Chromatin Structure through the Cell Cycle

STUDIES WITH REGENERATING RAT LIVER

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Liver nuclei were prepared through the first cell cycle in partially hepatectomized young rats showing 30% parenchymal cell synchrony. To determine if nucleosome structure altered during this period, liver nuclei from sham-operated rats were compared with nuclei isolated at various times after partial hepatectomy. These nuclei were exposed to deoxyribonuclease I (EC 3.1.4.5), deoxyribonuclease II (EC 3.1.4.6) or micrococcal nuclease (EC 3.1.4.7) and the nucleosome-associated DNA length was ascertained. In no case was a difference in the DNA lengths associated with nucleosome structure observed. Differences were observed with regard to the histones and their relative association with nuclear material. When nuclei from normal rat livers were incubated in hypo-osmolar medium 9% of histone 1 and 4% of the other histones were released. These released histones, unlike those remaining bound to the nuclei, showed high [^3H]adenosine and [^3H]acetate uptakes *in vivo*. [^{32}P]P_i uptake was also much greater into released than bound histones 1 and 3, but was not different for histone 2A. At 3.5–4.5 h after partial hepatectomy, the release of histone 1 was trebled and that of histone 4 doubled. By 13.5 h, when phosphorylation of the bound forms of histones 2A and especially 1 was increased, no further changes in histone release in hypo-osmolar medium were found. The released histones from partially hepatectomized livers had indistinguishable [^3H]adenosine uptakes from controls. The roles are discussed of phosphorylation and ADP-ribosylation in labilizing histone binding.

Changes in the post-synthetic modifications of histones and non-histones during the cell cycle are well known. Acetylation, phosphorylation and ADP-ribosylation may be expected to weaken interactions between proteins and DNA, but although morphological differences are apparent between interphase and metaphase cells, the patterns of DNA fragments released by nuclear attack on mitotic chromosomes and interphase nuclei are indistinguishable when examined by gel electrophoresis (Axel, 1976). On the other hand thermal stability, circular dichroism and dye-binding studies have shown correlations with the metabolic status in the cell cycle (for references, see Baserga, 1976).

In earlier experiments, we found (Fónagy *et al.*, 1977) that when thymus nuclei were incubated in a slightly hypo-osmolar medium, phosphorylated histone and non-histone proteins were released into solution. We have been concerned here to investigate possible relationships between the chemical proper-

ties of the released proteins and the amounts released at different times in the cell cycle in regenerating rat liver.

We have also attempted to see if DNA-protein interactions in nucleosomes obtained at the same time showed differences in morphology or nuclease digestibility from G₀-phase through S-phase, and to determine if the formation of poly(ADP-ribose) affects the quantity of DNA associated with liver nucleosomes.

Materials and Methods

Animals

Male rats of 70–90 g body wt. were kept from weaning in controlled lighting with a 12 h day. Partial hepatectomies were performed in the first hour of light; no further food was given to the animals from that time, but 10% (w/v) sucrose was available for drinking.

Nuclei

Nuclei were prepared either by the method of Chauveau *et al.* (1956) with 2.2 M-sucrose/5 mM-

Abbreviation used: DNAase, deoxyribonuclease.

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MgCl₂/10mM-Tris/HCl, pH 7.4 (Mg²⁺ nuclei) or by the bivalent ion-free method of Burgoyne *et al.* (1970).

Nuclease digests

Nuclei from regenerating, sham-operated or normal livers were suspended in the appropriate buffers (see below) at a concentration of 1mg/ml. For nuclease digestions, the DNA rendered acid-soluble at various time points was always monitored (Compton *et al.*, 1976). Samples were taken at 0, 2, 5, 10, 15 and 30min and the nuclease was inactivated by the addition of EDTA (50mM final concn.) or Tris buffer (50mM final concn.), pH 10, and placed on ice. Individual samples were exposed to DNAase-free ribonuclease, proteinase-K and sodium dodecyl sulphate, then extracted with phenol and chloroform/octan-1-ol, and finally precipitated with ethanol/sodium acetate (Compton *et al.*, 1976; Bellard *et al.*, 1976). Individual samples were taken up in 10mM-Tris (pH 7.5)/1mM-EDTA buffer, and the DNA concentrations determined optically by using the extinction coefficient of 22/mg of DNA per ml at 260nm; 1–3.5µg of DNA was used for electrophoresis.

