Volume 6 Number 3 March 1979 Nucleic Acids Research

Comparison of non histone proteins selectively associated with nucleosomes with proteins released during limited DNase digestions

N.Defer[+], M.Crepin[++], C.Terrioux[++], J.Kruh[+] and F.Gros[++]

[+]Institut de Pathologie Moléculaire, 24, rue du Faubourg Saint-Jacques, 75014 Paris, and
[++]Département de Biologie Moléculaire, Institut Pasteur, 28, rue du Docteur Roux, 75724, Paris Cedex 15, France

Received 8 November 1978

ABSTRACT

Cultured mammary cells from GR mouse were used to analyse proteins associated with the mononucleosomes and released by a short micrococcal DNase treatment of nuclei. On metrizamide density gradients, mononucleosomes appear to be heterogeneous according to their content of associated non-histone proteins. Proteins associated with the denser fraction (1.22 - 1.24 g/ml) were analysed by two dimensional electrophoresis and compared to the proteins released by DNase I treatment. All the proteins associated with mononucleosomes were also released by DNase I treatment. It could then be assumed that these proteins are associated with the active part of the genome. Additional proteins were released by micrococcal DNase treatment of the nuclei. They could be involved in a higher order organization of chromatin.

INTRODUCTION

Chromatin is composed of repeated units, the nucleosomes, made of histones and DNA segments of about 200 base pairs (1-3). Every class of DNA,including actively transcribed and inactive genes, was found to be present in nucleosome structure (4-6). However, active genes are in a more accessible form for DNAse I digestion (4-7). They are also preferably released by micrococcal DNase digestion.

It is usually admitted that the non-histone proteins (NHP) are involved in the regulation of gene expression, which raises the question of the specific association of individual NHP with active genes. The binding of NHP to nucleosomes has been controversial (8-11). recently we have established that phosphoproteins and protein kinases are at least partly associated with mononucleosomes isolated after a short micrococcal DNase digestion of rat liver-chromatin (12). Paul and Malcolm have shown that un-

fixed nucleo-proteins can be banded isopycnically in metrizamide according to the protein : nucleic acid ratio (10). We have then analysed by this method the chromatin fragments obtained after DNase digestion of the nuclei of GR cells labeled with (^{3}H) thymidine and (^{35}S) methionine. In this paper we show 1) the heterogeneity of the monomers according to their NHP content ; 2) the presence of NHP strongly associated to part of the mononucleosomes ; 3) that most of the proteins bound to the monomers are also released by short DNase I digestion of the nuclei.

MATERIALS AND METHODS

Isolation of Nuclei : Mouse mammary tumor cells (GR strain) grown as described elsewhere (13) were labeled with (^{3}H)-thymidine, 100 µCi/ml (60 Ci/mM) and (^{35}S)-methionine, 50 µCi/ml (800 Ci/mM)(Amersham). Growing cells were collected by centrifugation and washed once with PBS (0,14 M NaCl, 3 mM KCl, 9,5 mM Na_2PO_4, 1,5 mM KH_2PO_4). From these cells nuclei were prepared using a method previously described (14). During nuclei preparation, 1 mM phenylmethylsulfonyl fluoride (PMSF) was used to inhibit protease activity. After centrifugation on a 2 M sucrose cushion, the nuclei were washed several times by resuspension in RSB buffer (10 mM Tris-HCl pH 7,6, 0.3 M sucrose and 3 mM $CaCl_2$).

DNase I and Micrococcal Nuclease Digestion : The nuclear pellet was resuspended in RSB buffer containing 1 mM $CaCl_2$ at a DNA concentration of 20-100 A_{260}/ml and preincubated at 37°C for 5 min. Bovine pancreatic DNase I (Worthington DPFF) was added to a final concentration of 20 µg/ml at 37°C for various times under the conditions described by Weintraub and Groudine (4). In other experiments nuclei were treated with 200 units/ml of micrococcal nuclease (Worthington) as described by Shaw et al (15). Both nuclease digestions were performed in such conditions that 10 % of DNA became acid-soluble.

