- or tri-nucleosomes. Thermal-denaturation properties of control and phosphorylated mononucleosomes and core particles were very similar; removal of histone H1 and non-histone chromosomal proteins in 0.5 M-NaCl markedly increased the proportion of DNA 'melting' below 70°C.

The kinetics and extents of nuclease digestions in regenerating rat liver nuclei are indistinguishable through the first cell cycle (Caplan *et al.*, 1978), although enhanced sensitivity to digestion of newly replicated DNA by deoxyribonuclease I (Seale, 1975) and micrococcal nuclease (Hildebrand & Walters, 1976) has been detected after very short (less than 1 min) exposures to [^3H]thymidine. Similarly, with deoxyribonuclease II digestion and Mg^{2+} precipitation, nucleosomes have been isolated from transcriptionally active chromatin with properties different from those from inactive regions (Gottesfeld & Butler, 1977). Previous work with unfractionated chromatin showed variations in physical characteristics (transition temperature, c.d.) through the cell cycle (see Baserga, 1976). Nucleosomes were therefore prepared from regenerating rat livers in S-phase, so that their properties might be compared with those from livers in G_0 . The [^3H]thymidine-labelling patterns in the nucleosomes were markedly affected by the ages of the rats used. With older rats, mononucleosomes released from micrococcal-nuclease-digested lysed nuclei, isolated 5–15 min after injection of [^3H]thymidine, had higher specific radioactivities in their DNA than did the other nucleosomes. The mononucleosomes were heterogeneous (see also Varshavsky *et al.*, 1976), and contained both

200- and 140-base-pair DNA. From the ^{32}P content of histones H1, H2A, H3 and H4 it was evident that histones in new mononucleosomes were more phosphorylated than were those from rat nuclei in G_0 phase. Phosphorylation of normal rat liver nuclei *in vitro*, with subsequent digestion by micrococcal nuclease, caused the release of mononucleosomes from which phosphorylated histone H1 had been released, and in which the core histones H3 and H2A were phosphorylated.

Materials and Methods

Animals

Male Wistar rats were kept after weaning on a controlled 12 h light–12 h dark regimen, with food available in the dark. Timing was adjusted so that the animals were always partially hepatectomized in the first hour of light, when they were all well fed. After operation they were given 10% (w/v) sucrose to drink. The rats were killed by decapitation and the livers placed on ice after exsanguination.

Preparation of nuclei

Nuclei were isolated by a method using bivalent-ion-free media (Burgoyne *et al.*, 1970). They were

finally suspended in 50mM-Tris/HCl (pH 7.4)/1 mM-CaCl₂ and their concentration was adjusted to 3 mg of DNA/ml.

Micrococcal-nuclease digest (EC 3.1.4.7)

Usually, nuclei were digested for 10 min at 37°C with 100 units of micrococcal nuclease (Worthington Biochemical Corp., Freehold, NJ 07728, U.S.A.)/ml in an attempt to obtain a high proportion of mononucleosomes (Doenecke, 1977). The reaction was stopped by the addition of 4 vol. of 50mM-Tris/HCl (pH 7.4)/10mM-NaEDTA, the nuclei were sedimented (Noll *et al.*, 1975) and washed once again in Tris/EDTA. The washed nuclei were lysed in 5mM-sodium phosphate buffer (pH 6.8)/0.2mM-EDTA (sodium salt) (Doenecke, 1977); the nucleosomes released into the lysate were the chromatin fraction. DNA remaining in the pellet was examined in a number of experiments.

*Nucleosome sedimentation on isokinetic sucrose gradients (Finch *et al.*, 1975)*

Sometimes chromatin was made 0.5–0.6M in NaCl and left for 2 h at 0°C before being sedimented. In these cases the sucrose solutions contained 0.5 or 0.6M-NaCl. Sedimentation was for 16 h in a Spinco SW 27 rotor; chromatin containing 1–3 mg of DNA was placed on each gradient. After centrifugation, 1 ml fractions were collected and scanned at 260 nm. When appropriate, the nucleosomes were exhaustively dialysed in Sartorius membranes against 0.25mM-EDTA (sodium salt) at 0°C and, if necessary, concentrated by ultrafiltration in the sacs.

DNA isolation and gel electrophoresis

Samples were treated with deoxyribonuclease-free ribonuclease and proteinase K and deproteinized with phenol (Compton *et al.*, 1976; Bellard *et al.*, 1976). DNA recoveries were always checked, by using the molar absorption coefficient of 22 at 260 nm. DNA was separated electrophoretically on 2% polyacrylamide/agarose gels (Bellard *et al.*, 1976). After electrophoresis the gels were exposed to ethidium bromide (10 µg/ml) for 15 min, washed in water for 5 min, examined and photographed. Usually 3–5 µg of DNA was applied per slot.

When radioactivity determinations on the separated DNA were required, the negatives were enlarged to 10.8 cm × 14.8 cm and scanned with a Vitatron TLD 100 flying-spot scanner. The DNA bands were cut out, dissolved in 100-vol. H₂O₂/NH₃ (sp.gr. 0.880), and the specific radioactivity was determined by referring scintillation counts to the scan.

Electrophoretic separation of nucleosomes

In some experiments, dialysed concentrated nucleosomes that had been separated on the sucrose gradients were examined electrophoretically (Varshavsky *et al.*, 1976). The gels were stained successively with ethidium bromide and Coomassie Blue and photographed before radioactivity determinations.

Other radioactivity measurements

[³²P]P_i (100 µCi/100 g; 1.3 Ci/mmol), U-¹⁴C-labelled algal protein hydrolysate (10 µCi/100 g; 52 mCi/mg-atom) or [6-³H]thymidine (100 µCi/100 g; 23.2 Ci/mmol) (all from The Radiochemical Centre, Amersham, Bucks., U.K.) were given to rats intramuscularly or intravenously at various times before death. In experiments when the specific radioactivity of the nuclear samples was to be studied, nuclei were made 10% (v/v) in ice-cold trichloroacetic acid, washed five times and extracted for 40 min at 70°C in 10% trichloroacetic acid. After removal of the trichloroacetic acid with ether, DNA was measured (Burton, 1956). Blood was collected from three to six rats for each group at death, the plasma separated and its radioactivity determined. Catabolism of [³H]thymidine to ³H₂O was followed by drying the plasma in a vacuum desiccator and redetermining the radioactivity.