To determine the DNA repeat length above 150 base-pairs after using deoxyribonuclease or micrococcal nuclease, polyacrylamide gels containing 2% acrylamide and 0.5% agarose were run in an 11cm long slab apparatus (Bellard *et al.*, 1976). To investigate the DNA repeat pattern below 140 base-pairs, 98% formamide/6% acrylamide gels were run as described elsewhere (Oudet *et al.*, 1977). After electrophoresis, the gels were exposed to ethidium bromide (0.5µg/ml) for 30min, destained in water for 5min, and detected with short-wavelength u.v. light and photographed through a red filter.

The conditions for nuclease digestions are as follows. Micrococcal nuclease (Worthington) was used at 1000 units/ml at 20°C in a medium containing 1mM-CaCl₂, 0.25M-sucrose, 50mM-Tris/HCl, pH 7.5, 2mM-MgCl₂, 1mM-sodium bisulphite and 0.1mM-phenylmethanesulphonyl fluoride (Spadafora *et al.*, 1976). DNAase I at 300 units/ml was used at 37°C in a medium containing 0.25M-sucrose, 50mM-Tris/HCl, pH 7.5, 10mM-MgCl₂, 10mM-sodium bisulphite and 0.1mM-phenylmethanesulphonyl fluoride (Oudet *et al.*, 1977). DNAase II at 1000 units/ml was used at 37°C in a medium containing 10mM-Tris/HCl, 0.2mM-EDTA, pH 7.0, or pH 5.0, 0.1mM-phenylmethanesulphonyl fluoride and 1mM-sodium bisulphite or the same buffer plus 2mM-MgCl₂ (Altenburger *et al.*, 1976).

Poly(ADP-ribosyl)ation

The poly(ADP-ribosyl)ation reaction was performed by taking up nuclei in 0.25M-sucrose, 50mM-Tris/HCl, pH 7.5, 5mM-MgCl₂, 1mM-sodium bi-

sulphite and 0.1mM-phenylmethanesulphonyl fluoride. The reaction was started by adding 5mM-NAD⁺ with 0.5µCi of [³H]adenine-labelled NAD⁺ (5µCi/mmol, from New England Nuclear, Boston, MA, U.S.A.) and incubating at 37°C with occasional shaking. At various times up to 180min, small samples were withdrawn and mixed with 2ml of ice-cold 20% trichloroacetic acid. After at least 30min on ice, this material was filtered on a GC glass-fibre filter, the tube washed with 3×5ml of ice-cold 20% trichloroacetic acid and the filter finally washed with ethanol. After drying, the filter was put into 5ml of Omnifluor/toluene and counted for radioactivity in a Packard scintillation spectrometer. Snake-venom phosphodiesterase analysis of incorporated radioactivity was performed (Shima *et al.*, 1969; Miller, 1975) and confirmed that authentic poly(ADP-ribose) was formed in the presence and absence of nucleases.

