NUCLEAR PROTEINS

II. Similarity of Nonhistone Proteins in Nuclear Sap and Chromatin, and Essential Absence of Contractile Proteins from Mouse Liver Nuclei

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ABSTRACT

High resolution SDS slab gel electrophoresis has been used to examine the distribution of nonhistone proteins (NHP) in the saline-EDTA, Tris, and 0.35 M NaCl washes of isolated mouse liver nuclei. These studies led to the following conclusions: (a) all the prominent NHP which remain bound to DNA are also present in somewhat similar proportions in the saline-EDTA, Tris, and 0.35 M NaCl washes of nuclei; (b) a protein comigrating with actin is prominent in the first saline-EDTA wash of nuclei, but present as only a minor band in the subsequent washes and on washed chromatin; (c) the presence of nuclear matrix proteins in all the nuclear washes and cytosol indicates that these proteins are distributed throughout the cell; (d) a histone-binding protein (J2) analogous to the HMG1 protein of K. V. Shooter, G. H. Goodwin, and E. W. Johns (Eur J. Biochem. 47:263-270) is a prominent nucleoplasmic protein; (e) quantitation of the major NHP indicates that they are present in a range of 2.2×10^5–5.2×10^6 copies per diploid nucleus. Most of the electrophoretically visible NHP are probably structural rather than regulatory proteins; (f) actin, myosin, tubulin, and tropomyosin, if present at all, constitute a very minor fraction of the nuclear NHP. Contractile proteins constitute a major portion of the NHP only when the chromatin is prepared from crude cell lysates instead of from purified nuclei. These studies support the conclusion that there are no clear differences between many nucleoplasmic and chromatin-bound nonhistone proteins. Except for the histones, many of the intranuclear proteins appear to be in equilibrium between DNA, HnRNA, and the nucleoplasm.

Most studies of nonhistone proteins begin with chromatin purified by repeated washing in dilute buffers. The washes are usually discarded. Since the development of high-resolution SDS gel electrophoresis there have been few studies to examine whether the proteins removed by the nuclear washes represent a unique set of proteins or whether they are essentially identical to the nonhistone proteins that remain bound to DNA. Using a relatively low resolution SDS gel electropho-

resis technique, Comings and Tack (13) showed that in mouse liver nuclei the nuclear sap and nonhistone proteins exhibited many similar bands, suggesting that they were not distinct entities. The nuclei appeared to possess several classes of non-histone proteins, some of which were more tightly bound to DNA than others; but even the most tightly binding proteins could be removed with low ionic strength washes. Proteins removed in a 0.4 M salt wash were essentially identical to those remaining on the chromatin. Using high resolution Laemmli-type (29) slab gel electrophoresis, Comings and Harris (10) showed that the proteins removed by a 0.35 M wash of nuclei previously washed with 0.15 M NaCl were virtually identical to the nonhistone proteins remaining on the chromatin. Similar conclusions on the relationship of "loosely bound" 0.35 M NaCl wash proteins to nonhistone chromosomal proteins have been reached by others (23, 24, 28, 37).

A common procedure in the isolation of chromatin is to wash nuclei (or whole cell homogenates) twice in saline-EDTA (S-E) (0.075 M NaCl, 0.025 M EDTA) followed by several washes in dilute Tris (or water) (6, 14, 20, 34, 41, 53). With the use of high resolution slab gel electrophoresis, the following questions were investigated. (a) What percentage of the total of nuclear proteins is removed by these nuclear washes and how do these proteins compare electrophoretically to the nonhistone proteins on well-washed chromatin? (b) Are there any proteins which are significantly enriched in the nuclear sap when compared to the chromosomal nonhistone proteins? (c) How many of the major nonhistone proteins are present per genome when the whole nucleus is examined as opposed to well-washed chromatin?

We have also examined the nonhistone proteins of chromatin prepared from whole liver lysates. This frequently used technique for isolating chromatin involves successively washing a lysate of frozen whole tissue with saline-EDTA and dilute Tris (6, 18, 20, 21, 34, 53). The crude chromatin pellet is then centrifuged through 1.7 M sucrose, dialyzed against dilute Tris, sheared, and centrifuged to remove unsheared material. A potential problem with this technique is that the chromatin is exposed to cytoplasmic proteins during the initial homogenization. Although this results in a chromatin preparation which is adequate for many studies, the tendency for some types of cytoplasmic protein to bind to DNA (5, 9, 13, 39, 43-45, 51) suggests that the nonhistone proteins of such

chromatin may be different from those isolated from purified nuclei. To examine this, we have isolated chromatin by both techniques and compared the nonhistone proteins by slab gel electrophoresis.

MATERIALS AND METHODS

Isolation of Nuclei and Preparation of Nuclear Washes

Swiss mice were used. In all experiments the mice were killed by cervical dislocation immediately before use. The livers were removed and cut into small pieces in ice-cold TCMB buffer consisting of 10^{-2} M Tris, 10^{-4} M cadmium sulfate, 3×10^{-3} M magnesium chloride, 10^{-3} M sodium bisulfite, pH 7.0, 1 μg/ml soybean trypsin inhibitor. The cadmium sulfate, sodium bisulfite, soybean trypsin inhibitor, and pH 7 (instead of 8) were used to inhibit proteolysis. The lysate was then mixed with 1.2 vol of 2.4 M sucrose buffer and the nuclei were isolated as described previously (10).

