The non-histone proteins of the rat liver nucleus and their distribution amongst chromatin fractions as produced by nuclease digestion

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ABSTRACT

The search for proteins involved in maintaining higher order chromatin structures has led to a systematic examination of the non-histone proteins (NHP) of rat liver nuclei in the context of nuclease digestion studies. 40-45 % of the ^3H -tryptophan labelled NHP originally present could be removed by extensive washing in a "physiological" buffer, incubation at 37°C with or without nuclease and a further wash step. Nuclei at this stage had a remarkably constant NHP content (ca. $0.73 \mu\text{g}/\mu\text{g}$ DNA), independent of the degree of digestion with micrococcal nuclease or HaeIII. The solubilized chromatin produced by limited digestion with either nuclease contained $0.3\text{--}0.5 \mu\text{g}$ NHP/ μg DNA, this value falling to ca. 0.16 after more extensive cleavage. Insoluble chromatin fractions were between 2-fold (very limited digestion) and 16-fold (extensive digestion) richer in NHP than the corresponding soluble fractions. Gel electrophoresis revealed about 12 NHP bands in soluble fractions, the most prominent of M.Wt. 41,400, while the insoluble material had at least 50 components. These properties were independent of whether lysis of nuclei occurred in 0.2 or 50 mM ionic strength. The large disparity in NHP content between complementary soluble and insoluble chromatin fractions is considered in terms of chromatin organization in vivo and the possible role of NHP migration.

INTRODUCTION

Current models of higher order interphase chromatin organization in eukaryotes envisage the existence of quasi-independent, supercoiled "domains" or looped structures attached to a supporting matrix, each unit containing ca. $40\text{--}80 \times 10^3$ bp of DNA. These models have arisen from hydrodynamic studies of the folded interphase genome of *Drosophila melanogaster* treated with ethidium bromide and DNAase I (1), of HeLa nuclei treated with ethidium bromide and γ -radiation (2), of mouse FM3A cells depleted with SDS (3) and from nuclease digestion studies on rat liver nuclei (4). Looped domains have also been observed in electron micrographs of dehistonized HeLa metaphase chromosomes (5).

According to one version of the domain hypothesis (4), certain chromatin

fractions may be enriched in domain "attachment" points by nuclease digestion and it is of interest to determine the molecular species that form the sites of these constraints. Although opinions vary as to the possible involvement of RNA, it seems highly likely that some fraction of the non-histone proteins (NHP) plays a major role. Characterization of this protein class has revealed structural elements, as well as fractions with regulatory and enzymic activity (see 6 for review). Older work with NHP was mainly concerned with tissue and species variations or with their influence on the transcription of reconstituted chromatin (see 6 for review), while more recently, many studies of the small atypical HMG subfraction (e.g. 7-9) and the NHP found in isolated mononucleosomes (e.g. 10-12) have appeared. Only a little attention (e.g. 13) has yet been paid as to how the distribution of NHP in bulk chromatin is affected by nuclease digestion, especially from a quantitative standpoint. Such an analysis is presented in the following with particular regard for the NHP composition of soluble chromatin, since this form is the most widely studied, and of insoluble chromatin, where domain "attachment points" may be located after nuclease digestion (4).

MATERIALS AND METHODS

Preparation and labelling of rat liver nuclei. Nuclei were prepared essentially as described (14) using a homogenization buffer of 0.34 M sucrose, 65 mM KCl, 15 mM NaCl, 10 mM Tris-HCl, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, pH 7.4 and stored as frozen pellets. Nuclei with ^{14}C -labelled DNA and ^3H -labelled NHP were prepared as described (15) except that two 1.5 ml injections of 50 μCi ^{14}C -thymidine, 400 μCi ^3H -L-tryptophan, 0.15 M NaCl, 10 mM Na acetate, pH 7.0 were given 24 h apart. Typical specific activities obtained were ca. 250 ^3H dpm μg^{-1} NHP and ca. 700 ^{14}C dpm μg^{-1} DNA. Radioactive samples were combusted in an oxidizer (Oxymat, Deutsche Inter-technique, Mainz) to completely separate the ^3H and ^{14}C components before liquid scintillation counting. The conversion of ^3H dpm into NHP weight values was calibrated from DNA and total protein determinations of double-labelled material, from which a value of 1.1 $\mu\text{g}/\mu\text{g}$ DNA for the histone contribution was subtracted.