Nuclear incubation and histone isolation

To examine histone modification and release, 0.5ml of nuclear suspensions (Mg²⁺ nuclei, 2mg of DNA/ml) in 5mM-MgCl₂, 10mM-Tris/HCl, pH 7.4, 2mM-2-mercaptoethanol were added to 0.5ml of medium [100mM-Tris/HCl (pH 7.4)/4mM-ATP/1mM-MgCl₂]. The presence of ATP minimized protein phosphatase action during incubation. For determining the total histone content, 1ml of suspension was stopped at 0°C with 0.1ml of 60% HClO₄, and the histones were isolated as described below. Otherwise the suspensions were incubated for 20min at 37°C, brought to 0°C and centrifuged at 25000g for 5min. Proteins present in the supernatant are 'released proteins'. The nuclear pellet remaining after centrifuging was extracted twice with 0.5M-HClO₄, and the 'bound' histone 1 precipitated by the addition of 100% (w/v) trichloroacetic acid to give a final concentration of 25%. The 'bound' acid ethanol-soluble group of histones was then extracted in 4:1 (v/v) ethanol/1.25M-HCl (Johns, 1964). Large-scale preparations of liver histones were by methods previously described (Ord & Stocken, 1975). Sometimes crude histone 1 was removed from the released proteins after precipitation at 0.5M-HClO₄, followed by precipitation of the supernatant at 25% trichloroacetic acid. Histones 2A, 3 and 4 could then be selectively removed in acid ethanol.

Protein electrophoresis

Polyacrylamide-gel electrophoresis at pH 2.9 (Panyim & Chalkley, 1969) or with sodium dodecyl sulphate at pH 7.0 (Weber *et al.*, 1973) was performed with cylindrical or slab gels. After staining with Coomassie Brilliant Blue the gels were scanned in a Vitatron TLD 100 scanner; where appropriate, 1mm

slices were taken for determination of their radioactivity.

Quantitative estimation of histone released during incubation

Electrophoretic analysis showed that although the 'total' histone 1 contained the non-histone chromosomal proteins HMG-1 and -2 (Smith & Stocken, 1973; Shooter *et al.*, 1974), these were absent from the 'bound' histone 1 obtained after removing the 'released' protein. The difference in amounts of protein (Lowry *et al.*, 1951) between the total and bound histone 1 therefore represented the histone 1 released and all the non-histone proteins HMG-1 and -2. Polyacrylamide-gel electrophoresis of the released proteins at pH 2.9 showed the presence of proteins HMG-1 and -2, histone 1 and traces of the other histones (see Fig. 2). The amount of proteins HMG-1 and -2 was determined in two ways: earlier experiments (Ord & Stocken, 1975) had obtained quantitative recoveries of proteins HMG-1 and -2 after separation of the crude histone 1 on DEAE-cellulose at pH 7.4; in normal rat liver proteins HMG-1 and -2 were then found to be $11 \pm 1\%$ of the 0.5M-HClO_4 -soluble proteins. Secondly, quantitative analysis of the gel slices gave the relative proportions of histone 1 to proteins HMG-1 and -2, assuming colour equivalence. Good agreement was obtained between the two methods. With the amount of histone 1 release determined, the release of the other histones was calculated from the scans of the released proteins by comparison with histone 1, again assuming colour equivalence.

DNA synthesis

To check the timing of DNA synthesis and the extent of synchrony, [^3H]thymidine was given 30 min before death from 20.5 to 24.5 h after partial hepatectomy. DNA was isolated and measured by the method of Burton (1956) and its radioactivity determined with respect to the plasma ^3H pool. Additionally, smears were made from portions of the liver at death: radioautography allowed the number of radioactive nuclei to be counted. In other experiments the rats were given [^3H]thymidine 24 h after operation and killed between 28 and 31 h. Portions of the livers were fixed and sectioned for radioautography. The peak rate of DNA synthesis in the rats was between 20.5 and 21 h. Mitosis occurred between 29 and 30 h; 30% parenchymal cells showed [^3H]thymidine uptake and 16% of the mitoses were labelled. This balance between division and polyploidy after partial hepatectomy is as expected for rats of 80 g body wt.

Isotope uptake

[^{32}P]P_i (1–3 Ci/mmol), [G- ^3H]adenosine (38 Ci/mmol), [6- ^3H]thymidine (23.3 Ci/mmol) and [^3H]-

methyl acetate (551 mCi/mmol) were given to the rats intramuscularly 1–13 h before death. [γ - ^{32}P]ATP was prepared by the method of Post & Sen (1967). Unless otherwise stated, all isotopes were from The Radiochemical Centre, Amersham, Bucks., U.K. When comparisons were made between normal and partially hepatectomized rats, isotope uptakes were corrected for the specific radioactivities of the respective plasma or liver homogenate pools.