The white nuclear pellet was resuspended in 0.075 M sodium chloride, 0.025 M EDTA, 0.01 M Tris, pH 7.0 (S-E wash), by vortexing and, after 5 min, centrifuged at 500 g for 10 min. In all experiments 10 ml of wash were used per five mice. The supernate was removed and brought to 10^{-4} M phenylmethylsulfonyl fluoride (PMSF) by adding 0.01 vol 10^{-2} M PMSF in ethanol. This was then dialyzed for 24–48 h against two changes of 0.002 M EDTA and two changes of distilled water, then lyophilized (15). The second S-E wash and two or three subsequent Tris washes (0.01 M Tris, pH 7) were obtained the same way, except that the second and third Tris washes were centrifuged at 1,000 g for 10 min. The nuclei were finally washed once in 0.35 M NaCl, 10 mM Tris, pH 7.0. Aliquots of whole, unwashed, S-E-washed, Tris-washed, and 0.35 M NaCl-washed nuclei were removed for biochemical analysis, dialyzed, and lyophilized for electrophoresis. In some experiments, the washes were centrifuged at 100,000 g for 1 h and the supernates and pellets dialyzed and electrophoresed. To obtain total liver and cytoplasmic protein (Figs. 3 and 4) an aliquot of the liver lysate in TCMB buffer was taken before adding the 2.4 M sucrose. This served as total liver (Fig. 4). Part of this aliquot was centrifuged at 100,000 g for 1 h to give a pellet and supernate (cytosol).

Isolation of Chromatin from Whole-Cell Lysates

The procedure of Bonner (6, 20, 21) was used to isolate chromatin from frozen rat liver. The livers of five rats were frozen on dry ice. 10–20 g were broken into small pieces, placed frozen into a Waring Blender with 200 ml of saline-EDTA (0.075 M NaCl, 0.025 M EDTA, pH 8), and blended at 80 V for 1 min and at 45

V for 3 min. The lysate was strained through a double layer of Miracloth (Chicopee Mills, Inc., New York) to remove fibrous tissue and the filtrate centrifuged at 1,500 g for 10 min. The pellet was resuspended in 40 ml of saline-EDTA and centrifuged at 1,500 g for 10 min. This pellet was resuspended in 10 mM Tris, pH 8, and centrifuged at 4,000 g for 10 min. The pellet was again resuspended in 10 mM Tris, pH 8, and centrifuged at 12,000 g for 10 min. These Tris washes were repeated two more times. The final pellet was termed crude chromatin. This was resuspended in Tris buffer, stirred for 1 h, and then centrifuged through 1.7 M sucrose, 10 mM Tris, pH 8, at 50,000 g for 4 h. After this centrifugation, there was usually a small layer of opalescent material at the Tris-sucrose interface. The pellet was termed sucrose-washed chromatin. It was dialyzed against 10 mM Tris, pH 8, overnight, sheared in a Virtis homogenizer at 30 V for 90 s, and then centrifuged at 12,000 g for 30 min. The supernate was termed sheared chromatin and the pellet unsheared chromatin. The OD at 320 mm was 0.08 or less of the OD at 260 mm.

SDS Gel Electrophoresis

The method of electrophoresis was a modification of the Laemmli Tris-glycine SDS slab gel technique (2, 15, 29). Lyophilized samples were solubilized in 1% SDS, 5 × 10^{-2} M Tris, 2 × 10^{-3} M EDTA, 4 × 10^{-3} M dithiothreitol, 10% glycerol, pH 6.8, and centrifuged at 1,500 g for 10 min and the protein concentration of the supernate was determined by a TCA precipitation technique (12). 20-25 μg of protein were loaded in each well in 50 μl or less. 10-11% acrylamide gels were used for high resolution of the nonhistone proteins. The gels were electrophoresed with an Ortec pulsed DC power supply at 25 mA, 170 pulses/s, for approximately 3 h and the gels removed when the ion front had migrated to the end of the gel. The gels were stained in 0.05% Coomassie blue, 10% glacial acetic acid, and 25% 2-propanol overnight, then destained in several changes of 10% glacial acetic acid. The gels were photographed with Kodak Contrast Process Ortho (CPO) film (Eastman Kodak Corp., Rochester, N. Y.) through a yellow filter and printed on Kodak F2 paper. For densitometry scans the photographs were scanned with a Joyce-Loebl microdensitometer (Joyce, Loebl & Co., Burlington, Mass.). To obtain an estimate of the nonhistone protein:histone ratio, the area under histone and nonhistone proteins on the scans from 14% gels was cut out and weighed. Since the histone to DNA ratio is approximately 1.0, this also allowed an estimate of the total protein to DNA ratio. Examination of nuclei from which the histones had been removed by extraction with 0.2 HCl indicated that very few of the nonhistone proteins were being masked by the histone bands. To obtain an estimate of the relative amount of some of the nonhistone proteins, the areas under individual peaks were cut out and weighed and compared to the sum of the areas under all the other nonhistone proteins.

To determine whether the intensity of Coomassie blue staining of histone and nonhistone proteins was similar, a mixture of equal parts by weight of whole calf thymus histone and bovine serum albumin (BSA) was electrophoresed, stained, photographed, and scanned, and the area under the BSA and histones was cut out and weighed. By this procedure, when care was taken not to overload the gel, the BSA:histone ratio was 1:1. Urea gel electrophoresis was carried out by the technique of Orrick et al. (36) and Yeoman et al. (50).