Histone isolation and electrophoresis

Histones were isolated from the dialysed concentrated nucleosomes by precipitation at 0.5M-HClO₄. After re-extraction the supernatants were pooled and the histone-H1 group of proteins was precipitated at 25% trichloroacetic acid. After extraction in 0.5M-HClO₄, the 0.5M-HClO₄-soluble proteins were reprecipitated at 25% trichloroacetic acid. Core histones were subsequently extracted from the precipitated nucleosomes in 250mM-HCl. Protein was determined with bovine serum albumin as a standard (Lowry *et al.*, 1951) and examined electrophoretically on polyacrylamide gels in sodium dodecyl sulphate (Weber *et al.*, 1973) or acid urea at pH 2.9 (Panyim & Chalkley, 1969). Radioactivity in the histone bands was determined after solution at 50°C in H₂O₂/NH₃.

Phosphorylation in vitro

Nuclei were isolated from 400–500 g rats in the usual way. They were finally suspended in 55mM-Tris/HCl (pH 7.4)/11 mM-MgCl₂ and the concentration of nuclei was adjusted to 3.3 mg of DNA/ml. The sample to be phosphorylated was diluted with 0.1 vol. of 20mM-[γ-³²P]ATP (Post & Sen, 1967), made 1 mM in cyclic AMP and incubated with protein kinase (Sigma Chemical Co., St. Louis, MO, U.S.A.;

40 picomolar units/ml of nuclei) for 15 min at 37°C. Unlabelled ATP was added similarly to the controls, which were kept at 0°C. After phosphorylation the control and phosphorylated nuclei were sedimented at 1000g for 5 min, the nuclei resuspended in 50 mM-Tris/HCl (pH 7.4)/1 mM-CaCl₂ and treated with micrococcal nuclease as described above.

Measurements of T_m ('melting' temperature)

These were performed with a Beckman DK-2A spectrophotometer, with 0.25 mM-EDTA as blank. The concentration of the dialysed nucleosomes was adjusted to give an initial A_{260} of 0.5. The rate of heating was about 0.5°C/min; hyperchromicities were of the order of 35%.

Results

Recovery of nucleosomes

It is possible that the isolation procedure used here caused losses that especially effected S-phase nuclei (Marshall & Burgoyne, 1977). In spite of this, differences were detected between nucleosomes from

G₀- and S-phase nuclei. No significant variations were found in the recoveries of 'purified' nuclei from normal or regenerating rat livers in G₀- and S-phases. In G₀-phase the recovery was $83 \pm 2.2\%$ (S.D.) of the expected value (1 mg of DNA/g wet wt. of liver) (three experiments) and in S-phase, $73 \pm 17\%$ (11 experiments).

Major losses of DNA occurred after digestion by micrococcal nuclease. The recoveries were the same for normal and regenerating livers, but were only $46 \pm 14.5\%$ (10 experiments). Electrophoretic analysis of DNA in the washed digested nuclei showed a relative deficiency of mononucleosomes. Analysis of the DNA lost into the supernatant during 3 min digestion with micrococcal nuclease showed the presence of many classes of polynucleosome, but after 10 min digestion mainly mononucleosomes were detected with other products of molecular weight less than that of 140-base-pair DNA (Fig. 1a). Over 75% of [³H]thymidine radioactivity in this supernatant (15 min exposure to thymidine *in vivo*) was soluble in 5% trichloroacetic acid compared with less than 2% in the original undigested nuclei.

The washed nuclei were lysed in 5 mM-phosphate buffer (pH 6.8)/0.2 mM-NaEDTA. Recovery of DNA

Fig. 1. Gel electrophoresis of DNA after micrococcal-nuclease digestion of liver nuclei from normal and partially hepatectomized 400-500g rats in S-phase

Digestion was with 100 units of micrococcal nuclease/3 mg of DNA per ml for 10 min at 37°C. Preparation of the fractions, isolation of the DNA and electrophoretic procedures are described in the text. (a) (left to right) 5 µg of DNA/slot: 1, 2, DNA from original nuclei; 3, 4, DNA in supernatant released from nuclei after 10 min incubation with micrococcal nuclease; 5, 6, DNA in chromatin released after lysing the digested nuclei; 7, 8, DNA in the residual pellet. (b) (left to right) 5 µg of DNA/slot: 11, DNA from trinucleosome-peak nucleosomes separated on sucrose gradients; 12, DNA from dinucleosome-peak nucleosomes separated on sucrose gradients. 2 µg of DNA/slot: 9, DNA from ascending-edge mononucleosomes; 10, DNA from descending-edge mononucleosomes. (c) (left to right) 5 µg of DNA/slot: 13, as 9, re-run on 5% (w/v) acrylamide gel; 14, as 10, re-run on 5% acrylamide gel.

in lysates from washed nuclei was not affected by partial hepatectomy in the older rats ($36 \pm 6.6\%$) (five experiments), but appeared lower than in younger rats in S-phase ($62 \pm 15\%$) (five experiments). DNA remaining in the pellets after lysis was relatively deficient in mononucleosomes and had substantially greater amounts of DNA in polynucleosomes, with some DNA remaining at the top of the gels (Fig. 1a).