Nuclease digestion. Incubation buffer A contained 65 mM KCl, 65 mM NaCl, 10 mM Tris-HCl, 0.5 mM spermidine, 0.15 mM spermine, 0.2 mM Na_2EDTA , 0.2 mM Na_2EGTA , 5 mM 2-mercaptoethanol, 1 mM PMSF, pH 7.4, supplemented either

with 2 mM CaCl_2 for micrococcal nuclease digestion or with 10 mM MgCl_2 for HaeIII digestion. After washing (defined as 30-60s vortexing), nuclei (50-100 A_{260} units DNA/ml) were digested with either ca. 0.25 units of micrococcal nuclease (Worthington) or ca. 40 units of HaeIII (15) per A_{260} unit of DNA. Digestion was terminated with ice-cold buffer B (as buffer A except with 20 mM EDTA, 2 mM EGTA, no divalent cation and no 2-mercapto-ethanol; 0.2 % Triton X-100 was added where noted).

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out essentially as described (16) with a sample buffer of 62.5 mM Tris-HCl, 2.5 % SDS, 0.5 % dithiothreitol, 10 % glycerol, 20 mM Na_2EDTA , 1 mM PMSF, pH 6.8. Molecular weights were calculated from a plot of log M.Wt. versus mobility using as markers bovine serum albumin (68.000), glutamate dehydrogenase (53.000), glyceraldehyde-3-phosphate dehydrogenase (36.000), carbonic anhydrase (29.000), and cytochrome c. (11.700) (Boehringer Mannheim). Acetic acid/urea tube gels were run according to Panyim and Chalkley (17).

Metrizamide density gradients. Soluble chromatin samples containing ca. 10 A_{260} units of DNA were run on 41.5 % metrizamide gradients and their buoyant densities calculated as described (18). After exhaustive dialysis to remove metrizamide, all fractions were combusted to assay their DNA and NHP content.

RESULTS

The basic experimental sequence detailed below may be summarized as follows; washing of thawed nuclei, incubation (with or without nuclease) at 37° C, termination of digestion and further washing, nuclear lysis, and fractionation of the insoluble and soluble chromatin. At each step, the quantity of NHP removed or remaining with the DNA was determined from the ^3H counts, the DNA assayed from the ^{14}C counts, and the fractions examined on gels. The use of ^3H -tryptophan ensures specific labelling of the bulk of the NHP and allows a linear correlation between ^3H dpm and protein weight (19). This was regarded as a more accurate quantitative assay than chemical determination of NHP remaining after histone extraction or by integration of NHP bands on gels. The histones and HMG proteins 14 and 17 (20,21), lacking tryptophan, do not incorporate radioactivity. The amount of NHP found in frozen nuclei immediately after preparation was $1.38 \pm 0.12 \mu\text{g}/\mu\text{g}$ DNA (7 determinations). Quantities of NHP are reported below as percentages of this amount (unless stated otherwise).

Pre-incubation washes of nuclei. To remove more loosely bound NHP, thawed nuclei were repeatedly washed with ice-cold incubation buffer A, considered to be close to physiological composition and ionic strength. Three successive washes led to an overall removal of 18-22 % of the NHP, a fourth wash extracting very little extra. Inclusion of 0.2 % Triton X-100 in the second wash increased this figure to 28-32 % (Table I), due to removal of membrane proteins (22) and probably also to easier removal of other components. The third wash served mainly to remove the detergent before incubation with enzyme. The NHP content of the nuclei at this stage was $1.00 \pm 0.06 \mu\text{g}/\mu\text{g}$ DNA (4 determinations).

