The variation with age of nuclear phosphoproteins released during micrococcal-nuclease digestion and nucleosomal phosphoproteins in three cell types from rat liver

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(Received 1 October 1980/Accepted 10 October 1980)

The nucleosomal non-histone phosphoproteins, and phosphoproteins released during the digestion of nuclei by micrococcal nuclease, were studied in three rat liver nuclear populations, namely diploid stromal, diploid parenchymal, and tetraploid parenchymal nuclei, which were separated by zonal centrifugation, in 3-week-old rats in which the parenchymal cells contain diploid nuclei and in 2- and 4-month-old rats with increasing proportions of parenchymal tetraploid nuclei. Qualitative and quantitative differences in nucleosomal phosphoprotein band patterns were found among different types of nuclei and ages. More phosphoprotein bands were found in nucleosomes derived from parenchymal than stromal nuclei. The number of phosphoproteins released during micrococcal-nuclease digestion increased with age for parenchymal nuclei. The significance of these results, considered in conjunction with the increase of DNA repeat length and decrease of nuclease accessibility with age, is discussed.

Recent studies have shown that eukaryotic chromatin is organized in repeated units, called nucleosomes, that contain about 200 base-pairs of DNA per repeat. Each nucleosome contains a 'core particle' consisting of a histone octamer and a DNA strand 140–145 base-pairs in length [for reviews, see Kornberg (1977) and Felsenfeld (1978)]. Core particles are connected by linkers whose length may vary from 15 to 100 base-pairs of DNA among different organisms and tissues (Morris, 1976*a,b*; Spadafora *et al.*, 1976), among cells of the same tissue (Thomas & Thompson, 1977; Zongza & Mathias, 1979), and among different nucleosomes in the same cell (Johnson *et al.*, 1976; Lohr *et al.*, 1977; Martin *et al.*, 1977). Histone H1 is bound to linker DNA.

The presence and role of non-histone proteins in nucleosome structure has been studied by various groups. No non-histone proteins were found in nucleosomes from chicken erythrocytes (Oudet *et al.*, 1975) and trout testis (Honda *et al.*, 1975), but small amounts of non-histone proteins are present in the chromatin of these cells. Nucleosomes prepared

from chromatin or nuclei by using mild techniques and low ionic strength were found to contain some non-histone protein (Liew & Chan, 1976; Goodwin *et al.*, 1977; Staron *et al.*, 1977; Bakayev *et al.*, 1978; Defer *et al.*, 1978; Letnasky, 1978; Defer *et al.*, 1979; Gronow *et al.*, 1979). Phosphoproteins may be involved in the control of gene expression. Their presence in mononucleosomes has been reported by Defer *et al.* (1978), who also detected a nuclear protein kinase. We have reported (Zongza & Mathias, 1979) that there is a difference in repeat lengths and chromatin sensitivity to nucleases among the three rat liver nuclear populations [diploid stromal (2NS), diploid parenchymal (2NP) and tetraploid parenchymal nuclei (4NP)] and among different ages. In an attempt to understand the possible role of non-histone protein in determining differences in repeat length and the sensitivity of chromatin to nucleases, we undertook the study of phosphoproteins bound to mononucleosomes in the three liver nuclear populations, in 3-week-, 2-month- and 4-month-old rats. Interactions between histones and non-histone chromosomal proteins, and between these proteins and DNA, are probably altered or destroyed during digestion of chromatin with micrococcal nuclease. Consequently we have also examined the non-histone proteins that are released during micro-

Abbreviation used: SDS, sodium dodecyl sulphate.

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coccal-nuclease digestion. The nucleosomal phosphoprotein patterns obtained from different liver nuclei and ages presented both qualitative and quantitative differences. The number of phosphoproteins released during micrococcal-nuclease digestion increased with age for parenchymal nuclei. More phosphoprotein bands were found in nucleosomes derived from parenchymal nuclei than stromal nuclei.

