Conformational changes in rat liver chromatin after liver regeneration

H. SIMPKINS, L. M. THOMPSON, N. WALDECK, D. S. GROSS and D. MOONEY
*Department of Biological Chemistry and Pathology, University of California, Irvine, Medical Center,
101 City Drive South, Orange, CA 92668, U.S.A.*

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N-Pyrenemaleimide, a fluorescent probe that specifically labels histone H3 of rat liver chromatin *in situ*, was used to monitor the accessibility of histone H3 in chromatin isolated from rat liver at different times during regeneration. At times of maximum DNA synthesis (18–24 h after hepatectomy), the accessibility of the probe was found to be markedly (40–50%) increased. This increase is abolished, however, by treatment of the chromatin fibres with high salt (2 M-NaCl) or detergent. Tryptophan fluorescence was also enhanced at points of maximum DNA synthesis, suggesting that some non-histone tryptophan-containing protein was being synthesized. The polarization of the labelled histone H3 is not markedly altered, suggesting that fibre aggregation or dissociation does not occur. Mononucleosomes extracted from sham-operated and hepatectomized animals did not exhibit any difference in binding to the probe. Also, analysis of the chromatin protein by electrophoresis on detergent- and acid/urea/Triton-X-100-containing polyacrylamide gels showed no detectable difference in histone H3 : 1, H3 : 2 or H3 : 3 subclasses.

Although morphological differences are readily apparent between interphase and metaphase cells, the patterns of DNA fragments are identical (Axel, 1976). Dye-binding studies involving intercalating dyes have shown correlation with metabolic states in the cell cycle with cultured cells (Nicolini & Baserga, 1975*a,b*; Nicolini *et al.*, 1975), and changes in post-translational modification of chromatin proteins have been observed during the cell cycle with acetylation, phosphorylation, etc. (Nadler, 1976; D'Anna *et al.*, 1978; Gurley *et al.*, 1978). These latter modifications are generally thought to result in changes in DNA–histone, histone–histone and histone–non-histone-protein interactions.

In addition, chromatin proteins have been studied after hepatectomy, and changes in the histone/DNA ratio have been described (Garrard & Bonner, 1974). Coincident increases in DNA and histone synthesis have also been shown to occur after regeneration and are unrelated to high-mobility-group-protein synthesis (Keuhl, 1979). Thus we decided to investigate whether changes in the molecular structure of chromatin could be detected by a fluorescent probe that labels histone H3 *in situ* in active cells (e.g. rat liver thymocytes) as opposed to chromatin from quiescent cells (e.g., chick erythrocytes), where little binding is observed. The

probe is also sensitive enough to detect changes in histone interaction occurring with salt-induced fibre changes (Mooney *et al.*, 1980). Such a probe also has advantages over binding measured with [³H]*N*-ethylmaleimide, since only the bound probe is fluorescent. Urea-dissociated chick erythrocyte nucleosomes have been labelled with *N*-pyrenemaleimide (Zama *et al.*, 1976, 1978*a*) and *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine and iodoacetylaminofluorescein (Dieterich *et al.*, 1976). By this method of labelling, both histone H3 molecules are labelled and interactions between the two fluorescent molecules results. The possibility of structural changes being produced by this method of labelling is also likely, and two populations of labelled nucleosomes are observed under the electron microscope. However, the effect of denaturants (urea, pH, solvent) on the labelled nucleosomes has yielded interesting information (Zama *et al.*, 1978*b*). Thus a study was performed to investigate the reactivity of histone H3 thiol groups *in situ* and the degree of tryptophan (non-histone protein) fluorescence during liver regeneration after partial hepatectomy.

Materials and methods

Procedure for partial hepatectomy

Male Sprague–Dawley rats (150–250 g) were

Abbreviation used: SDS, sodium dodecyl sulphate.

67% hepatectomized under diethyl ether anaesthesia as described by Higgins & Anderson (1931). The livers were allowed to regenerate for 18, 24, 36 and 72 h time periods, after which the rats were killed and the livers removed. Control rats were sham-operated and killed at time intervals corresponding to those of the partially hepatectomized rats.