Preparation of Monomers of Nucleosomes : Nuclei at a concentration of 10^{8}/ml were incubated for 90 sec with 200 units/ml of micrococcal nuclease in RSB containing 1 mM $CaCl_2$, and then treated for 10 min with 10 mM EDTA. Analysis of the released particles on a 5-30 % sucrose gradient shows mainly mononucleosomes and few oligo-

T A B L E
=========

Comparison of proteins released by nuclease treatment and proteins associated with mononucleosomes

Peptides	Nuclease treatment		Mononucleosomes	
	DNase I	Micrococcal DNase	A	B
1	++	+	+	++
2	++	+++*	-	-
3	+	++*	++	++
4	++	+++*	++	+++
5	++	++*	+	-
6	++	++*	+	++
7	+	+	+	+
8	+++	+	+	+++
9	+	+	+	+
10	+	+	+	-
11	+	++	+	+
12	+	++	+	++
13	+	-	+	+
14	+	-	+	-
15	++	-	+	++
16	++	-	+	+
17	++	-	-	-
18	+	+	+	+
19	+	+	+	-
20	++	+	+	-
21	-	+	+	-
22	-	+	-	-
23	-	+	-	-
24	-	+	-	-

Relative intensity of each spot is indicated by the number of +. A and B respectively correspond to mononucleosomes obtained by Biogel column before and after purification on metrizamide gradient.

* proteins present in several spots corresponding to the same molecular weight but with different charges.

mers of digested chromatin. Chromatin subunits were purified on a Biogel A5 column (100 x 2,5 cm) as previously described (12). Three main peaks were obtained after elution from this column. Analysis on a sucrose gradient of the fraction eluted in the same volume

as catalase (MW 240.000) shows the presence of 11 S mononucleosomes containing 180-200 base-pair DNA.

Buoyant Density Centrifugation in Gradients of Metrizamide (2-(3 acetamido 5-N methylacetamido-2, 4, 6-triiodobenzamido -2-deoxy-D-glucose) : A 87 % w/w solution of metrizamide (Nyegaard & Co., A/S Oslo) was prepared by slowly adding 50 g of metrizamide to 35 ml of buffer Tris-HCl 10 mM pH 7.6 EDTA 1 mM , with stirring at room temperature. For centrifugation, chromatin subunits were mixed with metrizamide solution to obtain a concentration of 41 % metrizamide. Samples of 8 ml were centrifuged in 10 ml polycarbonate tubes in a fixed-angle 65 rotor at 80.000 g for 40 hrs at 5°C. Fractions were analysed for DNA and protein content by measuring respectively (^{3}H) thymidine and (^{35}S) methionine incorporated in cell culture. The refractive index of each fraction was measured and the density calculated using the relationship 3.50 n - 3.462 (10).

Acrylamide Gel Electrophoresis : Non-histone proteins associated with nucleosomes or released by DNase treatment were analysed on two-dimensional polyacrylamide gels (16). In this paper we only show autoradiographies of the 2D electrophoreses, since the amount of available material usually did not allow to obtain coomassie blue stained spots. Moreover O'Farrell has shown that all spots stained with coomassie blue are also present in autoradiogram performed after (^{35}S) methionine labeling (personnal communication). Supernatants or diluted samples were concentrated by precipitation with 3 volumes of acetone.

RESULTS

Separation of Chromatin Fragments by Metrizamide Gradients : Micrococcal nuclease digestion of GR cell nuclei produced the familiar pattern of DNA fragments seen in other systems (2, 17-19). Biogel chromatography of the nuclear digests resulted in the separation of the monomers from the dimers and trimers and from larger fragments of chromatin (15). Analysis of the total nuclear digests on metrizamide gradients (figure 1) shows the presence of two main components on the basis of their density : a major low