Experimental

Materials

Micrococcal endonuclease (EC 3.1.31.1) and ATP (adenosine 5'-triphosphate disodium salt) were purchased from P-L Biochemicals Inc., Milwaukee, WI 53205, U.S.A. Proteinase K, deoxyribonuclease I (EC 3.1.21.1, grade I) and ribonuclease (pancreatic) (EC 3.1.27.5) were purchased from Boehringer Corp. (London), Ealing, London W5 2TZ, U.K. Spermine and spermidine were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Ampholytes (pH range 3.5–10) were obtained from LKB Producter A.B., Stockholm, Sweden. Adenosine 5'-[γ - ^{32}P]triphosphate triethylammonium salt (specific radioactivity 3000 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of nuclei

Male Sprague-Dawley rats were fed on a GR3 E.K. diet, and had access to the food and water *ad libitum*. They were starved overnight before being killed. Livers were removed from 3-week-, 2-month- and 4-month-old rat that had been killed by cervical dislocation. Liver nuclei were prepared as described by Zongza & Mathias (1979), but in the presence of 0.1 mM-phenylmethanesulphonyl fluoride to prevent proteolysis.

Liver nuclei were separated in different classes by zonal centrifugation as described by Johnston *et al.* (1968). Nuclei, before digestion with micrococcal nuclease, were washed with buffer A [15 mM-Tris/HCl (pH 7.4)/60 mM-KCl/15 mM-NaCl/15 mM-2-mercaptoethanol/0.15 mM-spermine/0.5 mM-spermidine]/0.34 M-sucrose-2 mM-EDTA/0.2 mM-EGTA/0.1 mM-phenylmethanesulphonyl fluoride (Hewish & Burgoyne, 1973), and finally suspended in digestion buffer A/0.34 M-sucrose/0.1 M-phenylmethanesulphonyl fluoride. DNA analysis was carried out as described by Burton (1956).

[γ - ^{32}P]ATP labelling of nuclear proteins

For the labelling of nuclear proteins with ^{32}P the method described by Rickwood *et al.* (1973) was used. Fractionated nuclei were suspended

in incubation buffer [0.34 M-sucrose/0.1 M-Tris/HCl (pH 8.0)/0.01 M-MgCl₂/0.025 M-NaCl/0.1 mM-phenylmethanesulphonyl fluoride] at a concentration of 1×10^8 nuclei/ml. [γ - ^{32}P]ATP (20 $\mu\text{Ci}/10^8$ nuclei) in incubation buffer was added and the nuclei were incubated for 30 min at 37°C. At the end of the incubation, unlabelled ATP was added to a final concentration of 5 mM and nuclei were cooled on ice. The nuclei were pelleted by centrifugation at 1000 g for 10 min, washed once with 0.34 M-sucrose/0.1 M-Tris/HCl (pH 8.0)/1 mM-MgCl₂/0.025 M-NaCl/0.1 mM-phenylmethanesulphonyl fluoride and once with buffer A/0.34 M-sucrose/2 mM-EDTA/0.2 mM-EGTA and finally resuspended in digestion buffer. Samples were counted for radioactivity by measuring Čerenkov radiation in an Inter technique ABAC SL40 liquid-scintillation counter. Phosphoproteins were revealed after radioautography of the second-dimension gel.

Micrococcal-nuclease digestion and sucrose-gradient analysis of chromosomal fragments