Preparation of chromatin and mononucleosomes

Rat liver chromatin was prepared from sham-operated and partially hepatectomized rat liver by using the technique of Marushige & Bonner (1966). The isolated chromatin was purified by pelleting through 1.7 M-sucrose in 10 mM-Tris/HCl (pH 8.0)/1 mM-phenylmethanesulphonyl fluoride at 120 000 g for 90 min. The pellet was then washed once in storage buffer (10 mM-Tris/HCl (pH 8.0)/1 mM-phenylmethanesulphonyl fluoride/0.1 mM-EDTA and suspended in the same buffer to a chromatin protein concentration of 0.5–1.0 mg/ml.

Mononucleosomes were prepared from sham-operated- and partially hepatectomized-rat-liver nuclei by digestion with micrococcal nuclease (Worthington) at 220 units/ml. Nuclei were first suspended with a Pasteur pipette in 15 mM-Tris/HCl (pH 7.4)/60 mM-KCl/15 mM-NaCl/0.5 mM-mercaptoethanol/0.15 mM-spermine/0.5 mM-spermidine/0.35 M-sucrose/1 mM-phenylmethanesulphonyl fluoride and then incubated at 37°C for 3 min. The addition of 1 mM-CaCl₂ and micrococcal nuclease started the enzyme reaction and the digestion was allowed to proceed for 20 min to give a higher percentage of monomers when the reaction products were analysed by a sucrose-gradient centrifugation. The digestion was terminated with 2 mM-EDTA, pH 7.0, centrifuged at 4000 g for 15 min, the supernatant (S1) discarded, the pellet (P1) resuspended in 1 mM-EDTA (pH 7.0)/0.5 mM-phenylmethanesulphonyl fluoride with a Pasteur pipette, the nuclei swollen for 1 h and then centrifuged at 1800 g for 15 min before ultracentrifugation on the gradient. Measurement of the A_{260} of S1 and P1 showed that 80% of the digested DNA was liberated into the supernatant. Analysis of the mononucleosomes on a polyacrylamide gel, as described by Creusot & Christman (1980), gave a base-pair repeat of 210 ± 10 base-pairs. The protein composition of the mononucleosomes is shown in Fig. 4 (below).

Core histones were obtained as described by Phillips & Johns (1965) from whole chromatin or nucleosomes by extracting with 0.4 M-H₂SO₄ at 4°C for 30 min and centrifuging at 1000 g for 30 min. Ethanol (5 vol.) was added to the supernatant, which was then kept overnight at -20°C. Precipitated whole histones were collected by centrifugation at 2000 g for 30 min and dried *in vacuo*. Core histones were then purified by extraction with 0.5 M-HClO₄ at 0°C for 60 min. The resulting

precipitate was pelleted at 2000 g for 20 min and resuspended in 8 M-urea/10% (v/v) acetic acid/0.05% Pyronine Y/8% (v/v) 2-mercaptoethanol for acid/urea/Triton-X-100/polyacrylamide-gel analysis.

Preparation of N-pyrenemaleimide and chromatin-labelling conditions

The probe was synthesized as described by Weltman *et al.* (1973) from 3-aminopyrene. The final ethanol precipitate had m.p. 219°C (reported 223°C) [Found: C, 81.08; H, 3.88; N, 4.72. Calc.: C, 80.8; H, 3.74; N, 4.72 (Galbraith Laboratories, Knoxville, TN, U.S.A.)]. It was dissolved before labelling as a 100 µM solution in ethanol. Fluorescence was determined in Durrand and Perkin-Elmer 43A fluorescence spectrophotometers employing 1 cm quartz cells with emission and excitation polarizers. Whole chromatin or nucleosomes were made to react in storage buffer at the protein and probe concentrations stated in the respective Tables or Figure legends. The reaction, unless otherwise stated, proceeded at 25°C until completion, generally 10–20 min. Chromatin and nucleosome protein concentrations were determined as described by Lowry *et al.* (1951), with total calf thymus histone as standard.