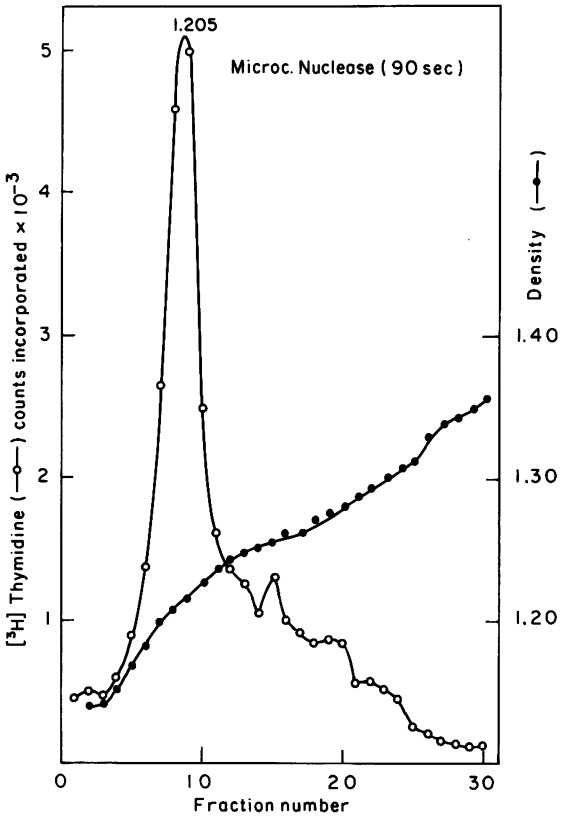

FIGURE 1 : Isopycnic banding of particles released by treatement of nuclei with micrococcal nuclease. The cells were labeled for 26 hrs with (3H) thymidine. Nuclei were incubated 90 sec. at 37°C with micrococcal nuclease (200 µ/ml) as described in the methods section ; the hydrolysate was centrifuged and the supernatant (0.5 to 2 ml) was mixed with the concentrated metrizamide solution to a final concentration of 41 %. After centrifugation at 80 000 g for 40 hrs, 0.2 ml fractions were collected : 20µl aliquots were used to determine the (3H) thymidine pattern.

density peak (1.205 g/ml) and a denser heterogenous fraction (1.22 to 1.25 g/ml). This figure shows the results obtained with the chromatin solubilized after limited micrococcal DNase digestion in which 10 % of DNA was rendered acid-soluble. The relative proportion of the denser fraction was found to be smaller when only 3 to 5 % of DNA was acid-soluble. Under these conditions a smaller quantity of monomers was released from chromatin. Mononucleosomes

have then been separated from larger fragments by Biogel A5 chromatography. The analysis of the prepurified monomers on metrizamide gradients (figure 2) resulted in the separation of three classes of particles : two lighter fractions (1.19 and 1.21 g/ml) and a denser fraction including 37 % of the mononucleosomes. When DNA extracted from each of these peaks was analysed on 5 % polyacrylamide gels (results not shown) it was found to contain 180-200 base pairs with the same heterogeneity for each peak.

The analysis of the larger chromatin fragments on metrizamide gradients (figure 2b) showed essentially one peak (1.205 g/ml). The material sedimenting at densities 1.22 - 1.25 g/ml could result from a further digestion of chromatin after Biogel chromatography by DNase remaining associated to the fraction as observed in experiments performed with rat liver chromatin (12). No DNase activity has been found associated with the monomer fraction.