Nuclear suspensions containing 1.5×10^8 nuclei/ml were digested with 20 units of micrococcal nuclease/ml in the presence of 1 mM-CaCl₂ until 10–15% of the DNA was rendered acid-soluble. Digestions were stopped by adding EDTA to a final concentration of 5 mM. Control samples were incubated for the same time in the presence of CaCl₂, but without micrococcal nuclease. EDTA was added to stop the digestion of chromatin by endogenous nuclease activity. Both micrococcal-nuclease-digested samples and control samples were centrifuged at 1000 g for 10 min. Supernatants (S₁) were kept for analysis of the proteins released during digestion. Pellets from the nuclei digested by micrococcal nuclease were lysed in 0.2 mM-EDTA (pH 7.4)/0.5% Triton (w/v)/0.2% sodium deoxycholate/0.1 mM-phenylmethanesulphonyl fluoride (Liew & Chan, 1976). Nuclei were allowed to swell for 15 min at 0°C and the lysate was centrifuged for 10 min at 2700 g to remove nuclear membranes and debris. The supernatant containing the nucleosomes was loaded on a 5–20% (w/v) linear sucrose gradient containing 0.2 mM-EDTA (pH 7.4)/0.1 mM-phenylmethanesulphonyl fluoride. Either 12 or 60 ml were used, the latter for preparative purposes. About 4–5 A_{260} units, in a volume of less 200 μl , were loaded on 12 ml gradients and up to 40 A_{260} units in less than 2 ml were loaded on 60 ml sucrose gradients. The 12 ml gradients were centrifuged for 13 h at 34 000 rev./min in a 6 $\times$ 14 ml SW Ti MSE rotor (r_{av} , 8.43 cm), and 60 ml gradients were centrifuged for 27 h at 23 000 rev./min in a 3 $\times$ 70 ml SW MSE rotor (r_{av} , 11.34 cm). After centrifugation, the gradients were collected in fractions (0.5–1 ml), and fractions comprising the mononucleosome peaks were combined. Nucleosomes were pre-

precipitated by adding 1 M-MgCl₂ to a final concentration of 10 mM, left for 30 min at 0°C and centrifuged at 2700 g for 30 min (Honda *et al.*, 1975; Liew & Chan, 1976).

Two-dimensional electrophoresis of proteins

Proteins were isolated from mononucleosomes by dissolving them in 0.1 M-Tris/HCl (pH 7.4)/2 mM-MgCl₂ followed by treatment with 5 µg of RNAase A/mg of DNA and 50 µg of DNAase I/mg of DNA for 90 min at 0°C. Solid urea to a final concentration of 9 M was added, followed by an equal volume of 9.5 M-urea/4% (w/v) Triton X-100/4% (w/v) ampholytes (pH 3.5–10)/10% (v/v) 2-mercaptoethanol/10.2% (w/v) SDS (O'Farrell, 1975). Proteins released during the digestion were precipitated with 3 vol. of ethanol at –10°C overnight and dissolved in the above buffer. Protein analyses were carried out as described by Lowry *et al.* (1951).

The O'Farrell (1975) two-dimensional electrophoretic system was principally used with modifications as described by Phillips (1976). The isoelectric focusing was performed in 5% (w/v)-polyacrylamide rod gels (0.7 cm diam. × 7 cm long) with ampholytes of pH range 3.5–10. The voltage was gradually increased to 200 V while maintaining the current at less than 1 mA/rod and continued for 8 h. The rod gels were cut longitudinally in half, equilibrated in equilibration SDS buffer [2.3% (w/v) SDS/0.5% (v/v) 2-mercaptoethanol/10% (v/v) glycerol/0.0625 M-Tris/HCl, pH 6.8] for 45 min at room temperature with five changes of equilibration buffer. After equilibration, the halves of the gels were fixed on top of the second-dimension slab gels [8 cm long; 10% (w/v) polyacrylamide; Laemmli (1970) electrophoretic system]. Electrophoresis was carried out at 10 mA/slab for 1 h, then at 20 mA/slab until the second ion front was 0.5 cm above the edge. Slab gels were stained overnight in 0.03% Coomassie Brilliant Blue in water/methanol/acetic acid (5:5:1, by vol.) and destained in water/methanol/acetic acid (6:3:1, by vol.). Stained gels were dried and radioautographed. The same amount of protein (300 µg) was applied for all samples on each gel and the amounts of radioactivity loaded varied from 10 000–100 000 c.p.m. The time of exposure to Kodak Kodivex X-ray film varied from 2 weeks to 1 day. Drawings of the positions of radioactive spots were made by illuminating the developed X-ray film from underneath and marking the outlines of the spots on tracing paper. The spots were identified by numbers. These numbers were assigned by comparison in separate runs of both the original X-ray film and the tracing from it, in such a way that a given number always represents a spot in a particular position relative to features common to all the patterns. The drawings do not give an indication

of the relative intensities of the spots, which vary over a wide range.