Results

Nuclease digestion of nuclei from regenerating rat livers

Nuclei from regenerating and sham-operated livers were prepared either by the method of Chauveau *et al.* (1956) or Burgoyne *et al.* (1970). These nuclei were exposed to DNAase I, DNAase II or micrococcal nuclease (Fig. 1b) and samples taken at various times, the DNA isolated and electrophoresed on agarose/polyacrylamide gels. At early times during digestion by micrococcal nuclease and DNAase II in the presence of MgCl_2 , normal rat liver nuclei show a 200 base-pair repeat pattern (cf. Altenburger *et al.*, 1976; Kornberg, 1977) (Fig. 1a). DNAase I gave a 10-base-pair repeat pattern up to 140 base-pairs (Oudet *et al.*, 1977; Kornberg, 1977). In all cases, no differences in the pattern from sham-operated and regenerating liver nuclei could be detected.

In addition, a study has been made of [^3H]thymidine uptake by regenerating rat livers in S-phase 15–60 min before death. Newly synthesized [^3H]DNA was analysed after nuclease digestion, on agarose/polyacrylamide gels by fluorographic techniques (Seale, 1976, 1977) or by scintillation counting or by nucleosome isolation on isokinetic sucrose gradients (Kornberg, 1977). No evidence could be detected of non-coincidence between the distribution of ^3H radioactivity and either ethidium bromide binding to the DNA on the gels or u.v. absorption at 260 nm.

Protein release from liver nuclei from control and partially hepatectomized rats during incubation in hypo-osmolar conditions

The polyacrylamide-gel-electrophoretic pattern (pH 2.9) of non-histone proteins released from liver nuclei during incubation (Fig. 2) was qualitatively different from that shown by thymus nuclei (Fónagy *et al.*, 1977), but the amounts of histone were similar (Table 1) except for the smaller release of histone 3. As with thymus, the released histones were sharply distinguishable metabolically from the bound proteins by their greater uptake of isotope (Table 2).

In liver, bound histones 1 and 4 were insignificantly available to modification in control nuclei

Fig. 1. Gel patterns of DNA from rat liver nuclei digested with DNAase II in the presence of 2 mM-MgCl₂ (a) or micrococcal nuclease (b)

Samples A to F represent 5 min of incubations while A' to F' represent 15 min. Sample A is the DNA from nuclei of sham-operated animals, while B-F represent samples from nuclei of livers from 14, 21, 25, 28 and 48 h post-hepatectomy animals. The slot marked 'O' is the zero time nuclelease sample of sample A, but was identical for B-F showing no digestion of DNA into smaller fragments. See the Materials and Methods section for other details.

Fig. 2. Polyacrylamide-gel electrophoretic analysis (pH 2.9) of isotope uptake *in vivo* into proteins subsequently released after incubating rat liver nuclei

Protein (25 µg) released from normal rat liver nuclei were applied to the gels. After staining and scanning of the gels, 1 mm slices were cut and their radioactivities determined by scintillation counting. Hatched areas show the densitometer scan. —, ³²P radioactivity after uptake for 1 h *in vivo* in normal rats. ---, [³H]acetate radioactivity after uptake for 1 h *in vivo* in rats partially hepatectomized 13.5 h before death. ●—●, [³H]adenosine radioactivity after uptake for 13.5 h *in vivo* in normal rats. The non-histone chromosomal proteins HMG-1 and HMG-2 migrated 3.5–4 cm, histone 1 4–4.5 cm, histone 3 4.5–4.7 cm, histone 2B 4.7–4.9 cm, histone 2A 4.9–5.5 cm and histone 4 5.5–6.0 cm.