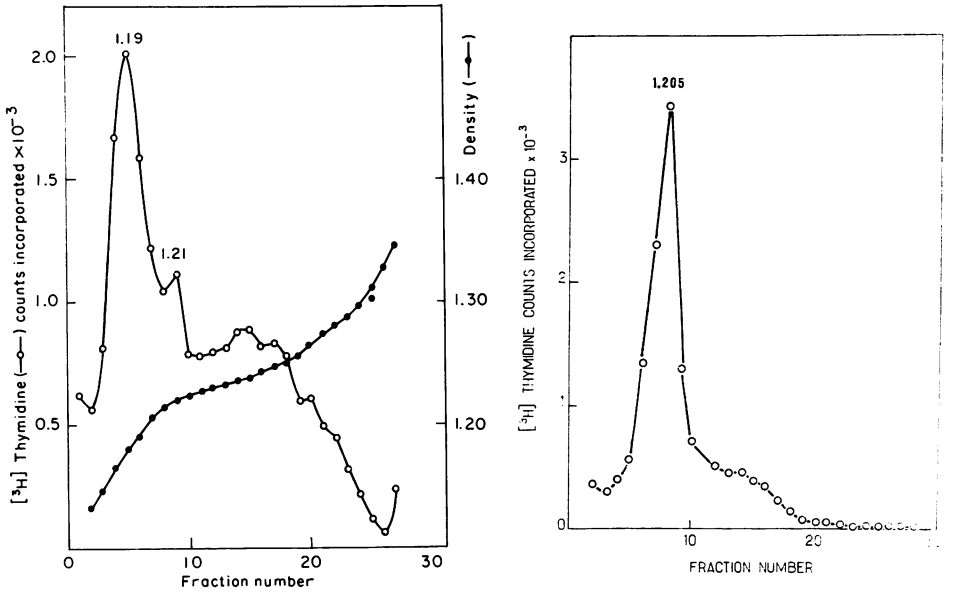

FIGURE 2 : Isopycnic banding of monocucleosomes and chromatin excluded from Biogel column. Cells and nuclei were treated as described under figure 1. After micrococcal DNase digestion, the supernatant was fractionated on a Biogel A5m column. Fractions corresponding to the excluded peak ant to mononucleosomes were collected, pooled and analysed on metrizamide gradient as described under figure 1. a) monocucleosomes ; b) excluded chromatin.

Analysis of the proteins bound to the monomers : Since mononucleosomes prepurified by gel filtration were found homogenous by electron microscopy and electrophoresis of DNA, the heterogeneity of the particles could result from bound proteins. The cells were therefore cultured in the presence of (^{35}S)-methionine and (^{3}H)-thymidine for 14 hrs at 37°C. The protein/DNA ratio measured by the (^{35}S/^{3}H)ratio was found higher in the denser fraction of the metrizamide gradient of mononucleosomes. Moreover, by two dimensional gel electrophoresis no detectable (^{35}S)) acidic proteins were found in the lighter components (1.19, 1.21 g/ml). The absence of radioactive spots in these fractions can be due to an absence of bound proteins after metrizamide gradient or to a very

FIGURE 3 : Two dimensional gel electrophoresis of proteins associated with the prepurified mononucleosomes. The prepurified mononucleosomes were prepared as described under figure 2 from cells cultured with (^{35}S) methionine. Fractions corresponding to the denser particles of the metrizamide gradient (1.22 - 1.25 g/ml) (figure 2a) were pooled, concentrated and suspended in the lysis buffer used to perform the electrophoresis. About 12 500 cpm of (^{35}S) methionine were loaded on the gel. Autoradiography of dry gel was obtained after two weeks of contact with X ray Kodirex film. Polypeptides of special interest (see table) are noted with arrow and numbered the polypeptide A represents Actin.

low content of methionine in the proteins. The electrophoretic pattern of the NHP extracted from the denser fraction of mononucleosomes (1.22 - 1.25 g/ml) shows at least 18 different polypeptides (figure 3). Most of them are acidic (pH 4.5 - 7.0) and their molecular weights were from 20 000 to 200 000. They correspond to some of the polypeptides (1,3,4,6,9,11,13,15,16,18) extracted from undigested nuclei (figure 4). Most of them are still present in the residual pellet left after micrococcal digestion of the nuclei (Results not shown).

Labelled proteins found in the denser fraction represent about 35 per cent of the total radioactive proteins submitted to gradient analysis. Most of the other proteins sedimented as free proteins (d = 1.28 g/ml) ; they includ unbound contaminant proteins as previously shown (12) and loosely bound proteins dissociated from protein-DNA complexes by metrizamide (20). The associated proteins here observed represent mostly the proteins strongly