Purification and electrophoresis of DNA

Samples were made 0.5% with respect to SDS. Proteinase K (self-digested for 1 h) was added to give

Fig. 1. Electrophoresis of DNA present in DS₁ fraction and in mononucleosomes isolated from sucrose-gradient analysis of chromatin fragments

Results shown are for diploid parenchymal nuclei from 2-month-old rats. (a) Mononucleosomes; (b) overall chromatin fragments; (c) DS₁ fraction.

a concentration of 200 $\mu\text{g/ml}$, and the mixtures were incubated for 2 h at 37°C. The concentration of SDS was increased to 1% and NaCl was added to 1 M. The samples were extracted twice with chloroform/isopentyl alcohol (24:1, v/v). DNA was precipitated with 2 vol. of 95% ethanol for at least 3 h at -40°C. The pellets were dried under N_2 and dissolved in DNA electrophoresis buffer diluted 100-fold (see below) to give an A_{260} value of about 38.

Gel electrophoresis of DNA samples was carried out in vertical 2.5% acrylamide (acrylamide/bis-

acrylamide, 19:1, w/w)/0.5% agarose slab gels (8 cm long) with the Tris/borate buffer system [0.9 M-Tris/HCl (pH 8.3)/25 mM-EDTA/0.88 M-boric acid] of Peacock & Dingman (1968). To 50 μl of the solutions of DNA, 10 μl of 60% (w/v) sucrose and 0.1% (w/v) Bromophenol Blue was added and 2 μl was applied to each slot. Gels were run at 75 V for about 1½ h or until the Bromophenol Blue marker was 1 cm above the bottom of the gel. Gels were stained in a solution of 3 μg of ethidium bromide/ml for 30 min. DNA was made visible in the gels under u.v. light and photographed through an orange filter.

Fig. 2. Radioautographs of two-dimensional gel electrophoresis of ^{32}P -labelled phosphoproteins released from liver nuclei of 3-week-old rats during micrococcal-nuclease digestion (DS_1) compared with control nuclei without exogenous nuclease (CS_1)

(a) 2NS- CS_1 (CS_1 fraction from 2NS nuclei); (b) 2NP- DS_1 (DS_1 fraction from 2NP nuclei); (c) 2NS- DS_1 (DS_1 fraction from 2NS nuclei); (d) 2NP- CS_1 (CS_1 fraction from 2NP nuclei). The positions of proteins used as molecular-weight markers, namely bovine serum albumin (68 000) and ovalbumin (43 000), are shown on the diagram for 2NP- CS_1 together with the pH gradient. These scales apply to all the parts of this Figure and of Figs. 3–6 and 10–12.

Results

Analysis of phosphoproteins released by micrococcal-nuclease digestion and endogenous-nuclease digestion

Fractionated nuclei from 3-week-, 2-month- and 4-month-old rats that had been labelled with ^{32}P were digested with micrococcal nuclease in the presence of Ca^{2+} until about 10–15% of the original DNA had become acid-soluble. To examine the release of proteins into the medium as a consequence of the digestion with micrococcal nuclease, nuclei after the digestion were centrifuged and the supernatants (DS_1) kept for analysis of DNA and proteins. Control nuclei, incubated in the presence of Ca^{2+} for the same time but with no micrococcal nuclease, were centrifuged and the supernatants (CS_1) kept for analysis of DNA and proteins. In this way the digestion due to endogenous nucleases during the period of micrococcal-nuclease action

could be monitored. Proteins released from control and nuclei digested by micrococcal nuclease were analysed by two-dimensional electrophoresis as described in the Experimental section.