Biochemical Analysis

The protein and DNA content of the washes and nuclei were determined by the Lowry (32) and diphenylamine techniques (7).

Contractile Proteins

Mouse myofibrils were isolated by the technique of Etlinger and Fischman (22). α- and β-tropomyosin (16, 17) were isolated by modifications (35, 49) of the technique of Bailey (3). Pig α-actinin, for use as an electrophoretic marker, was kindly donated by Dr. Goll.

RESULTS

The nomenclature and molecular weight of the mouse liver nonhistone nuclear proteins, as observed by Tris-glycine SDS gel electrophoresis, have been reported in the first paper in this series (10). The proteins were divided into groups A-J and the proteins within each group assigned a number from 1 to 10. The major nonhistone proteins, A10, B10, C10, etc., serve to separate each group. Fig. 1 shows a comparison of the proteins in the two (S-E) washes, two Tris washes, one 0.35 M NaCl wash, and final chromatin. The major proteins, A3, 4, 10, B4-5, 10, C10, D4, D10, E10, G10[1], H5, 10, I2, and I4, are common to all washes and final chromatin. The most striking difference between the first S-E wash and other washes was the presence of proteins in the E10 to F3 region which are cytoplasmic proteins (see Fig. 3). The nonhistone proteins in the Tris washes, 0.35 M NaCl wash, and final chromatin are virtually identical, differing only in the relative intensity of some of the bands (see also Fig. 4). A few micrograms of mouse myofibril were electrophoresed in one slot to show that myosin migrates in the region of protein A4 or 5. Actin comigrates with G10, which is especially prominent in the first S-E wash and present to a lesser degree in the other washes and on chromatin.

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[1] In Fig. 1, G10 did not show up well in the Tris and 0.35 M NaCl wash. This is seen better in Fig. 4.
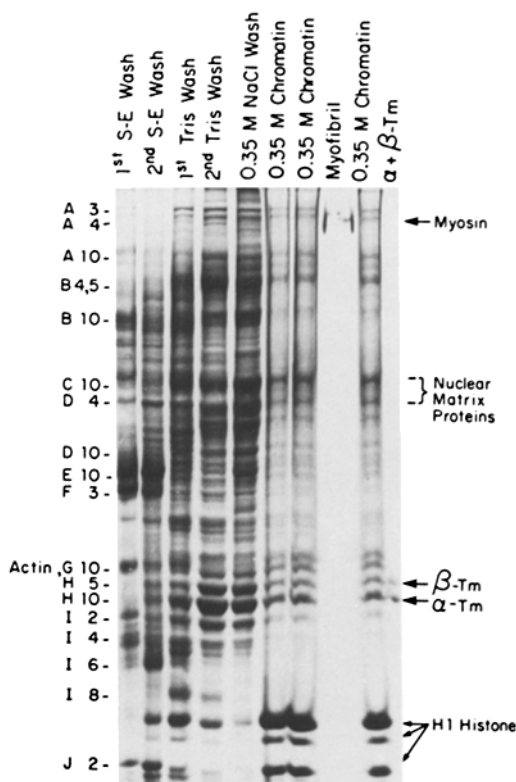
FIGURE 1 SDS slab gel electrophoresis in 11% acrylamide of the proteins of the saline-EDTA, Tris, and 0.35 M NaCl washes of mouse liver nuclei, compared to the washed chromatin (0.35 M chromatin), mouse myofibrils, and α- and β-tropomyosin. C10 and D4 are the major nuclear matrix proteins.

Mouse myofibril was placed on the outer slots. In this system actin stains well and provides a clear demonstration of the fact that it is abundant in the first S-E wash, as a cytoplasmic contaminant, but is rapidly washed out and not visible by this technique in the second Tris wash and 0.35 M NaCl wash or washed chromatin. The band between myosin and actin in the myofibril is α-actinin.

The percentage of protein removed in the different washes in five experiments was determined by Lowry assay (Table I). Of the total protein removed by washing, on the average 50% came out in the first S-E wash, 13% in the second, for 63% in the combined S-E washes. The Tris

α- and β-Tropomyosin (16, 17) consistently comigrated with the bands H10 and H5, respectively. Preliminary studies (see below) suggest that these are HnRNP proteins rather than α- and β-tropomyosin. Bands C10, D1, and D4, which occur in both the washes and are prominent on washed nuclei, have been identified as nuclear matrix proteins (1, 4, 38) and are discussed in detail in a subsequent paper.[2] J2 is prominent in the S-E washes and will also be discussed later.

The proteins in these washes were also examined by urea gel electrophoresis (Fig. 2). Here, the major bands are again very similar for the different washes and in the washed chromatin.