[^3H]Thymidine specific radioactivity of DNA in nuclei and nucleosomes in S-phase 21–22h after partial hepatectomy

Liver regeneration can occur in rats of all ages, but synchrony of parenchymal cells is 'tighter' in younger rats. In 90g rats, 30% of the parenchymal cells took up thymidine between 21 and 25h after partial hepatectomy, and the peak rate of synthesis was at about 21h. Ploidy increases after partial hepatectomy, and only 16% of the cells went into mitosis. Older rats have higher ploidy in G_0 -phase and show fewer mitoses after hepatectomy (not more than 5%). Thymidine is rapidly catabolized in liver, even in S-phase, so that after 1h, 70–80% of the ^3H radioactivity in the plasma of the rats was in $^3\text{H}_2\text{O}$ (Fig. 2);

Fig. 2. [^3H]Thymidine uptake into DNA from regenerating rat livers in S-phase

Rats were injected intramuscularly with [^3H]thymidine ($100 \mu\text{Ci}/100 \text{g}$ body wt.) 0.25–1h before death, 22h after partial hepatectomy. Blood was collected and the total radioactivity of the plasma measured. Radioactivity of $^3\text{H}_2\text{O}$ was also determined. Nuclei were isolated, digested with micrococcal nuclease and fractionated into chromatin and pellet as described in the text. For each determination, 10–12 large or 20–25 small rats were used. —, Specific radioactivity of DNA as c.p.m./μg, for 400–500g rats (▲) and 60–120g rats (△); □—□, % of [^3H]thymidine catabolized to $^3\text{H}_2\text{O}$; ●, pellets after lysis of washed nuclei from 60–120g rats; ○, chromatin after lysis of washed nuclei from 60–120g rats.

the catabolism may have been slightly faster in the younger rats. Predictably, the rapid catabolism of thymidine affected the apparent rates of thymidine uptake into DNA, especially in the livers of the smaller animals (Fig. 2). This, and the extent of recovery of DNA in the chromatin, appeared to influence the labelling patterns found in the nucleosomes.

[^3H]Thymidine specific radioactivity of the nucleosomes could be estimated after separating the nucleosomes on sucrose gradients or after gel electrophoresis. In nucleosomes isolated from young rats on sucrose gradients after 0.25 or 1h labelling, the specific radioactivity of mononucleosomes was lower than for dinucleosomes, which was lower than that for pentanucleosomes (Fig. 3). After 0.25h the differences were relatively small, 168 c.p.m./μg of DNA for mononucleosomes and 255 c.p.m./μg of DNA for tetranucleosomes, but after 1h exposure the polynucleosomes had specific radioactivities 2.5 times those of the mononucleosomes. With gel-electrophoretic analysis after 0.5h labelling (Fig. 4a) the same order of specific radioactivities was found. In the older rats after 1h (Fig. 4b) the specific radioactivities of the nucleosomes were approximately equal in all the preparations examined by gel electrophoresis. This was found too for mononucleosomes and dinucleosomes isolated on sucrose gradients from

Fig. 3. [^3H]Thymidine specific radioactivity in nucleosomes from livers of 60–120g rats in S-phase

Nuclei were obtained and nucleosomes prepared and analysed on isokinetic sucrose gradients as described in the text. After extensive dialysis against 0.25 mM-EDTA, pH 7.4, DNA was isolated from the separated nucleosomes (Compton *et al.*, 1976; Bellard *et al.*, 1976) and its specific radioactivity measured. △, After 1.0h exposure to [^3H]thymidine; ○, after 0.25h exposure to [^3H]thymidine.

Fig. 4. Electrophoretic analyses of the specific radioactivities of DNA in nucleosomes in S-phase

Nuclei were prepared, digested with micrococcal nuclease and separated into chromatin and pellet. DNA was then isolated and examined electrophoretically (see the Materials and Methods section). For 60-120 g rats, there were 20/group; for larger rats, 12-15/group. (a) 60-120 g rats, [^3H]thymidine injected 0.5 h before death: $\circ$, original nuclei; $\bullet$, washed nuclei; Δ , chromatin. (b) 400-500 g rats, [^3H]thymidine injected 1 h before death. Results are from original and washed nuclei, chromatin and pellets, $\pm$ S.E.M. (six to nine samples/point). (c) 300-350 g rats, [^3H]thymidine injected 0.25 h before death: $\circ$, original nuclei and pellet; $\bullet$, chromatin.

400-500 g rats in S-phase, after exposure to [^3H]thymidine for 0.5 h.

The low specific radioactivity for mononucleosomes compared with trinucleosomes in the chromatin isolated from young rats in S-phase suggested that, in these rats, thymidine catabolism rapidly decreased the specific radioactivity of the thymidine pool. Since the effect was less evident in older rats, thymidine was given to these rats for 5-15 min before death. With 15 min exposure after intramuscular injection, gel electrophoresis (Fig. 4c) indicated that the specific radioactivity of the DNA declined from the mononucleosomes through to the tetranucleosomes. Further, the specific radioactivity of the whole chromatin fraction analysed chemically (Burton, 1956) showed that this material had a higher [^3H]thymidine specific radioactivity than did the DNA remaining in the nuclear residue after lysis (Table 1). Altering the time for lysis from 5 to 60 min (with intermittent disruption by Pasteur pipetting) did not affect the extent of chromatin release. The greater [^3H]thymidine specific radioactivity of DNA in mononucleosomes released into the supernatant during digestion, or subsequently lysed as chromatin, compared with DNA in mononucleosomes retained in the nuclear pellet, was also found after [^3H]thymidine injection intravenously 5 or 10 min before death (Table 1).

Table 1. Specific-radioactivity analyses of liver DNA from 300-350 g rats in S-phase after 5-60 min exposure to [^3H]thymidine

Animals were given [^3H]thymidine intramuscularly (i.m.) or intravenously (i.v.). Nuclei were prepared and fractionated into chromatin and pellet as described in the text. ^3H specific radioactivity in the total DNA of the chromatin and pellet (c.p.m./μg of DNA) was determined by the method of Burton (1956). Gel-electrophoretic analyses are described in the Materials and Methods section. There were 10-12 animals per group. The mononucleosomes released into the supernatant during micrococcal-nuclease digestion (10 min, 37°C) had the same specific radioactivity as those subsequently released in the chromatin; their specific activity was therefore included.* Specific radioactivities are given in arbitrary units, as means $\pm$ S.E.M. for four to six determinations.