DS_1 and CS_1 fractions were also analysed for the presence of DNA. Very little DNA was found in DS_1 fractions (one-hundredth of the original DNA present in the sample) and none in CS_1 fractions. DNA from DS_1 was purified and analysed in 2.5% (w/v) polyacrylamide/0.5% agarose slab gels. Electrophoresis revealed the presence of DNA of mononucleosome size as the main component. Some subnucleosomal material was also present, and small fragments of DNA soluble in cold HClO_4 ran out of the gel (Fig. 1). The presence of mononucleosome in the DS_1 fractions could be explained by supposing that nuclei are partially disrupted during digestion and mononucleosome leaks out without any oligomer being able to be released. The suggestion that mild micrococcal-nuclease digestion causes dis-

Fig. 3. Radioautographs of two-dimensional gel electrophoresis of ^{32}P -labelled phosphoproteins released from liver nuclei of 2-month-old rats during micrococcal-nuclease digestion (DS_1) and from control nuclei (CS_1) (a) 2NS- CS_1 ; (b) 2NP- CS_1 ; (c) 2NS- DS_1 ; (d) 2NP- DS_1 (these fractions are defined in the legend to Fig. 2).

ruption of nuclei has been made by Tata & Baker (1978). No signs of lysed nuclei were found under the phase-contrast microscope. However, partial nuclear disruption that allowed the release of mononucleosome cannot be excluded. This could happen if micrococcal nuclease attacks chromatin that is in contact with the nuclear envelope, so that the structure of the nuclear membrane and subsequently its permeability is altered to permit the liberation of small particles, like subnucleosomal fragments and mononucleosomes, into the medium. Alternatively, these fragments might be released via the nuclear pores.

No DNA was found in CS₁ fraction. However, although endogenous nuclease does digest chromatin (Zongza & Mathias, 1979), the amount of DNA digested is very little, and no mononucleosome escapes into the medium.

Comparison of the radioautographs of phosphoproteins present in CS₁ fractions and DS₁ fractions from the three types of nuclei studied in the three ages shows the presence of some common bands (Figs. 2, 3, 4, 5 and 6). However, the relative amounts of the various bands and the appearance of different bands make each pattern distinct. The phosphoproteins present in S₁ fractions could be either nucleoplasmic proteins extracted by the digestion buffer or chromosomal phosphoproteins that are involved in the higher structure of chromatin and liberated through the action of the endogenous and micrococcal nucleases and proteins whose binding sites at the linker DNA were attacked by micrococcal and endogenous nucleases. The presence of mononucleosomes in DS₁ fractions may indicate that some of the phosphoproteins present in these fractions are nucleosomal. However, the DNA/protein ratio in DS₁ fractions was only 1:50 compared with 1:2 found for mononucleosomes.

Comparison of the phosphoproteins released in the digestion buffer during micrococcal-nuclease digestion of liver nuclear populations in the three ages studied shows that the number of these proteins increases with age for parenchymal nuclei. In DS₁ fractions from stromal nuclei, a small increase in the number of phosphoproteins is found between 3-week- and 2-month-old rats. The phosphoproteins present in CS₁ fractions are fewer than the phosphoproteins of DS₁ fractions.

In 3-week-old rats (Fig. 2) the phosphoproteins released in the digestion buffer are mostly acidic (pH 4–7) and in number vary from at least 10 (2NS-CS₁) to at least 22 (2NP-DS₁). In 2-month-old rats (Figs. 3 and 4) the number of phosphoproteins released in the digestion buffer varies from 20 (2NS-CS₁) to 37 (4NP-DS₁). Phosphoproteins occur at pH values up to 9, but most of the bands are in the acidic region. For 2-month-old rats, fewer phosphoproteins are released in the digestion buffer

Fig. 4. Radioautographs of two-dimensional gel electrophoresis of ³²P-labelled phosphoproteins released from liver nuclei of 2-month-old rats during micrococcal-nuclease digestion (DS₁) and from control nuclei (CS₁) (a) 4NP-CS₁ (CS₁ fraction from 4NP nuclei); (b) 4NP-DS₁ (DS₁ fraction from 4NP nuclei).