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[2] Comings, D. E., and T. A. Okado. 1976. Nuclear proteins. III. The fibrillar nature of the nuclear matrix. *Exp. Cell Res.* In press.
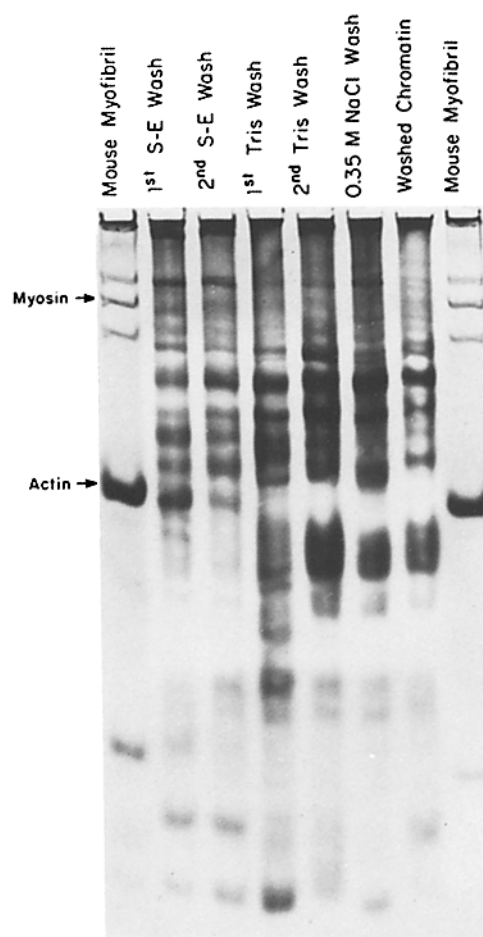

FIGURE 2 Urea gel electrophoresis of the saline-EDTA, Tris, and 0.35 M NaCl washes compared to the washed chromatin and mouse myofibrils. Actin is a prominent band in the first S-E wash. The band between myosin and actin in the myofibril is α-actinin.

TABLE I

Distribution of Proteins in Nuclear Washes*

Nuclear wash	Percentage of Protein
	mean ± SD
First saline-EDTA wash	50 ± 14
Second saline-EDTA wash	13 ± 2
First Tris wash	14 ± 9
Second Tris wash	11 ± 4
Third Tris wash	4 ± 0.3
0.35 M NaCl wash	8 ± 5

* Results of five experiments.

washes removed another 29% and the 0.35 M NaCl washes removed approximately 8%. The proteins recovered by washing constituted 35–45% of the total nuclear protein, or approximately 50% of the total nonhistone nuclear proteins.

An alternative method of examining whether the proteins that are easily washed out of the nucleus are significantly different from the chromosomal nonhistone proteins is to compare the electrophoretic profiles of the unwashed nuclei and those washed successively with saline-EDTA, Tris, and 0.35 M NaCl. These are shown in Fig. 3. Except for a few unique bands in the whole unwashed nuclei, the electrophoretic profiles of these different sets of nuclei are essentially identical. Coelectrophoresis with the supernate of cytoplasm centrifuged at 100,000 g for 1 h shows the presence of bands comigrating with nuclear matrix proteins C10, D1, and D4. Bands H5 and H10, presumptive ribonucleoproteins (see below), are totally absent from the cytosol. Proteins E10 and F3 (between D4 and G10) are very prominent in the cytosol and are the major proteins unique to the saline-EDTA washes of nuclei (Fig. 1). These are presumably cytoplasmic contaminants.

An estimate of the nonhistone protein:histone ratio of these nuclei was obtained by densitometric tracing (see Materials and Methods). This was felt to be more accurate than the separation of histones by acid extraction, since significant amounts of nonhistone proteins are also removed by acid (13). Since the histone:DNA ratio is approximately 1.0, the total protein:DNA ratio was obtained by adding 1.0 to the nonhistone protein:histone ratio. These protein:DNA ratios are shown in Table II and agree with biochemical analyses which indicate that the protein:DNA ratios generally range from 2.3 to 5.0 for whole liver nuclei (8, 26, 46–48, 52). In one experiment bio-

chemical determination of the protein:DNA ratios gave 2.6, 2.2, 1.8, and 1.6 for the unwashed and S-E-, Tris-, and 0.35 M NaCl-washed nuclei, respectively.
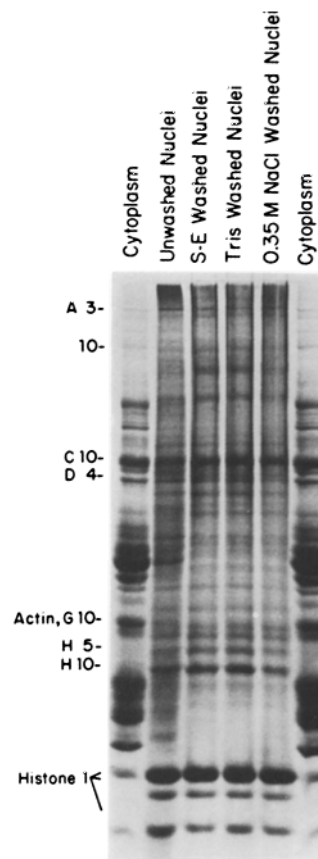

FIGURE 3 SDS gel electrophoresis in 11% acrylamide of a 100,000-g supernate of cytoplasm, unwashed nuclei, and nuclei successively washed in saline-EDTA, Tris, and 0.35 M NaCl. Bands comigrating with the nuclear matrix proteins C10 and D4 are also present in the cytosol. Bands H5 and H10, presumptive RNP particle proteins, are totally absent in the cytosol.

TABLE II

Nonhistone Protein:Histone Ratio and Protein:DNA Ratio of Nuclei Based On Densitometry*

Nuclei	NHP:Histone	Protein:DNA‡
	mean ± SD	
Whole unwashed nuclei	1.91 ± 0.21	2.91
Saline-EDTA-washed nuclei	1.44 ± 0.36	2.44
Tris-washed nuclei	1.32 ± 0.33	2.32
0.35 M NaCl-washed nuclei	1.15 ± 0.29	2.15

* See Materials and Methods. Based on three experiments.
‡ Based on a histone:DNA ratio of 1.0.