SDS/polyacrylamide and acid/urea/Triton X-100/polyacrylamide gels

The chromatin samples were monitored by SDS/polyacrylamide-gel electrophoresis and acid/urea/Triton X-100 gel electrophoresis. SDS/polyacrylamide gels were run by using a modification of the procedure used by Laemmli (1970). The reservoir buffer was 0.1% SDS/0.19 M-glycine/0.015 M-Tris/HCl, pH 8.2, the separating gel contained 12% (w/v) acrylamide/bisacrylamide (37.5:1) in 0.1% SDS/0.375 M-Tris/HCl, pH 8.8, and the stacking gel contained 4.2% acrylamide with the same acrylamide/bisacrylamide ratio, in 0.1% SDS/0.125 M-Tris/HCl, pH 6.8. The current was kept at 7.5 mA as the specimens migrated through the stacking gel and kept constant at 12.5 mA until the marker dye was within 0.5 cm of the bottom of the separating gel. The gels were then stained with 0.1% (w/v) Coomassie Blue in methanol/acetic acid/water (10:7:83, by vol.) and were destained with the same methanol/acetic acid/water mixture.

Core histones were analysed on acid/urea/Triton X-100/polyacrylamide gels by using a modification of the procedure followed by Alfageme *et al.* (1974). To give optimal resolution of histone species and subspecies, the system was developed with upper and lower gels. The lower separation gel contained 13.3% polyacrylamide (acrylamide/bisacrylamide, 150:1, w/w) in 5% acetic acid/5.8 M-urea/0.27% Triton X-100 and the upper gel was 4.2% poly-

acrylamide (acrylamide/bisacrylamide, 37.5:1, w/w) in 1% acetic acid/6.7 M-urea/0.62% Triton X-100. Gels were pre-electrophoresed at 200 V for 3–4 h until the current was equilibrated, then scavenged for free radicals with 1 M-cysteamine/HCl, 20 μ l per well, for 60 min by using a 5%-acetic acid/buffer system. Samples were electrophoresed after replacement of electrode buffer at a constant 7.5 mA until tracking dye entered the separation gel, then at a constant 11.0 mA for an additional 7 h. Gels were stained and destained as described for SDS/polyacrylamide gels.

Results

N-3-Pyrenemaleimide, a thiol-group-specific fluorescent probe, has been shown to primarily label histone H3 in intact rat liver chromatin, but not chick erythrocyte chromatin (Mooney *et al.*, 1980), however, mononucleosomes from both preparations do label. This suggests that only cysteine-96 in the histone octamer of rat liver chromatin is being labelled and that cysteine-110, which is also found in chick histone H3, is reactive but buried within the chromatin thread, thus being inaccessible to the probe. After nuclease digestion it becomes more accessible, accounting for the labelling of chick mononucleosomes. It also appears that only one of the two histone H3 molecules is labelled *in situ*, since treatment with 2 M-NaCl results in a 2-fold increase in fluorescence with the probe.

Labelling of chromatin isolated from partially hepatectomized and sham-operated rat liver shows a higher fluorescence intensity at 18–24 h after hepatectomy than at other time points owing to structural change in the chromatin fibre allowing greater accessibility of the probe to the histone H3 thiol groups. Saturation curves are presented in Fig. 1, showing fluorescence changes with increasing concentrations of label at constant chromatin concentration (Fig. 1a) and increasing chromatin concentrations at constant label concentration (Fig. 1b). As can be seen in the Figures, the reaction with the thiol groups of both the partially hepatectomized-rat and the sham-operated-rat chromatin is saturable. However, the fluorescence intensity of the hepatectomized-rat chromatin is approx. 50% higher than for the sham-operated-rat chromatin. In contrast, mononucleosomes from sham-operated-rat and partially hepatectomized-rat liver (Fig. 2) show no difference in fluorescence intensity.

The effect of time after hepatectomy was studied from 18 to 72 h, and it was found that the effect was maximal at 18–24 h (Fig. 3). This was found to hold for over eight different experiments. The increase varied at 18 h from 20 to 45%.

High salt concentrations (2 M-NaCl) or detergent (0.01% SDS) have been shown (Mooney *et al.*,

Fig. 1. *Chromatin and N-pyrenemaleimide saturation curves*

(a) Chromatin protein (0.3 mg) from rat liver nuclei 18 h after partial hepatectomy (O) and sham-operation in storage buffer was incubated with various concentrations of label (0.5–10 μ M) for 2 h at 4°C and the fluorescence (*F*) measured at an excitation wavelength of 342 nm followed by an emission wavelength of 377 nm. Fluorescence is expressed in arbitrary units. (b) 5 μ M-*N*-pyrenemaleimide was made to react with the indicated amounts of chromatin protein in storage buffer from hepatectomized (O) and sham-operated (Δ) animals. Each curve represents a single experiment that has been repeated several times to give the same results, with two determinations/point and three animals/group.