during the digestion of stromal nuclei with micrococcal nuclease compared with parenchymal nuclei. DS₁ fraction from diploid parenchymal nuclei has more bands in common with DS₁ fraction from tetraploid parenchymal nuclei than with DS₁ fraction from stromal nuclei. In 4-month-old rats (Figs. 5 and 6) the number of phosphoproteins released in the digestion buffer during digestion with micrococcal nuclease increases up to 43 for parenchymal nuclei. DS₁ fractions from diploid and tetraploid parenchymal nuclei have many common bands, whereas few bands are similar between stromal and parenchymal nuclei. Detailed identification of the bands is recorded in the diagrams of the radio-

Fig. 5. Radioautographs of two-dimensional gel electrophoresis of ^{32}P -labelled phosphoproteins released from liver nuclei of 4-month-old rats during micrococcal-nuclease digestion (DS_1) and from control nuclei (CS_1) (a) 2NS-CS_1 ; (b) 2NP-CS_1 ; (c) 2NS-DS_1 ; (d) 2NP-DS_1 (fractions are defined in the legend to Fig. 2).

autographs (Figs. 2, 3, 4, 5 and 6). The significance of these results is discussed below.

Sucrose-gradient analysis of ^{32}P -labelled nucleosomes

^{32}P -labelled nuclei were digested with micrococcal nuclease until 10–15% of the original DNA was rendered acid-soluble, and the chromatin fragments were analysed on sucrose gradients as described in the Experimental section. Fig. 7 shows the radioactivity and absorbance profiles for a typical gradient. As Fig. 7 shows, a considerable amount of radioactivity was present on top of the gradient. To examine the presence of phosphorylated proteins in the top of the gradient, appropriate fractions (600 000 c.p.m.) were pooled and precipitated with 3 vol. of ethanol overnight at -20°C . Very little radioactivity (about 1500 c.p.m.) was found in the pellet, the bulk of the radioactivity remaining in the supernatant. Thus this high radioactivity is probably due to free $[\text{P}^{32}\text{O}_4]^-$ or

$[\text{P}^{32}\text{P}]\text{ATP}$ released from a loosely bound form or present in the original lysate.

To confirm the association of phosphorylated non-histone proteins with nucleosomes, isolated nucleosomes were purified in a sucrose gradient (Fig. 8). Radioactivity remained mainly in the mononucleosome peak, but again some radioactivity was found on top of the gradient, probably because of contamination of the isolated nucleosome fraction with top fraction of the gradient. To examine the stable association of phosphoproteins with nucleosomes and the possibility of rearrangement of proteins during the sucrose-gradient centrifugation, isolated labelled mononucleosomes were mixed with isolated unlabelled oligomer and run again on sucrose gradients (Fig. 9). No radioactivity migrated to oligomer regions. The mononucleosome fraction was shown to contain only mononucleosomes by electrophoresis of purified DNA from this fraction in 2.5% acrylamide/0.5% agarose gels, where only a single band was observed (Fig. 1).

Fig. 6. Radioautographs of two-dimensional gel electrophoresis of ^{32}P -labelled phosphoproteins released from liver nuclei of 4-month-old rats during micrococcal-nuclease digestion (DS_1) and from control nuclei (CS_1) (a) 4NP- CS_1 ; (b) 4NP- DS_1 (fractions are defined in Fig. 4).

Two-dimensional electrophoresis of phosphoproteins bound to mononucleosomes

Mononucleosome-bound phosphoproteins were prepared as described in the Experimental section and analysed by two-dimensional electrophoresis. Mild digestions were necessary to avoid the liberation of non-histone proteins from mononucleosomes. Mononucleosomes prepared in this way contained histone H1, as revealed by SDS/polyacrylamide-gel electrophoresis (Zongza, 1980). Little or no H1 was found in the proteins that appear in the soluble phase during the course of the preparation of these mononucleosomes, indicating that a substantial proportion of the mononucleo-

Fig. 7. Profile of sucrose gradient of chromatin fragments produced by digestion of nuclei with micrococcal nuclease. Nuclear proteins of diploid parenchymal nuclei from 4-month-old rats were labelled with ^{32}P . Subsequently the nuclei were digested with micrococcal nuclease until 10% of the original DNA was rendered acid-soluble. —, A_{260} ; $\blacktriangle$, ^{32}P radioactivity.