Table 1. *Protein release from normal and partially hepatectomized rat liver nuclei*

Rats were sham-operated or partially hepatectomized 3.5–4.5 or 13–14 h before death. No detectable differences were seen between the two groups of partially hepatectomized rats and the results were therefore taken together. Nuclei were incubated at 37°C for 20 min in a hypo-osmolar solution under conditions described in the text. Released proteins were those present in the supernatant after the incubated nuclei had been brought to 0°C and centrifuged for 5 min at 25000g. Bound histone 1 was that present in the nuclear pellet after the removal of the released proteins. Total histone 1 and proteins HMG-1 and HMG-2 were those proteins present in the nuclear suspensions which were stopped by HClO₄ before incubation. The amounts of non-histone chromosomal proteins (HMG-1 and HMG-2) were reported earlier (Ord & Stocken, 1975), and confirmed in these experiments. Released histone 1, which was ADP-ribosylated, could then be calculated (see the text) as could the amounts of the other histones released during the incubation. Released proteins are expressed as µg of protein/mg of DNA or as % of total histones 1, 3, 2B, 2A or 4 ± s.e. (number of determinations given in parentheses).

	Control liver nuclei	Partially hepatectomized liver nuclei
Released protein	961 ± 190 (11)	1166 ± 87 (6)
Total histone 1, protein HMG-1 and HMG-2	180 ± 36.9 (9)	180 ± 50.6 (6)
Bound histone 1	139 ± 20.9 (9)	113 ± 18.9 (6)
Protein HMG-1 and HMG-2	25 ± 5.5 (11)	20 ± 10.2 (6)
Released histone 1 (1a)	16 (9%) (9)	47 (29%) (6)
Released histone 3, 2B, 2A	3.9 ± 1.5% (8)	4.7 ± 0.8% (4)
Released histone 4	3.4 ± 2.0% (8)	7.4 ± 1.9% (4)

Table 2. *Isotope uptake into histones from control and partially hepatectomized rat liver nuclei*

The preparation of nuclei, incubation conditions and isolation of histones are described in the text. [³²P]P_i and [³H]-acetate uptakes *in vivo* (a, c) are given as c.p.m./nmol. Transfer of [^γ-³²P]ATP is given as mmol of γ-P transferred/mol of protein ± s.e. To determine the radioactivity of the released proteins, ³²P]P_i and ³H adenosine uptakes were obtained after isolating the histones from the soluble proteins by classical methods. For [³H]acetate uptake and for [^γ-³²P]ATP transfer the results were obtained by radioactivity counting of the gel. Neither [^γ-³²P]ATP transfer nor [³H]adenosine uptakes *in vivo* differed between control livers and those after partial hepatectomy; the latter results, which are expressed as the ratio of ³H radioactivity of the released: bound histones, are the means of two experiments which did not differ by more than 5%.

(a) [³² P]P _i uptake <i>in vivo</i> (c.p.m./nmol)						
	Released histones			Bound histones		
Histone	Control	6 h post hepatectomy	13.5 h post hepatectomy	Control (4)	3.5–4 h post hepatectomy (3)	13.5 h post hepatectomy (3)
H1	29.7 ± 5.2 (3)	35.0	32.7	2.5 ± 1.4	6.9 ± 1.9	13.6 ± 3.6
H3	176	116	—	10.8 ± 1.3	19.4	12.6 ± 5.7
H2A	25.6, 14.1	33.3	—	18.5 ± 3.6	20.9, 0.6	30.8 ± 25.6
(b) [γ - ³² P]ATP uptake <i>in vitro</i> (c.p.m./nmol)						
	Released histones (4)		Bound histones (4)			
H1	26.8 ± 8.0		2.5 ± 1.2		—	
H3	55.1 ± 9.4		9.8 ± 3.0		6.0	
H2B	32.0 ± 16.0		—		550	
H2A	24.7 ± 8.3		9.7 ± 2.2		1081	
H4	34.3 ± 3.7		1.5 ± 0.0		726	
(c) [³ H]Acetate uptake <i>in vivo</i> (c.p.m./nmol)						
	Released histones (1)		Bound histones (1)			
H1	—		—		—	
H3	—		6.0		—	
H2B	550		—		—	
H2A	1081		14.1		—	
H4	726		0		—	
(d) [³ H]Adenosine uptake <i>in vivo</i> (ratio of ³ H radioactivity in released and bound histones)						
	Labelling time (h) ...	1 (2)	4.5 (2)	13.5 (2)		
	H1	4.4	12.0	17.9		
	H3	13.9	26.9	26.6		
	H2A	2.1	20.5	4.5		