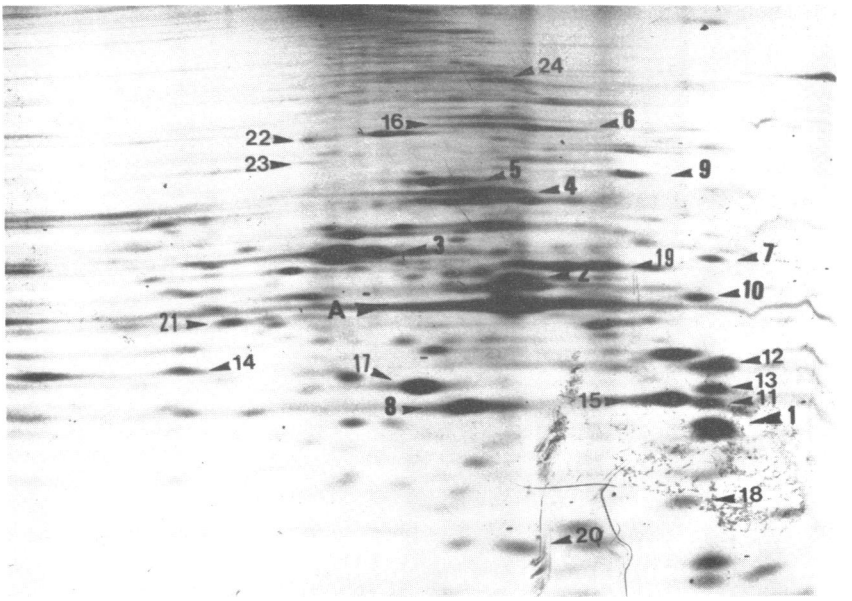

FIGURE 4 : Two-dimensional gel electrophoresis of non histone proteins from non digested nuclei. Nuclei, prepared as described in the methods, from (^{35}S) methionine labeled cells, were suspended in the lysis buffer of the electrophoresis. About 120,000 cpm we re loaded on the gel. Autoradiography was obtained after three days of contact with X ray Kodirex film.

bound to the particle. Figure 5 shows the electrophoretic pattern of proteins associated to the mononucleosomes eluted from the biogel column. Some additional spots were found as compared to the denser fraction of the metrizamide gradients (5,10,14,19,20,21). The proteins corresponding to these spots may be either contaminants or loosely bound proteins. Moreover the denser fraction of the metrizamide gradient is enriched in proteins of the groups 1,4,6,8, 12,15 as compared to proteins of the groups 3,7,9,11,13,16,18, the relative amount of which was not modified after gradient purification.

Comparison of proteins released by limited DNase I and micrococcal DNase digestions : Limited digestion of liver and erythrocytes nuclei by DNase I resulted in a preferential loss of the active sequences (4). DNase treatment of the GR cell nuclei leads to a rapid digestion of more than 75 % of the DNA. Figure 6 shows the two dimensional electrophoretic pattern of proteins released by DNase I where 10 % of DNA has become acid-soluble. Figure 7 shows the 2D-electrophoretic pattern of the proteins released by

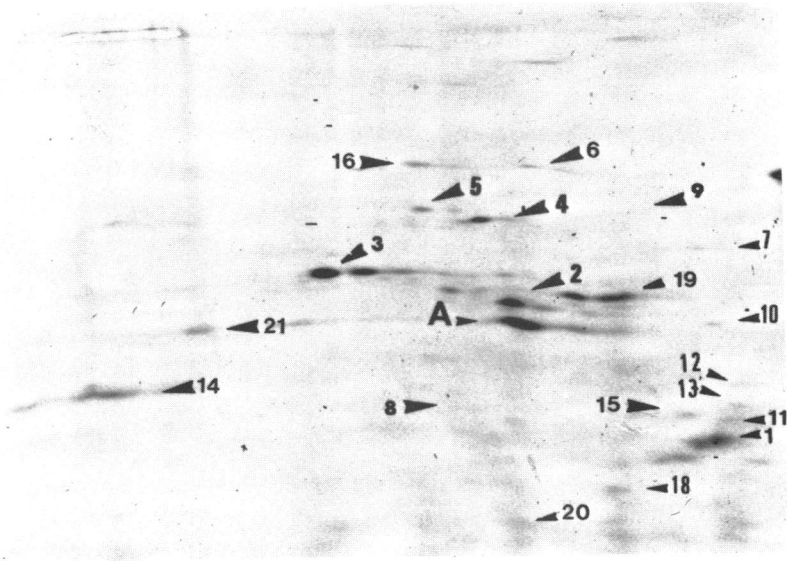

FIGURE 5 : Two dimensional gel electrophoresis of proteins associated with mononucleosomes. Mononucleosomes prepared by biogel chromatography as described in the Methods Section, were concentrated by acetone precipitation.