1980) to enhance the reactivity of *N*-pyrenemaleimide with rat liver chromatin. High salt disrupts the histone octamer, thus exposing the histone H3 cysteine groups. Treatment with 2 M-NaCl gave a 2–3-fold increase in fluorescence (Table 1) and eliminated the previously observed difference. Addition of 0.01% SDS to the reaction mixture resulted in a 6-fold increase and also eliminate the previous fluorescence increase of the 24 h hepatectomy.

Fig. 2. *N*-pyrenemaleimide saturation curve of rat liver chromatin and nucleosomes

Chromatin (10 μ g of protein) from 24 h partially-hepatectomized- (●) and sham-operated (○) -rat livers, as well as nucleosomes from the same hepatectomized- (▲) and sham-operated (△) -rat livers were made to react to completion in storage buffer at the indicated *N*-pyrenemaleimide concentrations. Reactions were monitored at an emission wavelength of 377 nm and an excitation wavelength of 342 nm. Fluorescence (*F*) is expressed in arbitrary units. Each curve represents a single experiment that has been repeated several times to give the same results, with two determinations/point and three animals/group.

The effect of the *N*-pyrenemaleimide label on the intrinsic tryptophan fluorescence of the non-histone proteins of partially hepatectomized-rat and sham-operated-rat chromatin was determined. Chromatin isolated from rat liver 24, 36 and 72 h after partial hepatectomy was compared in Fig. 3; this shows a marked increase in tryptophan fluorescence of the chromatin preparation 24 h after hepatectomy. An increase in tryptophan fluorescence in rat chromatin 24 h after hepatectomy when compared with the zero-time control, with rats 24 h after sham-operation and with rats 72 h after hepatectomy was also observed (Table 2). This would suggest that there is either a greater accessibility to the tryptophan residues or a greater number of tryptophan-containing non-histone proteins being synthesized 24 h after hepatectomy than at the latter regeneration times.

Quenching of tryptophan fluorescence by iodide has been used with membranes to determine which tryptophan residues are external and in contact with

Fig. 3. *N*-pyrenemaleimide and tryptophan fluorescence of chromatin protein

After regeneration of partially-hepatectomized-rat liver at the indicated time points, *N*-pyrenemaleimide and tryptophan fluorescence of whole chromatin (0.3 mg of protein) from these livers and from sham-operated livers at the same time points were measured and the fluorescence ratio hepatectomized/control (F_H/F_C) was calculated. *N*-Pyrenemaleimide fluorescence (○) at a label concentration of 5 μ M was monitored at an emission wavelength of 377 nm, after excitation at 342 nm, and tryptophan fluorescence (●) was measured at an emission wavelength of 328 nm and an excitation wavelength of 290 nm. Each point represents an average ratio for at least three separate experiments with the indicated margins of error.

the solvent (Imae *et al.*, 1979). When the reaction took place in 0.12 M-NaI, no decrease in fluorescence was observed, indicating that the non-histone tryptophan residues are not accessible to solvent. The 24 h-after-hepatectomy sample maintained the original increase over the other samples; therefore the observed difference was not due to alteration in the exposure of the non-histone tryptophan residues to solvent.

The quenching of tryptophan fluorescence with *N*-pyrenemaleimide was also observed. As Table 2 shows, *N*-pyrenemaleimide does quench the fluorescence of all the samples tested. On the presumption that the increase in tryptophan fluorescence of the chromatin 24 h after hepatectomy is due to an additional non-histone tryptophan-containing protein(s), this sample should quench pro-

Table 1. *Effect of NaCl and SDS on N-pyrenemaleimide fluorescence of chromatin obtained from sham-operated and hepatectomized animals*

Fluorescence was observed at an emission wavelength of 377 nm and an excitation wavelength of 342 nm. The concentration of chromatin protein was 10 µg and the *N*-pyrenemaleimide concentration 500 nM for each sample. Fluorescence was measured and compared for each in 10 mM-Tris/HCl (pH 8.0)/1 mM-phenylmethanesulphonyl fluoride/0.2 mM-EDTA.

$$R = \frac{\text{Fluorescence intensity of chromatin from rats 24 h after hepatectomy}}{\text{Average fluorescence intensity of 0h-control chromatin and chromatin from rats 24 h after sham-operation}}$$

R represents results obtained from the following experiment $\pm$ s.d. of *R* values obtained from two other similar experiments.