(Table 2, *a-d*); bound histones 3 and 2A showed [^{32}P]P_i incorporation (Table 2, *a-b*), but little [^3H]-adenosine uptake (Table 2, *d*). The extent of ^{32}P uptake *in vivo* into histone 2A was not different between the released and bound proteins (Table 2, *a*). That this difference was observed with phosphorylation *in vitro* is possibly due to the release of histones under the conditions of incubation and their consequential availability to phosphokinase and phosphatases (Fónagy *et al.*, 1977). Adenosine uptake into the released proteins (Fig. 2 and Table 2*d*) is consistent with our earlier studies on adenine uptake and ADP-ribosylation (Ord & Stocken, 1977). There the histones had not been separated into released and bound fractions and ADP-ribosylation of histone 2A was then only seen after incubation with NAD, and was not detected on histone 4.

Histone 1 and the non-histone proteins HMG-1 and -2 were isolated from the released proteins by classical methods. U.v. analysis then showed that these proteins contained about 1 mol of adenine/mol of protein. Protein HMG-1 dominated the electrophoretic pattern at pH2.9; the histone 1 present in the released proteins also migrated significantly more slowly than bound histone 1 (Fig. 3). All the proteins HMG-1 and HMG-2 were released during incubation, and chromatographic separation of bound histone 1 on DEAE-cellulose and Bio-Rex 70 (Smith & Stocken, 1973) confirmed that all the proteins HMG-1 and HMG-2 and all the ADP-ribosylated histone 1 had been released into the hypo-osmolar incubation medium. The ethanol/HCl-soluble histones 2A, 3 and 4 could also be recovered from the released proteins.

The results indicate that the released proteins carry more than one modification. We suggest that simple phosphorylation provides seryl phosphate groups to act as acceptors for ADP-ribose. If ADP-ribosylation occurs, the proteins dissociate under hypo-osmolar conditions, although from quantitative recoveries of histone 1 (Table 1) and histones 2A, 3 and 4 (not shown) from all the nuclei examined in these experiments, and from the constancy of the nuclear digest patterns, complete histone dissociation does not occur *in vivo*.

Partial hepatectomy tripled the release of histone 1 and doubled that of histone 4 (Table 1). This was seen at 3.5h, the earliest time examined (bound histone 1 now $112 \pm 19.2 \mu\text{g}/\text{mg}$ of DNA) and was not significantly different 13–14h after operation (Table 1), when the phosphate contents of histones 1 and 2A are greatly increased (Thrower *et al.*, 1975). Partial hepatectomy increased [^{32}P]P_i uptakes *in vivo* into bound histones 1 and 2A (Table 2*a*), especially at 13.5h, consistent with the expected prereplicative increase in ^{32}P specific radioactivity of these proteins (Thrower *et al.*, 1975). [^3H]Adenosine uptakes were unchanged, as were the adenine contents of the non-

Fig. 3. Polyacrylamide-gel electrophoresis (pH2.9) of 0.5M-HClO₄-soluble material from released and bound proteins of liver nuclei incubated in hypo-osmolar conditions

The supernatant released during nuclear incubation was made 0.5M in HClO₄. The soluble material was brought to 25% in trichloroacetic acid; 10 μg of protein was applied to the gel. The nuclear pellet from which the soluble proteins had been released during incubation was similarly treated; 2 μg of protein was applied to the gel. ----, Acid (0.5M-HClO₄)-soluble proteins released during nuclear incubation. —, 0.5M-HClO₄-soluble protein remaining bound to the nuclei. Protein HMG-1 ran at about 3.5 cm, HMG-2 at about 3.8 cm; histone 1 ran between 4.2 and 4.5 cm.

histone chromosomal proteins HMG-1 and HMG-2 and histone 1 released into the incubation medium. [^3H]Acetate uptake by the released protein was increased 13.5h after partial hepatectomy (Fig. 2); the uptakes into histones 1, 2A and 4 at this time are probably associated with the onset of histone biosynthesis and *N*-terminal acetylation.