In Fig. 1a, B and C, gel patterns of the proteins removed by the first and second (Triton) washes are shown. The bulk of the proteins have M.Wts. between 30.000 and 90.000. In agreement with earlier observations (22), the principal qualitative effect of including Triton was to extract proteins with M.Wts. of 51.400 ± 1.400 (averaged from 5 gels). On certain gels, weaker extra bands were seen in the region of 31-33.000.

Incubation of nuclei and post-incubation washing. After washing, nuclei were suspended in buffer A and incubated at 37°C for up to 45 min with micrococcal nuclease, with HaeIII and without enzyme respectively. (Enzyme levels used here led to $<5\%$ release of acid soluble DNA counts and ca. 60-80 % chromatin solubilization in both cases after 45 min incubation and nuclear lysis - see below and ref. 4). Aliquots were removed at intervals from 1-45 min, the nuclei spun off (2.500 g, 5 min, 4°C) and the

Table I. The distribution of ^3H -NHP and ^{14}C -DNA counts for a typical experiment where nuclei were extensively digested with micrococcal nuclease.

Fraction	% of NHP originally in nuclei	% of DNA originally in nuclei
1st wash	8	b
2nd wash (+0.2% Triton)	17	b
3rd wash	3	b
4th wash	≤ 2	b
Released after incubation at 37°C and further wash	12	4
Released by lysis alone	ca. 1-2 (ca. 2-3) ^a	ca. 0.5-1.0
Soluble chromatin	10 (17) ^a	78
Insoluble chromatin	47 (81) ^a	17

a. Expressed as % of NHP entering lysis step b. Negligible

supernatants (S) removed for NHP assay. The nuclei were then immediately washed in 10 vols. (based on the original aliquot) of stop buffer B + 0.2 % Triton, spun down again and the NHP of this second supernatant (S') also determined. Although the amount of NHP released into S increased with time and digestion, as reported previously, e.g. for Friend cell nuclei (23), the sum of NHP found in S and S' was quite constant for all samples, regardless of incubation time or nuclease action, and represented a further 10-12 % of the original NHP (Table I). Note that a specific release of HMG 1 and 2 (7,9) representing probably ca. 1-2 % of the total counts at most would be difficult to detect here. By this stage, a total of 40-45 % of the original NHP had been removed (Table I).

The gel pattern of material released upon incubation alone at 37° C is shown in Fig. 1a, D. It is very similar to that of proteins in the first pre-incubation wash (Fig. 1a, B) with a relative intensification of bands at ca. 96.000, 83.000, 70.000, 41.000, 33.000, 25.500, 17.500 and 13.000

Figure 1. (a) 14 % SDS gel of (A) marker proteins; (B) NHP removed by first wash; (C) NHP removed by second (Triton) wash; (D) NHP released merely upon incubation at 37° C; (E) NHP released by dilution into buffer B after incubation. (b) 16 % SDS gel of (A) NHP released upon nuclear lysis; (B) marker proteins. M.Wts. $\times 10^{-3}$.

M.Wt. The doublet centred at 25.500 corresponds to the expected positions of HMG 1 and 2 in this gel system (24), however no obvious relative intensification of this doublet was seen in samples that had been digested. The extra protein released by dilution into stop buffer B + Triton after incubation (Fig. 1a, E) shows a similar but weaker pattern.

Lysis of the nuclear pellets. After a further wash in buffer B to remove Triton (this removed almost no extra NHP), aliquots of the nuclei themselves were assayed for NHP and DNA content. Consistent with the above results, the ratio of residual NHP to DNA was also independent of incubation times between 1 and 45 min and the degree of nuclease digestion. Analysis of a large number of samples (Table II) shows a remarkably constant amount of NHP (ca. 0.73 $\mu\text{g}/\mu\text{g}$ DNA) accompanying the nuclei into the lysis step.