Pulse (min)	^3H Specific radio- activity of DNA		Gel-electrophoretic separation; specific radioactivity of mononucleosomal DNA	
	Chromatin	Pellet	Chromatin*	Pellet
60, i.m.	188	288	418 $\pm$ 76	418 $\pm$ 76
15, i.m.	82	56	29 $\pm$ 1.8	19 $\pm$ 1.6
10, i.v.	25	10	33 $\pm$ 4.4	18 $\pm$ 3.6
5, i.v.	—	—	62 $\pm$ 5.9	27 $\pm$ 3.6

The higher specific radioactivity of DNA in released chromatin compared with that in the residual nuclei after short periods of exposure to [^3H]thymidine is ascribed partly to the greater proportion of mononucleosomes in the former fraction (Fig. 1a) and partly to the selective release of highly labelled mononucleosomes into the chromatin.

The rapidity of nucleosome assembly made any precursor relationship for the specific radioactivities of DNA in mononucleosomes compared with di- or oligo-nucleosomes appear very transient, and even with 10 min pulses intravenously it was not invariably found (Table 2, cf. Table 1). Further, the high-specific-radioactivity mononucleosomes were only detected by chemical or electrophoretic analyses either of the DNA released into the medium during nuclear digestion or of DNA subsequently lysed as chromatin, and were not found in mononucleosomes subsequently isolated from that chromatin on sucrose

gradients (Table 2). Specific radioactivities of these dialysed mononucleosomes showed good agreement when determined directly or after electrophoretic analysis (Table 2). It was tentatively concluded that mononucleosomes containing high-specific-radioactivity DNA were released both in the micrococcal-nuclease digests and in chromatin, but that during sedimentation on sucrose gradients the high-specific-radioactivity DNA could become dissociated, possibly concomitantly with a loss of histone H1 (see below).

Evidence consistent with such a dissociation was obtained with preparations of chromatin sedimented on sucrose gradients with or without 0.5M-NaCl (Figs. 5a and 5b). The 0.5M-NaCl dissociates histone H1 and some NaCl-soluble non-histone proteins from the nucleosomes; our resulting mononucleosomes had no remaining histone H1 (Gaubatz & Chalkley, 1977). A small amount (3–5% of DNA applied to gradient) of non-diffusible DNA was also dissociated (Fig. 5b). With 400g rats labelled intramuscularly with [^3H]thymidine for 30 min, this dissociated DNA had a specific radioactivity about 10 times that in the NaCl-treated mononucleosomes.

Table 2. *Specific radioactivity of DNA in nucleosomes of chromatin from 300–350g rat livers 22h after partial hepatectomy*

Groups of 10–12 animals were given 100 μCi of [^3H]thymidine/100g body wt. intravenously as indicated. Nuclei were digested with micrococcal nuclease for 10 min at 37°C, treated and analysed as described in the text. [^3H]Thymidine specific radioactivity is expressed as c.p.m./ μg of DNA. The specific radioactivities of DNA in the chromatin and pellet were determined after electrophoretic separation of the isolated DNA. Nucleosomes were obtained after isokinetic sucrose gradient separations; the concentration of their DNA was determined optically on the nucleosomes* or on their DNA†. The specific radioactivity was confirmed by electrophoretic analysis‡.

Source of DNA	Base-pair length of DNA ...	^3H Thymidine specific radioactivity	
		200	400
5 min pulse, intramuscularly			
Pellet		159	159
Chromatin		344	159
Dialysed nucleosomes*		101	168
Dialysed nucleosomes, DNA isolated†		85	183
Dialysed nucleosomes, DNA isolated‡		85	200
10 min pulse, intramuscularly			
Pellet		142	142
Chromatin		142	142
Dialysed nucleosomes*		164	198
Dialysed nucleosomes†		165	135
Dialysed nucleosomes,‡ re-run on 5% gels		112	—

Table 3. *^3H Lysine and ^{32}P P_i uptakes into proteins and DNA of nuclei from control and partially hepatectomized rat livers*

Seven control and 16 partially hepatectomized 90g rats were given [^{32}P]P_i and [^3H]lysine intramuscularly 1 h before death. Nuclei were prepared, digested and separated into chromatin and pellet as described in the text. Nucleosomes were then obtained from the chromatin by isokinetic sucrose-gradient fractionation. The specific radioactivity of the DNA was obtained from A_{260} determinations and is given as ^{32}P c.p.m./mg of DNA relative to the ^{32}P radioactivity of plasma. Total protein was determined in 0.1% sodium dodecyl sulphate. Other samples were precipitated at 0.5M-HClO₄ and the core histones extracted in 250mM-HCl. [^3H]Lysine specific radioactivity of the proteins is given as c.p.m./mg of protein relative to the ^3H radioactivity of plasma. The values are the means of duplicate determinations, which agreed to $\pm 3\%$.

	^{32}P relative specific radioactivity of DNA	^3H Lysine relative specific radio- activity of protein	
		Sodium dodecyl sulphate- soluble	250mM- HCl- soluble
Control			
Pellet	2	11	2.4
Mononucleosomes	3	4	5.5
22h after partial hepatectomy			
Pellet	6	16	5.6
Mononucleosomes	12	29	13

Fig. 5. Sedimentation of liver nucleosomes from partially hepatectomized rats in S-phase

(a) 350g rats: $[{}^{32}\text{P}]\text{P}_i$ was given intramuscularly 17h before death and $[{}^3\text{H}]$ thymidine 10min intravenously before death. Nucleosomes were isolated and separated on isokinetic sucrose gradients. —, A_{260} ; ····, $[{}^3\text{H}]$ thymidine; ---, $[{}^{32}\text{P}]\text{P}_i$. (b) 400–500g rats: nucleosomes were isolated and separated on sucrose gradients with and without 0.5M-NaCl. —, No NaCl; ----, +0.5M-NaCl (only shift of A_{260} at top of gradient shown). Hatched area, protein released by 0.5M-NaCl. There were 10–12 rats/group.