Fig. 8. Sucrose-gradient analysis of ^{32}P -labelled mononucleosome fraction

Mononucleosome fraction was isolated by a sucrose-gradient analysis of ^{32}P -labelled chromatin fragments (as in Fig. 7) and centrifuged again in a sucrose gradient. —, A_{260} ; $\blacktriangle$, ^{32}P radioactivity.

some fraction contained the H1-binding site in the spacer DNA. Radioautographs of the phosphoproteins of mononucleosomes are shown in Figs. 10, 11 and 12. The number of phosphoproteins bound to mononucleosomes, from all nuclei and ages studied, varied from 15 to 20. They extend from pH 4.5 to 9. Comparison of the nucleosomal phosphoproteins among the three different liver nuclear types in the same age and among the different ages in the same nuclear type shows that more common bands can be found among the different nuclear types of the

Fig. 9. Sucrose-gradient analysis of a mixture of ^{32}P -labelled mononucleosome and unlabelled oligomer fractions

^{32}P -labelled mononucleosome and unlabelled oligomer fractions were isolated by sucrose-gradient analysis of ^{32}P -labelled and unlabelled chromatin fragments respectively (as in Fig. 7), combined and re-centrifuged in a sucrose gradient. —, A_{260} ; ▲, ^{32}P radioactivity.

same age than among different ages. In 2- and 4-month-old rats (Figs. 11 and 12), mononucleosomes from stromal nuclei have fewer phosphoprotein bands in common with tetraploid parenchymal than with diploid parenchymal nuclei.

The phosphoprotein band patterns from mononucleosomes are quite different from those of respective DS_1 fractions. Fewer bands are found in mononucleosomes than in DS_1 fractions. Only few bands are common between DS_1 fractions and mononucleosomes of the same type of nucleus and age. These common bands could be a consequence of the presence of small amounts of mononucleosome DS_1 fractions. The different phosphoprotein patterns for both mononucleosomes and DS_1 found in the different nuclear types and ages are in agreement with the proposition that phosphoproteins play a regulatory role in gene expression (Kamiyama *et al.*, 1972; Stein *et al.*, 1974; Stott & Williamson, 1978). Different phosphoprotein-regulation patterns and ^{32}P incorporation have been reported for developing rats (Guguen-Guilouzo *et al.*, 1979).

Discussion

The present results confirm the presence of non-histone proteins in nucleosomes and also show that phosphoproteins of nucleosomes vary among different types of nuclei of the same tissue and of different age. Different sets of phosphoproteins are released during digestion from different nuclei. Some

Fig. 10. Radioautographs of two-dimensional gel electrophoresis of ^{32}P -labelled phosphoproteins associated with mononucleosomes derived from liver nuclei of 3-week-old rats

(a) Mononucleosome fraction from 2NS nuclei; (b) mononucleosome fraction from 2NP nuclei.

of these proteins could be nucleoplasmic and soluble in the digestion buffer. However, the released proteins may also include proteins whose binding sites to chromatin were attacked by micrococcal nuclease and endogenous nuclease and hence appear in the soluble phase. Also, chromosomal proteins that contribute to the higher orders of structure of chromatin, whose interactions with histones and DNA are destroyed because of the digestion, would be released. A few of these proteins may be nucleosomal, since DS_1 fractions contain mononucleosomes.

Many differences were found in phosphoproteins bound to mononucleosomes of various nuclei and ages and in phosphoproteins released during

Fig. 11. Radioautographs of two-dimensional gel electrophoresis of ^{32}P -labelled phosphoproteins associated with mononucleosomes derived from liver nuclei of 2-month-old rats

(a) Mononucleosome fraction from 2NS nuclei; (b) mononucleosome fraction from 2NP nuclei; (c) mononucleosome fraction from 4NP nuclei.