Under our experimental conditions phosphorylation by the nuclear kinase(s) *in vitro* was indistinguishable 4 and 13.5h after partial hepatectomy from that in normal liver nuclei. This suggests that in G₁-phase the increased phosphorylation of histones *in vivo* may be effected by a kinase that is not retained by the nuclear preparation, in contrast with the increased activity of a nuclear kinase phosphorylating histone 1, detectable in these nuclei in S-phase (Siebert *et al.*, 1971).

ADP-ribosylation and nuclease digestion patterns

Using nuclei isolated from normal rats, the effects of poly(ADP-ribose) formation *in vitro* on nucleosome structure was investigated. The incorporation

of [^3H]adenine from NAD into nuclei was followed as trichloroacetic acid precipitable material (Fig. 4). In all cases, exposure to nuclease resulted in a 10–41% increase in the rate of ADP-ribosylation. Analysis of the snake-venom phosphodiesterase hydrolysate of this trichloroacetic acid precipitable material indicated that the average chain length of newly formed poly(ADP-ribose) was 3.1–3.4, and that about 0.37 mol of ADP-ribose was incorporated/h per mol of histone.

With all conditions used, and variations of bivalent cation between 0.5 and 10 mM and of NAD from 0.5 to 5 mM, no differences in the DNAase I, DNAase II and micrococcal nuclease digestion patterns could be detected between nuclei from controls and those showing poly(ADP-ribose) formation. In experiments involving DNAase II with MgCl_2 present in the poly(ADP-ribosyl)ation reaction, the 200-base-pair and not the 100-base-pair repeat pattern was observed (cf. Fig. 1b). A variety of methods was used to remove the Mg^{2+} from nuclei in unsuccessful attempts to detect the 100-base-pair repeat pattern; these included addition of 50 mM-EDTA, dialysis against 10 mM-Tris/HCl, pH 8, and dialysis against 50 mM-EDTA, pH 7.5. The ADP-ribosylation reaction was therefore conducted for 1 h at 37°C in the absence of

MgCl_2 using nuclei prepared by the method of Burgoyne *et al.* (1970), followed by exposure to DNAase II also in the absence of MgCl_2 . The trichloroacetic acid-precipitable radioactivity was 100- to 450-fold less than the ADP-ribosylation in the presence of Mg^{2+} , but showed an increase in radioactivity with time; nuclease treatment did not stimulate this rate. Furthermore, the snake venom phosphodiesterase pattern indicated minimal, if any, formation of poly(ADP-ribose), but perhaps a small amount of the monomer.

Discussion

Nuclease digestion patterns have been used to characterize the structural complexity of nucleosomes. The amount of DNA associated with nucleosomes seems to vary as a function of the transcriptive (Thomas & Thompson, 1977) or replicative (Seale, 1976, 1977) activity of a group of cells or tissue. Two variables that affect cell activity and/or nucleosome structure have been investigated here: effects of regeneration and ADP-ribosylation *in vitro*. Regeneration involves relatively substantial modifications of nuclear proteins, including phosphorylation, acetylation and ADP-ribosylation, as well as amounts of non-histone proteins. No differences in the DNAase I, DNAase II or micrococcal nuclease patterns could be detected, however. Three interpretations are possible: first, these major and relatively massive chemical modifications and alterations in tissue activities do not produce modifications in nucleosome structure; secondly, the level or type of modification is below the resolution of the techniques used or, thirdly, the conditions under which the nuclease experiments are performed preclude detection of any changes.