One of the implications of Fig. 3 is that many of the prominent nonhistone proteins are present in greater numbers per nucleus than would be estimated on the basis of just examining purified chromatin. By determining the relative area under some of these more prominent proteins and knowing the molecular weight (10), the number of proteins per diploid nucleus (6×10^{-12} g DNA) could be determined. These are given in Table III. The values range from 220,000 copies of A3 to 5,200,000 copies of H10.

The protein in the washes could be: (a) free; (b) bound to RNA; or (c) bound to DNA released during the washing procedure. The last could be ruled out as a significant factor by the fact that

biochemical analysis showed little DNA in the washes. To determine whether the nuclear wash proteins were free or bound to RNP particles, the washes were centrifuged at 100,000 g for 1 h and both the pellet and the supernate electrophoresed. In the same set of experiments a mouse liver was perfused and homogenized in TCMB buffer (see Materials and Methods). An aliquot of this homogenate was electrophoresed to represent total cell protein. This homogenate was also centrifuged to give a 100,000-g supernate and pellet. Biochemical analyses indicated that 80% or more of the nuclear wash proteins were in the 100,000 g supernates. The electrophoretic results are shown in Fig. 4. This verifies that the protein in the

TABLE III

Estimation of the Number of Molecules of Major Nonhistone Proteins (NHP) per Mouse Liver Diploid Nucleus

Protein	Molecular weight (10)	Moles:Nucleus $\chi \times 10^{-18}$ $\chi =$	Molecules:Nucleus $\chi \times 10^6$ $\chi =$	% of total NHP
A3	252,000	0.33	0.22	0.80
A4	237,000	0.44	0.28	0.95
A9	182,750	0.42	0.25	0.67
A10	173,250	0.74	0.45	1.1
B4, 5	145,000	1.31	0.80	1.7
B10	109,000	1.25	0.76	1.2
C10	68,000	5.0	3.1	3.0
D1	67,000	3.6	2.2	2.0
D4	65,000	2.4	1.5	1.5
D7	63,250	3.7	2.3	2.1
D10	58,000	3.4	2.1	1.7
E10	53,700	4.0	2.4	1.9
F3	52,500	4.9	3.0	2.2
G3	47,800	3.0	1.8	1.2
G10	42,000	4.7	2.9	1.7
H2	40,800	4.9	3.5	1.7
H5	37,000	5.4	3.3	1.7
H10	33,000	8.6	5.2	2.5
I2	32,500	6.7	4.1	1.9
I4	30,500	7.9	4.9	2.1
I6	29,500	7.2	4.4	1.8
I8	29,000	8.3	5.1	2.1

Based on a NHP:histone ratio of 1.91 and a total protein:DNA ratio of 2.91 for whole unwashed nuclei, assuming a diploid DNA content of 6×10^{-12} g and a nonhistone protein content of 11.5×10^{-12} g.
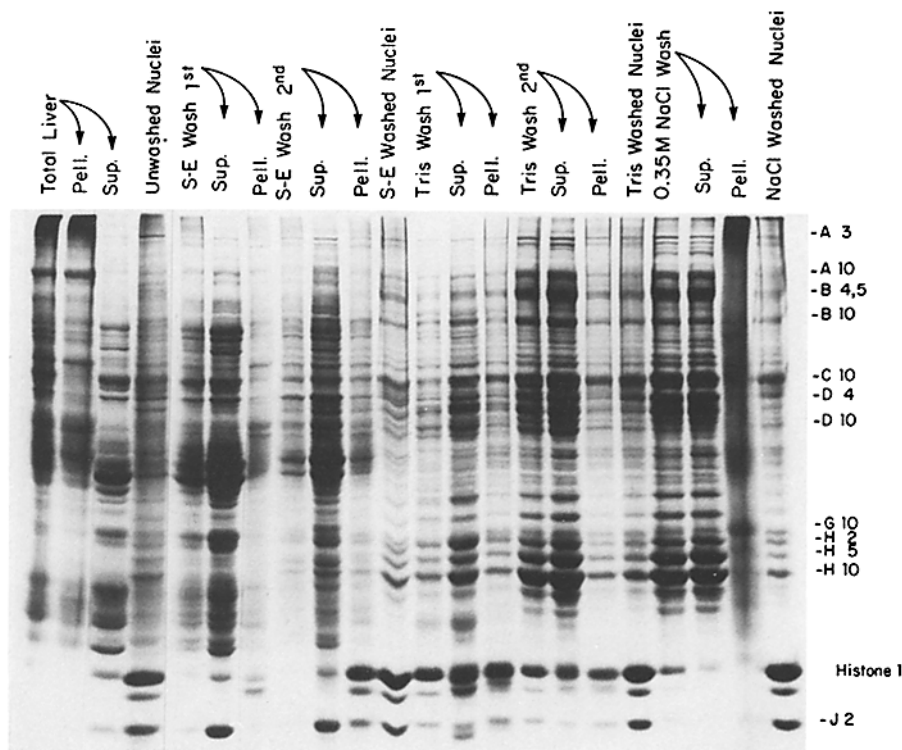
FIGURE 4 SDS slab gel electrophoresis in 11% acrylamide of whole liver homogenate, saline-EDTA, Tris, and 0.35 M NaCl washes and their 100,000 g × 1 h supernates and pellets.

washes and the protein in the 100,000-g supernates were very similar.