Nuclei were lysed overnight at 4°C in the standard buffer of 0.2 mM EDTA, pH 7.4 (25) or, as noted below, in a buffer containing 50 mM NaCl, 5 mM EDTA, 5 mM Tris, pH 7.4. The latter was used for comparison following reports (26,27) that below an ionic strength of ca. 10-20 mM, chromatin undergoes a structural transition to a more decondensed state. It was examined whether this transition was accompanied by changes in the NHP content of the chromatin. The EDTA concentration of the NaCl-containing buffer was raised to 5 mM as it was found that lysis in 50 mM NaCl, 0.2 mM EDTA led to a poor yield of soluble chromatin. The extra EDTA raised the yield to values only slightly less than those obtained in the absence of NaCl.

After lysis, the suspension was centrifuged at 10,000 g for 15 min to yield a supernatant fraction of soluble chromatin and a pellet of insoluble chromatin. Control experiments (no enzyme) showed (a) that the maximum amount of DNA released into the lysis supernatant after a 40 min incubation

Table II. NHP compositions of washed and variously incubated nuclei.

Nuclei	NHP/DNA weight ratio	No. of samples
+ micrococcal nuclease incubated 1-35 min	0.75 $\pm$ 0.04 ^a	10
+ HaeIII incubated 2-45 min	0.73 $\pm$ 0.04 ^a	14
no enzyme incubated 1-45 min	0.72 $\pm$ 0.08 ^a	10

a. Standard deviation

at 37° C was ≤ 1.0 % of the total, showing the almost complete suppression of double-stranded endogenous nuclease activity, and (b) that about 3 % of the total NHP that goes into the lysis step was released into solution by the act of lysis alone (Table I), independent of the time of prior incubation at 37° C. The gel pattern of this minor but complex fraction showed at least 40 different proteins (Fig. 1b, A). The same was observed if lysis took place in 50 mM NaCl, 5 mM EDTA rather than 0.2 mM EDTA buffer. In the quantitative description of soluble chromatin below (Fig. 2), all values have been corrected for this background of NHP released by lysis alone by analyzing all samples in parallel, with and without enzyme, for a given incubation time, and subtracting out the corresponding counts.

Properties of the soluble chromatin fraction. Soluble chromatin fractions resulting from increasing extents of nuclease digestion were characterized by their NHP and DNA content. The results of five separate incubations with lysis in 0.2 mM EDTA (3 with HaeIII and 2 with micrococcal nuclease) are shown in Fig. 2a. In all cases, the average NHP/DNA ratio of the soluble chromatin fell with increasing digestion from initial values generally between 0.3 and 0.5 to a value of ca. 0.18 μg NHP/ μg DNA after ca. 50 % of the total chromatin had been rendered soluble by either enzyme. The overall amount of NHP in the soluble fraction however increased with increasing solubilization (Fig. 2b), showing that chromatin released later is not completely devoid of NHP. Possible interpretations of these curves are considered below. Extensive kinetic data for chromatin produced by lysis in 50 mM NaCl or higher were not gathered, but the general tendency for the

Figure 2. (a) NHP/DNA ratio of soluble chromatin as a function of the degree of chromatin solubilization after nuclear lysis. ●—● digestion with micrococcal nuclease, □—□ digestion with HaeIII, nuclei lysed in 0.2 mM EDTA in both cases. ■—■ digestion with micrococcal nuclease and lysis in 50 mM NaCl, 5 mM EDTA. (b) Overall amount of NHP in soluble fraction as a function of solubilization. Symbols as for (a).

average NHP/DNA ratio to drop with increasing solubilization was also observed (see the one curve for lysis in NaCl in Fig. 2a), and values for extensively digested chromatin were approximately the same.