Modification of nucleosome morphology was not apparent under our conditions. The technology used is easily capable of detecting a 10% change in the amount of DNA associated with the nucleosomes. Such a 10% change would result in a 20-base-pair shift in the repeat frequency of nucleosomes. The agarose/polyacrylamide gel system can resolve 10-base-pair differences. If only 10–20% of the nuclei are involved in the ADP-ribosylation or regeneration response and these nuclei exhibited a 10% shift in the amount of DNA associated with the nucleosome, we would expect a 'smearing' of the monomer or multimer DNA bands. This was not observed either in visually inspected gels, optically scanned gels, or in gels subjected to fluorographic analysis. We must therefore conclude that although the nuclease and gel technology are capable of detecting relatively small differences in nucleosome structure, under the conditions used here no such differences could be detected.

Fig. 4. Formation of poly(ADP-ribose) in the presence or absence of micrococcal nuclease as determined by the trichloroacetic acid-precipitability of radioactivity originating in [^3H]NAD

Isolated rat liver nuclei were incubated for various times after which they were brought to 0°C and made 20% (final) trichloroacetic acid. After 60 min of incubation, micrococcal nuclease was added (arrow) to a portion of incubating nuclei and after various time periods these samples were precipitated with trichloroacetic acid on ice. The trichloroacetic acid-precipitable material was trapped on glass-fibre filters, washed with ethanol and estimated. ○, Micrococcal nuclease; ●, control.

The identity of the nuclease digest patterns contrasts with the increased dissociation of some of the nuclear proteins in the hypo-osmolar incubation conditions. Histones released from normal liver nuclei were highly modified; the extent of their release (4% of core histones and 9% histone 1) is consistent with current estimates for the proportion of transcriptionally active DNA. Their greater release (5–7 and 29% respectively) by 3.5h after partial hepatectomy would then correlate with well-established increases in transcriptional activity early in G₁-phase.

Simple phosphorylation, like acetylation (Davie & Candido, 1977), may not be sufficient to labilize histone–DNA interactions in the tests used here. The results suggest that for increased transcriptional or replicative activity multiple and possibly co-operative modifications of the same protein are required.

There is a temporal correlation between the first peak of cyclic AMP production in regenerating liver (Thrower *et al.*, 1975), the increased phosphorylation of histone 1, and its increased release from incubated nuclei 3.5h after partial hepatectomy. As the specific radioactivity of [³H]adenosine in the released histone 1 is unchanged, more ADP-ribosylation of histone 1 must have occurred. Amounts of poly(ADP-ribose) polymerase are not thought to increase early in G₁-phase (Hilz & Stone, 1976). The increased ADP-ribosylation of histone 1 may therefore be consequential upon the increase in serine phosphorylation, but by 13.5h, when phosphorylation of histone 1 is greatly increased, no further release of the protein was detectable. This suggests either that the activity of the poly(ADP-ribose) polymerase became limiting, or that other factors, which may include the presence of non-histone proteins, are additionally involved.

Partial hepatectomy causes a profound change in the activity in the remaining liver cells as compared with sham-operated controls. As shown here, this change in activity is accompanied by an increase in the release of proteins from isolated nuclei without affecting the repeat pattern of the DNA associated with histones in nucleosomes. These observations might indicate that the histone octamer shifts away from and then reassociated with DNA, as determined by the chemical modifications of the nuclear proteins. We would suggest that the release of various proteins (modified histones for example) may be the result of this activity. If nucleosomes are mainly but not exclusively associated with transcriptionally inactive DNA sequences, the identity of the nucleosome-digestion patterns seen here would indicate that DNA–histone interactions in liver cell chromatin were quantitatively indistinguishable regardless of the state of activity of this tissue. Further, the shifts in transcriptive and replicative activity probably require the modification of certain nuclear proteins associated with previously active or newly active

sequences; such modifications have no effect on the quantity of DNA associated with individual stable nucleosomes.

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