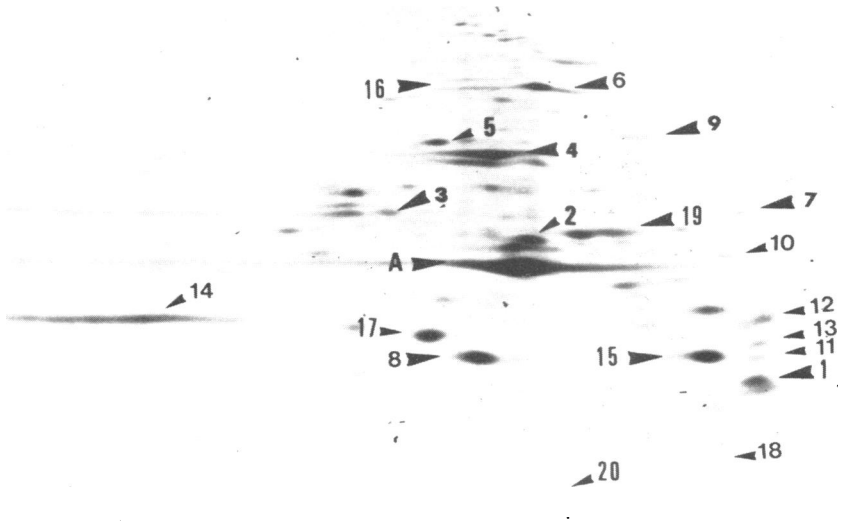

FIGURE 6 : Two dimensional gel electrophoresis of nuclear non-histone proteins released by DNase I digestion. Nuclei from (^{35}S) methionine labeled cells weretreated with DNase I to give 10 % perchloric acid soluble material. After digestion, nuclei were pelleted and the supernatant precipitated with acetone. 80,000 cpm were loaded on gel electrophoresis and the dry gel was developed for 4 days.

micrococcal DNase under the same conditions. Most of the proteins released by both nucleases were found identical (spots 1-12). Nevertheless some differences were observed : a) some spots were preferentially present in one digest (for example 13-17 spots in DNase I digest, and 21-24 spots in micrococcal DNase digest) ; b) for many common spots the relative intensity is different in the two digests ; c) some single spots (2-6) in the DNase I digest appear as several spots in the micrococcal DNase digest (same molecular weight but different charges). These last spots could correspond to post-translationally modified proteins. A control experiment (figure 8) shows that very few proteins are spontaneously released from nuclei incubated under the same conditions but in the absence of DNase. Therefore non-chromatin proteins or spontaneously released proteins do not contribute significantly to the pattern of the proteins released from chromatin