The curves of Fig. 2a suggest that micrococcal nuclease or HaeIII releases a NHP-rich fraction early in the digestion. However they could simply result if lysis of digested nuclei released an extra amount of unbound NHP relative to that detected by lysis of undigested nuclei, i.e. if the correction for background NHP counts described above were inadequate. It was thus necessary to determine how much of the measured amount of NHP represented material that was actually bound to the soluble chromatin. Nuclei were digested with micrococcal nuclease, the pellet split and the two halves lysed in 0.2 mM EDTA and in 50 mM NaCl, 5 mM EDTA respectively, yielding ca. 60 % solubilization in both cases. The soluble chromatin fractions were centrifuged to equilibrium in their respective buffers on metrizamide density gradients, where free protein bands at a much higher buoyant density than that bound to DNA (18). This technique was considered more reliable than sedimentation velocity experiments where aggregated NHP can cosediment with chromatin fractions without actually being bound to them. The peak fractions (Fig. 3) contained NHP to the extent of 0.16 $\mu\text{g}/\mu\text{g}$ DNA in both the low and higher ionic strength cases - only ca. 5 % lower than

Figure 3. Metrizamide gradient profiles of soluble chromatin. Nuclear lysis and centrifugation in (a) 0.2 mM EDTA and in (b) 50 mM NaCl, 5 mM EDTA. $\blacktriangledown$ — $\blacktriangledown$ ^{14}C -DNA counts, Δ — Δ ^3H -NHP counts. O—O metrizamide density.

the values measured before centrifugation. The buoyant densities of the peak fractions were 1.23 and 1.20 g/ml respectively (Fig. 3a and 3b), the variation probably being due to salt-induced hydration differences. No free protein (expected to band at $\rho \geq 1.28$ (13)) was observed, although free NHP fractions do not band readily (13) and recovery of counts from the gradient was about 70 % for the low ionic strength case and only ca. 45 % with NaCl present. Moreover a minor chromatin peak banding at a somewhat higher density, as has been reported for more extensively cleaved chromatin from tissue culture cells (13,12), was not seen.

Soluble chromatin was further investigated by column chromatography. Since any free NHP might aggregate, the agarose pore size was chosen to be as large as possible, while still small enough to ensure that the chromatin was almost completely excluded. The O.D. and radioactivity profiles of chromatin lysed in 0.2 mM EDTA and passed over Sepharose 6B equilibrated in the same buffer are shown in Fig. 4a and b. The average NHP/DNA ratio for the main chromatin peak from fractions 9 to 17 in Fig. 4b was ca. 0.15 - about 10-15 % lower than the value before chromatography. The material at

Figure 4. Chromatography on Sepharose 6B of soluble chromatin. (a) optical density profile $\blacksquare$ — $\blacksquare$ A260, $\square$ — $\square$ A230 (b) radioactivity profile $\blacksquare$ — $\blacksquare$ ^{14}C -DNA counts, $\square$ — $\square$ ^3H -NHP counts.

the tail end of the peak had a somewhat higher relative content than that at the front. Although a small peak was seen with a high A_{230}/A_{260} ratio (Fig. 4a), no enrichment of ^3H counts was found under it (Fig. 4b), and gel analysis revealed only faint histone bands with no NHP visible.

The very small drop in the NHP/DNA ratio of soluble chromatin after density equilibrium centrifugation or column chromatography, together with the inability to detect significant quantities of unbound protein in both cases supports the idea that nearly all of the NHP observed in the soluble chromatin fraction is in fact bound, and that chromatin released early in the digestion is genuinely enriched in NHP relative to the material released later.

The NHP of the soluble chromatin fraction was examined by gel electrophoresis. To avoid any possible loss of these proteins by prior denaturing extraction techniques, the lyophilized chromatin was taken up directly into the SDS-containing gel buffer. A weak but reproducible pattern of a limited number of NHP bands could be seen on overloaded gels (Fig. 5a). The most prominent (estimated very roughly at 20-30 % of the total) was of M.Wt. 41.400 ± 200 (5 gels) very close to that of actin (28). Other major bands were seen at 51.000 ± 200 , 62.700 ± 200 and 75.400 ± 300 (3 gels). Occasionally, ca. 12-15 bands could be detected. The major bands were also visible in each of the fractions 10-14 constituting the main chromatin peak after passing the material over Sepharose 6B (Fig. 4a) and in chromatin produced by lysis in 50 mM NaCl (data not shown). Note that the linear correlation between counts and protein weight assumed in calculating the figure of 0.16 μg NHP/ μg DNA, may not be strictly justified if the number of components is as limited as the gels suggest.