This does not answer the question of whether the supernatant proteins are free or bound to RNA which does not pellet at 100,000 g for 1 h. This will be examined in detail in a subsequent paper.

In the first and second S-E-wash supernates, there was a prominent band at position J2. In 11% gels, this comigrates with, but is distinct from the most rapidly migrating of the three histone 1 proteins.[3] Since J2 is present in only trace amounts in the cytoplasm, this protein appears to be truly enriched in the nucleoplasm. This is a histone-binding protein[3] and appears to be identical to the HMG1 protein of Shooter et al. (42).

To examine the relationship between the proteins of the total cell lysate and the proteins of the final sheared chromatin, aliquots were taken at each step in the preparation of chromatin and

[3] Connor, B. J., and D. E. Comings. 1976. Nuclear proteins. IV. Histone bining proteins. Manuscript in preparation.

compared by SDS slab gel electrophoresis. Fig. 5 shows a typical experiment for the isolation of chromatin from rat liver. The saline-EDTA supernate (S-E sup) represents the protein of the supernate of the total cell lysate after homogenization in a Waring Blendor. This represents primarily cytoplasmic proteins. The next four slots show aliquots of the Tris washes. The crude chromatin is the pellet before centrifugation through sucrose. After centrifugation through 1.7 M sucrose the chromatin was dialyzed, sheared, and centrifuged at 12,000 g for 30 min to produce a supernate of sheared chromatin and a pellet of unsheared chromatin. The sheared chromatin was most enriched in histone, and the OD 320 mm/260 mm was 0.08. Most of the major nonhistone protein bands of the sheared chromatin were also present in the other washes, including the first saline-EDTA wash. The 220,000 molecular weight component comigrated with myosin, and the 42,000 molecular weight band comigrated with actin. The latter band is relatively large in the Tris washes and chromatin, and may represent the tendency for actin to bind to many different structures (19). By
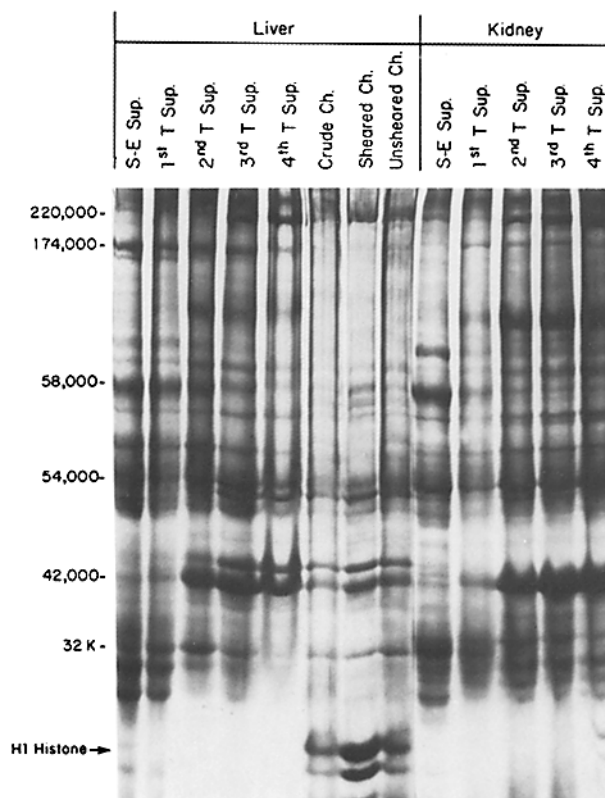
FIGURE 5 SDS slab gel electrophoresis of the proteins of the saline-EDTA and four Tris washes of chromatin prepared from whole cell lysates of rat liver chromatin. Ch = chromatin. Similar washes of a kidney lysate are shown on the right. See text for details. 10% acrylamide gel.

binding to the crude chromatin pellet, it may have become enriched over its concentration in the cytoplasm. Washes of a kidney preparation were included to show the many similarities in proteins of the kidney and liver (12). Fig. 6 shows the washes obtained during preparation of chromatin from the rat brain and kidney. Again, the bands present in the chromatin were also present in the washes including the saline-EDTA whole cell supernate. The proteins at 220,000, 52,000, and 42,000 daltons were especially prominent chromatin nonhistone proteins.

These results strongly suggest that when chromatin is prepared by homogenizing cells, the exposure of the chromatin to cytoplasmic proteins allows many of them to bind to the chromatin, with the result that the final chromatin is severely contaminated with polypeptides that are not naturally present to this degree in the intranuclear chromatin. If this is the case, the electrophoretic profile of the chromatin prepared from whole cell