With 90g rats and 15 min labelling, when the specific radioactivity of the $[{}^3\text{H}]$ thymidine pool was just starting to fall, the specific radioactivities were 193 c.p.m./ μg for the dissociated DNA and 219 for that in the mononucleosomes. At 60 min, when the thymidine pool in these young rats was almost unlabelled, the specific radioactivity of mononucleosomes prepared in the absence of NaCl was 258 c.p.m./ μg compared with 354 c.p.m./ μg of DNA in the presence of 0.5M-NaCl, relatively unlabelled DNA having been removed.

Histone phosphorylation in 'new' nucleosomes

Histone and DNA synthesis occur together in S-phase, although the replication of histone octamers may be conservative (Leffak *et al.*, 1977). That new histones were associated with new DNA was shown from labelling studies either with amino acids given 10–15 min and $[{}^3\text{H}]$ thymidine for 5–10 min before death (results not shown) or with $[{}^3\text{H}]$ lysine and $[{}^{32}\text{P}]\text{P}_i$ given 1 h before death (Table 3). Mononucleosomes depleted of histone H1 (see below) were isolated from lysed chromatin released from control or partially hepatectomized rats; only the sodium dodecyl sulphate-soluble proteins from the young

control rats showed $[{}^3\text{H}]$ lysine radioactivity. In S-phase, uptake of both radioisotopes increased, the mononucleosomes showing higher specific radioactivities in both DNA and proteins than in the pellets.

New histones, especially histones H1 and H4, are phosphorylated after their biosynthesis; histone H2A is also continuously phosphorylated in rat liver in interphase (Ord & Stocken, 1975). At 1 h after exposure to $[{}^{32}\text{P}]\text{P}_i$, chromatin released from young rat livers in S-phase had a higher ${}^{32}\text{P}$ radioactivity per mg of histone H1 than did the residual nuclear pellet. In the chromatin, the specific radioactivities of histone H1 and the core histones were greater in the mononucleosomes than in the di- and tri-nucleosomes (Table 4). When the core histones were examined by acid/urea/polyacrylamide-gel electrophoresis ${}^{32}\text{P}$ was found with histones H2A, H3 and H4. With high-specific-radioactivity histone H1-deficient chromatin from the older rats, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the histones from mono- and di-nucleosomes showed the presence of a ${}^{32}\text{P}$ -labelled band running immediately behind histones H2B and H3 which was believed to be poly(ADP-ribosyl)ated histone H3 (Ord & Stocken, 1977) (Fig. 6). This component comprises 5% of the

Table 4. [32 P]P_i uptakes into histones from regenerating rat liver chromatin in S-phase

The animals were given [32 P]P_i intramuscularly 1 or 17 h before death and were killed 22 h after partial hepatectomy. Nucleosomes were isolated from chromatin by sedimentation on sucrose gradients. Results from the 90 g rats (20 per group) were obtained after selective extraction of histone H1 with 0.5 M-HClO₄ and of core histones with 250 mM-HCl. The data for the individual histones were obtained after acid/urea/polyacrylamide-gel electrophoresis of the core histone fraction. With the nucleosomes from the 350 g rats (10 per group), the total protein of the nucleosomes was extracted and examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Specific radioactivity is expressed as c.p.m./mg of protein.

	Rat wt. (g)	Length of pulse (h)	Base-pair length of DNA in nucleosomes ...	Specific radioactivity of protein			
				140	200	400	600
Histone H1	90	1	—	—	2090	430	130
Core histones	90	1	—	—	650	160	0
Core histones	90	17	—	—	1444	1040	780
Histone H3	90	17	—	—	3500	0	0
Histone H2B/H2A	90	17	—	—	2500	1000	1000
Histone H4	90	17	—	—	3000	0	0
Histone H1	350	17	—	—	—	154	—
Poly(ADP-ribosyl)ated histone H3	350	17	—	152	125	116	—

Fig. 6. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteins from rat liver nucleosome preparations

Nucleosomes containing about 10 μ g of DNA were dissolved in 6 M-urea/1% (w/v) sodium dodecyl sulphate/1% (w/v) 2-mercaptoethanol, and applied to each slot. Left to right: (a) 350 g rats, 22 h after partial hepatectomy. 1, 2, Proteins from descending edge of the mononucleosome peak off sucrose gradient. The band running more slowly than the main histones is in the position of poly(ADP-ribosyl)ated histone H3. Note also the absence of histone H1. 3, 4, Proteins from the ascending edge of the same mononucleosome peak. (b) Liver nuclei from normal rats, phosphorylated *in vitro*. 1, 2, Core histones from ascending- and descending-edge 140-base-pair DNA core particles released from phosphorylated nuclei during micrococcal-nuclease digestion and separated on Sephadex G-200 (see also Fig. 7a). Note absence of histone H1. 3, Histones from dinucleosomes from the same nuclei, released as chromatin and separated on sucrose gradients. Histone H1 present. (c) Chromatin released from normal liver nuclei phosphorylated *in vitro* and separated on sucrose gradients. 1, Histones from mononucleosome peak. 2, Histones from dinucleosomes. Note the presence of histone H1 in both samples and traces of non-histone proteins running more slowly than histone H1.

histone H3 of rat liver nuclei and in G₁/S-phases includes newly synthesized histone H3 (Ord & Stocken, 1975).