Examination of soluble chromatin with NHP/DNA ratios higher than 0.16 (i.e. material released after a relatively short digestion in Fig. 2) failed to reveal bands other than the above, unless the degree of solubilization was so limited (below 5 %) that the very small amount of NHP released by lysis alone (Fig. 1b) could still be seen superimposed upon the chromatin pattern. Thus the "early" chromatin produced by these enzymes under the present buffer conditions was not detectably enriched in any particular NHP fractions.

Properties of the insoluble chromatin fraction. The progressive release by nucleases of soluble chromatin with a much lower NHP/DNA ratio than the original nuclei at lysis should lead to an enrichment in the NHP content of

Figure 5. (a) 14 % SDS gel of (A) marker proteins and (B) the proteins of soluble chromatin.

(b) 14 % SDS gel of (A) proteins extracted directly from unwashed nuclei; (B) the proteins of the insoluble chromatin fraction; (C) the proteins extracted from (B) with 0.6 M NaCl; (D) the proteins remaining insoluble after NaCl extraction of (B), and (E) marker proteins. M.Wts. $\times 10^{-3}$

the insoluble chromatin pelleted after lysis. This was confirmed and quantitated in the experiment of Fig. 6. At the earliest time point (1 min) the insoluble chromatin contained about twice as much NHP per weight of DNA as the corresponding soluble fraction. After the longest incubation times, ($\geq 80\%$ of the total chromatin solubilized), this factor had risen to ca. 16-fold, the NHP content of the insoluble fraction being $\geq 3.0 \mu\text{g}/\mu\text{g DNA}$.

The gel pattern was correspondingly complex (Fig. 5b, B) containing at least 50 bands. The most prominent were found in regions corresponding to M.Wts. of about 80-100,000, 65-72,000, and 50-55,000 and at $45,300 \pm 500$, $41,700 \pm 300$, $40,300 \pm 400$, $36,600 \pm 200$, $34,900 \pm 300$, and $33,800 \pm 300$.

Figure 6. NHP/DNA ratio of the insoluble chromatin fraction as a function of the degree of chromatin solubilization after nuclear lysis. $\square$ - $\square$ digestion with micrococcal nuclease, $\blacksquare$ - $\blacksquare$ digestion with HaeIII.

(averaged from 5 gels of different preparations). Comparison with the NHP extracted directly from whole nuclei (Fig. 5b, A) showed little qualitative difference. Although relative intensities were somewhat different, the overall distribution was similar despite the fact that the insoluble chromatin contained less than half the amount of NHP originally in the nuclei (Table I).

In an attempt to further fractionate the insoluble chromatin in a gentle manner, pellets were extracted with 0.7 M NaCl (19). Depending on the degree of digestion leading to the original insoluble chromatin pellet, up to ca. 95-98 % of the DNA/histone could be solubilized together with ca. 20 % of the NHP leaving behind almost exclusively NHP. Fig. 5b shows the gel pattern of the latter material (gel D) as well as of the smaller amount of NHP dissociated by NaCl along with the DNA/histone (gel C). No simple fractionation was achieved, both patterns showing numerous identical bands, although the NHP extracted by NaCl (gel C) is relatively impoverished in bands in the 34-37,000, 50-55,000, 65-72,000 and 90-100,000 M.Wt. regions.

Distribution of the HMG proteins. The four main HMG proteins constitute less than 3 % of the total NHP content (29) and both HMG 14 and HMG 17 lack tryptophan (20,21). Specific release of these fractions, e.g. upon nuclease digestion (7,9), would therefore escape detection in the above radioactivity experiments. Aliquots from each of the stages described above were thus examined on acetic acid/urea gels for HMG content after selective precipitation with TCA (29). Bands corresponding to HMG 14 and 17 were seen in the wash extracts, in the material released during incubation, and in both