Fig. 12. Radioautographs of two-dimensional gel electrophoresis of ^{32}P -labelled phosphoproteins associated with mononucleosomes derived from liver nuclei of 4-month-old rats

(a) Mononucleosome fraction from 2NS nuclei; (b) mononucleosome fraction from 2NP nuclei; (c) mononucleosome fraction from 4NP nuclei.

digestion. The difference in electrophoretic patterns between phosphoproteins released during digestion and proteins bound to nucleosomes indicates that distinct sets of non-histone chromosomal proteins are involved in the structure of chromatin subunits

and via attachment to linker DNA with higher orders of chromatin structure. The re-centrifugation in sucrose gradients of ^{32}P -labelled purified mono-

nucleosomes with unlabelled oligomers ruled out the possibility that rearrangement of non-histones could take place during centrifugation. The present results cannot exclude the possibility that nucleosomes may be heterogeneous in their protein content. It is possible that, under our experimental conditions (mild digestion), we have released the most accessible nucleosomes, some of which may be components of active genes. Inactive nucleosomes could contain only small amounts of non-histone proteins, as suggested by Gottesfeld *et al.* (1975) and reported by Letnasky (1978). That mononucleosomes are heterogeneous was recently demonstrated in a report by Defer *et al.* (1979). They found that, on metrizamide gradients, mononucleosomes appear to be heterogeneous according to their content of associated non-histone protein. It is very likely that some well-defined proteins could vary from one nucleosome to another, since it is admitted that non-histone proteins include proteins playing a specific role in gene activation.

The number of phosphoprotein bands was about the same in nucleosomes derived from diploid stromal and parenchymal nuclei from 3-week- and 2-month-old rats and then decreased for both diploid and tetraploid parenchymal nuclei at the age of 4 months. Nucleosomes from diploid and tetraploid parenchymal nuclei had more common bands than did parenchymal and stromal nuclei, as would be expected, because of their differences in metabolic activity (Johnston & Mathias, 1972). The procedure that we have used reveals only those proteins that are phosphorylated under the conditions that are employed for the labelling *in vitro*. It does not necessarily represent the pattern of activity in the intact tissue. However, the existence of differences between the pattern of labelling in stromal and parenchymal nuclei indicates that the labelling *in vitro* has some physiological significance.

The number of phosphoproteins released into the digestion buffer during micrococcal- and endogenous-nuclease digestion increases with age. This could indicate either that as the animals age the non-histones become more loosely bound to chromatin and therefore are removed more easily from chromatin during digestion, or more phosphoproteins are associated with linker DNA. The significance of phosphorylation in achieving this loose binding is apparent. *In vivo*, however, other chemical modifications of non-histones may also be responsible for the alteration of their charge and consequently of their interactions with DNA and histones. Non-histone chromosomal proteins have been found tightly bound to active parts of the genome (Wakabayashi *et al.*, 1973; Wang *et al.*, 1976; Gottesfeld & Butler, 1977), thus probably inducing a change in the conformation of chromatin necessary for the transcription. The transcriptional

activity of the genome, on the other hand, declines with age, and at the same time the ratio of non-histone proteins to DNA is decreased (Gershey & Kleinsmith, 1969; Zhelabovskaya & Berdyshev, 1972).

We have found that the accessibility of chromatin to nucleases decreases with age and that the DNA repeat length increases (Zongza & Mathias, 1979). Since more phosphoproteins are released in the digestion buffer as the animal ages, it seems probable that more non-histone proteins are attached to spacer DNA as the cell becomes older. The variation, on the other hand, of the phosphoprotein patterns among different nuclear classes of liver and among different ages, supports the suggestion that phosphoproteins are involved in the control of gene expression (Stein *et al.* 1974; Letnasky & Wenzel, 1977; Stott & Williamson, 1978), because different parts of the genome should be activated in different nuclei at different ages.

However, the present results cannot indicate the exact role of non-histone chromosomal proteins in nucleosome structure and chromatin organization and regulation. Detailed analysis and isolation of the non-histone proteins present in mononucleosomes would be very helpful in understanding the organization of chromatin. Chemical modifications of the non-histone proteins should also be studied extensively to allow the determination of possible changes in interaction of non-histone chromosomal proteins with DNA and histones.

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