lysates should be different from that prepared from isolated nuclei. To examine this, the experiment shown in Fig. 7 was carried out. Nuclei were isolated from rat liver and then washed in the same manner as the whole cell lysates, once in saline-EDTA, and three times in dilute Tris, to produce a crude chromatin preparation. This preparation was then centrifuged through 1.7 M sucrose, and the pellet termed "sucrose-washed chromatin." This pellet was sheared and centrifuged at 12,000 g for 30 min to give "sheared chromatin" in the supernate and a pellet of "sheared pellet chromatin." These preparations were coelectrophoresed with the chromatin prepared from whole cell lysates and with a mouse myofibril preparation. As shown in Fig. 7, the nonhistone proteins in the chromatin preparation from whole cells (whole cell sheared chromatin) showed a profile significantly different from those in the chromatin from whole nuclei. In the whole cell chromatin the myosin and actin bands are very
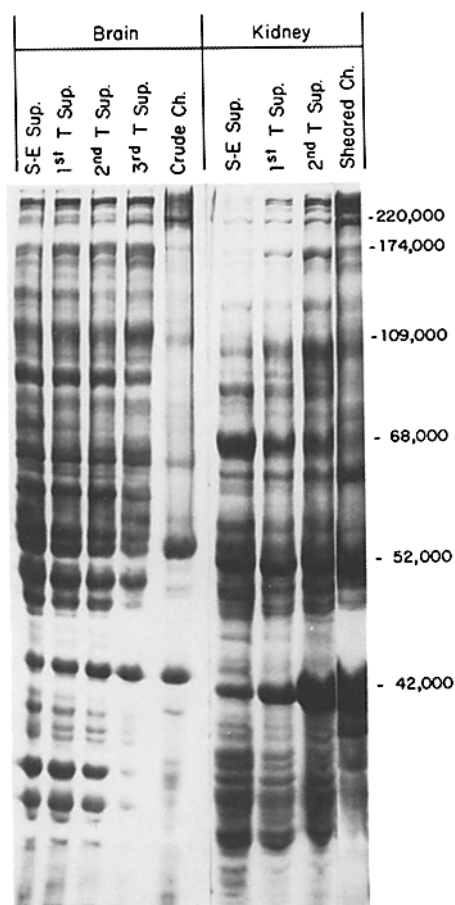
FIGURE 6 Electrophoresis of the proteins of saline-EDTA and Tris washes of brain and kidney chromatin prepared from whole cell lysates. Molecular weights are shown on the right. 10% acrylamide.

prominent. Also prominent are bands at 52,750 daltons corresponding to the prominent saline-EDTA wash proteins, and a band migrating just before actin. In the chromatin from whole nuclei, a triplet of proteins of 65,000–68,000 daltons, representing nuclear matrix proteins,[2] and a triplet of H2, H5, and H10 proteins is prominent. Preliminary results suggest that H5 and H10 are not α- and β-tropomyosin.

To attempt to identify further the proteins associated with whole cell chromatin, the sheared whole cell chromatin was coelectrophoresed with a preparation of mouse brain tubulin (not shown). In Fig. 7, for the whole cell sheared chromatin there is a set of three proteins at 52,000–54,000 daltons. The lighter two bands comigrated with tubulin.

DISCUSSION

The major conclusions of this study are as follows.

(a) All the prominent electrophoretically detectable nonhistone proteins which remain bound to DNA after extensive washing of nuclei in SE, Tris, and 0.35 M NaCl are also present in somewhat similar proportions in the nuclear washes. The first SE wash of the nuclei shows the greatest variation in distribution of proteins and here the most striking differences are in several cytoplasmic proteins in the 50,000–55,000 mol wt range.

(b) After two saline-EDTA washes, the electrophoretic profile of the whole nuclei is very similar to that of nuclei washed with Tris and 0.35 M sodium chloride.

(c) G10, a protein which comigrates with actin, is prominent in the first SE wash but present as only a moderate-sized band in the final chromatin. This is even more striking by urea gel electrophoresis. Here, a band comigrating with actin is very prominent in the first SE wash but missing in the Tris and 0.35 M sodium chloride washes and in the washed chromatin.

(d) Several proteins are enriched in the 100,000-g supernate of the S-E washes. These include C10, D4, E10, F3, G10, and J2. Of these, C10 and D4 are nuclear matrix proteins.[2] These are also prominent in a 100,000-g supernate of cytoplasm and all of the washes, suggesting that these proteins are distributed throughout the cell and are not restricted to the nuclear matrix. E10 and F3 are very prominent in the cytoplasm and appear to be cytoplasmic contaminants. G10, which comigrates with actin, is a prominent cytoplasmic protein and is enriched in the first S-E wash of nuclei. It is, however, still present as a moderate-size band even in the well-washed chromatin. Electrophoresis in urea gels strongly suggests that this chromatin-bound G10 is no longer actin (Fig. 2). J2, a histone-binding protein, appears to be a true nucleoplasmic protein. It is virtually absent from the cytoplasm and is much enriched in the 100,000-g supernate of the first and second S-E washes of nuclei (Fig. 4). It has a molecular weight of approximately 25,000 daltons and appears to be identical[3] to the HMG1 protein of Shooter et al. (42).