Phosphorylation of nucleosomes *in vitro*

To investigate any relation between phosphorylation and the structure of the nucleosome we decided to examine the properties of nucleosomes from livers in G₀-phase which had been phosphorylated *in vitro*. Preliminary experiments with isolated mononucleosomes indicated little 32 P incorporation into their proteins, but if the nuclei were incubated with [γ - 32 P]ATP, 32 P uptake into histones occurred (Caplan *et al.*, 1978). As expected, histone H1 released from the nuclei during the phosphorylation showed marked 32 P incorporation (Caplan *et al.*, 1978; Letnansky, 1978). After phosphorylation the sedimented and resuspended nuclei, when digested with micrococcal nuclease in the usual way, released 32 P-labelled mononucleosomes (Fig. 7a) that had substantially higher specific radioactivities than did the mono- or di-nucleosomes subsequently released during lysis (Fig. 7b) (three experiments; relative specific radioactivities: mononucleosomes in supernatants 7.0 ± 1.1 ; mononucleosomes from gradients, 1.0; dinucleosomes from gradients, 0.56 ± 0.05).

Properties of isolated nucleosomes

Theoretically expected amounts of histone H1 are recovered in whole nuclei isolated from regenerating rat livers. If these nuclei are subsequently incubated in hypo-osmolar medium, up to 29% of the histone H1 may be released in the ADP-ribosylated form (Caplan *et al.*, 1978). The non-histone proteins HMG-1 and HMG-2 are also released during incubation in hypo-osmolar medium (Caplan *et al.*, 1978); these proteins were not detected in the 0.5 M-HClO₄-

Fig. 7. Phosphorylation of normal rat liver nuclei *in vitro*; mononucleosome isolation

Normal rat liver nuclei from 400–500g rats were prepared and incubated with [γ - ^{32}P]ATP for 15 min at 37°C as described in the Materials and Methods section. Proteins released during incubation were removed after centrifugation and the sedimented and resuspended nuclei digested with micrococcal nuclease for 10 min at 37°C. (a) Supernatants released during nuclease digestion were dialysed exhaustively against 0.25 mM-EDTA, pH 7.4, and concentrated in their sacs. Then 1.2–1.5 mg of DNA was applied to Sephadex G-200 columns (100 cm $\times$ 1 cm²) and eluted in 0.25 mM-EDTA. Fractions (2 ml) were collected every 0.25 h and the A_{260} (----) and ^{32}P radioactivity (—) determined. The void volume was 60 ml. The mononucleosome peak was in tubes 42–45; histone H1 was contained in the protein eluted in tubes 95–100. (b) The chromatin subsequently released by lysis was analysed on sucrose gradients. Fractions (1 ml) were collected, their A_{260} values read (----) and the ^{32}P radioactivity (—) was determined. Mononucleosomes were present in tubes 21–24 and dinucleosomes in tubes 17–19 (see also Fig. 8).

soluble proteins from any of the nucleosome preparations studied here.

The ratios of histone H1 to DNA were normal in the supernatants released during micrococcal-nuclease digestion and in the lysed chromatin (Table 5), but the histone could have become dissociated from the nucleosomes. That this can occur, especially with mononucleosomes, has been reported previously (Varshavsky *et al.*, 1976; Gaubatz & Chalkley, 1977), and Letnansky (1978) has also observed that the released histone H1 was highly phosphorylated. In our experiments, loss of histone H1 from mononucleosomes was variable. With young rat livers in S-phase, in an experiment where 85% of the DNA of the washed nuclei was recovered as chromatin, histone H1 was easily detectable in the mononucleosomes from the lysed chromatin, relatively more being recovered in di- and tri-nucleosomes (Table 5). In some older rats, only traces of histone H1 were detected in the mononucleosomes recovered from

sucrose gradients (Table 5), but in others (used for data in Figs. 5, 6 and 9) little or no deficiency of histone H1 was detected. Histone H1 was found in di- and tri-nucleosomes recovered from sucrose gradients. In contrast, only traces of histone H1 were found on the mononucleosomes released from G₀- or S-phase nuclei during digestion with nuclease (Figs. 6 and 7a).

The presence of two main classes of mononucleosome, one containing 200-base-pair DNA and core histones and the second 140-base-pair DNA, core histones and no histone H1, was confirmed with mononucleosomes isolated from rat liver chromatin of rats given [^{32}P]Pi, 17 h and [^3H]thymidine 1 h before death, 22 h after partial hepatectomy. When the ascending and descending halves of the mononucleosome peak from the sucrose gradients (Fig. 5a) were analysed separately, the two classes of nucleosomes overlapped, but DNA from the descending peak migrated slightly more rapidly than did DNA from

Table 5. *Composition of nucleosomes from regenerating rat liver nuclei in S-phase*

Nuclei were prepared and digested and the chromatin was lysed in the usual way. The first section (1) of the Table shows the ratios of protein to DNA in nucleosomes released during micrococcal-nuclease digestion and in the chromatin subsequently released by lysis. Amounts of DNA were determined optically after ribonuclease, proteinase and phenol treatments. Micrococcal nuclease (100 units/ml; 3 mg of DNA/ml) was given for 3 or 10 min at 37°C. In the second section (2) of the Table, the chromatin is from 90 g rats (see also Fig. 3 and Table 4). In these experiments the proteins were successively extracted from the same batches of dialysed concentrated nucleosomes, but as the DNA was not determined in the same samples, the data have been normalized to 0.2 for the crude histone-H1 fraction, soluble in 0.5M-HClO₄. In the third section (3), '140 > 200' nucleosomes containing more 140-base-pair DNA material were from the descending side of the mononucleosome peak and those with more 200-base-pair DNA than 140 ('200 > 140') were from the ascending side: '200'-base-pair DNA was 196 ± 5 base-pairs.