Fluorescence intensity of *N*-pyrenemaleimide-labelled chromatin obtained from livers of rats:

Solvent	At 0h (control)	24h after sham-operation	24h after hepatectomy	72h after hepatectomy	<i>R</i>
(a) Storage buffer	1.09	1.04	1.40	1.10	1.3 $\pm$ 0.1
(b) 2 M-NaCl	3.34	3.37	3.38	3.25	1.0 $\pm$ 0.1
(c) 0.01% SDS	6.19	6.67	5.46	5.13	0.9 $\pm$ 0.1

Table 2. *Effect of NaI and N-pyrenemaleimide on tryptophan fluorescence of rat liver non-histone chromatin proteins*

Tryptophan fluorescence of chromatin was measured at an emission wavelength of 328 nm with an excitation wavelength of 290 nm. Measurements were performed for the indicated time points in 10 mM-Tris/HCl/1 mM-phenylmethanesulphonyl fluoride, 0.2 mM-EDTA, pH 8.0, and 0.12 M-NaI in storage buffer and 500 nM-*N*-pyrenemaleimide at a protein concentration of 10 µg. *R* is defined as in Table 1.

Fluorescence intensities of chromatin prepared from livers of rats:

Solvent	At 0h (control)	24h after sham-operation	24h after hepatectomy	72h after hepatectomy	<i>R</i>
(a) Storage buffer	0.51	0.49	0.79	0.49	1.6 $\pm$ 0.1
(b) 0.12 M-NaI	0.50	N.D.	0.75	0.51	1.5 $\pm$ 0.1
(c) 500 nM- <i>N</i> -pyrene- maleimide	0.34	0.33	0.59	0.34	1.8 $\pm$ 0.1
Degree of quenching [i.e. (c)/(a)]	0.67	0.67	0.75	0.69	

portionately more than the others if the protein lies in close proximity to histone H3 and equally to the others if not. Table 2 shows that the degree of quenching of the 24 h-after-hepatectomy sample is slightly less than the other samples, i.e., 0.75 as compared with 0.67, indicating that any newly synthesized non-histone protein does not move proximal to the labelled H3.

The mobility of a fluorescent probe in the chromatin lattice may be determined by fluorescence polarization. Table 3 indicates the polarization constants for chromatin and nucleosomes isolated from rat liver 24 h after partial hepatectomy and sham-operated rat liver. All of the above exhibit polarization; sham-operated-rat and hepatectomized-rat chromatin are equivalent to each other, as are the nucleosomes to each other. This indicates that the greater fluorescence intensity of the chromatin 24 h after hepatectomy over the chromatin 24 h after sham-operation is not a result

Table 3. *Fluorescence polarization of chromatin and nucleosomes*

Rat liver chromatin and nucleosomes (10 µg of chromatin protein) were labelled at saturating concentrations of probe (200 nM-*N*-pyrenemaleimide) in storage buffer. Fluorescence intensities were measured with polarizers at 0°/0° (V_v), 90°/0° (V_H), 90°/90° (L_H) and 0°/90° (L_v). The polarization constant (*P*) was described by:

$$\frac{V_v - L_v(V_H/L_H)}{V_v + L_v(V_H/L_H)}$$

Polarizations are given as means $\pm$ s.d., with the numbers of determinations in parentheses.

Sample	<i>P</i>
Rat liver chromatin	
24 h after sham-operation	0.21 (3) $\pm$ 0.04
24 h after hepatectomy	0.21 (3) $\pm$ 0.04
Rat liver nucleosomes	
24 h after sham-operation	0.07 (2) $\pm$ 0.02
24 h after hepatectomy	0.05 (2) $\pm$ 0.02

of increased mobility of the probe within the chromatin lattice, but of increased fluorescence per binding site.