(e) To obtain a preliminary estimate of whether some of these proteins are bound to HnRNA, the nuclei were washed with STM (0.1 M NaCl, 0.001 M MgCl₂, 0.01 M Tris) at pH 7.0, then at pH 8.0 by the technique of Samarina et al. (40). Electro-
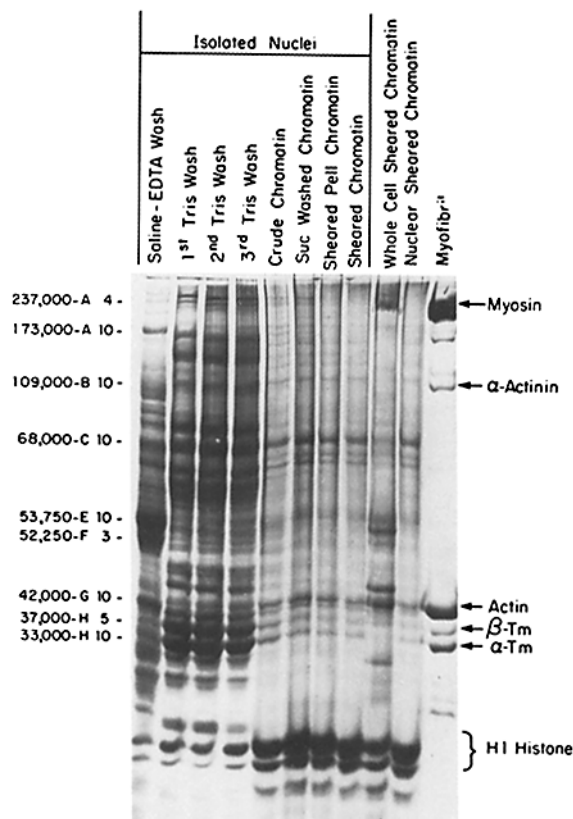
FIGURE 7 Electrophoresis of saline-EDTA and Tris washes of isolated rat liver nuclei, and crude chromatin, sucrose washed chromatin, and sheared chromatin from nuclei. These are compared to the proteins of sheared chromatin isolated from whole cell lysates (whole cell sheared chromatin) and proteins of mouse myofibrils. 10% acrylamide.

phoresis of the pH 8.0 wash, which is enriched in RNP proteins, shows that H5 and H10 are very prominent. These 33,000- and 37,000-mol wt proteins are presumably analogous to the 34,000- and 38,000-mol wt proteins found associated with the 30S RNP particles (33). The results of electrophoresis of purified RNP particles and a comparison of their proteins and the proteins of high-speed supernates of nuclear washes will be presented in a subsequent paper.

Since many of the proteins present in well-washed chromatin are also present in the nuclear washes, estimates of the number of specific non-histone proteins per nuclei based on washed chromatin alone will be somewhat less than the estimate of their true number. For example, Garrard et al. (25) examined the nonhistone proteins of purified sheared rat liver chromatin and concluded that the proteins ranged in frequency from 8.4 ×

10^3 to 3.4×10^6 copies per diploid nucleus. When calculated on the basis of electrophoretic profile of whole nuclei, the number of major nonhistone proteins ranged from 2.2×10^5 to 5.2×10^6 copies per nucleus. Presumably, most of these major nonhistone proteins, visualized by electrophoresis, are structural rather than regulatory proteins. The large number of molecules per nucleus is consistent with this conclusion. On the basis of analogies with bacterial systems, repressor proteins are probably present in the 5×10^2–5×10^4 copy per nucleus range (31).

The study of NHP chromatin from whole cell lysates was stimulated when we were asked to compare electrophoretically the proteins of a sample of such chromatin and those of the chromatin preparations we were obtaining from whole nuclei. The electrophoretic profiles of these two preparations were so different that we felt that the

two techniques required closer examination. Some of the most prominent proteins in the chromatin prepared from whole cells comigrate with myosin, actin, and tubulin, proteins which are especially prominent in the cytoplasm. Of these, actin tends to adhere nonspecifically to many different structures (19). Our conclusions from this study are as follows: (a) for the study of nonhistone proteins, chromatin is best isolated from purified nuclei. For tissues such as calf thymus, where the cells have a minimum amount of cytoplasm, the difference between the nonhistone proteins of chromatin isolated from whole cell and those from isolated nuclei will be less than with tissues possessing large amounts of cytoplasm, such as liver; (b) comparative studies of the chromatin nonhistone proteins from different tissues may actually examine tissue-specific cytoplasmic proteins unless the chromatin is isolated from purified nuclei.

The problem of cytoplasmic contamination of chromatin prepared from whole cell lysates was also demonstrated by studies showing that "chromatin" histone protease is actually a contaminant originating from damaged mitochondria (27). This protease was present on chromatin isolated from whole cells but not on chromatin isolated from purified nuclei. It has recently been suggested that up to 35% of the nonhistone proteins of rat liver chromatin (isolated predominately by the whole cell lysate technique) consist of myosin, actin, tubulin, and tropomyosin (18). When isolated nuclei are used as the starting point for chromatin isolation, as in the present experiments, myosin, actin, and tubulin are essentially absent, and bands H5 and H10, which comigrate with β- and α-tropomyosin, appear to be HnRNA-associated proteins. Thus, in our studies, myosin, actin, tubulin, and tropomyosin constitute at most 2%, and probably much less, of the nonhistone proteins of chromatin prepared from purified nuclei. This essential absence of contractile proteins in the nucleus is not due to loss by proteolysis. The technique that we use for nuclear isolation and electrophoresis results in negligible proteolysis (15). This conclusion is consistent with the observations of H. Busch of Baylor College of Medicine, Houston, Texas (personal communication), using two-dimensional gel electrophoresis, that purified contractile proteins do not comigrate with rat liver nonhistone proteins isolated from purified nuclei and nucleoli. We had considered the possibility that constitutive heterochromatin might be condensed by an actin-myosin rigor-type interaction,

but electron microscopy of the kangaroo rat nuclei washed with ATP provides no evidence for this type of mechanism (11). Contractile proteins are prominent in the nuclei of *Physarum* (30). However, unlike most eukaryotes, this organism has an intranuclear mitosis, and actin, myosin, tubulin, and tropomyosin are present as part of the intranuclear spindle apparatus.

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