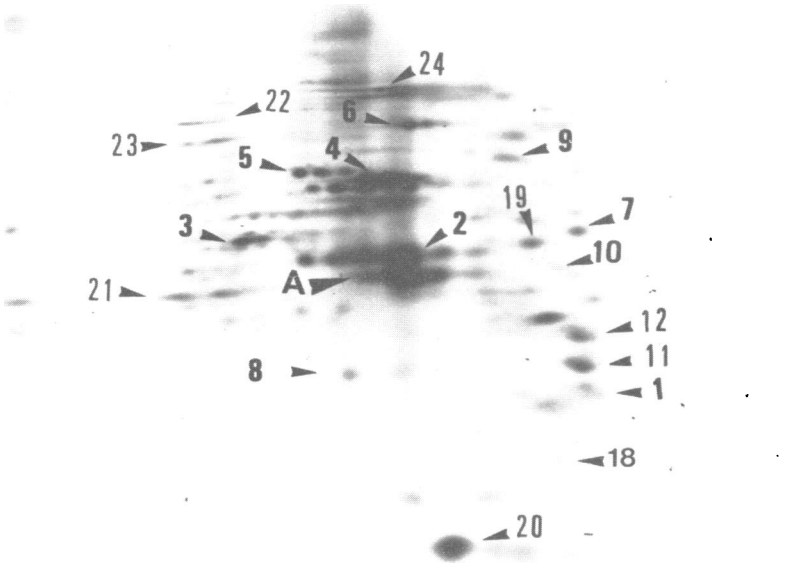

FIGURE 7 : Two dimensional gel electrophoresis of nuclear non histone proteins released by micrococcal nuclease digestion. Nuclei from (^{35}S)labeled cells were treated with micrococcal nuclease to give 10 % perchloric acid soluble material. As described in the methods, the proteins released in the supernatant were precipitated with acetone and analysed by 2D electrophoreris. Input 95 000 cpm and autoradiography developed after 3 days of contact.

by DNase digestion.

DISCUSSION

Among the proteins released by DNase I or micrococcal digestion we have to consider different classes : proteins associated with chromatin particles or to small DNA fragments and free proteins which have been released because their binding sites have been attacked by DNase or because of the modification of chromatin superstructure. Although most of the proteins released by both nucleases are identical, differences were found in the relative intensity of some spots, suggesting that, in limited DNase digestions, both DNase I and micrococcal DNase release initially the same regions of the chromatin. Vidali et al. (21) have reported

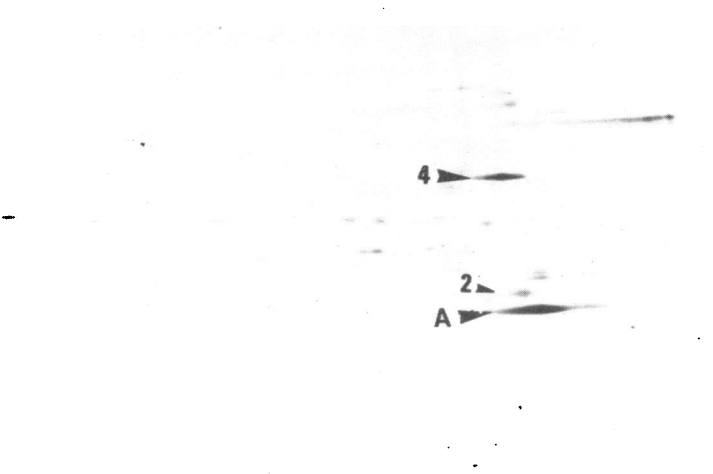

FIGURE 8 : Proteins spontaneously released from nuclei. Nuclei from (^{35}S) labeled cells were incubated at 37° C in the same conditions as described under figure 6 and 7 except that DNases were absent. The released proteins were analysed by 2D electrophoresis. Input 14 000 cpm and autoradiography developed after 3 days of contact.

that a short DNase I treatment of duck erythrocyte chromatin released a specific set of NHP which were found also bound to monomeres of nucleosomes. Since to obtain the same percentage of acid-soluble DNA, more chromatin has to be digested by micrococcal DNase, it is likely that spots with higher relative intensity in micrococcal digests correspond to proteins bound to some parts of chromatin possibly implicated in the higher structure of the chromatin (spots 2,4,11,12, and 21,24). The polypeptides preferentially found in the DNase I digests may correspond to proteins bound to sequences actively transcribed (spots 1,8,13,17). Some of these proteins (1,8,13,15, 16) were found bound to the denser mononucleosomes. Thus we have to consider the possibility that the binding of the proteins to chromatin and to chromatin subunits might be non-specific and might have occured either during nuclei isolation or during hydrolysis of the chromatin. The follo-

wing arguments, however, suggest that this is not the case :
1) The binding of the proteins to chromatin particles was sufficiently tight to have survived the non equilibrium conditions of gel filtration and of metrizamide gradients.
2) The relative intensity of the polypeptide spots in figure 3 does not reflect the relative intensity of the same spots from the total micrococcal DNase extract (figure 6).
3) Some proteins bound to the nucleosomes correspond to very minor spots only detected in the DNase I digest (spots 13,15,16).

Most of the proteins released by micrococcal DNase digestion of the nuclei were not found present in the purified nucleosomes. They may be proteins associated to extra nucleosomal core structures or to a higher order of structure of the chromatin.