Chromatin preparations were monitored for purity on SDS/polyacrylamide gels and core histones were analysed on acid/urea/Triton X-100/polyacrylamide gels (Fig. 4). Neither gel system showed significant differences between proteins from rats 24 h after hepatectomy and sham-operated rats,

although an occasional additional band did appear in hepatectomized-rat chromatin analysed by SDS/polyacrylamide-gel electrophoresis. The differences observed in the reactivity of *N*-pyrenemaleimide with the chromatin were independent of whether this protein was present or not, as were differences in tryptophan fluorescence. Since only a few non-histone proteins possess tryptophan, it appears that a minor non-histone tryptophan-containing protein or

Fig. 4. *SDS/polyacrylamide- and acid/urea/Triton X-100 polyacrylamide-gel electrophoresis*

Whole chromatin preparations (10–20 μ g of protein) from rat livers 24 h after sham-operation (a) and partial hepatectomy (b), as well as mononucleosomes (c) from rat livers 24 h after sham-operation, were run on SDS/polyacrylamide gels as described in the Materials and methods section. Core histones (10–20 μ g of protein) from whole chromatin 24 h after sham-operation (d) and after partial hepatectomy (e) were also run on acid/urea/Triton X-100/polyacrylamide gels as described in the Materials and methods section. Analysis of the acid/urea/Triton X-100/polyacrylamide-gel bands is based on those described in Zweidler (1978). M1, M2, M3 and NF represent minor histones.

proteins is synthesized in too small a quantity to be detected by one-dimensional detergent/polyacrylamide-gel electrophoresis.

Conclusion

Our results show changes in chromatin fibres isolated from rat liver during regeneration. It is noteworthy that the maximal effect is observed at 16–24 h after hepatectomy, which has been shown to be the time of maximum DNA and histone synthesis (Keuhl, 1979). Earlier times after hepatectomy were not employed owing to a report (Majumdar *et al.*, 1967) that they reflect the stress caused by the operative procedure. It is apparent that the increased accessibility to the probe is due to the quaternary structure of the octamers in the chromatin fibre and is not an intrinsic property of the nucleosomal subunit (since isolated subunits did not show the effect). The difference is also not due to an increased amount of histone H3, since our data and those of others (Garrard & Bonner, 1974) show no overall change in the relative amount of histone H3. In addition, no significant alteration in the subclasses of histone H3 was detected with acid/urea/Triton X-100/polyacrylamide-gel systems and dissociation of the chromatin fibre by high salt or detergent abolished the effect. The binding of [³H]*N*-ethyl maleimide was not studied, since experimental difficulties preclude its use with intact chromatin fibres. Nucleosomes, which can be labelled (Wang & Candido, 1978), however, did not show any change with the fluorescent probe.

Other authors have shown no alteration in nucleosomal chain length after hepatectomy (Caplan *et al.*, 1978). However, it is well known that the rate of nuclear-protein phosphorylation (D'Anna *et al.*, 1978; Gurley *et al.*, 1978) and acetylation (Nadler, 1976) is increased with DNA synthesis, and thus it is fair to speculate that although the compositional changes are too small overall to be detected by the acid/urea/Triton X-100/polyacrylamide-gel system, changes in histone–histone and histone–DNA interactions occur during liver regeneration, resulting in an increased accessibility to the probe. It is also noteworthy that the tryptophan data strongly suggest that one or more tryptophan-containing non-histone proteins are synthesized concurrent with normal DNA synthesis. These proteins may interact with histone H3 in such a way as to influence the probe's binding site, or they may have no such interaction at all. The latter hypothesis is suggested by the degree of tryptophan quenching by the *N*-pyrenemaleimide probe, which is not markedly altered by the rate of liver regeneration.

These results are provocative in the light of our previously reported work (Mooney *et al.*, 1980), which showed that the probe did not bind to any

great extent to chromatin derived from a quiescent cell (chick erythrocyte) but was markedly increased when made to react with chromatin from active (rat liver thymocyte) cells. Further studies on other systems *in vitro*, such as tissue-culture cells and yeast, are required to determine if a similar effect is observed. It is also noteworthy that the effect of increased susceptibility to the probe, but no detectable alteration in polarization, is similar to the change we observed with the salt-induced fibre change at 60mM-salt noted previously (Mooney *et al.*, 1980).

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