	Base-pair length of DNA	Proportion by weight to 1 part of DNA	
		0.5M-HClO ₄ -soluble proteins	250mM-HCl-soluble proteins
(1) Total nucleosomes released into supernatant after 3 min digestion (330 g rats used)	—	0.19	0.99
Total nucleosomes released into supernatant after 10 min digestion	—	0.21	0.71
Total nucleosomes in chromatin, 3 min digestion	—	0.20	1.43
Total nucleosomes in chromatin, 20 min digestion	—	0.19	1.47
(2) Chromatin separated on sucrose gradient (90 g rats used)	200	0.2	1.45
	400	0.2	1.23
	600	0.2	1.07
(3) Chromatin separated on sucrose gradient (350 g rats used)	140 > 200	0.06	0.96
	200 > 140	0.07	0.97
	400	0.20	1.38

the ascending edge on 2% (w/v) polyacrylamide-gel electrophoresis (Fig. 1b); with 5% gels, 140- and 200-base-pair DNA were clearly separated (Fig. 1c). No histone H1 could be detected with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of protein from the descending edge of the mononucleosome peak (Fig. 6).

Mononucleosome subclasses

Further evidence for different classes of mononucleosomes came from analyses of nucleosomes obtained from nuclei that had been phosphorylated *in vitro* (see above). Histone H1 is released into the supernatant in the phosphorylating conditions used here; this supernatant was therefore removed and the sedimented nuclei were resuspended in 50 mM-Tris/HCl (pH 7.4)/1 mM-CaCl₂, for micrococcal-nuclease digestion. Material released during digestion was passed over Sephadex G-200; mononucleosomes were eluted immediately after the void volume and dissociated histone H1 appeared a little later (Fig. 7a). The ³²P specific radioactivity of this histone H1 was 30 c.p.m./μg of protein, compared with 6 c.p.m./μg of protein for histone H1 extracted from the total chromatin released after lysis and analysed before its separation on sucrose gradients.

When the proteins of these fractions were examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, core particles without histone H1, obtained either from the mononucleosomes released during micrococcal-nuclease digestion or from the descending side of the mononucleosome peak from the sucrose gradients, showed a pronounced band running more slowly than histone H3 in the position of poly(ADP-ribosyl)ated histone H3 (Ord & Stocken, 1977). This was strongly radioactive, resembling the ³²P-labelled histone H3 component detected in mononucleosomes from S-phase regenerating liver, described above (Fig. 6). The core histones from histone H1-depleted mononucleosomes or 140-base-pair DNA core particles showed 3-fold greater ³²P uptake compared with core histones from di- and tri-nucleosomes, which still retained histone H1 (three experiments: ³²P radioactivity of core histones in histone H1-depleted particles, 79, 9, 33 counts; nucleosomes with histone H1, 25, 0, 17 counts).

When nucleosomes released during digestion, or recovered from sucrose gradients, were examined electrophoretically (Fig. 8) at least four components were detected; in order of decreasing mobility these were thought to be 140-base-pair particles, 140-base-pair particles with ADP-ribosylated histone H3, 200-base-pair particles without histone H1, but probably

Fig. 8. Gel-electrophoretic analysis of nucleosomes from rat liver

Nucleosomes corresponding to $3\mu\text{g}$ of DNA were applied to each slot. The gels were stained successively with ethidium bromide and Coomassie Blue. Base-pair lengths were allocated after isolation and electrophoresis of nucleosomal DNA using PM2 DNA digested with endonuclease R *Hae*III as standard. Left to right: (a) Nuclei from normal liver phosphorylated *in vitro*. 1-3, Nucleosomes from chromatin, separated on sucrose gradients (see Fig. 7b). 4-7, Nucleosomes released during micrococcal-nuclease digestion and separated on Sephadex G-200 (see Fig. 7a). 1, Phosphorylated nuclei, dinucleosomes. 2, Phosphorylated nuclei, mononucleosomes. 3, Normal nuclei, mononucleosomes. 4, Descending edge, phosphorylated nuclei. 5, Ascending edge, phosphorylated nuclei. 6, 7, As 4, 5, control nuclei. (b) Slots 3-5 of above, to show nucleosome subclasses (see the text). The major band in gel 3 corresponds to 200-, and in gel 4 to 140-, base-pair DNA. (c) Mononucleosome subclasses released from phosphorylated nuclei during micrococcal-nuclease digestion. The major (slowest) band contains 200-base-pair DNA.

with ADP-ribosylated histone H3, and 200-base-pair DNA with histone H1 and with unmodified histone H3. Other species of mononucleosomes and core particles with different modifications can be postulated, and may have been seen in some experiments (Fig. 8).

Thermal-denaturation studies on rat liver mononucleosomes

No significant differences were detected in thermal-denaturation transition temperatures (Fig. 9a, $81 \pm 0.8^\circ\text{C}$) between mononucleosomes from control rats in G_0 -phase and mononucleosomes from young rats in S-phase, although histones in the latter were substantially phosphorylated (Table 4). These mononucleosomes contained predominantly 200-base-pair DNA and were not greatly deficient in histone H1 (Table 5). The curves were monophasic (Woodcock & Frado, 1975) in contrast with the biphasic 'melting' curves with a temperature of 74°C , reported for core particles obtained from chick erythrocyte nuclei in

0.65M-NaCl (Weischet *et al.*, 1978). In the absence of NaCl, $6 \pm 1.3\%$ DNA 'premelted' at less than 70°C . When chromatin containing these mononucleosomes was sedimented in 0.5M-NaCl (Fig. 5b), stripped mononucleosomes, still containing theoretically expected amounts of core histones (Table 5), but without histone H1 or 0.5M-NaCl-soluble non-histone proteins, now 'melted' at $77 \pm 1^\circ\text{C}$ and had an enhanced amount of 'premelted' DNA ($44.5 \pm 7.9\%$; Fig. 9b) (cf. Weischet *et al.*, 1978).

Mononucleosomes containing new DNA, recovered from chromatin of old rats in S-phase, which were deficient in histone H1 (Table 5) with predominantly 140-base-pair DNA ('core particles'), also 'melted' at 81°C , and had insignificantly more 'premelted' material ($8.9 \pm 5.3\%$). Mononucleosomes from normal rats (440g body wt.), which had been left with ATP at 0°C , as controls for the experiments on phosphorylation *in vitro*, and which contained 200-base-pair DNA but no histone H1, 'melted' at $79 \pm 0.6^\circ\text{C}$ and had 18-19% DNA 'melting' at less than 70°C .