the soluble and insoluble chromatin fractions (Fig. 7 A-F). Although the relative distribution amongst these fractions was not quantitated, the ratio of HMG 14 to HMG 17 was not the same in each case (Table III). Relative to the ratio found for these proteins extracted directly from liver by 5 % PCA (30), soluble chromatin had a similar value while insoluble chromatin was somewhat enriched in HMG 14. Washing and incubation appeared to release HMG 17 more easily than HMG 14. As with the ^3H -tryptophan labelled NHP, about the same amount of HMG 14 and 17 was released by incubation at 37°C and a subsequent wash with buffer B + Triton whether nuclease was present or not (Fig. 7, B and C), although the HMG 14/HMG 17 ratio of material released was somewhat higher after nuclease digestion. No bands corresponding to HMG 1 or 2 were found in the position just behind H1 reported for other tissues (31) on different acidic gels in any of the fractions of Fig. 7, although it cannot be excluded that they comigrate in the H1 position under the present conditions.

Figure 7. Acetic acid/urea gels of the HMG proteins (A) in pre-incubation nuclear washes; (B) released upon incubation at 37°C in the presence of micrococcal nuclease; (C) as (B) without nuclease; (D) in soluble chromatin after extensive digestion; (E) in insoluble chromatin after extensive digestion; (F) in insoluble chromatin after no digestion; (G) extracted directly from rat liver with 5 % perchloric acid; (H) as (G) with histones added.

Table III. Ratios of HMG 14 to HMG 17 as measured from scans of the gels in Fig. 7.

Fraction	HMG 14/HMG 17
Nuclear washes	0.18
Released by incubation (15 min) without enzyme	0.25
Released by incubation (15 min) with micrococcal nuclease	0.33
Soluble chromatin	0.83
Insoluble chromatin	0.89
5 % PCA extract of liver	0.82

Effect of omitting pre-incubation wash steps or of pretreating with RNAase. Analysis of chromatin pellets after lysis of thoroughly washed and unwashed nuclei incubated at 37° C without enzyme for the same time revealed that the NHP content of the latter was ca. 8-10 % higher than that of the former (i.e. ca. 0.81 µg NHP/µg DNA rather than nearer 0.73). Thus, while the majority of loosely bound NHP is in any case released from unwashed nuclei during incubation and dilution into buffer B + Triton, a significant fraction is eventually found associated with the chromatin. It was not determined how this fraction would distribute itself between the soluble and insoluble chromatin fraction, had digestion of the chromatin taken place.

About 10 % of the nucleic acid content of normal rat liver nuclei is RNA (32), most of which is bound to protein. This RNP fraction thus forms a significant part of the total NHP content. It was asked whether pre-treatment of nuclei with RNAase led to a release of RNP proteins not otherwise removed in the wash steps. Two equal aliquots of nuclei were incubated at 37° C - one with 0.33 units (33) of DNAase free pancreatic RNAase per 10 A₂₆₀ units of DNA, and the other without enzyme. The presence of RNAase led, as expected, to a rapid release of digested RNA into solution; however the release of NHP was retarded. Thus, after 1 h, the amount of NHP released during incubation and the subsequent wash step was only ca. 60 % of the control value without RNAase. As above, the extra NHP was found associated with the chromatin after nuclear lysis. Although it is not clear why these proteins should be more easily washed out if the RNA is left intact, this and the previous experiment further stress the problem of assessing exactly what is genuine chromosomal NHP.

DISCUSSION

Identification of the NHP involved in maintaining chromatin domains is a part of the wider problem of defining which NHP fractions are actually bound to chromatin *in vivo*. Earlier studies (e.g. 34-36) have approached this question, but since the advent of nuclease digestion technology, little further work has been done. The present work has attempted to put such considerations on a more quantitative basis.