Our results confirmed those obtained by Paul and Malcolm with unfractionated chromatin from mouse Friend cell. (10) i.e. NHP were unevenly distributed along the chromatin. After chromatin fractionation we observed an heterogeneity of the monocleosomes which banded at the same density as the fragments studied by Paul and Malcolm. Electrophoretic heterogeneity of the monomers according to the size of their DNA and their protein content has previously been reported (22,23).

In our conditions we cannot exclude that some proteins could have dissociated from the mononucleosomes during the centrifugation in metrizamide gradient : our results, however, reflect an heterogeneity in the density of the monomers according to their protein contents. Now it will be of interest to attribute a biological function to these classes of monomers. Very recently Miller et al (23) have shown that the accessibility to DNase I of the globin gene in induced and non induced Friend cells was of the same order suggesting that the expression of the gene depends on other factors, such as associated proteins. GR cell contain an integrated virus (Mouse Mammary Tumor Virus) which is inducible by glucocorticoid hormones.

REFERENCES

1. Oudet, P., Gross-Bellard, M. & Chambon, P. (1975) Cell 4, 281-300.

2. Noll, M. (1974) Nature 251, 249-251.
3. Kornberg, R.D. (1974) Science 184, 868-871.
4. Weintraub, H. & Groudine, M. (1976) Science 193, 848-856.
5. Axel, R., Cedar, H. & Felsenfeld, G. (1975) Biochemistry 14, 2489-2495.
6. Bellard, M., Gannon, F. & Chambon, P. (1977) Cold Spring Harbor Symposium. Quant. Biol. XLII, 779-791.
7. Garel, A. & Axel, R. (1976), Proc. Nat. Acad, Sci US 73, 3966-3970.
8. Liew, C.C. & Chan, P.K. (1976). Proc. Nat. Acad. Sci.US 73, 3458-3462.
9. Lacy, E. & Axel, R. (1975) Proc. Nat. Acad. Sci. US 72, 3978-3982.
10. Paul, J. & Malcolm, S. (1976). Biochemistry 15, 3510-3515.
11. Goodwin, G.H., Woodhead, L. & Johns, E.W. (1977), FEBS Letters 73, 85-88.
12. Defer, N., Kitzis, A., Levy, F., Tichonicky, L., Sabatier, MM & Kruh, J. (1978) Eur. J. of Biochemistry, 88, 583-591.
13. Ringold, G.M., Lasfargues, E.Y., Bishop, J.M., & Varmus, H.E. (1975) Virology 65, 135-147.
14. De Pomerai, D.I., Chesterton, C.J. & Butherworth, Ph.W. (1974) Eur. J. Biochem. 46, 461-471.
15. Shaw, B.R., Corden, J.L., Sahasrabuddhe, C.G. & Van Holde,K.E. (1974), Biochem. Biophys. Res. Commun. 61, 1193-1198.
16. O'Farrell, P.H., (1975). J. Biol. Chem. 250, 4007-4009.
17. Ollins, A.L., Senior, M.B., & Ollins, D.E. (1976) J. Cell Biol. 68, 787-792.
18. Johnson, E.M., Littau, V., Allfrey, V.G., Bradbury, E.M. & Mathews, H.R. (1976). Nucl. Acids Res. 3, 3313-3329.
19. Lohr, D. & Van Holde K.E. (1975) Science 188, 165-166.
20. Gattoni, R., Stevenin, J. & Jacob, M. (1977) Nucleic Acid Res. 4, 3931-3941.
21. Vidali, G., Boffa, L.C., and Allfrey, V.G. (1971) Cell 12, 409-415
22. Bakayev, V.V., Bakayeva, T.G. & Varshavsky, A.J. (1977). Cell 11, 619-629.
23. Pospelov, V.A., Svetlikova, S.B., & Vorobev, V.L. (1977) FEBS Letters, 74, 229-233.
24. Miller, D.M., Turner, B., Nienhuis, A.W., Axelrod, D.E. & Gopalakrishnan, T.V. (1978) Cell 14, 511-521.