Fig. 9. First-derivative 'melting' curves for DNA in mononucleosomes from liver nuclei of normal or partially hepatectomized rats

Mononucleosomes were isolated on sucrose gradients from nuclei from sham-operated or partially hepatectomized rats 22h after operation. After exhaustive dialysis against 0.25M-NaEDTA, pH7.4, the 'melting' curves were determined. (a) Nucleosomes isolated in the absence of NaCl: —, control nuclei; ----, nuclei from partially hepatectomized rats. (b) Nucleosomes isolated in the presence of 0.5M-NaCl. In (b) no differences were detected in thermal denaturation between mononucleosomes from control or partially hepatectomized rats. The results were therefore superimposed.

These results indicate only small differences in thermal characteristics between mononucleosomes with 200-base-pair DNA carrying histone H1 and non-histone proteins, and core particles with 140-base-pair DNA, no histone H1 and probably lesser amounts of non-histone proteins. The most marked effect was caused by 0.5M-NaCl, which removed all of the histone H1 and substantial amounts, but not all, of non-histone proteins. This led to pronounced destabilization of a significant fraction of the DNA, which was perhaps mainly due to loss of the non-histone proteins, since mononucleosomes with 200-base-pair DNA, but without histone H1, were less obviously destabilized.

Discussion

Selective release of mononucleosomes from normal and regenerating rat liver nuclei occurred in the experiments reported here. We ascribe this partly to restricted release of polynucleosomes from higher orders of structure in heterochromatin compared with euchromatin. In older rats, where parenchymal-cell synchrony after partial hepatectomy is less than in younger rats, a smaller proportion of chromatin will be euchromatic in S-phase at the time the animals are killed. Although a precise description of the replicating site is not available, it is known that some heterochromatic regions of nuclei replicate late, and it is likely that at least local 'decondensation' is required for replication to proceed. DNA replicating forks have been detected in Chinese-hamster ovary cells, and there is a time lag before the new DNA is assembled into chromatin subunits (Seale, 1975;

Hildebrand & Walters, 1976): additionally, eukaryotic replicon lengths are thought to be longer than the 200–1000-base-pair lengths of DNA analysed in our experiments.

A further basis for the selective release of mononucleosomes was inferred from the experiments with G_0 -phase nuclei phosphorylated *in vitro*. ADP-ribosylated histone H1 and other proteins are released from nuclei under the phosphorylating conditions used here (Fónagy *et al.*, 1977; Caplan *et al.*, 1978). After these released proteins had been removed, micrococcal-nuclease digestion caused more phosphorylated histone H1 and phosphorylated core particles (140-base-pair DNA) to leak out of the nuclei. The specific radioactivities of the leaked histone H1 and core proteins were substantially greater than for histones subsequently released in chromatin. Also, substantial ^{32}P uptake into histones H3 and H2A was only detected in core particles, without histone H1, suggesting that phosphorylation of liver core histones in G_0 -phase and early G_1 -phase only occurs in chromatin where the attachment of histone H1 is already altered by ADP-ribosylation. In resting liver about 10% of histone H1 molecules are modified in this way (Caplan *et al.*, 1978). Other evidence that histone phosphorylation in G_0 -phase and early interphase is not random, but is restricted to certain regions in chromatin, has been discussed elsewhere (Ord & Stocken, 1975).

The extent of phosphorylation *in vitro* was apparently small, not exceeding 0.03 mol of phosphate incorporated/mol of released histone H1; for the other histones the incorporation was less. No differences were detected between control and phosphoryl-

ated nuclei in the amounts of DNA released during micrococcal-nuclease digestion or as chromatin. The properties of the core particles and mononucleosomes were also very similar from control and phosphorylated nuclei. [^{32}P]P_i is known to exchange rapidly into histone H2A in resting liver (Ord & Stocken, 1975). We think that the phosphorylation *in vitro* serves as a marker for a class of nucleosomes containing phosphorylated histones, which are preferentially released during micrococcal-nuclease digestion. The binding of ADP-ribosylated histone H1 in these nucleosomes is weakened compared with unmodified histone H1; histone H1 is then lost in hypotonic medium. This predisposes interbody regions between the core particles to nuclease attack. This attack is, however, not restricted to regions with modified histone H1, since, depending on the conditions of the digestion, very much more DNA is released as mononucleosomes than could be associated with ADP-ribosylated histone H1, and the kinetics of digestion are not detectably changed when ADP-ribosylation of histone H1 is increased 3-fold after partial hepatectomy (Caplan *et al.*, 1978).

With older rats in S-phase, short periods of exposure to [^3H]thymidine allowed the detection of core particles and mononucleosomes enriched in newly synthesized DNA and histones, which were released during micrococcal-nuclease digestion and in chromatin. These nucleosomes contained phosphorylated histones. It is not possible from our experiments to determine whether histones in the 'new' nucleosomes were phosphorylated before or after the assembly of the completed particle, but the presence of ^{32}P in histone H4, which is only found in livers in G₁/S interphase when new histone H4 is synthesized, and the successive dephosphorylation of the histones from mono- to tri-nucleosomes, are consistent with phosphorylation of new histones before the formation of the nucleosomes, followed by dephosphorylation as the nucleosomes 'mature'.

Evidence is accumulating supporting the location of histone H1 in the interbody regions, where replication may originate. We have previously suggested (Caplan *et al.*, 1978) that phosphorylation of histone H1 may serve as a site for ADP-ribosylation, which facilitates the release of this histone from chromatin, thus exposing possible initiating sites for replication. Details of the subsequent packaging of the newly replicated DNA are unknown, but if interbody regions become associated with histone H1 after the 140-base-pair DNA is 'coiled round its core histones' and if this association requires modified histone H1, which is relatively easily dissociated, the lability of the newly formed mononucleosomes may be explained.

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