The initial cold wash steps with a "physiological" buffer can only remove a certain fraction of the total NHP, probably made up largely of loosely bound cytoplasmic contaminants, nuclear sap, RNP and membrane proteins. However a substantial further fraction with similar electrophoretic properties is released upon incubation at 37° C. Since the same amount of NHP was extracted from control as from digested nuclei, if a post-incubation wash step was included, it appears that digestion in itself only enhances the partial release of a certain NHP fraction which can in any case be quantitatively removed by washing after incubation for as short as 1 min. Although NHP is known to be quite susceptible to proteolytic degradation, the constant amount that was extracted in the above manner from samples incubated anywhere between 1 and 45 min argues against a non-specific release of breakdown products. The radioactivity experiments do not exclude specific release of HMG proteins upon micrococcal nuclease digestion, but no such effect was observed upon gel analysis. This resembles the situation found for HMG 1 and 2 of mouse brain nuclei (9) but differs from the case of foetal calf thymus (9). The failure of this enzyme to specifically release HMG 14 and 17 is in agreement with other reports (7,37).

The domain hypothesis of chromatin organization (4) postulates that after nuclease digestion domain attachment points should be enriched in the insoluble chromatin fraction; experimentally, the latter was about 16-fold richer in NHP than the corresponding soluble chromatin fraction after extensive cleavage. It is possible that in vivo, certain areas of chromatin are indeed much richer in NHP and hence so hydrophobic that they cannot be solubilized. However, the very high total protein/DNA ratio of insoluble chromatin after extensive digestion (about 4.5:1) makes it unlikely that all the NHP would be directly bound to DNA; much of it could be in matrix-like structures that serve as anchor points for domains. Alternatively, nuclease scission itself might induce migration of NHP from their original sites to others eventually found in the insoluble fraction. The small amount of NHP consistently found with the soluble chromatin would then represent

just the most tightly bound fractions least susceptible to displacement. Migration of NHP could also occur during the nuclear lysis step, where binding constants might be quite different at low ionic strength. However, as no significant differences were seen in the NHP of chromatin produced in 0.2 mM EDTA or in 50 mM NaCl, it is likely that changes in ionic strength are more important for nucleosomal conformation and histone interactions (26,27) than for the gross distribution of NHP.

The present results suggest that nuclease digestion may not be a satisfactory approach to characterizing the proteins of domain attachment points. If cleavage does induce NHP migration, much of the protein of the insoluble chromatin might have been originally associated with DNA found elsewhere and be unrelated to domain constraints. Even after NaCl extraction, the remaining NHP contains numerous species, although the more prominent bands at ca. 71.000, 68.000 and 65.000 M.Wt. probably correspond to the three major matrix proteins (38). A similar complexity has been observed by Sanders (39) after direct extraction of micrococcal nuclease digested nuclei with NaCl up to 0.6 M.

Using either enzyme, soluble chromatin produced early in digestion was on average richer in NHP than later released material (Fig. 2a). The work of Gottesfeld *et al.* (see 40) with DNAase II suggests that this enrichment may signify the early release of a transcriptionally active fraction, however no attempt was made here to characterize the chromatin in this way. Recent experiments (41,42) indicate that micrococcal nuclease is capable of preferentially recognizing active chromatin, although Weintraub and Groudine (43) found no such effect in their original study. Nothing is yet known about the mode of attack of HaeIII. Even if the soluble chromatin produced here by short digestion did represent all or part of an active fraction, its low NHP content relative to the insoluble chromatin remaining behind suggests that it may well not be released with its full *in vivo* NHP complement. Alternatively, the above result could be due entirely to a progressive artefactual migration of NHP from the soluble chromatin to the insoluble material, as described above.

The similarity in the NHP gel patterns of whole nuclei, insoluble chromatin, and of the fractions removed by washing is consistent with earlier reports (34,35,44) that most NHP species can be only partially removed by such treatment. This is in keeping with the notion that, depending upon their function, many NHP have multiple binding sites in nuclei *in vivo* with widely varying dissociation constants. The remarkably constant amount of NHP found

in nuclei after the pre- und post-incubation wash steps described here, independent of nuclease digestion, argues strongly however for the efficiency and completeness of these procedures in removing all but an in some way specific NHP fraction. Such nuclei might therefore represent well-defined material suitable for further studies.

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ABBREVIATIONS

NHP, non-histone protein(s); HMG, high mobility group protein(s); EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis (β -aminoethyl ether)-N,N'-tetraacetic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

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